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INHIBITION OF CYANOGENESIS BY TANNINS

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Abstract—During isolation of two biosynthetic types of cyanogenic glycosides from *Carica papaya*, weak cyanide tests were obtained from initial fractions. Upon final purification, strongly positive cyanide tests were obtained. Pretreatment of extracts to remove polyphenolics alleviated inhibition of cyanogenesis, which led us to suspect that tannins were inhibitory agents. Qualitative and quantitative measures of inhibition were made using standard cyanogenic glycosides and polyphenolics. Cyanogenesis was inhibited quantitatively when condensed tannins (quebracho, wattle, and chestnut), or hydrolyzable tannin (tannic acid) were added. When tannins were precipitated from the reaction mixture, cyanide tests proceeded optimally. These results stress the need to interpret negative cyanide tests with care and indicate possible ecological synergisms between plant defensive chemicals.

Key Words—Tannins, cyanogenic glycosides, *Carica papaya*, β-glucosidase, tetraphyllin B, prunasin, amygdalin, enzyme inhibition.

INTRODUCTION

Tannins are widespread in plants, and their ingestion has been shown to have adverse effects on a variety of herbivores. Increased tannin ingestion has been correlated with decreased larval and pupal growth as well as increased larval and pupal mortality in *Operophtera brumata* (Feeny, 1968). These larval lepidopterans showed decreased fitness with addition of 1% condensed tannin to the diet (Feeny, 1968). Some species of acridid nymphs fed greater than 10% dry weight condensed tannin suffered reduced weight and survival as adults (Bernays et al, 1981). Lindroth and Batzli (1984) examined growth of *Microtus ochrogaster* on tannin-enriched diets and found survival and growth rates to be inversely related to tannin concentration.

It has been suggested that tannins function by reduction of foodstuff diges-

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tibility due to their ability to complex with nutritive proteins and digestive enzymes (Feeny, 1970, 1976; Levin, 1976; Rhoades and Cates, 1976; Rhoades, 1979). While it is difficult to separate antifeedant from direct effects, it is true that tannins complex with and precipitate proteins. Details of association and disassociation of these complexes have been described (Mejbaum-Katzenellenbogan et al., 1959). Tannin–protein complexes have been found to be very stable (Van Buren and Robinson, 1969) due to covalent condensations as well as hydrogen bonding (Loomis and Battaile, 1966). More recently the binding specificities of tannin and protein interactions have been measured using a competitive binding assay (Hagerman and Butler, 1981). The observed stability of tannin–protein complexes has been corroborated by structural elucidation of binding sites in protein complexes with proanthocyanidins and gallic acid esters (McManus et al., 1983). Reaction mechanisms have been reviewed by Van Sumere et al. (1975).

Support for the idea of a direct action of tannins on digestive enzymes has been assembled in several in vitro studies. Inhibition of enzyme activity by oak tannins has been shown for trypsin hydrolysis of casein protein (Feeny, 1969) and α-amylase hydrolysis of starch (Gadal and Boudet, 1965). An influence of tannins upon enzyme action has been implied for cellulases and pectinases involved in the ripening of fruits in some early studies (Barnell and Barnell, 1945; Hathway and Seakins, 1958). Wattle tannin and tannic acid have been tested for their ability to inhibit enzyme activity in order to elucidate a possible role of tannins in fruit ripening (Goldstein and Swain, 1965). This study demonstrated precipitation of several enzymes by tannins, including a commercially available β-glucosidase fraction, as measured by loss of enzyme activity.

Cyanogenic glycosides are known toxins (Jones, 1962, 1981) which have been demonstrated to be ecologically important deterrents to herbivory (Angseesing and Angseesing, 1973; Cooper-Driver and Swain, 1976; Bernays et al. 1977; Woodhead and Bernays, 1978). Their deterrency is manifested only in the release of hydrogen cyanide upon enzymatic hydrolysis.

Carica papaya L. (Caricaceae) contains the cyanogenic glycosides tetraphyllin B and prunasin (Spencer and Seigler, 1984a). During isolation of these two biosynthetic types of cyanogenic glycoside, only weak cyanide tests were obtained. This phenomenon had previously been noted during isolation of prunasin from *Passiflora edulis* (Spencer and Seigler, 1983). Prunasin is known to be hydrolyzed efficiently by a β-glucosidase specific for aromatic cyanogenic glycosides (Hosel, 1981). Tetraphyllin B is efficiently hydrolyzed by a β-glucosidase specific for cyclopentenoid cyanogenic glycosides (Spencer and Seigler, 1982a, 1984b). Upon final purification of the cyanogenic glycoside fractions, rapid hydrolysis took place with addition of appropriate enzyme fractions, and positive cyanide tests were obtained. Pretreatment of the extracts to remove polyphenolics alleviated inhibition of cyanogenesis, which led us to suspect that tannins were inhibitory agents.

The present study was carried out in order to determine the potential for the existence of a synergistic interaction between the protein-precipitating tannins and the enzyme-mediated production of cyanide within tissues of a single plant.

METHODS AND MATERIALS

Plant Materials. Dried leaves of *Carica papaya* L. were purchased commerically (Corn Country Products, Champaign, Illinois). Fresh leaves of *P. quadrangularis* L. were obtained from cultivation at the University of Illinois, Urbana. Voucher specimens are on file at the university herbarium. Fresh leaves of *Polypodium californicum* L. were obtained from cultivation at the University of California, Irvine. Fresh material of *Adenia digitata* Engl. was obtained from Abbey Gardens, Carpenteria, California.

Isolation of Tannins from Carica. Dried leaves of *Carica papaya* (35.1 g) were soaked in 80% MeOH homogenized. The mixture was covered and refrigerated for 12 hr. The mixture was then filtered and the solid material was extracted twice with 80% MeOH, filtered, and the procedure repeated using Me_2CO and finally distilled H_2O. The filtrate was then concentrated under vacuum to a thick syrup. This was brought up to volume in 100 ml of MeOH, and 10 ml of this solution was then diluted to a total volume of 100 ml and read colorimetrically at 725 nm.

Isolation of Glycosides. Prunasin was isolated from leaves of *Polypodium californicum* as previously described (Spencer and Seigler, 1983; Spencer, Seigler, and S. Whitmore, unpublished). Tetraphyllin B was isolated from *Adenia digitata* as previously described (Spencer and Seigler, 1982a,b). Amygdalin was obtained commercially (Calbiochem).

Qualitative Determination of Cyanide. Emulsin was obtained commercially (Sigma). Emulsin is a partially purified β-glucosidase enzyme fraction capable of hydrolyzing aromatic cyanogenic glycosides (Hosel, 1981). *Passiflora quadrangularis* enzyme preparation was made by grinding 100 g of fresh leaves in 500 ml Me_2CO followed by filtration and rinsing with additional Me_2CO. The solid in the filter was dried under vacuum conditions and suspended in 500 ml against pH 6.8 buffer. The final product was concentrated under vacuum to 50 ml and its activity tested using fresh plant material according to the Feigl-Anger method. *Passiflora quadrangularis* enzyme preparation efficiently hydrolyzes cyclopentenoid cyanogenic glycosides (Spencer and Seigler, 1984b).

HCN released by β-glucosidase hydrolysis of cyanogenic glycosides was tested using the Feigl-Anger method (Feigl and Anger, 1966). Strips of Whatmann 3 MM paper were soaked 1–2 min in 1% 4,4-tetramethyldiaminediphenyl methane (tetrabase) in $CHCl_3$ and 1% (w/v) copper ethylacetoacetate in $CHCl_3$ and were dried. The strips turn blue upon exposure to HCN. One milliliter each

of a 1.0 M solution of cyanogenic glycoside and the corresponding enzyme (1 mg/ml for emulsin, a concentration of similar activity of the other preparations) were placed in a vial with Feigl-Anger strip held in place above the liquid with a stopper. Amygdalin with emulsin, prunasin with emulsin, and tetraphyllin B with *P. quadrangularis* enzyme preparation each gave positive cyanide tests as indicated by the Feigl-Anger method. Enzyme specificity for each substrate was shown to be in accordance with previous reports (Spencer and Seigler, 1984b).

Quantitative Determination of Cyanogens. Quantitative measurements of cyanide were made using a modified Lambert procedure (Lambert et al., 1975). Standard NaCN solution was prepared as 1.0 ml 0.01 M NaCN in 1.0 M NaOH diluted to 50 ml with 1.0 M NaOH. Trial tubes contained 0–500 μl NaCN each, then made up to a volume of 1.0 ml with 0.1 M NaOH. Acetic acid, 0.5 ml 1.0 M, was then added to each trial tube. Succinimide–*N*-chlorosuccinimide (5.0 ml), made up in the proportion 2.5 g succinimide to 0.25 g N-chlorosuccinimide per liter of distilled water was then added. Finally 1.0 ml of barbituric acid–pyridine reagent, comprised of 12.0 g barbituric acid and 120 ml pyridine made up to a final volume of 400 ml with distilled water, was added to each tube. Five dilutions each of triplicate samples were vortexed and the optical density read after a 10-min period at 580 nm. A standard curve was then prepared.

Qualitative tests for HCN were carried out using 1.0 ml of 1.0 M solution of the cyanogenic glycoside and 1.0 ml of the appropriate enzyme or enzyme preparation (see above) placed together in the outer well of a Warburg flask while 0.5 ml 0.1 M NaOH was placed in the center well. Flasks were stoppered tightly and incubated at room temperature for 12 hr. The contents of the center well were then removed with a Pasteur pipet and diluted 1:20 with 0.1 M NaOH. Appropriate dilutions were made (as for the standard NaCN solution) and determinations of cyanide made using the modified Lambert method.

Quantitative Determination of Tannins. Quebracho tannin from *Schinopsis balansae* Engl. was obtained from the Van Dyke Supply Company, Woonsocket, South Dakota; wattle tannin from *Acacia mearnsii* DeWild and chestnut tannin from *Castanea sativa* L. were the gifts of D.S. Seigler. Tannic acid, quercetin, and rutin were purchased commercially (Sigma).

Total phenolics in the above polyphenol preparations were determined by the Folin-Denis method (Folin and Denis, 1915; Folin and Ciocalteau, 1927; Rhoades, 1977). The Folin-Denis reagent was prepared by adding 41.25 g $Na_2WO_4 \cdot 2H_2O$, 8.25 g $20MoO_3 \cdot 2H_3PO_4 \cdot 48H_2O$, and 20 ml 85% phosphoric acid to 300 ml distilled water. The mixture was refluxed 2 hr, cooled, and diluted to 1 liter in distilled water. A blank of 1.0 ml 80% MeOH + 1.0 ml Folin-Denis reagent + 1.0 ml Na_2CO_3 (1.0 M) was prepared with vigorous shaking upon each addition. Absorbance was measured at 725 nm. Polyphenolic preparations ranging from 10^{-7} g/ml MeOH to 0.10 g/ml in 80% MeOH were measured at 725 nm.

Inhibition of Hydrolysis of Cyanogenic Glycosides by Tannins. Tannins were added to Warburg flasks containing the three cyanogen sources and corresponding β-glucosidase fractions. Six flasks were used for each run, the first containing no tannin and the rest making up a range of tannin concentration from 0.01 mg/ml to 100mg/ml. Tannin (1.0 ml), 1.0 ml of cyanogenic glycoside (474 mg amygdalin; 295 mg prusasin; 287 mg tetraphyllin B), and 1.0 ml enzyme preparation were placed in the outer well, with 0.1 M NaOH (0.5 ml) placed in the center well. Amounts of active β-glucosidase added were not precisely measured, but each enzyme preparation was adjusted in volume so that 1.0 ml completely hydrolyzed 1.0 ml of a 1.0 M solution of substrate in 12 hr. After 12 hr, the contents of the center wells were removed and treated as previously described. Tests were also carried out after tannins were precipitated with a saturated caffeine solution and with a 1.0% gelatin solution (Segleman and Farnsworth, 1969). The mixture was centrifuged and additional enzyme added. A final assay for cyanide was made using the Feigl-Anger method.

RESULTS AND DISCUSSION

Feigl-Anger cyanide tests (Feigl and Anger, 1966) were positive for all standard enzyme–cyanogenic glycoside mixtures. In the presence of excess quebracho, wattle, or chestnut tannin as well as tannic acid, results of all Feigl-Anger tests were negative. Extracts of *Carica papaya*, which contains tetraphyllin B and prunasin (Spencer and Seigler, 1984a), also failed to yield cyanide upon treatment with enzyme fractions known to hydrolyze these cyanogenic glycosides. In the presence of the flavonoids quercetin or rutin, all Feigl-Anger tests were positive. Upon precipitation of tannins by casein, caffeine, or gelatin, addition of enzyme to both standard and samples of treated extract gave positive tests for cyanogenesis. These results indicate that the presence of tannins inhibited enzymatic hydrolysis of cyanogens, while the presence of flavonoids did not.

The degree of inhibition of cyanogenesis in vitro was tested quantitatively by varying the amount of tannin added to each cyanogenic glycoside–enzyme mixture and subsequently measuring the amount of cyanide released after 12 hr.

Figure 1 shows the degree of inhibition of hydrolysis of amygdalin by emulsin in the presence of tannin. Wattle, chestnut, and quebracho tannins and tannic acid all inhibited hydrolysis. Quebracho tannin was found to be twice as active as either wattle or chestnut tannin. Tannic acid was found to be approximately three times as active an inhibitory agent as was the same concentration of any of the condensed tannins. Significant inhibition occurred upon addition of tannins in concentrations representing 0.03–3.0% of the reaction mixture in all cases. Halving the amount of amygdalin present in the reaction mixtures resulted in a decreased inhibitory effect of the tannins. Analysis of these diluted mixtures yielded straight line plots reflecting incompletion of hydrolysis.

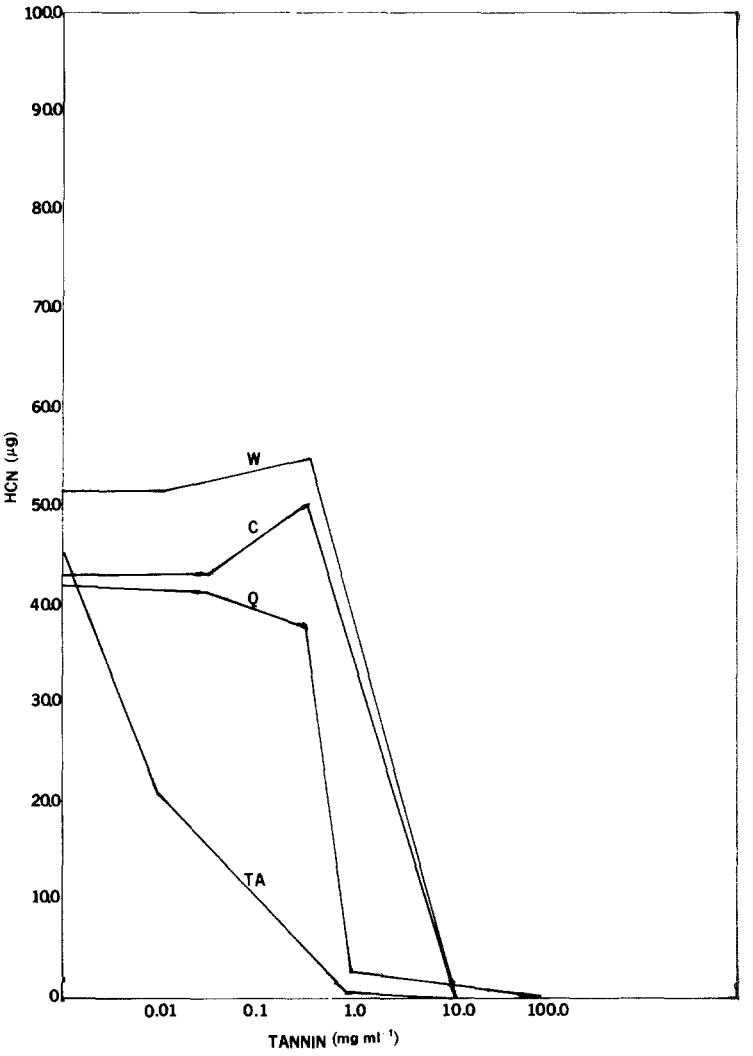

FIG. 1. Inhibition of hydrolysis of amygdalin by emulsin upon addition of tannin. TA = tannic acid; Q = quebracho tannin; C = chestnut tannin; W = wattle tannin.

Figure 2 represents the degree of inhibition of hydrolysis of prunasin by emulsin in the presence of tannins. Wattle, chestnut, and quebracho tannins all inhibited hydrolysis. Chestnut tannin was found to be approximately 1.5 times as active an inhibitory agent as wattle tannin. The threshold of inhibition occurred with 1.0 mg/ml tannin, below which point the lower concentrations of tannin no longer significantly inhibit cyanogenesis, as was found for amygdalin.

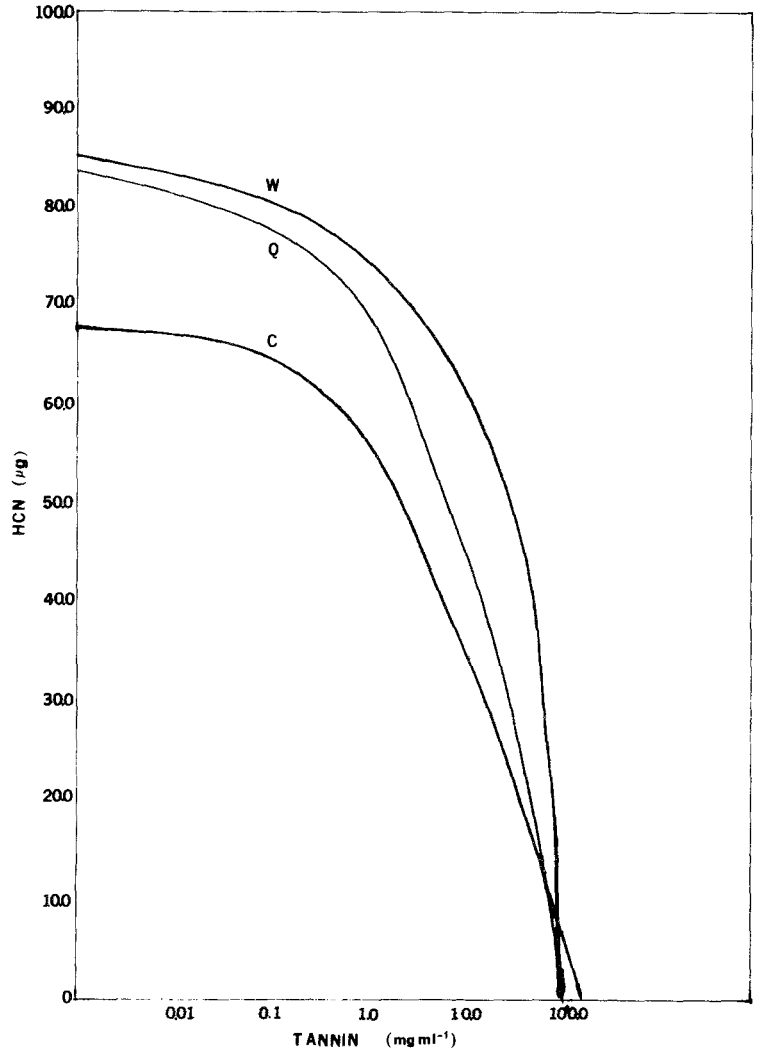

FIG. 2. Inhibition of hydrolysis of prunasin by emulsin upon addition of tannin. W = wattle tannin; Q = quebracho tannin; C = chestnut tannin.

Significant inhibition again occurred at tannin concentrations representing 0.3–3.0% of the reaction mixture.

Wattle, chestnut, and quebracho tannins all inhibited hydrolysis of tetraphyllin B by *P. quadrangularis* enzyme preparation (Figure 3). Thresholds of inhibition occurred at 1.0 mg/ml tannin added to the reaction mixture. Complete inhibition occurred at tannin concentrations above 3.0% of the reaction mixture.

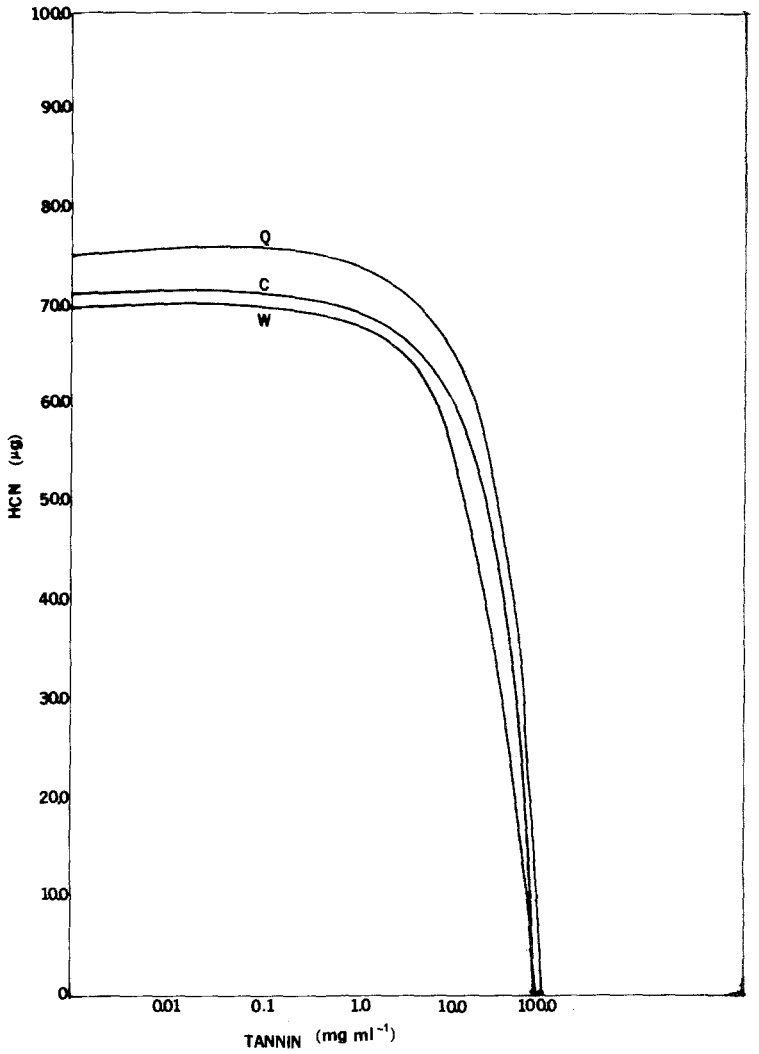

FIG. 3. Inhibition of hydrolysis of tetraphyllin B by a *Passiflora quadrangularis* enzyme preparation. Q = quebracho tannin; C = chestnut tannin; W = wattle tannin.

Figures 1–3 all show inhibition of cyanogenesis by added tannins. A similar threshold response was found in all reactions, except that of the emulsin hydrolysis of amygdalin in the presence of tannic acid where some degree of inhibition was found at all levels. The three condensed tannins were found to show differential inhibitory activity against a given enzyme preparation. No one condensed tannin was found to have the greatest inhibitory effect on all cyanogenic glycosides. Tannic acid, however, was found to have a greater inhibitory effect

than any of the other tannins. This indicates that the structural form and type of tannin used in ecological experimentation may affect the results obtained.

A quantitative measurement of the total phenolics present in leaf extracts of *Carica papaya* was made using the Folin-Denis (Folin and Denis, 1915; Folin and Ciocalteau, 1927; Rhoades, 1977) technique. Standard absorbance–concentration curves were prepared for all of the tannins used in this study (Figure 4). Comparison of total phenolics in *C. papaya* with these standard curves shows our samples to contain approximately 2.3% dry weight polyphenolics. This re-

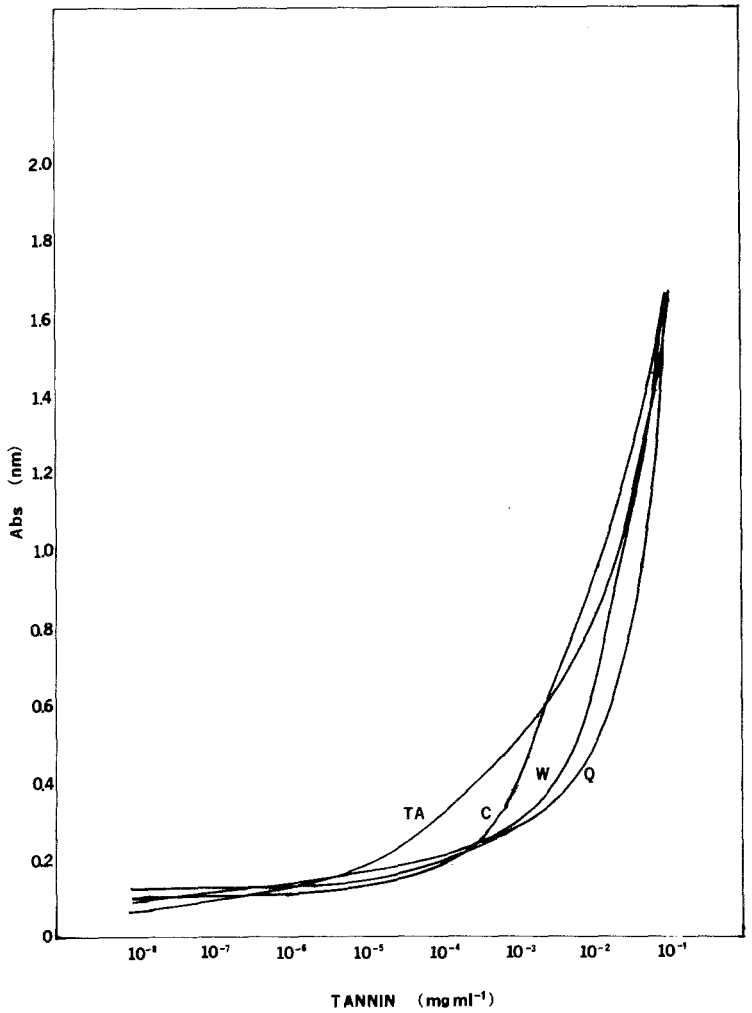

FIG. 4. Standard absorbance curves prepared by the Folin-Denis colorimetric method. TA = tannic acid; C = chestnut tannin; W = wattle tannin; Q = quebracho tannin.

sult is in a similar range to those of Feeny (1970) and Feeny and Bostock (1968), who found 0.5–5.0% dry weight polyphenolics in oak leaves (*Quercus robur* L.). We also measured polyphenolics in *Quercus agrifolia* Nee and found them to be present as 2.17% dry weight. The cyanogenic glycosides of *C. papaya* have been isolated as 0.019% dry weight (Spencer and Seigler, 1984), indicating that sufficient tannin is present to inhibit cyanogenesis if the tannin and enzyme were allowed to react. It was also found that addition of polyphenolics extracted from *C. papaya* in fact inhibited cyanogenesis when added to prunasin–emulsin and tetraphyllin B–glucosidase mixtures in the same manner as did the tannins from other sources.

Alternative models to the digestibility reduction theory of tannin function have been proposed, including direct toxicity (Bernays, 1980). It has been found that, although tannins have the potential for reducing digestibility of proteins in foodstuffs, the detergency of insect gut fluid may interfere with tannin precipitation of dietary proteins (Martin and Martin, 1984). Studies of the actual effects of phenolics upon digestion have not shown a clear function (Williams, 1959; Jung and Fahey, 1983). Regardless of their mechanism of action, we feel it important to consider their interaction with other secondary metabolic systems.

It is not clear how tannins and cyanogenic glycosides contained in the same plant would interact in natural situations of herbivory. It would, of course, not be expected that β-glucosidases would be precipitated by tannins in normal plant metabolism. These enzymes are probably isolated compartmentally within plant tissues (Kojima et al., 1979).

It is possible that herbivores which are deterred by cyanogenic glycosides do not ordinarily encounter significant amounts of tannins during feeding. Conversely, those herbivores that are not deterred may feed to such a manner that contact between tannin and the glucosidases responsible for toxification is maximized. An inverse relationship between tannin and cyanide content in *Lotus corniculatus* has recently been described (Ross and Jones, 1983).

Our data show that negative test results for cyanogenic glycosides can be caused by the concurrent pressence of tannins. We have shown that tannins inhibit quantitatively the β-glucosidase-mediated hydrolysis of cyanogenic glycosides in vitro. We suggest that in certain ecological situations tannins may interfere with the release of HCN from the hydrolysis of cyanogenic glycosides and thereby reduce their toxic effects.

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