though a number of these compounds were highly toxic to rats. Poisoning in a substantial number of these cases was cholinergic. The *O,S,S*-trialkyl phosphorodithioates, therefore, may be included with the *O,O,S*-trialkyl phosphorothioates and *O,S*-dialkyl alkylphosphonothioates as compounds that appear to be toxic by a cholinergic mechanism but are poor in vitro anticholinesterases. These observations raise the possibility of an activation step of some kind in the mode of action of these compounds. The absence of correlation between k_i values and rat oral toxicity also suggests that structural features might play a role in this proposed activation process.

The similarity in hydrolysis rates of the phosphorodithioates and OOS-Me (7) was surprising since the dithioates would be expected to be substantially more susceptible to hydrolysis owing to less $d\pi$–$p\pi$ interaction between the two sulfur atoms and phosphorus (Murdock and Hopkins, 1968). The similarity in hydrolysis rates, however, is consistent with the poor anticholinesterase activity of the trialkyl phosphorothioates and phosphorodithioates.

Registry No. 1, 22608-53-3; 2, 22082-34-4; 3, 4104-02-3; 4, 99559-84-9; 5, 13194-48-4; 6, 74125-01-2; 7, 2953-29-9; acetylcholinesterase, 9000-81-1.

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Phenolic Acid Content of Food Plants and Possible Nutritional Implications[1]

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The *p*-coumaric, ferulic, and caffeic acid contents of alfalfa, cabbage, and spinach were determined by gas chromatography after derivatization with *N,N*-bis(trimethylsilyl)trifluoroacetamide. Total phenolic acids were estimated by extraction with 1% NaOH containing 0.5% $NaBH_4$. Free or nonesterified acids were extracted with 80% methanol. Alfalfa contained the highest concentration of total *p*-coumaric acid (1 mg/g) and of free caffeic acid (0.68 mg/g). Lower levels of free caffeic acid were found in cabbage and spinach but none in wheat bran. Wheat bran had the highest content of ferulic acid (4.4 mg/g). Only the free form of caffeic acid was found in alfalfa and cabbage, probably because the alkaline treatment destroyed all that was present.

Fiber-rich plant foods stimulate the growth rate of immature guinea pigs fed purified diets (Ershoff, 1957; Reid and Mickelson, 1963; Lakhanpal et al., 1966; Singh et al., 1968; Knehans et al., 1979). They also protect against radiation damage (Calloway et al., 1963) and salmonellosis (Nabb and O'Dell 1964). The growth stimulant in alfalfa and other plants studied is water insoluble and associated with cell wall components and holocellulose prepared from the plant. It is unstable to alkaline solutions and loses activity with long-term dry storage, presumably by oxidation (Knehans et al., 1979).

The mechanism by which fibrous plant foods and the water-insoluble residues prepared from them stimulate growth is unknown but fibrous products do change the intestinal microflora of the guinea pig (Knehans and O'Dell, 1980). Recent observations (Johanning et al., 1984) have shown that fibrous residues prepared from alfalfa, cabbage, and spinach impair the growth rate of an intestinal anaerobe, *Bacteroides ovatus*, by removal of hemin from the medium. Alkaline treatment of the fiber-rich residues decreases their antibacterial action (Johanning et al., 1984) as well as the guinea pig growth stimulating activity. The active component may stimulate the growth rate by an antibiotic effect on certain intestinal microflora, but this is unknown. In any case its chemical properties suggest a compound that is easily hydrolyzed by alkaline solution or is prone to oxidation in alkaline solution or both.

Some of the polyphenolic acids are covalently linked to cell wall components and possess chemical properties similar to the growth stimulant found in alfalfa and its water-insoluble components. Caffeic acid, 3,4-dihydroxycinnamic acid, is widely distributed in plants and is highly sensitive to air oxidation, particularly in alkaline solution. Although there is not good evidence that they are esterified to cell wall components, several phenolic acids, including

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the cis and trans forms of ferulic and *p*-coumaric acids, are esterified to cell wall polysaccharides (Hartley and Jones, 1977). Treatment of ryegrass with a crude cellulose releases a series of carbohydrate esters of ferulic and *p*-coumaric acids (Hartley, 1973; Hartley et al., 1973). Phenolic acids also exist in some plants in the free form and/or as esters of small compounds, e.g., chlorogenic acid is an ester of caffeic and quinic acids. However, Jung et al. (1983) found no free, ether-soluble phenolic acids in alfalfa hay, soybean stover, bromegrass hay, or corn stalkage.

Free phenolic acids such as *p*-coumaric, ferulic, and caffeic acids can be separated and estimated by gas–liquid chromatography (Vande Casteele et al., 1976), or high-performance liquid chromatography (HPLC) (Hartley and Buchan, 1979). HPLC has the advantage that derivatization is not required and the compounds can be detected electrochemically (Felice et al., 1976). The major analytical problem relates to the determination of esterified phenolic acids. Sodium hydroxide is commonly used to saponify the ester linkage, but such an alkaline solution accelerates oxidative destruction of polyphenols. Attempts to prevent oxidation include the use of nitrogen gas (Jung et al., 1983) and the use of $NaBH_4$ (Schmidtlein and Herrmann, 1975), but the effectiveness of these precautions is not clear, particularly as regards caffeic acid.

The objective of this study was to determine the concentrations of the common phenolic acids found in food plants that stimulate the growth rate of guinea pigs fed purified diets. The chemical forms of *p*-coumaric, ferulic, and caffeic acids in alfalfa, cabbage, spinach, and wheat bran were also sought.

MATERIALS AND METHODS

Plant Materials. Fresh cabbage, spinach, and alfalfa were frozen and lyophilized before maceration. The dry material was ground to pass a 2-mm screen and stored at 4 °C. Water-insoluble residue (WIR) was prepared by extracting the powder 10 times with hot water (Johanning et al., 1984).

Analytical Methods. The phenolic acids were separated and estimated by gas–liquid chromatography (GLC) after silylation according to Vande Casteele et al. (1976). The ratio of peak height to an internal standard was compared to the ratio of standard compound relative to the same concentration of internal standard. Salicylic acid was first used as the internal standard, but it was unsatisfactory in presence of $NaBH_4$. When $NaBH_4$ was used in the extraction procedure, boric acid complexed with the salicylic acid standard and interfered with its silylation. Subsequently, *p*-hydroxybenzoic acid was used as the internal standard.

Standard curves were prepared from ethyl acetate solutions of *p*-coumaric, ferulic, and caffeic acids and aqueous methanolic solutions of chlorogenic and quinic acid. The internal standard was added before the solution was taken to dryness in a screw-capped tube with a stream of N_2. The dry residue was silylated with *N,N*-bis(trimethylsilyl)trifluoroacetamide (BSFTA) according to Gehrke et al. (1969). BSTFA (0.2 mL) was added and heated at 125 °C for 20 min. The solution was cooled in ice and stored in the refrigerator until injected into the chromatograph.

Chromatography was performed with a Perkin-Elmer Sigma 3B chromatograph using a column packed with Chromosorb W-HP, 80–100 mesh, coated with 1.5% methylsilicone (SE-30) and 1.5% phenyl methyl silicone (SE-52). The gas flow rates for N_2, H_2, and air were 25, 30, and 30 mL/min, respectively. The operating temperatures were as follows: injector, 80 °C; detector, 285 °C; initial, 80 °C; final, 285 °C with a ramp rate of 5 °C/min. For most measurements the attenuator sensitivity was set at 400.

Phenolic acids were extracted from acid (pH <4) aqueous solutions with 2 volumes of ethyl acetate (EtOAc). The extract was taken to dryness in vacuo, dissolved in 1 mL of EtOAc, transferred to a screw-capped vial, and taken to dryness under N_2. The residue was silylated as above. For 80% methanol extracts, the solution was taken to dryness in vacuo at room temperature; the residue was dissolved in absolute alcohol (50 mL), filtered, and again taken to dryness. This residue was dissolved in 4 mL of methanol, transferred to a vial, taken to dryness under N_2, and silylated.

All analyses were run in duplicate, and the mean concentration was calculated from the standard curve based on the aliquot of the sample extract injected.

Extraction of Plant Materials. Two methods of extraction were used, one designed to determine total phenolic acids and the other to determine free or unesterified acids. Presumably the difference measures chiefly esters of polysaccharides but would include soluble esters such as chlorogenic acid.

Since it is well-known that caffeic acid is extremely labile to oxidation in alkaline solutions such as used to hydrolyze ester-bound phenolic acids, the method of Schmidtlein and Herrmann (1975) was employed to determine total phenolic acids. This method uses NaOH containing $NaBH_4$, which serves as a reducing agent. As will be shown in the results (Figure 3), $NaBH_4$ affords some protection of standard caffeic acid solutions but does not fully protect caffeic acid against destruction during hydrolysis of natural product. For routine analyses, 0.40 g of dry lyophilized plant material was refluxed for 10 min with 40 mL of deoxygenated (N_2) 1% NaOH containing 0.5% $NaBH_4$. The solution was cooled in an ice bath, adjusted to pH 2.5 with concentrated HCl, and centrifuged at 1850*g* for 20 min. An aliquot, usually 20 mL, was extracted with EtOAc and silylated as described above.

To determine free phenolic acids, 0.40 g of dry plant material was first extracted twice with 40 mL of chloroform to remove lipids. This was followed by refluxing with 40 mL of 80% methanol for 30 min. The methanol-soluble fraction was dehydrated and silylated as described above.

RESULTS AND DISCUSSION

Methodology. The separation of BSTFA derivatives achieved by this system is illustrated by Figure 1. The retention time for the *p*-hydroxybenzoic acid derivative was less than 15 min, and for caffeic acid, 25 min. Quinic, *p*-coumaric, and ferulic acids appeared between 20 and 25 min, and chlorogenic acid appeared at approximately 40 min. The peak heights of *p*-coumaric and ferulic acids were nearly the same and slightly less than that of *p*-hydroxybenzoic acid. Ferulic acid was somewhat less sensitive followed by chlorogenic acid. The quantitative relationships of the peak height ratios to concentration are shown in Figure 2.

Caffeic acid is a highly unstable compound; aqueous solutions undergo rapid oxidative degradation standing in air. Chromatograms (not shown here) of solutions allowed to stand at room temperature for 24 h revealed three or more additional peaks and a loss of 50% of more of the original peak height. Standing in 1% NaOH for 24 h led to total loss of the caffeic acid peak.

In preliminary trials to test stability of the acids, the $NaBH_4$ concentration was varied from 0.1 to 0.7% in 1.0% NaOH, and the solutions were heated to boiling for 10 min. As shown in Figure 3 sodium borohydride partially pro-

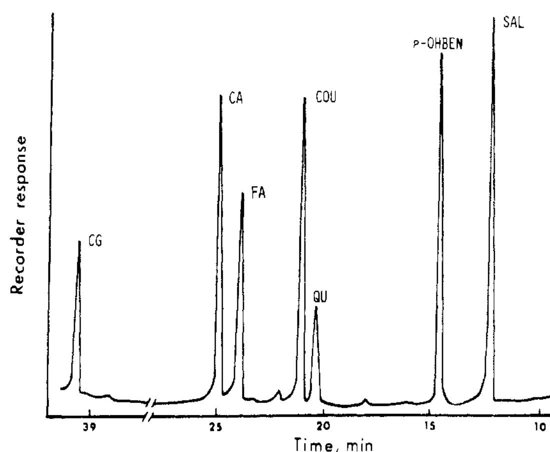

Figure 1. Gas-liquid chromatograph of BSTFA derivatives of phenolic acids. Normally salicylic (SAL) or *p*-hydroxybenzoic acid (*p*-OHBEN) served as internal standards. Key: QU, quinic acid; COU, *p*-coumaric acid; FA, ferulic acid; CA, caffeic acid, CG, chlorogenic acid. A $^{1}/_{10}$-mg portion of each acid was silylated with 0.2 mL of BSTFA, and 5 μL was injected into the chromatograph with the attenuator set at 400.

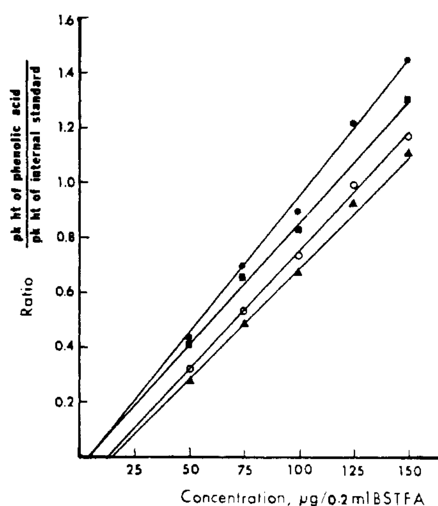

Figure 2. Standard curves for phenolic acids. The peak height of the acid relative to the internal standard (0.1 mg/0.2 mL of BSTFA) plotted vs. the concentration in 0.2 mL of BSTFA: ferulic acid (○); *p*-coumaric acid (■); caffeic acid (●); chlorogenic acid (▲).

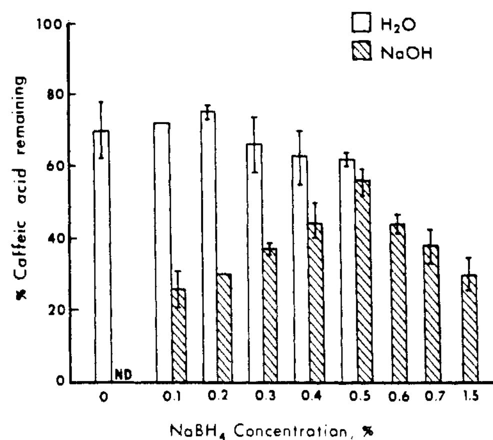

Figure 3. Protective effect of $NaBH_4$ additions to aqueous and 1% NaOH solutions of caffeic acid. A 0.01-mg portion of the acid was dissolved in 30 mL of the respective solutions, deoxygenated, and heated to boiling for 10 min.

tected sodium hydroxide solutions of caffeic acid against degradation with maximum protection occurring at 0.5%. Recovery at this level of $NaBH_4$ was 55–60%. When added to water without NaOH, it afforded 75% recovery, but $NaBH_4$ alone will not release maximal quantities of the acids from plant materials. While $NaBH_4$ is a good reducing agent its degradation in aqueous solution gives rise to an alkaline solution that is counterproductive as regards protection of phenolic acids.

Ferulic and *p*-coumaric acids were quite stable in 1% NaOH with or without $NaBH_4$, recovery being 90% or higher (data not shown).

Analysis of Plant Materials. Four plant products, the whole edible portion and the water-insoluble residues of alfalfa, cabbage, spinach, and wheat bran, were extracted with 1% sodium hydroxide containing 0.5% $NaBH_4$. This treatment was assumed to hydrolyze any ester bonds involving the phenolic acids under study. On the basis of our stability data, the NaOH–$NaBH_4$ extraction procedure would not destroy *p*-coumaric and ferulic acids but would destroy most, if not all, of the caffeic acid. Aqueous methanol extracted free acids with minimal destruction

Table I. Phenolic Acid Content (mg/g Dry Weight) of Plant Products as Determined by Two Extraction Procedures

plant fractn	extrctn method	*p*-coumaric acid	ferulic acid	caffeic acid
		A. Alfalfa		
whole	NaOH–$NaBH_4$[a]	1.0	2.1	ND[d]
whole	80% MeOH[b]	I[c]	0.78	0.68
WIR	NaOH–$NaBH_4$	0.12	0.10	ND
WIR	80% MeOH	0.15	0.05	0.06
		B. Cabbage		
whole	NaOH–$NaBH_4$	0.20	0.22	ND
whole	80% MeOH	I	0.04	0.08
WIR	NaOH–$NaBH_4$	0.10	ND	ND
WIR	80% MeOH	0.10	0.05	0.08
		C. Spinach		
whole	NaOH–$NaBH_4$	0.21	1.13	ND
whole	80% MeOH	0.24	0.53	ND
WIR	NaOH–$NaBH_4$	0.14	0.47	ND
WIR	80% MeOH	0.13	0.43	0.06
		D. Wheat Bran		
whole	NaOH–$NaBH_4$	0.26	4.35	ND
whole	80% MeOH	0.16	0.52	ND
WIR	NaOH–$NaBH_4$	0.14	2.83	ND
WIR	80% MeOH	0.18	0.48	ND

[a] Samples were refluxed 10 min with deoxygenated 1% NaOH containing 0.5% $NaBH_4$. [b] Samples were refluxed 30 min with deoxygenated 80% methanol. [c] Interference. The *p*-coumaric acid peak could not be separated from other major unknown peaks. [d] Not detected.

of caffeic acid. The phenolic acid contents of the plant extracts, determined by these methods and expressed as milligrams/gram of dry product, are presented in Table I. Wheat bran had the highest concentration of total ferulic acid, followed by alfalfa, which in turn had the highest content of *p*-coumaric acid. In general, ferulic acid occurred in higher concentrations than *p*-coumaric acid. The ferulic and *p*-coumaric acid contents of alfalfa appear to be highly variable. Jung et al. (1983) found 0.77 and 0.63 mg/g of the acids, respectively, compared to 2.1 and 1.0 mg/g in this study. Hartley (1983) found none of either acid in alfalfa stems.

Nothing can be said about the total caffeic acid content of the products analyzed because none was detectable after NaOH–$NaBH_4$ extraction. Although the quantity lost during 80% methanol extraction is unknown, there was a detectable amount in the extracts obtained from alfalfa and cabbage, but none from wheat bran. Methanolic extraction would not break ester bonds, and thus it detected

Table II. Estimation of Free (Unesterified) and Total Phenolic Acid (mg/g Dry Weight) in Alfalfa, Cabbage, Spinach, and Wheat Bran

plant product	*p*-coumaric		ferulic		caffeic	
	total[a]	free[b]	total	free	total	free
alfalfa	1.0	*c*	2.1	0.78 (37)[b]	ND[d]	0.68
cabbage	0.20	*c*	0.22	0.04 (18)	ND	0.08
spinach	0.21	0.24 (100)	1.13	0.53 (47)	ND	ND
wheat bran	0.26	0.16 (60)	4.35	0.52 (12)	ND	ND

[a] Total refers to the acid in the 1% NaOH–0.5% $NaBH_4$ extract. [b] Free refers to the acids in the 80% methanol extract (mg/g); percent of total given in parentheses. [c] Could not be separated from other compounds on the column. [d] Not detected so that there is no estimate of total.

only free or loosely bound acids. Alfalfa contained by far the highest concentration of free caffeic acid. On the basis of the relationship between free and total ferulic acid in alfalfa, one might expect the total caffeic acid to be 2.7 times the free levels or 1.8 mg/g. A similar extrapolation for cabbage would indicate a total caffeic acid content of 0.4 mg/g. Aqueous methanol extracted other substances that interfered with the determination of *p*-coumaric acid so that no reliable values were obtained for the level of the free acid in alfalfa and cabbage.

The water-insoluble residues were prepared by repeated (10×) extractions with hot water, a process that might be expected to remove unesterified phenolic acids. Contrary to expectation, methanolic extraction detected approximately the same concentration of *p*-coumaric and ferulic acids in the WIRs as did NaOH–$NaBH_4$ extraction, suggesting that they were not covalently bound. The exception was ferulic acid in the wheat bran residue, in which less than 20% was methanol soluble. There was a minute quantity of caffeic acid in the methanolic extracts of the alfalfa, cabbage, and spinach WIRs. In general, the concentrations of total cinnamic acid derivatives in the residues were appreciably less than the total in the whole plant products. This is particularly notable in the case of alfalfa in which the *p*-coumaric and ferulic acid concentrations ranged from 8- to 20-fold higher in the whole product than in the WIR. No phenolic acids were found in WIRs that had been treated overnight with 1 N NaOH at room temperature (data not shown). The data suggest that most of the esterified acids were associated with water-soluble components of the leafy plants.

It is worthy of note that no quinic or chlorogenic acid was detected in alfalfa or wheat bran. Quinic acid was found in cabbage WIR (0.21 mg/g) and spinach WIR (0.25 mg/g), and a trace of chlorogenic acid was detected in the aqueous methanol extract of whole spinach.

On the basis of the assumption that NaOH-$NaBH_4$ extraction measures the total concentration of a phenolic acid and 80% methanol extraction measures the unesterified portion, the distribution of *p*-coumaric and ferulic acid in the products is summarized in Table II. Such a calculation is not possible for caffeic acid because none was found in the NaOH–$NaBH_4$ extract. Obviously all of it was destroyed by the alkaline solution because some of the unesterified form was found in the methanolic extract. The percentage lost in the latter is not known. Whether there was no esterified caffeic acid or that it was totally destroyed is also unknown. The proportion of free ferulic acid ranged from 12 to 47%, and the percentages of free *p*-coumaric acid in wheat bran and spinach were 60 and 100%, respectively.

Unfortunately the methodology used here did not permit evaluation of the total and thus the esterified caffeic acid in the products analyzed. Nevertheless the two most reliable and consistently active sources of the guinea pig growth stimulant, alfalfa and cabbage, contained the highest concentrations of the unbound form of caffeic acid. Wheat bran, which contained none, was inactive in the guinea pig growth assay (Knehans et al., 1979). The WIR of alfalfa, which is a source of growth activity, contained a trace of free caffeic acid, but not after it was treated with NaOH solution. Since NaOH treatment causes loss of biological activity (Knehans et al., 1979; Johanning et al., 1984), caffeic acid is a reasonable candidate for the growth stimulant. Alkaline hydrolysis of plant products does not destroy *p*-coumaric and ferulic acids readily, but it almost totally destroys caffeic acid. Another technique for hydrolysis without oxidation is needed to measure esterified caffeic acid. Clearly the addition of $NaBH_4$ to the basic hydrolysis medium does not fully protect caffeic acid from oxidative destruction.

Registry No. Caffeic acid, 331-39-5; *p*-coumaric acid, 7400-08-0; ferulic acid, 1135-24-6.

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