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## Binding of Dietary Anions to Vegetable Fiber

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Binding of chenodeoxycholate and decanoate to alcohol-insoluble residue (AIR) of carrot, cabbage, broccoli, and onion was demonstrated. Binding of chenodeoxycholate to freeze-dried calcium pectate gel under conditions used for vegetable AIR was observed and supports the idea that binding of bile acids and fatty acids to vegetable fiber occurs through salt linkages to calcium pectate of the plant cell wall. Such binding may possibly be beneficial to human health by lowering blood cholesterol levels and by reducing the risk of colon cancer.

Vegetable fiber in the human diet has been implicated in lowering serum levels of cholesterol (Selvendran, 1978, 1985; Jenkins et al., 1979; Robertson et al., 1980; Chen et al., 1981; Judd and Truswell, 1982; Nakamura et al., 1982). Several studies have shown that vegetable fiber can bind bile acids and thereby prevent their reabsorption in the small intestine (Birkner and Kern, 1974; Kern et al., 1978; Selvendran, 1978; Robertson et al., 1980; Hoagland and Pfeffer, 1986, 1987). To replace lost bile acids, the human body draws upon its cholesterol reserves in the low-density lipids of blood serum. Selvendran (1978) obtained evidence that pectin in the cell wall material of parenchymatous tissue of mature runner bean pods was involved in binding of cholate. Further investigations have suggested that binding of bile acids to carrot fiber largely occurs through calcium salt linkages to the calcium pectate of residual cell wall (Hoagland and Pfeffer, 1986, 1987). Since calcium pectate is widely distributed in plants (Jarvis, 1982), the binding of bile acids to alcohol-insoluble residue (AIR) of several vegetables was determined. In addition, since calcium pectate is implicated in binding of bile acids, binding of other dietary anions that have a great affinity for calcium ions (fatty acids and phytate) may also be possible. This investigation was undertaken to extend the range of interactions of dietary fiber with anionic components of the human diet and to detail some of the chemistry that underlies the nutritional benefits of dietary fiber. The interaction between vegetable fiber and oxalate

was also investigated to clarify the involvement of calcium pectate in binding.

### EXPERIMENTAL SECTION

**Materials.** Chenodeoxycholic acid and sodium phytate were obtained from Sigma Chemical Co. Redistilled decanoic acid was a gift from Raymond Bistline. Citrus pectin was obtained from U.S. Biochemical Corp. Oxalic acid and KCl were Baker analyzed reagent grade. 4-(2-Hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) was obtained from Aldrich Chemical Co. Acetonitrile was the HPLC-grade product of Burdick & Jackson. Other chemicals were reagent grade.

**Preparation of Vegetable Alcohol-Insoluble Residue (AIR).** Washed vegetables, purchased from a local supermarket, were cut into small pieces and minced in a blender with water (300 mL/200 g of vegetable). The mixture was frozen for at least 24 h to disrupt the plant cells and then thawed. Water-soluble material was removed by vacuum filtration of several washings with water on a medium sintered-glass filter. The wet residue was then extracted with absolute ethanol (1 L/200 g of original vegetable) by refluxing for 5 h. The material was filtered and washed several times with water. The AIR was rapidly washed with dilute (10:1) water-ammonium hydroxide and water to neutralize the fiber with negligible deesterification. A slurry of the AIR was then freeze-dried to give yields ca. 3% of the starting weight of material.

**Reversed-Phase HPLC.** A Du Pont Zorbex ODS, 4.6-mm i.d.  $\times$  15-cm length column was used with a mobile phase of pH 7.2, 0.02 M phosphate-acetonitrile (67:35 (v/v)) as previously described (Hoagland and Pfeffer, 1987; Parris, 1977). Peak detection was recorded with an Apple IIe computer running CHROMATOCHART software by Interactive Microware.

**Gel Permeation Chromatography.** Chromatography was performed with a Synchron Synchronapak GPC 100, 4.6-mm i.d.  $\times$  25-cm length column and a mobile phase of pH 7.20, 0.01 M

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**Table I. Binding of Chenodeoxycholate to Vegetable AIR**

source of AIR	method	mg/mL	extract pH	binding/100 mg of AIR	
				mg	$\mu\text{mol}$
broccoli	GP <sup>a</sup>	1	6.78	0.96 $\pm$ 0.09	2.45
cabbage	GP	1	6.23	0.93 $\pm$ 0.01	2.37
carrot	GP	2	6.56	1.53 $\pm$ 0.03	3.90
cabbage	GP	2	5.95	1.56 $\pm$ 0.01	3.98
broccoli	GP	2	6.57	1.98 $\pm$ 0.01	5.05
carrot	RP <sup>b</sup>	2	7.25	1.39 $\pm$ 0.05	3.55
onion	RP	2	7.25	0.59 $\pm$ 0.08	1.51
cabbage	RP	2	7.26	1.54 $\pm$ 0.01	3.93
calcium pectate gel	GP	2	7.55	1.83 $\pm$ 0.17	4.67

<sup>a</sup>0.01 M phosphate, pH 8.08; 30-min contact time at room temperature. <sup>b</sup>0.05 M imidazole, pH 7.50; 30-min contact time at room temperature.

phosphate buffer flowing at 1 mL/min at room temperature. A Du Pont 870 pump and Series 8800 gradient controller in isocratic mode and a Waters R401 differential refractometer were used along with the data acquisition system described above.

**Measurement of Binding.** Freeze-dried AIR was exposed to a test solution of bile acid or fatty acid (1 or 2 mg/mL per 100 mg of AIR) at room temperature for 30 min (Hoagland and Pfeffer, 1987). The solution was expressed from the fiber by filtration through a 0.22- $\mu\text{m}$  Millipore filter. The concentration of bile acid or fatty acid in the solution before and after contact with the fiber was determined by HPLC. Binding was calculated from the measured concentration difference as previously described (Hoagland and Pfeffer, 1987).

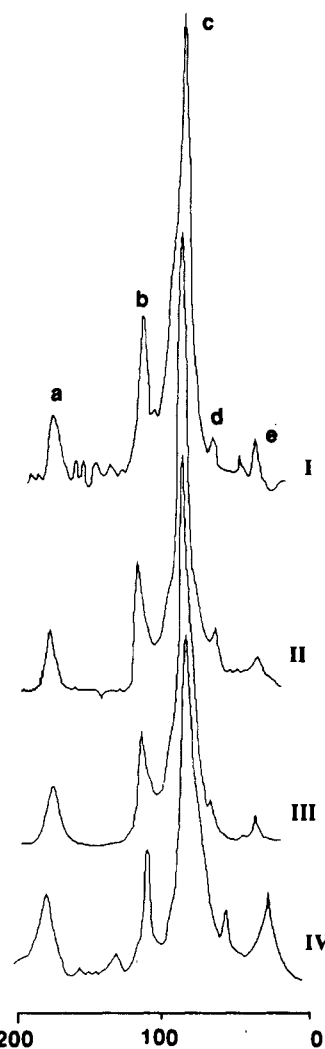
**Solid-State CPMAS <sup>13</sup>C NMR Spectroscopy.** CPMAS <sup>13</sup>C NMR spectra were acquired with a JEOL FX60QS NMR spectrometer operating at 15.04 MHz. The parameters had the following values: <sup>1</sup>H decoupling rf irradiation field strength, 11 G; contact time, 0.5 s; recycle time, 1.5 s; spectral width, 8000 Hz; sampling rate, 2K data points, filled to 4K; spinning, 2.1 kHz; standard, methyl carbons of hexamethylbenzene, 17.36 ppm. No spinning sidebands were observed.

**Calcium Ion Titration.** Calcium ion concentration was measured with a Radiometer F2002 calcium ion selectrode and PHM84 research pH meter. Titrations were performed with a Radiometer TTT80 titrator and ABU80 autoburet with a 10-mL syringe. An enclosed 150-mL titration cell was maintained at constant temperature (25 °C) with a Forma Scientific Model 70 bath and circulator and bathed in a slow stream of nitrogen to prevent absorption of carbon dioxide. The procedure of Graf (1983) was adopted for calibrating the electrode and for titrations. The calcium-specific electrode was calibrated with standard CaCl<sub>2</sub> solutions (Radiometer). Three solutions containing the possible combinations of 10 mg of sodium phytate and 100 mg of pectin were prepared with 100 mL of 0.10 M KCl, 0.025 M HEPES, pH 7.25 buffer. Each solution and buffer alone was titrated with 0.20 M CaCl<sub>2</sub>. Portions (50 mL) of the pectin-phytate solution were withdrawn during the titration and analyzed by GPC.

## RESULTS

**Vegetable AIR.** The AIR prepared from onions, cabbage, and broccoli gave similar <sup>13</sup>C solid-state CPMAS NMR spectra (Figure 1) dominated by peaks associated with pectin. The broad carbonyl peak at 180 ppm includes resonances for carboxylic and ester carbonyls. Some of the ester carbonyl peak reflects the presence of acetyl groups, since an acetyl methyl group is indicated by the peak at 22.1 ppm. Methyl esterification of some galacturonyl groups is indicated by the methyl carbon peak at 55 ppm.

**Chenodeoxycholate Binding.** The results listed in Table I show that the AIR of each vegetable tested bound chenodeoxycholate under conditions that have been applied to carrot AIR (Robertson et al., 1980; Hoagland and Pfeffer, 1986). Onion AIR had the smallest capacity to bind, which may be related to a low content of pectin (Redgwell and Selvendran, 1986). Broccoli AIR was the most effective material tested to date. Freeze-dried calcium pectate bound chenodeoxycholate to an extent comparable to vegetable AIR (Table I).



**Figure 1.** Solid-state CPMAS <sup>13</sup>C NMR spectra of vegetable AIR: (I) carrot; (II) cabbage; (III) onion; (IV) broccoli. Carbon peaks: (a) carbonyl; (b) anomeric; (c) methylene (-CHOH-); (d) methoxy; (e) methyl of acetyl.

**Table II. Binding of Decanoate to Vegetable AIR**

source of AIR	mg/mL <sup>a</sup>	extract pH	binding/100 mg of AIR	
			mg	$\mu\text{mol}$
carrot	1	6.29	0.66 $\pm$ 0.02	2.89
carrot	2	5.60	1.83 $\pm$ 0.01	8.03
cabbage	2	5.99	1.60 $\pm$ 0.03	7.02
broccoli	2	6.86	1.82 $\pm$ 0.01	7.98

<sup>a</sup>0.01 M phosphate, pH 8.53, gel permeation.

**Decanoate Binding.** The data in Table II indicate that vegetable AIR can bind decanoate at physiological pH

Table III. Binding of Oxalate to Vegetable AIR

source of AIR	mg/mL <sup>a</sup>	extract pH	binding/100 mg of AIR	
			mg	μmol
carrot	1	6.25	0.21 ± 0.01	2.33
cabbage	2	5.58	1.02 ± 0.02	11.33
broccoli	2	6.72	0.30 ± 0.04	3.33

<sup>a</sup>0.01 M phosphate, pH 8.53, gel permeation.

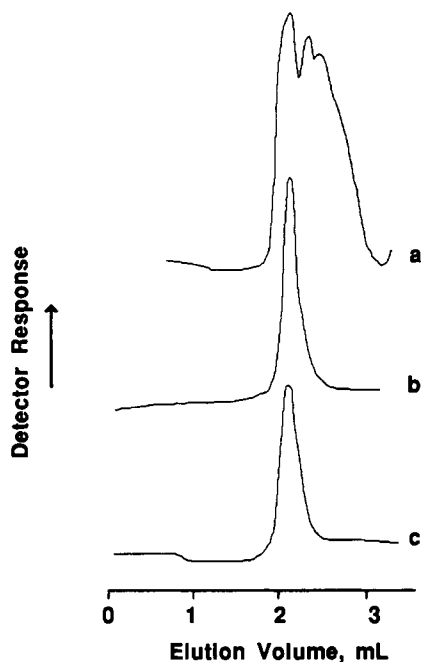


Figure 2. Gel permeation chromatograms of pectins extracted from carrot AIR during measurement of binding: (a) oxalate; (b) decanoate; (c) phosphate buffer control.

(Table I). In comparison with the moles of bound bile acid in Table I, the binding of decanoate was more extensive.

**Oxalate Binding.** The data in Table III suggest that vegetable AIR did bind oxalate. However, the gel permeation chromatograms in Figure 2 show that some degradation of the vegetable AIR occurred. Cabbage AIR removed an unusual amount of oxalate from solution (Table III).

**Phytate Binding.** Sodium phytate was titrated with  $\text{CaCl}_2$  by itself and in the presence of a 10-fold weight excess of citrus pectin. In the absence of pectin, precipitation of insoluble species of  $(\text{Ca})_n\text{phytate}$ , where  $n > 2$  (Graf, 1983), was observed at 0.45 mM  $\text{Ca}^{2+}$ . When pectin was present, only a haze was detected. Samples of the titrated solution containing pectin were chromatographed, and two chromatograms in Figure 3 suggest that some kind of interaction between phytate and pectin occurred. The peak area of the excluded material (pectin) increases slightly while the peak area for phytate decreases. The solution applied to the column at 1.29 mM  $\text{Ca}^{2+}$  was hazy, without discernible particles of precipitated calcium phytate.

#### DISCUSSION

**Calcium Pectate in Vegetable AIR.** The spectra in Figure 1 emphasize the similarity of the material prepared from a variety of vegetables and reflect the wide distribution of pectin in plant cell walls. Pectin has been isolated and characterized from carrots (Aspinall et al., 1983; Stevens and Selvendran, 1984a), onions (Mankarios et al., 1980; Redgwell and Selvendran, 1986), and cabbage (Stevens and Selvendran, 1984a). In each investigation a calcium chelator was employed to extract a major fraction

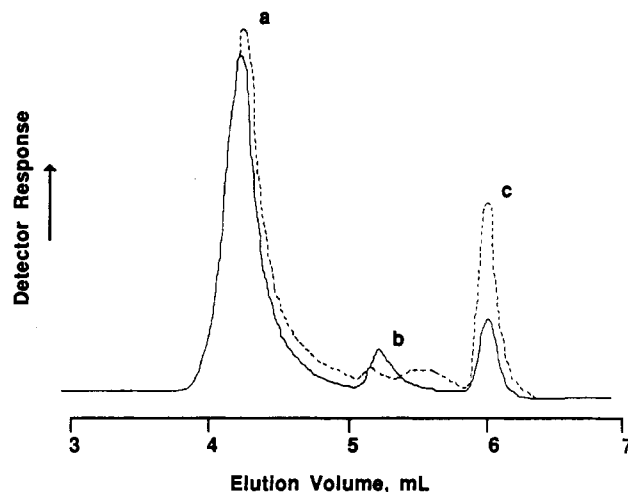


Figure 3. Gel permeation chromatograms of pectin (a), phytate (b), and calcium chloride (c) at  $\text{Ca}^{2+}$  concentrations of 0.24 mM (—) and 1.29 mM (---).

of pectin thought to be held in the middle lamella and within the primary cell wall by calcium junction zones (DeMarty et al., 1984; Rees, 1982). Mild alkali must be employed to remove from the cell wall residual pectin that may be linked to ferulic acid, hemicellulose, cellulose, or protein (such as extensin) by ester linkage (Jarvis, 1982; Redgwell and Selvendran, 1986).

**Binding of Bile Acids to Vegetable AIR.** Supporting evidence for the idea that bile acids bind to calcium pectate in vegetable AIRs was obtained by submitting a freeze-dried calcium pectate gel to the binding assay. The binding of chenodeoxycholate to this material, as indicated in Table I, is comparable to that for the vegetable AIRs. These results add support to previously reported experimental evidence that points to binding of bile acids to calcium pectate in carrot AIR (Hoagland and Pfeffer, 1986).

Interactions between calcium salts and carboxylate-bearing substances occur in other systems (Nancollas, 1983). Calcium salt linkages are reported to occur between poly(methyl methacrylate) and tricalcium silicate (Sugama et al., 1979). Insoluble calcium phosphate has been shown to bind bile salts near neutral pH (Van der Meer and De Vries, 1985). The dihydroxy bile salts are bound more tightly than glycocholic acid. Binding occurs at free calcium ion concentrations (1 mM) that are much lower than required for precipitation of calcium bile salt (12 mM). The binding in this case likely involves interactions between the carboxylate group of the bile salt and a calcium ion of the calcium phosphate.

Selvendran (1978) found that depectinated cell wall material (CWM) from the parenchyma of mature runner beans had a reduced ability to bind cholate (0.49% w/w) compared to 1.35% for whole CWM. Binding of cholate to CWM increased at lower pH, and Selvendran pointed out that increased ionization of pectin and cholic acid would favor their electrostatic repulsion. Calcium ions reduce electrostatic repulsion and allow binding to occur at pH values near 7 and above. Birkner and Kern (1974) observed appreciable binding of chenodeoxycholate to AIR of celery (3.1%), corn (2.2%), lettuce (1.5%), potato (2.0%), and string beans (5.5%) at pH 8.0, whereas apple or kidney bean AIR exhibited little binding capacity. Story et al. (1982), following earlier studies (Kritchewsky and Story, 1974), demonstrated that wheat bran and alfalfa could bind deoxycholate in phosphate buffer at pH 8.0. In the case of wheat bran, 50% ethanol-extracted material

showed the greatest capacity for binding deoxycholate (3.43%). This capacity was reduced to 0.76% upon treatment with sodium chlorite to extract lignin. Alfalfa was more extensively investigated and exhibited greater binding for deoxycholate (2.95%) than for cholate (1.22%) under comparable conditions (Story et al., 1982). Treatment of alfalfa with either ammonium oxalate or EDTA to remove any calcium pectate had little effect on bile acid binding capacity, whereas delignification with sodium chlorite markedly reduced binding capacity. Binding of bile acids to alfalfa and wheat bran appears to involve hydrophobic interactions with lignin, and calcium pectate does not appear to have a major role in binding of bile acids to these materials.

**Cholesterol Metabolism.** In humans much of the newly synthesized cholesterol occurs in the ileum and is exported in the high-density lipoproteins (HLP). Reduction of bile acid resorption in the ileum stimulates the synthesis of cholesterol and its release into the bloodstream in the HPL fraction. Concurrently, the liver receives a message to step up synthesis of bile acids and responds by increasing its number of receptor sites for low-density lipoproteins (LDL) in order to gain access to the cholesterol circulating in this fraction of the blood. About two-thirds of newly synthesized bile acids comes from circulating serum cholesterol, and the rest derives from cholesterol stored, synthesized *de novo*, or ingested. The net effect, then, is a lowering of total serum cholesterol and an increase in the ratio of HDL to LDL. These are conditions strongly associated with reduced risk of heart disease. Serum cholesterol levels are subject to several feedback controls that have extended response times and exhibit large individual variability (Bergström, 1961; Packard and Shepherd, 1982).

Carrots (200 g) added to the diet of five human subjects resulted in a significant lowering of blood cholesterol levels (Robertson et al., 1980). In addition, fecal content of both bile acid and calcium increased and alcohol-insoluble residue prepared from these carrots was shown to absorb bile acids (Robertson et al., 1980). The observed average increase in total fecal bile acid excreted was 0.13–0.27 g. Human subjects consuming 15 g/day of citrus pectin in a gel exhibited an average increase in total fecal bile acids of 0.17 g (Kay and Truswell, 1977). This amount of pectin is over twice the pectin content of 200 g of carrots. Given a fiber content of 3% for carrots and a total binding capacity of 4 g/100 g of AIR (Hoagland and Pfeffer, 1986), an increase in total fecal bile acids of 0.24 g could be expected if the binding process was near 100% efficient. In addition, based on an AIR calcium content of 1%, an increase in fecal calcium of 60 mg would be expected from 200 g of carrots (Robertson et al., 1980). The observed increases were from 60 to 84 mg of Ca/day. It would appear that calcium pectate in the cell wall could account for the observed effects in the carrot study and is significantly more effective in increasing fecal bile acid excretion than pectin fed directly.

Interpretation of pectin feeding studies is made difficult by variation in the degree of methylation, the source of pectin, its state of degradation, and the form in which it is fed. Reddy et al. (1980) found that rats fed a diet supplemented with citrus pectin had higher fecal levels of dihydroxy bile acids than controls. Carrageenan, on the other hand, resulted in higher fecal levels of all bile acids (Reddy et al., 1980). Perhaps in this case Carrageenan blocks resorption sites for bile acids. The hypercholesterolemic effect of pectin fed to rats was increased by calcium added to the diet (Bock and Ranhotra, 1984).

Even though free calcium levels in living systems are maintained at low levels by many control mechanisms, in feeding studies with pectin some of the hypercholesterolemic effects may be attributed to calcium pectate that is expected to form whenever pectin is exposed to ionic calcium. Free calcium in the gut ranges from 0.3 to 5 mM (Carey et al., 1983). Calcium pectate in solution can bind lipid micelles, and the resulting complexes are retained on a 3- $\mu$ m Millipore membrane filter (Falk and Nagyvary, 1982). Calcium salt linkages between pectin carboxylate groups and fatty acid carboxylates may hold this complex together.

Pectin may also reduce serum cholesterol levels by releasing in the colon fermentation products that signal the liver to reduce the production of LDL. Fermentation of pectin itself is about 90% complete, while in the case of the carrot study, judging by the increase in fecal calcium content, calcium pectate in the plant cell wall may not be as available for fermentation, even though breath hydrogen increased and remained at an elevated level after carrot supplementation ceased (Robertson et al., 1980).

**Decanoate Binding.** Free fatty acids in the human colon may promote colon cancer (Newmark et al., 1983). If vegetable fiber can bind fatty acids, then the risk of colon cancer may be reduced. The results in Table II demonstrate that vegetable AIR did bind decanoate under physiological conditions. Since fatty acids have a strong affinity for calcium ions, decanoate very likely binds to vegetable AIR through salt linkages with the calcium pectate in the residual cell wall. Another plausible benefit that vegetable fiber may offer to human health would arise from bacterial fermentation of vegetable fiber in the colon. The release of calcium ions in the colon would lower the local levels of long-chain fatty acids by formation of insoluble fatty acid calcium salts. In addition, short-chain fatty acids produced by fermentation of pectin in vegetable fiber would lower the pH of the colon and reduce the solubility of long-chain fatty acids.

**Oxalate Binding.** The data in Table III establish an interaction between oxalate and vegetable AIR. However, in this case calcium oxalate may be precipitated within the AIR and only appear to be binding. This is suggested by the gel permeation chromatogram in Figure 2. When carrot fiber is treated with buffer alone, material is extracted that elutes at the void volume. This material has been shown by solid-state  $^{13}\text{C}$  NMR spectroscopy to be highly methylated pectin and represents no more than 1% of the AIR (Hoagland and Pfeffer, 1987). This pectin fraction may become available for extraction as a result of freeze-drying the AIR. When the carrot AIR is exposed to the same buffer with oxalate present, additional excluded material is extracted as shown by the chromatogram in Figure 2. Furthermore, when the carrot fiber is exposed to the same buffer with decanoate, the peak at the void volume is essentially the same as that observed for buffer alone. The binding of bile acids or decanoate does not result in the release of any more excluded material than that released by buffer alone.

**Phytate Binding.** The chromatographic results in Figure 3 suggest that phytate may interact strongly enough with pectin in the presence of calcium ions to elute with the excluded species in the solution. The retardation of crystallization of insoluble species of calcium phytate by pectin may not be unique. The crystallization of calcium carbonate in animal species has been reported to be controlled by anionic polysaccharides not unlike pectin (Borman et al., 1982; Fichtinger-Schepman et al., 1981). However, when carrot AIR was exposed to a 2 mg of

phytate/mL of solution near neutral pH, no indication of binding was observed. Indeed, the concentration of phytate in solution after exposure to freeze-dried carrot AIR was slightly elevated, suggesting that the material was taking up the solution with some exclusion of phytate. The large negative charge of phytate near pH 7 and its molecular size may block its entry into the residual cell wall network of cellulose, hemicellulose, and calcium pectate. Phytate has been considered to be marginally nutritionally detrimental in cases of low zinc intake (Maga, 1982; Sharma, 1986). Calcium pectate of vegetable fiber would appear to have little effect in sparing any potential adverse interaction between dietary phytate and zinc.

**Summary.** Evidence has been presented that adds to earlier results that firmly suggest that the calcium pectate in vegetable fiber has at least two active roles in human nutrition. The binding of bile acids through calcium salt linkages to calcium pectate in the ileum acts to lower blood cholesterol levels. The binding of free fatty acids to vegetable fiber in the colon may reduce the risk of colon cancer. Vegetable fiber appears not to interact with phytate but to interact with oxalate to release material presumed to be pectin. The evidence presented argues for the continued recommendation of moderate levels of vegetables in the human diet.

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**Registry No.** Calcium pectate, 12672-40-1; chenodeoxycholic acid, 474-25-9; decanoic acid, 334-48-5; oxalic acid, 144-62-7.

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