

## Cobinding of Bile Acids to Carrot Fiber

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Cobinding of bile acids to carrot cell wall residue, an experimental model for fiber, was demonstrated by reversed-phase high-performance liquid chromatography. Binding ranged from 1 to 2 g of dihydroxy bile acid/100 g of cell wall residue from pH 8.0 to 6.0. The proton relaxation time of bound chenodeoxycholate, determined by solid-state CPMAS  $^{13}\text{C}$  NMR spectroscopy, was similar to that for cell wall residue and indicates that binding is very tight. The binding order found was chenodeoxycholate > deoxycholate >> cholate. Protons are released during binding, and the extent of binding appears to be related to the calcium content of the cell wall residue. We propose a mechanism for binding that involves  $\text{Ca}^{2+}$  salt linkages between pectin in the cell wall residue and a bile acid.

Vegetable fiber in the diet can produce lowering of blood cholesterol levels (Kern et al., 1978; Jenkins et al., 1979). In vitro investigations have shown that certain vegetable fibers can absorb bile salt/acids under physiological conditions (Kern et al., 1978; Selvendran, 1978; Robertson et al., 1980). In vivo binding of bile salts/acids to dietary fiber blocks reabsorption. The body must draw upon its pool of cholesterol for synthesis of any bile acids lost through binding to dietary fiber. This process is presently thought to account for the hypercholesterolemic effect observed for vegetable dietary fiber. Birkner and Kern (1974) found that vegetable fiber exhibits binding for dihydroxy bile acids that is greater than that for the trihydroxy bile acid cholic acid and that binding is inversely related to pH. Material that is like dietary fiber from carrot can be prepared as an alcohol-insoluble residue (AIR) of walls of ruptured cells. Since carrot AIR has appreciable capacity to bind bile acids (Robertson, et al., 1980) and since its composition has been reported (Aspinall et al., 1983), we selected this material to investigate the chemical nature of such binding.

### EXPERIMENTAL SECTION

**Preparation of Carrot Alcohol-Insoluble Residue.** Washed, truncated carrots were minced in a blender with water (100 mL; 200 g of carrot). The mixture was gradually frozen. After at least 24 h in the freezer, the material was thawed and washed sequentially with water and ethanol. Solid material was recovered by filtration through a medium sintered-glass filter and then stirred in refluxing ethanol (1 L; 200 g of original carrot) for 4 h. After recovery by filtration, the material was washed with acetone and then stirred in water (1 L; 200 g of original carrot). The pH was adjusted to between 7 and 8 with either dipotassium phosphate or dilute ammonium hydroxide. After the mixture was stirred for 1 h and the pH stabilized, the material was washed with water. A slurry, prepared with water (10 mL; 1 g of residue), was freeze-dried. The

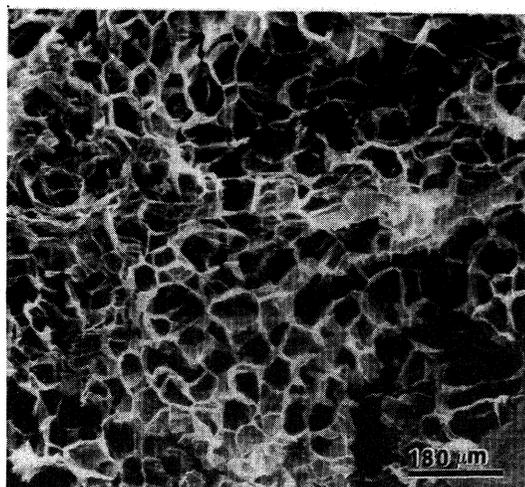
yield of alcohol-insoluble residue (AIR) was typically 3%. The sources of AIR's were as follows: commercial Canadian carrots, AIR I; laboratory greenhouse-grown Nantes Strong Top, AIR II; commercial Massachusetts carrots, AIR III.

**High-Performance Liquid Chromatography (HPLC) of Bile Acids.** 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) to separate the bile acids (Parris, 1977). A 1.0-mL flow rate was maintained with a Du Pont 870 pump and Series 8800 gradient controller in isocratic mode. Peaks were detected with a Waters R401 differential refractometer. Detector response was monitored with a Hewlett-Packard 3390A integrator. Standard bile acid solutions were made up in 0.05 M phosphate buffer, 2 mg each/mL. The final pH was obtained by microliter addition of acetic acid. The concentration of each bile acid was found to be directly related to either peak height or area, with negligible deviation only at low concentration (less than 0.2 mg/mL).

**Measurement of Binding.** The bottom of the barrel of a 10-mL glass syringe was covered with a disk of Whatman No. 2 filter paper cut from a sheet with a cork borer of appropriate diameter. The syringe was fitted with a Swinny adaptor containing a 13-mm, 22- $\mu\text{m}$  Millipore filter. Carrot AIR, 0.25 g, was packed into the barrel and was then completely wetted with 2.5 mL of 0.05 M phosphate buffer containing bile acid(s), 2 mg/mL, at the desired pH. After 30 min of contact, some of the solution was expressed from the fiber by slowly applying pressure to the syringe plunger. The pH of the filtrate, collected in a 3-mL Reacti-Vial, was measured with a small- (6-mm) diameter combination electrode. The concentration of bile acid(s) before and after contact with AIR was then determined from peak heights and/or areas after HPLC. Percent binding (g bile acid/100 g of fiber) was calculated from the decrease in bile acid concentration in the test solution after exposure to the AIR.

**Binding Equation.**  $[1 - (\text{Ht}/\text{Hs})] \times [\text{g BA}/\text{mL solution}] \times [\text{mL solution}/\text{g AIR}] \times 100 = \% \text{ binding (g BA}/100 \text{ g fiber)}$ . Key: Ht = peak height, bile acid test

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**Figure 1.** Scanning electron micrograph of carrot AIR, 300 $\times$ . Material is residue from cell walls.

solution; Hs = peak height, bile acid standard solution (before contact with AIR); BA = bile acid. Peak areas may also be used.

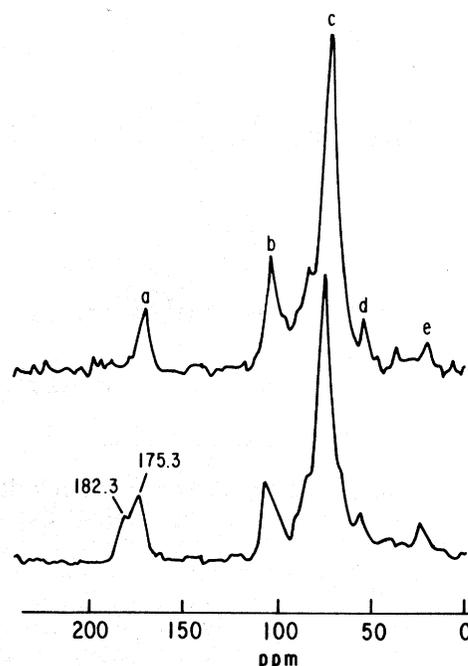
**Solid-State NMR Spectroscopy.** Cross-polarization, magic-angle spinning (CPMAS)  $^{13}\text{C}$  NMR spectra were obtained with a JEOL FX60QS NMR spectrometer operating at a  $^{13}\text{C}$  frequency of 15.04 MHz. The  $^1\text{H}$  decoupling irradiation field strength was 11 G, the contact time was 0.5 s, and the recycle time was 1.5 s. A spectral width of 8000 Hz and a sampling rate of 2K data points, zero filled to 4K, were used throughout. Chemical shifts were assigned relative to the hexamethylbenzene (solid) methyl resonance at 17.36 ppm. Samples were spun at approximately 2.1 kHz. No spinning sidebands were noted.

Peak intensity measurements were used for the calculation of relaxation times  $T_{1\text{H}}$ . Each frequency domain spectrum was obtained with 15–20 Hz of computer line broadening. Values of  $T_{1\text{H}}$  were determined by observation of the  $^{13}\text{C}$  magnetization via cross-polarization in the  $180-t-90^\circ$  pulse sequence (Sullivan and Marciel, 1982).  $T_{1\text{H}}$  values were calculated by a two-parameter experimental fit of the relaxation data. The uncertainty in the calculated values was generally found to be  $\pm 10\%$ .

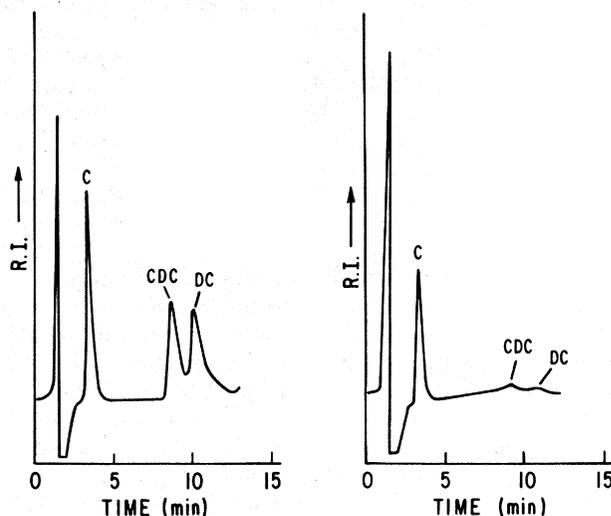
**Calcium Analysis.** AIR was ashed in an electric muffle furnace at 600  $^\circ\text{C}$  for 2 h. The ash after weighing was dissolved in 4 N nitric acid, and the calcium concentration was determined by atomic spectroscopy using standard methods.

## RESULTS AND DISCUSSION

The scanning electron micrograph of carrot AIR in Figure 1 shows that this material is cell wall remnant with much of the honeycomb structure intact. Carrot AIR has been shown by Aspinall et al. (1983) to consist mostly of pectin with small proportions of galactans and arabinogalactans. The CPMAS  $^{13}\text{C}$  NMR spectrogram of carrot AIR in Figure 2 contains resonances expected for material rich in pectin. The carbonyl peak at 175.3 ppm derives from both the ester and carboxylate groups of pectin. The ester content can be estimated by the area under the methoxy carbon peak at 55 ppm. Binding of bile acids to carrot fiber was first reported by Robertson et al. (1980). We have corroborated this finding using an independent procedure that employs HPLC and permits measurement of binding of bile acid mixtures. A solution of bile acids before and after 30-min contact with dry carrot AIR gave the HPLC responses shown in Figure 3. The binding of both deoxycholate and chenodeoxycholate is much greater than for cholate. Binding was also demonstrated by



**Figure 2.** Solid-state, CPMAS  $^{13}\text{C}$  NMR spectra of carrot AIR I before (top) and after  $1/3$  contact with  $^{13}\text{C}$ -24-chenodeoxycholate solution, pH 7.20 at room temperature. Pectin carbonyl peak is at 175.3 ppm, and ionized chenodeoxycholate carbonyl peak appears at 182.3 ppm.



**Figure 3.** Reversed-phase chromatograms of bile acid solution: 2 mg each of cholate (c), chenodeoxycholate (CDC), deoxycholate (DC) per mL of 0.05 M phosphate, pH 7.20 before (left) and after  $1/2$ -h contact at room temperature with carrot AIR I.

**Table I.** Proton Spin Relaxation Times via Carbon Resonances for  $^{13}\text{C}$ -24-Chenodeoxycholate Bound to Carrot AIR I<sup>a</sup>

substance	$T_{1\text{H}}$ , ms
carrot AIR I	83
amorphous unbound chenodeoxycholate	127
chenodeoxycholate bound to AIR I	80

<sup>a</sup> Solid-state 15-MHz CPMAS  $^{13}\text{C}$  NMR spectroscopy.

treating carrot AIR with  $^{13}\text{C}$ -24-chenodeoxycholate under typical binding conditions. The CPMAS  $^{13}\text{C}$  NMR spectrum of carrot AIR with bound labeled chenodeoxycholate in Figure 2 reveals the presence of the bile acid by the downfield shoulder of the carbonyl peak at 182.3 ppm. The proton spin relaxation time for bound chenodeoxycholate, as shown in Table I, is similar to that for carrot

**Table II. Cobinding of Bile Acids to Carrot AIR I<sup>a</sup>**

trial	bile acid	% binding <sup>b</sup>
1	cholate	0.48 ± 0.05
2	deoxycholate	1.84 ± 0.10
3	chenodeoxycholate	1.90 ± 0.06
4	cholate	0.71 ± 0.14
	deoxycholate	1.86 ± 0.05
5	deoxycholate	1.80 ± 0.04
	chenodeoxycholate	1.86 ± 0.06
6	cholate	0.59 ± 0.03
	deoxycholate	1.84 ± 0.05
	chenodeoxycholate	1.92 ± 0.05

<sup>a</sup>2 mg each of bile acid/mL of 0.05 M phosphate, pH 7.20; 1/2-h contact time at room temperature. <sup>b</sup>Milligrams of bile acid/100 mg of AIR.

**Table III. Cobinding of Chenodeoxycholate (CDC) and Deoxycholate (DC) by AIR I from Different Regions of Carrot<sup>a</sup>**

region	% CDC <sup>b</sup>	% DC
skin and xylem	1.81 ± 0.01	1.69 ± 0.01
pith	1.22 ± 0.02	1.01 ± 0.01

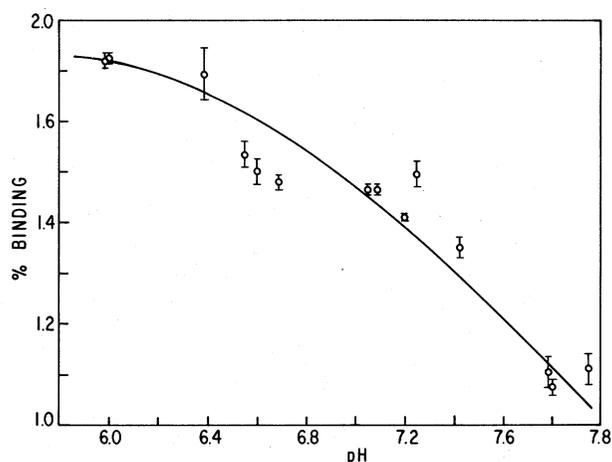
<sup>a</sup>2 mg of cholate, chenodeoxycholate, and deoxycholate/mL of 0.05 M phosphate, pH 7.17; 1/2-h contact time at room temperature. <sup>b</sup>Milligrams of bile acid/100 mg of AIR I.

AIR and is much shorter than the time for amorphous chenodeoxycholate. In the cross-polarization process the observed relaxation times of the carbon resonances are dependent on the directly bonded proton population. In a heterogeneous mixture each component exhibits its own characteristic relaxation time (proton  $T_{1H}$  value): carrot AIR, 83 ms; pure chenodeoxycholate, 127 ms. However, in phase homogeneous complexes rapid spin diffusion between associated components gives rise to common relaxation behavior, as seen in the complex of CDC bound to carrot AIR (Table I). Note that the proton pool of the large AIR polysaccharide dominates the relaxation of the bound chenodeoxycholate molecule. In a control experiment we observed that a physical mixture of chenodeoxycholate and carrot AIR have proton relaxation times characteristic of each individual component. An example of this phenomenon is given in a recent study of wood cellulose in which the phase homogeneous nature of a lignin-carbohydrate complex exhibits the convergence of proton  $T_{1H}$  values (Gerasimowicz et al., 1985).

The HPLC method developed can be used to evaluate cobinding of bile acids to fiber. The results of binding of combinations of cholate, deoxycholate, and chenodeoxycholate to carrot AIR in Table II reveal that (1) cobinding does occur and (2) the cobinding appears to be noncompetitive. The data establish that the dihydroxy bile acids bind more strongly than cholate. In every test that we have performed chenodeoxycholate was bound to a greater extent than was deoxycholate.

We also examined AIR prepared from different parts of carrot. The outer region, comprised largely of skin and xylem, yielded an AIR that cobinds chenodeoxycholate and deoxycholate significantly more than core- or pith-derived AIR (Table III). These two AIR's had similar CPMAS <sup>13</sup>C NMR spectra and proton relaxation times. The reason for this difference is presently unclear, and we can only note at this time that the outer region of the carrot is the area of active growth.

The filtrate obtained from measurement of binding usually had a pH lower than that of the solution used in the assay. In Figure 4 the extent of binding is correlated with the pH of the filtrate. This pH could be manipulated both by adjustment of the test solution pH and by pretreatment of the AIR with either dilute ammonia or acetic



**Figure 4.** Plot of observed binding of chenodeoxycholate to carrot (AIR II) as a function of pH of solution (2 mg of CDC/mL of 0.05 M phosphate, pH 7.0–7.2) recovered after 1/2 h of contact at room temperature. Different pH values were obtained by pretreatment of AIR II with dilute ammonia or acetic acid, followed by washing with water, prior to freeze-drying.

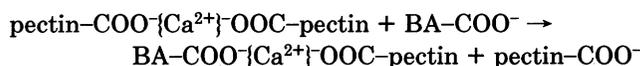
**Table IV. Cobinding of Chenodeoxycholate (CDC) and Deoxycholate (DC) to AIR from Different Batches of Carrots**

fiber	pH	% CDC <sup>a</sup>	% DC	% Ca
AIR III	6.77	1.62 ± 0.08	1.56 ± 0.15	0.92
AIR I	6.69	1.36 ± 0.03	1.04 ± 0.08	0.67
AIR III	7.00	1.17 ± 0.03	1.06 ± 0.05	0.92
AIR I	7.09	0.96 ± 0.04	0.25 ± 0.05	0.67

<sup>a</sup>Milligrams of CDC/100 mg of AIR.

acid (see the Experimental Section). Apparently, binding of bile acids to carrot AIR releases protons and is favored by initially low pH of the test solution and of the treatment of the fiber. These observations suggest that binding of bile acids to carrot AIR is a chemical process and very likely involves carboxylate groups. Selvendran (1978) has reported greater binding of bile acids to fiber at lower pH.

AIR's prepared from different types of carrots have different capacities for cobinding bile acids (Table IV). This is not unexpected, since Robertson et al. (1980) found different water holding (13–32 g/g) and cationic exchange (1.1–2.4 mg/g) capacities for AIR's from different types of carrots, as well as from the same type of carrot at different age. A possible clue to understanding the nature of the observed binding of bile acids was found in the calcium content of the AIR's in Table IV. The cationic exchange capacity of carrot AIR probably derives from the high content of pectin (Aspinall et al., 1983), much of which is calcium pectate, a recognized structural component of plant cell walls (DeMarty et al., 1984). We now propose that binding of bile acids to carrot AIR involves formation of Ca<sup>2+</sup> salt linkages between pectin carboxylate group and the carboxylate group of a bile acid (BA) by the following reaction:



This reaction would produce pectin carboxyl groups, which are stronger acids than bile acids, and would thereby cause a net release of protons. The calcium content of carrot AIR in Table IV is large enough to allow noncompetitive binding of bile acids through salt linkages, provided that the calcium sites are all available. In this case, the binding would depend upon the dissociation constant of the pectin-calcium-bile acid complex.

Carrot AIR serves as a working model for carrot fiber, since it is largely composed of undigestible cell wall material. Several properties of carrot AIR have now been identified that make it an increasingly attractive food additive. The large water holding capacity of carrot AIR is very likely responsible for the reduced fecal transient time reported by Robertson et al. (1979) for human subjects who ingested carrots as a dietary supplement. The cationic exchange capacity of carrot AIR might be used to enhance ingestion of trace minerals.

Blood levels of cholesterol could be expected to be lowered by ingested carrot AIR since this material binds bile acids. In this regard, Bergstrom (1961) has estimated that human beings excrete about 0.8 g of fecal bile acids/day. A 100-g portion of ingested carrot containing about 3% insoluble fiber that can cobind a total of at least 3% of bile acids under physiological conditions could be expected to increase fecal bile acids by about 90 mg or over 10% of the normal level. To maintain the bile acid pool of 3-5 g (Bergstrom, 1961), the conversion of cholesterol to bile acids would have to increase by over 10% its measured rate of 0.7 g/day. Bound bile acids would also be unavailable for absorption of dietary cholesterol. Robertson et al. (1979) reported a significant 11% reduction in serum cholesterol levels of adults who ingested 200 g of raw carrot/day for 3 weeks. They also observed an increase in fecal bile acids, as well as fats.

Carrot AIR could also be a source of dietary calcium that would be released upon breakdown of calcium pectate in the intestinal tract. The release of calcium in the colon could prevent the reported adverse effects of free fatty acids (Newmark et al., 1983). Carrot AIR has potential applications in the food industry as a baking ingredient or a meat extender.

#### ACKNOWLEDGMENT

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**Registry No.** Chenodeoxycholate, 474-25-9; deoxycholate, 83-44-3; cholate, 81-25-4.

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