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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 cheno-
deoxycholate, determined by solid-state CPMAS ^{13}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 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 con-
ditions (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 pre-
pared 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 me-
dium sintered-glass filter and then stirred in refluxing
ethanol (1 L; 200 g of original carrot) for 4 h. After re-
covery 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 di-
potassium 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 Cana-
dian carrots, AIR I; laboratory greenhouse-grown Nantes
Strong Top, AIR II; commercial Massachusettes carrots,
AIR III.

**High-Performance Liquid Chromatography
(HPLC) of Bile Acids.** A Du Pont Zorbex ODS, 4.6-mm
i.d. × 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 de-
viation 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-μm 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 phos-
phate buffer containing bile acid(s), 2 mg/mL, at the de-
sired 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 de-
termined 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. $[1 - (Ht/Hs)] \times$ [g BA/mL solu-
tion] × [mL solution/g AIR] × 100 = % binding (g
BA/100 g fiber). Key: Ht = peak height, bile acid test

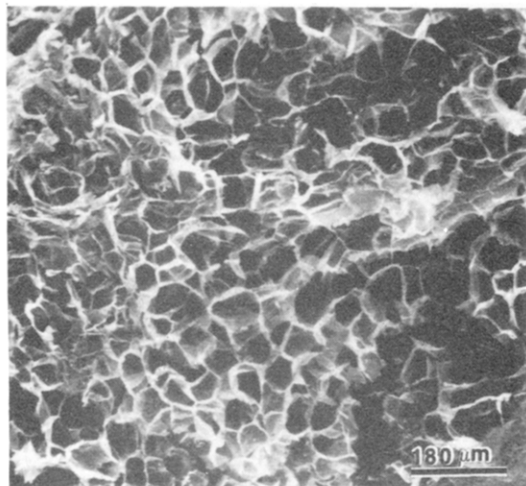Eastern Regional Research Center, USDA—ARS,
Philadelphia, Pennsylvania 19118.

Figure 1. Scanning electron micrograph of carrot AIR, 300×. 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}C NMR spectra were obtained with a JEOL FX60QS NMR spectrometer operating at a ^{13}C frequency of 15.04 MHz. The ^{1}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_{1H}. Each frequency domain spectrum was obtained with 15–20 Hz of computer line broadening. Values of T_{1H} were determined by observation of the ^{13}C magnetization via cross-polarization in the 180–t–90° pulse sequence (Sullivan and Marciel, 1982). T_{1H} values were calculated by a two-parameter experimental fit of the relaxation data. The uncertainty in the calculated values was generally found to be ±10%.

Calcium Analysis. AIR was ashed in an electric muffle furnace at 600 °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}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
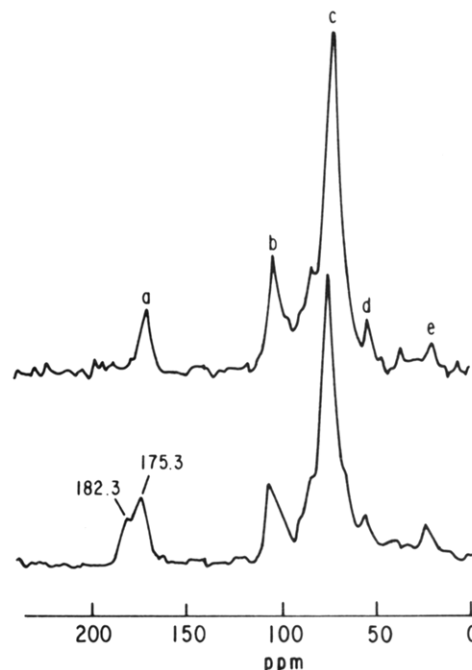

Figure 2. Solid-state, CPMAS ^{13}C NMR spectra of carrot AIR I before (top) and after $^{1}/_{3}$ contact with [^{13}C]-24-chenodeoxycholate solution, pH 7.20 at room temperature. Pectin carbonyl peak is at 175.3 ppm, and ionized chenodeoxycholate carbonyl peak appears at 182.3 ppm.
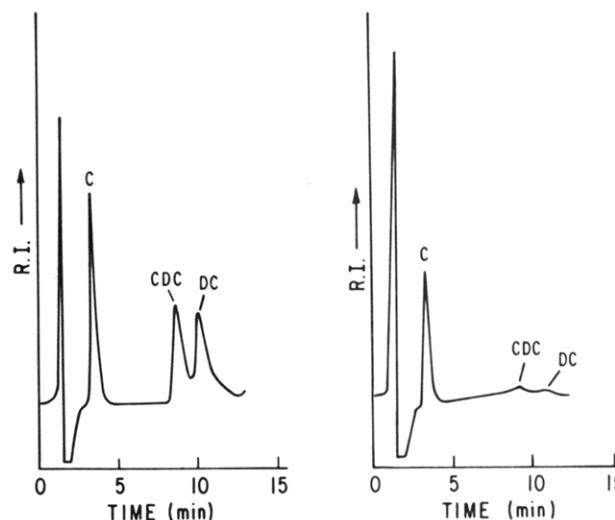

Figure 3. Reversed-phase chromatograms of bile acid solution: 2 mg each of cholate (c), chenodeoxycholate (CDC), deoxycholate (DC) per mL of 0.05 M phosphate, pH 7.20 before (left) and after $^{1}/_{2}$-h contact at room temperature with carrot AIR I.

Table I. Proton Spin Relaxation Times via Carbon Resonances for [^{13}C]-24-Chenodeoxycholate Bound to Carrot AIR I[a]

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

[a] Solid-state 15-MHz CPMAS ^{13}C NMR spectroscopy.

treating carrot AIR with [^{13}C]-24-chenodeoxycholate under typical binding conditions. The CPMAS ^{13}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[a]

trial	bile acid	% binding[b]
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

[a] 2 mg each of bile acid/mL of 0.05 M phosphate, pH 7.20; $^1/_2$-h contact time at room temperature. [b] 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[a]

region	% CDC[b]	% DC
skin and xylem	1.81 ± 0.01	1.69 ± 0.01
pith	1.22 ± 0.02	1.01 ± 0.01

[a] 2 mg of cholate, chenodeoxycholate, and deoxycholate/mL of 0.05 M phosphate, pH 7.17; $^1/_2$-h contact time at room temperature. [b] 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 ^{13}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
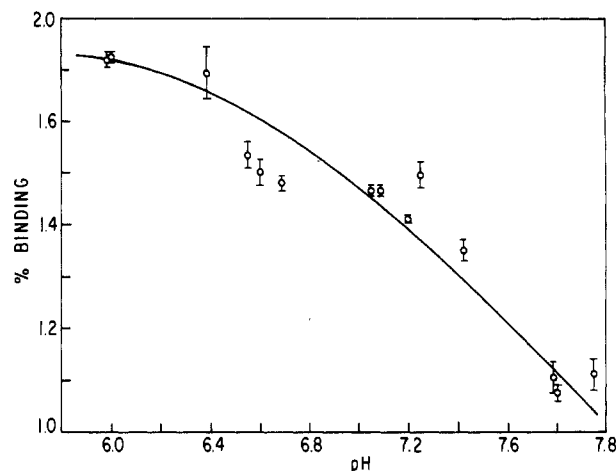

Figure 4. Plot of observed binding of chenodeoxycholate to carrot (AIR II) as a function of pH of solution (2 mg of CDC/mL of 0.05 M phosphate, pH 7.0–7.2) recovered after $^1/_2$ h of contact at room temperature. Different pH values were obtained by pretreatment of AIR II with dilute ammonia or acetic 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[a]	% 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

[a] 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^{2+} salt linkages between pectin carboxylate group and the carboxylate group of a bile acid (BA) by the following reaction:

pectin–COO⁻{Ca²⁺}⁻OOC–pectin + BA–COO⁻ →
BA–COO⁻{Ca²⁺}⁻OOC–pectin + pectin–COO⁻
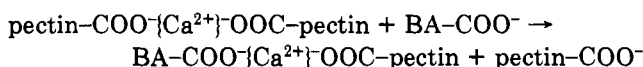
This reaction would produce pectin carboxyl groups, which are stronger acids than bile acids, and would thereby cause a net release of protons. The calcium content of carrot AIR in Table IV is large enough to allow noncompetitive binding of bile acids through salt linkages, provided that the calcium 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.

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Dietary Fiber and Starch Contents of Some Southeast Asian Vegetables

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Dietary fiber and its components, as well as starch, have been determined in those vegetables that are extensively grown and consumed in Southeast Asia, by the Southgate procedure. The richest sources of total dietary fiber among the 24 vegetables examined were *Pithecellobium jiringa* (stinkbean), *Hibiscus esculentus* (lady's finger), and *Pisum sativum* var. macrocarpon (snow pea).

Standard collations of foodstuff assays such as that of Paul and Southgate (1978) include "dietary fiber", but in the case of even this comprehensive work there is no breakdown of fiber components and single figures only are available, giving no idea of the variance associated with the assays. Ross et al. (1985) have presented extensive analyses of fiber components of Western vegetables, based on 4–16 assays/sample. Some Chinese vegetables were

estimated for dietary fiber (residue remaining after starch digestion, with corrections for protein and ash) in Australia by Wills et al. (1984), with single figures only given. Lund and Smoot (1982) presented analyses of cellulose, hemicelluloses, lignin, and cutin in various tropical fruits, but in only two tropical vegetables, the sweet potato and the yam.

More analyses of fiber components in tropical vegetables are required, not the least of which are those in the diets of the very extensive Southeast Asian populations, with the added purpose of complementing the epidemiological studies of cancers, heart disease, and metabolic disorders.

This paper presents the starch and fiber contents of some common Southeast Asian vegetables available in Singapore. As most of the foods are imported from around the region, these results may be taken as representative

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