Molecular Interactions with Dietary Fiber Components. Investigation of the Possible Association of Pectin and Bile Acids

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The interactions of bile acids and their conjugates with pectins of different degrees of methylation in the pH range of 3.5–7.3 were investigated by 360-MHz ^1H NMR spectroscopy. No molecular interactions were observed; i.e., no chemical shift changes or preferential broadening of specific resonances was apparent. The spin–lattice relaxation times (T_1) remained constant for bile salt CH_3 groups 18, 19, and 21 over the concentration range of the pectins studied. However, values for T_2, the spin–spin relaxation times as determined by Lorentzian line widths, doubled, primarily because of the effects of increased viscosity and/or micelle association. ^2H NMR spectroscopy studies conducted under different temperature and pH conditions were used to evaluate the molecular interactions beteen bile salts and pectin labeled with ^2H ($C\text{–}OC^2H_3$). The results indicated that pectin $\text{–}C\text{–}OC^2H_3$ groups undergo rapid isotropic motion that remains unaffected by added bile salts. Studies in which different food grade pectin samples were dialyzed to equilibrium with bile acids/salts in the pH range of 6.0–7.3 revealed binding of from 20 to 100 μg bile of acid/salt to 5 mg of pectin. Purification of the pectin samples was accomplished by centrifugation of the contaminating diatomaceous earth filter aid and by removal of low molecular weight materials by conventional tubing dialysis prior to equilibrium dialysis experiments. After the centrifugation supernatant solutions retained the capacity to bind bile acids, but dialysis of all pectin samples examined resulted in no binding by the retentate.

Numerous feeding and epidemiological studies (Spiller et al., 1978) have shown an inverse correlation between the nonnutritive fiber content of the diet and the levels of circulating cholesterol, blood lipids and lipoproteins in man (Kay and Truswell, 1977; Chen and Anderson, 1979). The studies suggest that as the amount of fiber consumed is increased, the amount of bile acids excreted is increased, which may require that more cholesterol be converted to bile acids. Also, dietary fiber may decrease cholesterol absorption. These effects could explain the observed lowering of circulating cholesterol levels. Eastwood and Hamilton (1968) proposed that lignin-containing noncarbohydrate plant residue was responsibe for removal of bile acids via a "hydrophobic binding" mechanism. From in vitro binding studies, they concluded that the more hydrophobic a bile acid is, the stronger the binding to the plant fiber residues. Story and Kritchevsky (1976) examined the binding of fiber as alfalfa, bran, cellulose, and wood lignin to bile acids and found similar trends; however, the hydrophobicity correlation was not as strongly supported. The conflicting results of these two studies suggest that a fiber component(s) other than lignin may play an important role in the removal of bile acids or in the prevention of their reabsorption.

Pectin and other soluble gel-forming polysaccharides have consistently been shown to effect hypocholesteremic changes in humans (Kay and Truswell, 1977; Miettinen and Tarpila, 1977; Chen and Anderson, 1979; Keys et al., 1961; Jenkins et al., 1979) as well as in animals (Riccardi and Fahrenbach, 1967; Leveille and Sauberlich, 1966; Ershoff and Wells, 1962; Kelley and Tsai, 1978). Unlike cellulose and lignin, pectin is extensively degraded by flora in the large intestine (Holloway et al., 1978). Consequently, any suppressive effect that pectin exerts on the enterohepatic recirculation of bile acids probably occurs within the distal segment of the small intenstine (terminal ileum), where 90–95% of the active bile acid transport takes place (Lack and Weiner, 1971).

Two opposing observations of the effect of pectin on active transport of bile acid and bile acid conjugates have been reported. Leveille and Sauberlich (1966) found that a 0.18% pectin solution inhibited active transport of the conjugate taurocholate in everted rat ileum sacs. Contrary to this, Kiriyama et al. (1974) were unable to duplicate this inhibition of cholate transport, even at pectin concentrations of 0.5%. The latter authors suggest that the activity of pectin may be highly dependent upon the physical characteristics of the preparations, i.e., molecular weight of the polymer, degree of methylation, and inherent viscosity.

Further evidence of pectin binding bile acid activity has been reported by Selvendran (1978). Studies of in vitro bile acid adsorption to well-characterized cell wall material derived from pectin-containing parenchymatous and lignified tissues (parchment layers) of mature runner bean pods were made. The findings indicate that in the absence of lignin, which is found mainly in the parchment tissue, parenchyma still adsorbed almost 10% (w/w) of bile acids. This binding increased with decreasing pH. Subsequent removal of the pectic substances from the parenchyma drastically diminished the binding of this cell wall fiber, suggesting that pectin (in an unionized form) rather than lignin is responsible for the observed bile acid association.

From our understanding of the reported physiological effects of pectin, at least three plausible mechanisms can be advanced to explain the observed inhibition of the enterohepatic recirculation of bile acids. The first is the direct, short-range chemical "binding" of the bile acids to the polymer backbone. The second is the reduction of bile acid diffusion, resulting from restricted mobility due to polymer cross-links (Brown, 1979). The third involves the interaction of pectin with bile acid transport sites within the intestinal mucosa in a manner that decreases the active

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absorption of bile acids. In the present work, we used NMR spectroscopy and equilibrium dialysis to assess the operational significance of the first two mechanisms for increased bile acid excretion from the digestive tract.

EXPERIMENTAL SECTION

Materials. Citrus pectin samples were obtained from Sunkist Growers, Corona, CA (50% OCH_3 content), Bulmers Ltd., Hereford, England (72 and 37% OCH_3 content), and Sigma Chemical Co., St. Louis, Mo (50% OCH_3 content).

Bile acids and conjugates were obtained from the Sigma Chemical Co. and checked for purity by TLC and high-pressure LC.

Methods. *NMR Spectroscopy.* ^1H NMR spectra were recorded at 360 MHz on a Brüker WH 360 pulse Fourier transform spectrometer at 37 °C. ^2H NMR spectra were recorded at 55.26 MHz with the same instrument. Spin–lattice relaxation times (T_1) were measured by the inversion–recovery method $(180°–\tau–90°)_n$ and spin–spin relaxation times (T_2) by the width-at-half-height technique. The latter was accomplished by a computer program, ABCUS, which performs a nonlinear regression by use of the Gauss–Newton iteration to fit digitized resonances which satisfy a Lorentzian line shape. For proton spectra, chemical shifts relative to external Me_4Si were reported. Deuterium resonances relative to external $CDCl_3$ taken to be 7.24 ppm on the Me_4Si scale were reported. Fifty percent methoxypectin obtained from Sunkist Growers was utilized for all ^1H and ^2H NMR studies. All samples were dialyzed and solutions were filtered before spectra were obtained.

Generation of Deuteriodiazomethane. The Deutero-Diazald Prep Set (Aldrich Chemical Co., Inc.) was used for this synthesis. To a flask containing dry ether (20 mL) at 25 °C, deuterioethanol (CH_3CH_3OD, 25 mL) and a 30% solution of sodium deuterioxide in deuterium oxide (20 g) were added; then in the following 30 min, 10 g of diazald (Aldrich Chemical Co., Inc.) in 75 mL of dry ether in a separatory funnel was added with stirring. The mixture was stirred for an additional 15 min to allow for complete exchange of deuterium for hydrogen. Then the flask was immersed in a water bath at 60 °C, and the ether and deuteriodiazomethane were rapidly distilled to a collection flask in a dry ice–acetone bath, which contained 40 mL of dry ether. For elimination of the possibility of explosion when preparing deuteriodiazomethane, glassware without ground glass joints should always be used. Furthermore, ethereal solutions of deuteriodiazomethane should be stored below 0 °C. Always wear rubber gloves and work in a well-ventilated hood with this material; it is highly toxic.

Preparation of Pectin Deuteriomethyl Ester. Calcium-free lemon pectin was prepared by stirring 1 g of lemon pectin (Sunkist Growers, Inc.) in 500 mL of water at room temperature for 3 days with IR-170 (HT, 20–40-mL) resin (Dow Chemical Co., Inc.). The resin was removed by filtration, and the filtrate was lyophilized.

a 20-mL portion of the ethereal deuteriodiazomethane, which had been stored for 1 day at −8 °C, was added over a 2-h period to a suspension of the filtrate (200 mg) in dry ether (20 mL) contained in a dry ice–acetone bath. The mixture was then filtered and washed with cold ether and with cold acetone. The derivatized pectin (214 mg) was stirred into water (25 mL), and the mixture was centrifuged at 25000*g* for 20 min to remove the polymethylene byproduct. Lyophilization yielded the deuteriomethylated pectin.

Determination of Pectin Methoxyl Content. The degree of esterification (DE) of the commercial lemon pectin before and after deionization and of the deuteriomethylated product was determined by the method of Wood and Siddiqui (1971). In this method, methanol released by saponification is oxidized and measured as the chromophore produced by reaction of the oxidation product formaldehyde with pentanedione and ammonia.

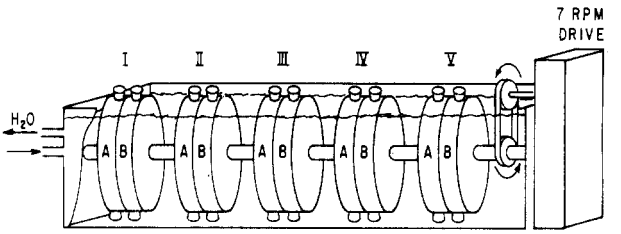

Figure 1. Equilibrium dialysis apparatus in a constant-temperature bath. Half-cell volume = 1.0 mL; membrane surface area = 4.5 cm^2; Q factor = 4.5.

The commercial pectin had a DE of 0.46, its deionized form, 0.57, and the deuteriomethylation product, 0.77, wherein all free carboxyl groups were esterified.

Confirmation of the degree of methylation of diazomethane-treated pectin was made by ^{13}C NMR spectroscopy of pectin gels (250 mg/2 mL of ^2H_2O) examined at 65 °C. A typical spectrum consisted of 15 000 transients at a repetition rate of 1.2 s. The ratio of nonesterified carboxyl groups to esterified carboxyl groups was determined from the ratio of the area of the free carboxyl group peak at 172.8 ppm to the area of the carbomethoxyl group peak at 171.3 ppm or to the area of the methoxyl group peak at 53.7 ppm.

Purification of Commercial Pectin Preparations. Samples (1.0 g) of Sunkist lemon pectin and Bulmers citrus pectin were stirred with water (100 mL) for 2 h. The turbid mixtures were centrifuged at 22000*g* for 30 min. Each clear supernatant solution was passed through a 0.45-μm Millipore filter and then lyophilized to yield a white product. The centrifugate from each sample was washed twice with water, twice with absolute ethanol, and twice with ether and recentrifuged after each washing. Sunkist lemon pectin yielded 0.58% insoluble residue, Bulmers low-methoxy (DE = 0.37) pectin, 2.1%, and Bulmers high-methoxy (DE = 0.72) pectin 0.33%. This residual material was diatomaceous earth (as determined by microscopic observation), which is routinely used in the pectin industry as a filter aid (Hull et al., 1953). Pectin preparations were also purified prior to equilibrium dialysis experiments by conventional tubing dialysis. Aqueous solutions (1%) of pectin were dialyzed 48 h against distilled water by dialysis tubing (Spectrum Medical Industries, Inc.) with a molecular weight cutoff of 12μtc000. Contents of the tubing were either freeze-dried directly or centrifuged and Millipore filtered as described above.

Evaluation of Pectin–Bile Acid Interactions by Equilibrium Dialysis. A five-cell dialyzer (Spectrum Medical Industries, Inc.) (Figure 1) with a half-cell volume of 1.0 mL, membrane (12000) molecular weight cutoff, Spectrum Medical Industries, Inc.) surface area of 4.5 cm^2, and a Q factor (ratio of surface area to half-cell volume) of 4.5 was used in all dialysis experiments. The dialyzer was immersed in a circulating, constant-temperature bath at 37 °C.

Except where described otherwise, dialysis experiments were conducted in Krebs–Ringer phosphate buffer, which was prepared by mixing 0.154 M sodium chloride (100 parts), 0.154 M potassium chloride (4 parts), 0.100 M calcium chloride (3 parts), 0.154 M magnesium sulfate (1 part), and 0.16 M phosphate buffer, pH 7.3 (21 parts).

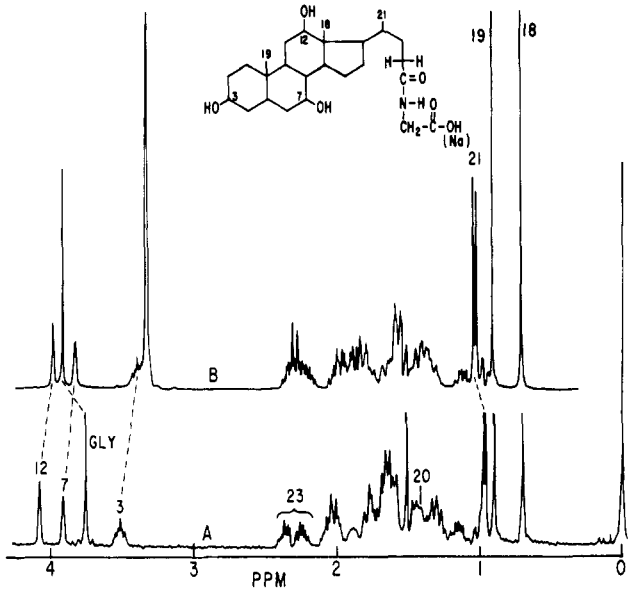

Figure 2. (A) 360-MHz ^1H spectrum of glycocholate in D_2O, pH 6.3, 3.5 mg/mL, 37 °C; (B) 360-MHz ^1H spectrum of glycocholic acid in $C^2H_3O^2H$, 3.5 mg/mL, 37 °C.

Solutions of either cholic acid (Sigma Chemical Co.) or glycocholic acid (Sigma Chemical Co.) at concentrations of 0.25 mg/mL Krebs–Ringer phosphate buffer were added to one half-cell (A) of the dialyzer, and buffer alone was added to the other half-cell (B) in control experiments to determine rates of equilibration without added pectin (Figure 1). Appropriate levels of a pectin preparation dissolved in the bile acid–buffer solution were added to the other four A half-cells. The other B half-cells again contained buffer alone, so that accumulation of bile acid in these B compartments could be compared to that in the control cell B containing no pectin. At selected times, 0.1-mL aliquots were withdrawn from the half-cell B which at t_0 contained only buffer. These aliquots were analyzed for bile acid concentration by a modification of the spectrophotometric assay described by Mosbach et al. (1954). In some experiments, the bile acid solution was placed in the other half-cell B coupled to the pectin solution in A, and the rate of bile acid migration to the pectin-containing half-cell was determined.

RESULTS AND DISCUSSION

NMR Studies. In testing for possible binding to pectin of cholic/glycocholic acid, the most prevalent bile acid/salt released into the gut, we chose a pH of 6.3, corresponding to the average value found in the small intestine (Bowen et al., 1974). Interactions beyond this point are not significant, since 90–95% of the bile acids are reabsorbed within the small intestine. Selvendran's (1978) in vitro studies suggest that the carboxylate groups (of both bile acid and pectin) in the unionized form play an important role in bile acid binding. Because these carboxyl groups range in pK values from 4 to 4.5, they are largely ionized within this region of the gut and therefore are unable to ineract through hydrogen bonding. Alternatively, binding could be effected through the association of bile salt hydroxyl groups with pectin carbomethoxyl or hydroxyl groups. If bile salts such as glycocholate were considered, the amide group could provide an additional active polar binding site.

We examined the high-field ^1H NMR spectra of glycocholate alone and with pectin to detect interaction at specific sites on the bile salt molecule. Figure 2 shows the 360-MHz ^1H spectra of glycocholate in (A) ^2H_2O and (B) $C^2H_3O^2H$ at 37 °C. The concentration of the bile salt at pH 6.3 in ^2H_2O is above its critical micelle concentration (cmc) (Small, 1971). The resolution of the two C-23 proton resonances in spectrum A of Figure 2 gives a clear indication of asymmetry of the side chain, induced perhaps by intramolecular association with one or more hydroxyl groups on the ring. Other resonances which are clearly observed include all three CH–OH methine protons, the glycine methylene group, and the 18, 19, and 21 methyl groups. Spectrum B of Figure 2 shows the effect on the chemical shift when glycocholate is dissolved in $C^2H_3O^2H$ to produce a solution which does not contain micelles (Small, 1971). In this spectrum, the asymmetry of the C-23 protons is masked by other overlapping resonances; however, we do see a decided upfield shift of all CH–OH methine protons due to a decrease in the hydrophobic micelle interactions associated with an aqueous environment. In contrast, the glycine methylene protons and the C-21 methyl group proton resonances of the side chain undergo significant deshielding resulting from a freer state of reorientation. The two spectra, parts A and B of Figure 2, represent extreme solution environments for bile salts and serve as models for different associated states. With the exception of nonspecific line broadening throughout, the spectrum of glycocholate in the presence of pectin, 1:5 (w/w), respectively, at pH 6.3 showed no change in chemical shift positions relative to those observed for spectrum of Figure 2. This suggests that the micellar state of the bile salt in the presence of the polysaccharide is preserved and that no major interaction sites are directly observable. To extract more information concerning the possible association and binding of glycocholate to the polymer, we determined the characteristic T_1 spin–lattice and T_2 spin–spin relaxation times of selecteed bile salt protons. If a small molecule such as glycocholate binds strongly to a large polymer, those protons closely associated with the binding should exhibit a shortening of their characteristic T_1 values; that is, the intimate contact induced on the proton of the bile salt by the structure of the polymer can cause transfer of energy and facilitate reorientation of the bile salt proton to its original equilibrium magnetization state. On binding to the polymer, bile salt protons should also have decreased T_2 spin–spin values because of the dependence of T_2 values on slow tumbling motions related to the effective molecular correlation time, T_{ceff} (the weighted average time required for rotation of the molecule through 1 rad in random, infinitestimal steps). Consequently, when small molecules bind to large polymers, the small molecules adopt the slow reorientational motion of the large structure, thereby reflecting this interaction in a shortening of the characteristic T_2 values. Table I contains the measured T_1 values for the methine CH–OH, glycine, and methyl group resonances of glycocholate in the presence and absence of pectin 1:5 (w/w respectively). While the methyl group resonance T_1 values remained constant, the values for the methine and glycine groups increased by 30–50%. This is true for spectra of glycocholate in the presence of 50% OCH_3 pectin as well as fully methylated pectin (~100% OCH_3 content prepared by diazomethane treatment). Such an observation is unexpected from an interacting system and is probably attributable to dominant viscosity effects, since at high magnetic fields such as 360 MHz, protons having effective correlation times in excess of 10^{-9} rad^{-1} experience an increase in T_1 values with increasing viscosity (Poole and Farach, 1971). (The relative viscosity of the pectin-containing solutions has a value of 8.) In contrast, the methyl resonance T_1 values do not reflect this viscosity effect

Table I. Spin-Lattice T_1 and Spin-Spin T_2 Relaxation Times for Glycocholate (GC) ± Pectin[a]

	H_3	H_7	H_{12}	H_{Gly}	CH_3-18	CH_3-19	CH_3-21
	T_1 Values, s						
GC, 3.5 mg/mL	0.41	0.29	0.26	0.65	0.38	0.38	0.39
GC, 3.5 mg/mL, plus pectin, 16.7 mg/mL	0.51	0.51	0.42	1.01	0.37	0.40	0.40
GC, 3.5 mg/mL, plus methylated pectin, 16.7 mg/mL	0.77	0.54	0.57	1.00	0.38	0.40	0.38
	T_2 values,[b] m s						
GC, 3.5 mg/mL	–	58	63	106	79	79	–
GC, 3.5 mg/mL, plus pectin, 16.7 mg/mL	–	35	45	39	45	45	
GC, 3.5 mg/mL, plus methylated pectin, 16.7 mg/mL	–	39	40	40	48	46	–

[a] Determined at pH 6.3; values are ±5%. [b] Calculated from $W^{1/2}$.

because their effective correlation times are much faster than 10^{-9} s rad^{-1} as a result of rapid segmental reorientational motion. T_2 values, derived from line widths (Table I for glycocholate in the presence of pectin, do mirror the effect of viscosity, since uniform line broadening is found throughout the spectrum. Evidently, the large overall effect of viscosity on the ^{1}H relaxation times in pectin solutions eclipses and expected smaller selective effects from interactions with bile acids and thereby precludes the demonstration of binding.

In feeding studies, methoxy content of pectin has shown a direct correlation with the amount of bile acid excreted (Kay and Truswell, 1977; Ebihara et al., 1979); therefore, we decided to examine the relaxation phenomenon of the pectin OCH_3 group resonance. Such experiments minimize ambiguities associated with large viscosity changes, since small amounts of bile salts added to pectin solutions contribute little to viscosity. One problem which persists, however, is the difficulty of interpreting the proton relaxation results. Unfortunately, since proton relaxation is not completely governed by a reorientational mechanism, we still must be able to identify those contributions from dipolar interactions with neighboring protons, collisions between neighboring molecules, and narrowing due to rotational isomerization, as well as spin and rotational diffusions, to isolate those effects of binding. At present, this is an insurmountable task. Alternatively, ^{2}H is a nucleus well suited for the measurement of binding sites (Glasel et al., 1973; Bernstein et al., 1979). Since ^{2}H has a nuclear spin of 1, the interaction of the electric quadrupole moment with the molecular electric field gradient surrounding the nucleus is the dominant mechanism for nuclear relaxation; consequently, intra- and intermolecular dipolar interactions can be neglected in this measurement. $^2HT_1$ relaxation is exclusively determined by the motion of the particular molecular segment under consideration. From the relationship

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{3}{8}\left(\frac{e^2qQ}{\hbar}\right)^2 T_{c,eff}$$

where $e^2qQ/\hbar$ is the quadrupole coupling constant for the C–^{2}H bond and is equal to 170 KHz, T_1 = spin–lattice relaxation time, T_2 = spin-spin relaxation time, and $T_{c,eff}$ = weighted-average reorientation time through 1 rad, $T_{c,eff}$ can be calculated from the observed T_1 or T_2 value under the conditions of extreme line narrowing, i.e., when $T_1 = T_2$ and the line shape is Lorentzian. If molecular motion is slow and anisotropic (the rotational correlative time for the tumbling molecules, $T_{c,rot} \geq 10^{-6}$ s rad^{-1}) relative to the quadrupole coupling, we observe two lines whose spacing in hertz corresponds to a relative ordering parameter. If motion is rapid and isotropic ($T_{c,rot} \leq 10^{-6}$ s rad^{-1}), we observe a single Lorentzian line, the line width of which

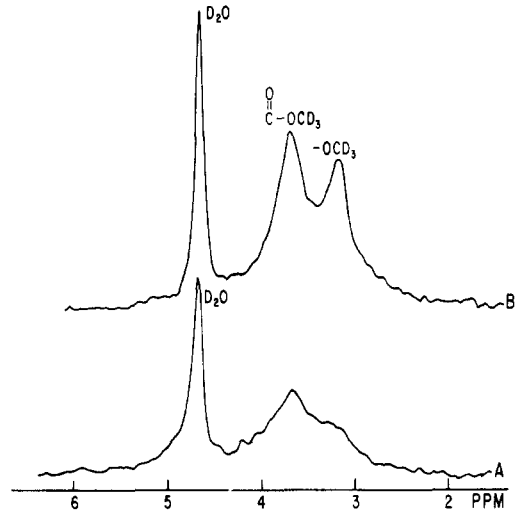

Figure 3. (A) 55.26-MHz ^{2}H spectrum of 40 mg/2 mL pectin containing 32% OC^2H_3 as the ester, pH 3.5, obtained after 500 transients, repetition time 1.6 s; (B) same as (A) except pH 6.3.

($W^{1/2}$) is a measure of the T_2 relaxation time from the relationship

$$W^{1/2} = 1/(\Pi T_2)$$

We may calculate the $T_{c,eff}$ from

$$T_{c,eff} = \frac{8/(3\Pi W^{1/2})}{(e^2qQ/\hbar)^2}$$

Deuteriodiazomethane-treated pectin was deionized, dialyzed, and subsequently used to examine the motional state of its OC^2H_3 moieties. Figure 3 shows the ^{2}H spectra of the deuterated product at 55.26 MHz under two different pH conditions. In addition to the deuterated carbomethoxyl resonance at δ 3.77, corresponding to the position of the C–OCH_3 group in the ^{1}H spectrum, we also observed an upfield resonance centered at δ 3.33, suggestive of –OC^2H_3 ether groups. Verification of this latter assignment was made from the 360-MHz ^{1}H spectrum of proteodiazomethane-treated pectin (Figure 4B), compared to the spectrum of the untreated 50% OCH_3 pectin (Figure 4A). In addition to the C–OCH_3 group resonance in the proton spectrum (Figure 4B) at δ 3.77, we observe two additional resolved OCH_3 resonances at δ 3.36 and 3.31, representing ether groups at ring positions 2 and 3 of the galacturonic acid residues and possibly an ether group at the C-6 hydroxy of the neutral residues (Gros et al., 1971). We were unable to suppress this concurrent etherification reaction produced by diazomethane. Consequently, we utilized the modified polymer containing both labeled groups for our studies. Resolution of the C–OC^2H_3 resonance was achieved by computer Lorentzian line fitting (see Experimental Section).

A comparison of the ^{2}H spectra of the labeled pectin at pH 3.5 and pH 6.3 in Figure 3 clearly shows line broad-

Table II. Deuterium Correlation Times

	pH	Δ1/2, Hz	$T_{c,eff}$, s rad^{-1} [a]		
			-C(=O)-OC^{2}H$_3$	^{2}H-11,12	NHCH(C^{2}H$_3$)$_2$
pectin	3.5	20.0	2.1×10^{-10}		
pectin	6.3	14.8	1.1×10^{-10}		
pectin plus glycocholate (5:1)	6.3	16.8	1.2×10^{-10}		
pectin plus KR[b] buffer	7.3	25.2	1.8×10^{-10}		
pectin plus KR buffer plus glycocholate (5:1)	7.3	25.2	1.8×10^{-10}		
fetuin					1.2×10^{-10}
[11,12-^{2}H]chenodeoxycholate	7.3	207		1.5×10^{-9} (9.6×10^{-11})[c]	

[a] ±10%. [b] Krebs-Ringer. [c] $CHCl_3$.

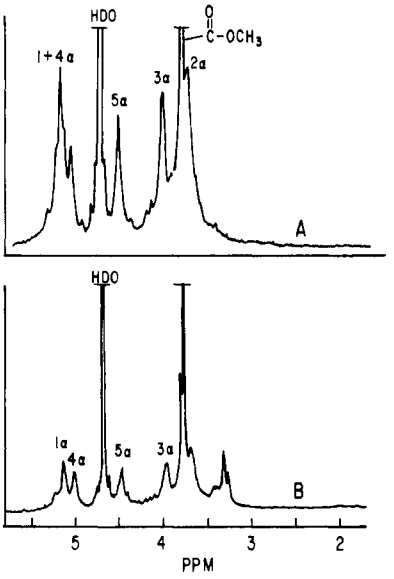

Figure 4. (A) 360-MHz ^{2}H spectrum of citrus pectin containing 50% OCH_3 as the ester; (B) same as (A) except material was treated with CH_2N_2 to give a fully esterified product.

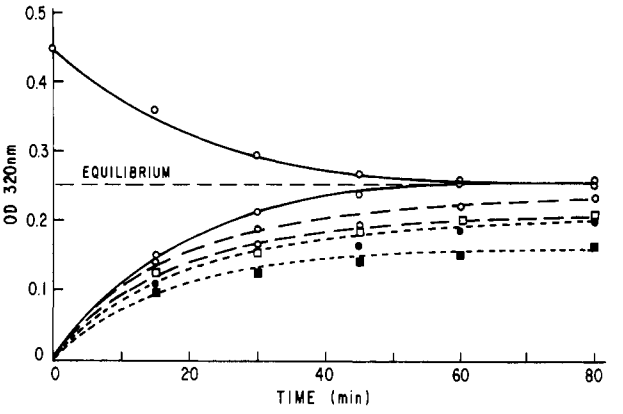

Figure 5. At t_0, control cells (O—O) contained 0.25 mg/mL glycocholic acid in Krebs-Ringer buffer at pH 7.3 in one half-cell and only buffer in each matching half-cell. Experimental cells contained pectin as well as the glycocholic acid as follows: (O--O) high-methoxy (72%) Bulmers pectin, 2.5 mg/mL; (□--□) high-methoxy (72%) Bulmers pectin, 5.0 mg/mL; (●---●) low-methoxy (37%) Bulmers pectin, 2.5 mg/mL; (■---■) low-methoxy (37%) Bulmers pectin, 5.0 mg/mL. Rates of appearance of glycocholic acid in opposing half-cells that contain only buffer at t_0 were measured.

ening, indicative of decreased mobility due to restrictions by the polymer network formed at low pH. This line broadening translates into a doubling of the $T_{c,eff}$ (Table II). A similiar increase in pectin chain-ordering aggregation at low pH has recently been qualitatively detected with circular dichroism (Davis et al., 1980). Note that the value of the $T_{c,eff}$ for the pectin OC^{2}H$_3$ groups at pH 6.3 is comparable to the value measured for the reorientation rate of the acetone-reduced lysine C^{2}H$_3$ group of the glycoprotein fetuin (Bernstein et al., 1979) (Table II). This is not surprising, since the dominant effect of reorientation in both of these spin-labeled moieties is derived from localized segmental motion. In contrast, [11,12-^{2}H]-chenodeoxycholate, a molecule that aggregates in water to form a micelle of molecular weight 8000 (Small, 1971), has a $T_{c,eff}$ which is approximately 1 order of magnitude slower. In this example, reorientation of the ^{2}H nucleus is dependent on the motion of the aggregate and not the freely rotating segment appended to a large structure. When [11,12-^{2}H]chenodeoxycholate is dissolved in chloroform, no micellar aggregation is observed (Small, 1971). Thus, rapid tumbling of the monomeric species results in a $T_{c,eff}$ similar to that observed for a freely reorienting segment. Glycocholate added to 5-fold excess pectin has little or no effect on $T_{c,eff}$ of the deuterons of the carbomethoxyl group. A 72% increase in reorientation time is noted, however, when the ionic strength of the solution is raised to 0.15 N with Krebs–Ringer buffer. This suggests that the polymer chains self-associate more strongly in a higher ionic strength medium. No effect on $T_{c,eff}$ is observed when glycocholate is added to the pectin solutions containing Krebs–Ringer buffer. These results indicate that the carbomethoxyl group is not directly involved in any binding of glycocholate to pectin, since such an interaction is expected to produce severe broadening of the deuterium resonance resulting from a decrease in $T_{c,eff}$ of several orders of magnitude (Glasel et al., 1973).

Equilibrium Dialysis Experiments. The first equilibrium dialysis experiments measured the effects of pectin methoxyl ester content and of pectin concentration on the binding of glycocholic acid. Figure 5 shows that Bulmers low-methoxy (37%) pectin binds glycocholic acid twice as effectively as does Bulmers pectin with a methoxy content of 72%. Also, the degree of binding is proportional to the concentration of pectin. At equilibrium (80 min), 5 mg of low-methoxy pectin bound 102 μg of glycocholic acid, while 2.5 mg bound 61 μg.

As shown in Figure 6, 2.5 mg of Sunkist lemon pectin binds 20 μg of cholic acid. This result is obtained whether cholic acid at t_0 is in the same compartment as the pectin or in the matching half-cell.

In all dialysis experiments, use of buffer solutions of sufficient ionic strength was essential to minimize Donnan effects. Such effects would be especially detrimental in these studies, since we were examining the interactions of a nondiffusible anionic polymer with diffusible anions. The Krebs–Ringer buffer minimized Donnan effects, but in phosphate buffer alone at equilibrium substantially more cholic acid was present in the half-cell opposite to pectin; i.e., negative binding was suggested. Even in Krebs–Ringer buffer, Donnan effects can be significant,

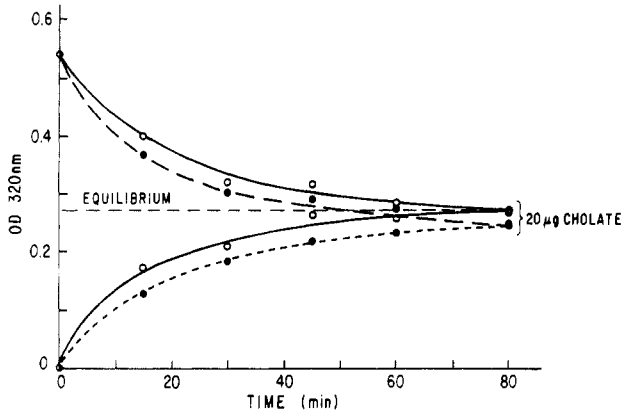

Figure 6. At t_0, control cell (O—O) contained 0.25 mg/mL cholic acid in Krebs–Ringer buffer at pH 7.3 in one half-cell and only buffer in each matching half-cell. Experimental cells contained 2.5 mg/mL Sunkist lemon pectin and 0.25 mg/mL cholic acid: (●---●) rate of disappearance of cholic acid from opposing half-cells that contain cholic acid plus buffer at t_0; (●——●) rate of appearance of cholic acid in opposing half-cells that contain only buffer at t_0.

as shown in experiments with the polyanionic pectin derivative, polygalacturonic acid. With polygalacturonic acid, the equilibrium concentration of cholic acid was greater in the half-cell not containing this polymer, as would be expected with Donnan contributions.

Comparisons were made of the efficiency of binding of glycocholic acid by Bulmers low-methoxy pectin at pH 6.0 and 7.3. Nearly twice the quantity of bile acid was bound at the higher pH. This is somewhat unexpected, since at the higher pH more ionic groups would be present in both the polymer and the bile acid.

The capacities of various commercial pectin preparations to bind glycocholic acid were compared. The Bulmers low-methoxy pectin possessed twice the capacity of any other preparation examined. Efforts were made to purify this material prior to making structural modifications in order to understand the nature of the interaction. The off-white-colored materials did not lose their color or binding capacity upon repeated reprecipitations from alcohol, the usual method of pectin purification. When 1% solutions of pectin were centrifuged at 22000g, however, a significant pellet was formed from the low-methoxy pectin which accounted for 2.1% of the sample weight. After the supernatant was passed through a 0.45-μm Millipore filter and lyophilized, the resulting white material, when examined by equilibrium dialysis, bound glycocholic acid at levels comparable to those in the pectin preparation before purification. At equlibrium, the bile acid levels in the half-cells containing the pectin preparations more than doubled the levels in the opposing half-cells. The centrifugable material present in all commercial pectins examined apparently is not essential for the binding of bile acids. This material is mainly colloidal diatomaceous earth, which is routinely used in the pectin industry as a filter aid.

Significantly, no binding of glycocholate was shown by equilibrium dialysis when the pellet obtained by centrifugation was vortexed into a solution of this bile salt in buffer. Moreover, no lowering of the concentration of free sodium glycocholate was observed when an equal amount of the pellet residue was added to an aqueous solution of this bile salt. Apparently, the binding of bile acids by commercial pectin samples is a result of the cooperative efforts of both pectin and the contaminant, and the binding capacity of the commercial pectin increases with increasing amounts of contaminant present. These results have additional importance, since the feeding studies demonstrating hypocholesteremic effects of pectins included similarly contaminated food grade preparations (Leveille and Sauberlich, 1966; Kay and Truswell, 1977). Furthermore, the amount of excess bile acid excreted per day (~200 mg) due to the ingestion of 15 g of Bulmers citrus pectin per day (Kay and Truswell, 1977) closely corresponds to the amount of bile acid we observe bound to the unpurified low-methoxy pectin preparation (20 mg/g of pectin). Other feeding studies using different sources of food grade pectin (Keys et al., 1961; Jenkins et al., 1975; Miettinen and Tarpila, 1977) have demonstrated less excretion of bile acids per weight of pectin fed.

Equilibrium dialysis experiments also were conducted with other preparations derived from Bulmers low-methoxy pectin. The material obtained after conventional tubing dialysis was totally devoid of capacity to bind glycocholic acid, either with its diatomaceous earth contaminant present or after its removal by centrifugation. These results suggest that native high molecular pectin does not bind bile acids but that the binding demonstrated in the equilibrium dialysis experiments is due to a low molecular weight (less than 12000) nondiatomaceous earth contaminant of commercial pectin preparations. These studies are supported from our dialysis studies with pure grapefruit pectin, isolated in the laboratory, from grapefruit albedo. This preparation did not bind bile acids.

Future studies will be concerned with isolating and characterizing the factor present in commercial pectin preparations and further evaluating its binding properties. The levels of bile acid excretion in feeding experiments may be related to the level of binding component in each of the respective pectin samples. In order to resolve this issue and to assess the physiological activity of the binding component, we will conduct feeding experiments with both dialyzed and nondialyzed pectin samples.

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Nutrient Utilization by Human Subjects Consuming Fruits and Vegetables as Sources of Fiber

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Apparent digestibilities of energy, nitrogen, and fat and balances of calcium, magnesium, zinc, and copper were determined for 12 men consuming 4 diets containing different amounts of fruits and vegetables. Mean intakes of neutral detergent fiber (NDF) from the four diets were 1.9, 10.1, 19.4, and 25.6 g/day. The diets were consumed for 3 weeks each in a 4 × 4 Latin square design. Urine and feces were collected during the last 7 days of each dietary period. Mean bowel transit time was not affected by the level of fiber intake. Number of defecations and fecal weight increased with fiber. Energy, nitrogen, and fat fecal excretions increased and apparent digestibilities decreased as fiber increased. Increased levels of fiber intake did not affect balances of calcium, magnesium, and copper. Zinc balance decreased, but remained positive, as fiber increased. Mean balances for these minerals were positive on all four diets.

In most studies of the effects of fiber on humans, cereal fibers were fed. In two studies carried out in this laboratory, the effects of diets containing fruits and vegetables as sources of fiber were studied. In an earlier study (I) (Kelsay et al., 1978), 2 diets were fed to 12 men in a crossover design for 26 days each. A low fiber diet containing fruit and vegetable juices was compared with a higher fiber diet containing fruits and vegetables. Mean intakes of neutral detergent fiber (NDF), as determined by the Goering and Van Soest (1970) method were 4.9 and 24.9 g/day on the low and higher fiber diets, respectively.

In study I, the inclusion of fruits and vegetables in the diet decreased bowel transit time and increased number of defecations and fecal weight. The higher fiber diet also increased fecal excretions and decreased apparent digestibilities of energy, nitrogen, and fat (Kelsay et al., 1978). Fecal excretions of calcium and zinc were greater on the higher fiber diet, resulting in negative balances that were significantly lower than those on the low fiber diet. Although magnesium and copper fecal excretions were not significantly different on the two diets, probably due to small differences in intakes of these two minerals, magnesium and copper balances were negative on the higher fiber diet and were significantly lower than those on the low fiber diet. Iron and phosphorus balances were not affected by the inclusion of fruits and vegetables in the diet (Kelsay et al., 1979a,b).

In the second study (II), reported here, the low fiber diet was compared to three diets containing increasing levels of fiber in fruits and vegetables. Since mineral balances were negative on the higher fiber diet fed in study I, we wished to determine the effects of increasing levels of fiber on mineral balances and to define the maximum level of fiber intake at which subjects would be in mineral balance.

EXPERIMENTAL SECTION

Twelve men 35–49 years of age participated in the study. Body weights ranged from 65.5 to 111.0 kg and heights from 162.0 to 187.5 cm. The subjects consumed four diets for 3 weeks each in a 4 × 4 Latin square design. Diet 1 was a low fiber diet and diets 2, 3, and 4 contained increasing amounts of fruits and vegetables. Diet 2 had half the amounts of fruits and vegetables in diet 3, and diet 4 had 1.5 times the amounts of fruits and vegetables in diet 3. Mean NDF intakes on the four diets, as analyzed by the American Association of Cereal Chemists' (AACC) modification of the NDF method (AACC, 1978), were 1.9, 10.1, 19.4, and 25.6 g/day for diets 1, 2, 3, and 4, respectively. In this method, residual starch is removed by the use of amylase, and the resulting NDF values are lower than those obtained by the original method. Caloric in-

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