preferably exchanged as determined by the ^1H NMR coupling patterns.

The results in Tables I and II show that although the overall amounts of deuterium incorporated with either method are similar, the platinum-catalyzed exchange gives a distribution of labeling which is less specific than the acid-catalyzed reaction. Thus, higher levels of multiple exchange are observed with the platinum method than with the acid catalysis method. This may be an advantage when a labeled compound is to be used in biological experiments where the lability of the isotopic label is of concern. For example, under the back-exchange conditions noted in Table I (footnote *c*), the label at position 3 of compound **2** can be removed, thus eliminating the possibility of loss of label from this position under biological conditions. The additional deuterium introduced at positions 5 and 7 by the platinum-catalyzed exchange would thus somewhat compensate for the lost label at position 3. The platinum method is also useful in incorporating isotopic label at positions which are unaffected by acid catalyzed exchange conditions. Thus, positions 2 and 7 of compound 1 and positions 5 and 7 of compound **2** are exchanged by the platinum method but not under acid catalysis.

An additional consideration in comparing the two methods is the stability of the substrate toward the reaction conditions. In these experiments it was noted that the products from the more prolonged, sulfuric acid catalyzed reactions with compounds **2** and **3** showed some evidence of side reactions on the basis of the appearance of peaks of minor impurities in the NMR spectra. There was no evidence for the production of side products during the prolonged platinum catalysis reactions.

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Fractionation and Study of Chemistry of Pectic Polysaccharides

The heterogeneous nature of grapefruit (*Citrus paradisi*) pectin was investigated by employing ion-exchange chromatographic techniques. Four chemically distinct pectic polysaccharides were resolved. These polysaccharides eluted sequentially with 0.025, 0.1, 0.25, and 0.5 M sodium phosphate buffer, pH 6.0, and comprised 13, 5, 62, and 20%, respectively, of the total pectin subjected to ion-exchange chromatography. No striking differences in the galacturonic acid content, which ranged from approximately 70 to 80% of these pectic polysaccharides, were observed. However, the neutral sugar composition of these pectic polysaccharides was found to be different. These studies affirm the heterogeneous nature of pectin and suggest that meaningful evaluation of the dietary role of pectin could only be achieved by being aware of this heterogeneity.

Experimental findings of several investigators consistently suggest that pectin when supplemented in the diet causes lowering of the serum cholesterol level in man as well in several laboratory animals (Kay and Truswell, 1977; Mokady, 1973; Lin et al., 1957). In view of the heterogeneous nature of pectin, it is possible that, of a number of polysaccharides found in pectin, a single pectic polysaccharide may be uniquely responsible for the said nutritional role of dietary pectin (Baig and Cerda, 1980). It follows, therefore, that the fractionation of dietary pectin and subsequent nutritional studies on individually resolved pectic polysaccharides of known physicochemical characteristics may result in the elucidation of the elusive biochemical basis by which dietary pectin causes lowering of cholesterol levels. Described in this report are the fractionation and chemical characterization of several pectic polysaccharides found in commercially prepared grapefruit pectin. These studies serve as a stepping stone toward our understanding of the biochemical basis by which pectin and its various polysaccharidic components may cause lowering of cholesterol levels.

MATERIALS AND METHODS

Grapefruit pectin was a gift from Lykes-Pasco Co., Dade City, FL. This commercially available pectin was extracted from the grapefruit albedo by first working the albedo with deionized distilled water, followed by extraction of pectin from water-washed albedo with a hot (176 °F) solution of HCl, pH 1.6, for 45 min. The pectin thus extracted with acid was recovered by precipitation with 70% (v/v) isopropyl alcohol. Before use in experiments described in this report, this commercially available pectin was reprecipitated 3 times by using 70% (v/v) ethanol, dialyzed against deionized distilled water, and lyophilized.

DE-52 Diethylaminoethylcellulose Ion-Exchange Chromatography. A column (1.5 × 24 cm) was equilibrated with 0.025 M sodium phosphate buffer, pH 6.0, and 250 mg of pectin dissolved in 100 mL of equilibrating

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Table I. Fractionation of Pectin on the DE-52-cellulose Column

fraction	eluent	polysaccharides recovered, %
I	0.025 M sodium phosphate	12.98
II	0.100 M sodium phosphate	4.77
III	0.250 M sodium phosphate	62.17
IV	0.500 M sodium phosphate	20.05

Table II. Methoxyl Content of Various Pectin Fractions

	methoxyl content, %
pectin (unfractionated)	8.9
fraction I	8.7
fraction II	14.85
fraction III	10.77
fraction IV	4.42

buffer was loaded onto the column. The column was first washed with 100 mL of equilibrating buffer and then eluted successively with 250 mL each of 0.05, 0.10, 0.25, 0.50, and 1.0 M sodium phosphate buffer, pH 6.0. Individual fractions were collected and the column eluate was monitored by assaying uronic acid content. Fractions constituting various pectin polysaccharide peaks were pooled, dialyzed against deionized distilled water, and lyophilized.

Chemical Analysis of Pectic Polysaccharides. Unfractionated pectin and pectic polysaccharides recovered following ion-exchange chromatography as described above were hydrolyzed with 2 N trifluoroacetic acid at 121 °C for 2 h in sealed ampules (Albersheim et al., 1967) Trifluoroacetic acid was removed by vacuum evaporation, the hydrolyzed material was loaded onto a Dowex 50 H^+ resin (AG50W-X8, 100–200 mesh) column (1 × 5 cm) following its dissolution in deionized distilled water, and the effluent recovered was loaded onto a Dowex 1 formate resin (AG1-X2, 200–400 mesh) column (1 × 5 cm). Neutral sugars were eluted from the column by washing it with 4 volumes of deionized distilled water. For elution of acidic sugars held on the formate resin, the column was eluted with 4 volumes of 6 N formic acid. The acidic and neutral sugar components thus separated by ion-exchange chromatography were further resolved into individual sugar components by descending paper chromatographic techniques. For separation of neutral sugars, the solvent ethyl acetate–pyridine–water (8:2:1 v/v) was used (Williams and Bevenue, 1953). Acidic components were separated by using the solvent ethyl acetate–pyridine–acetic acid–water (5:5:1:3 v/v) (Fischer and Dorfel, 1955). Paper chromatograms were developed with aqueous acetone containing silver nitrate, followed by a dip in alcoholic potassium hydroxide (Trevelyn et al., 1950). The contrast between developing sugars and the paper background was preserved by dipping chromatograms in 10% sodium thiosulfate in water.

The relative proportion of neutral sugar components found in pectin or its various pectic polysaccharides was determined by gas–liquid chromatography. Alditol acetate derivatives of the neutral sugar components were prepared according to the method described by Lehnhardt and Winzler (1968). Analysis was performed by using a Hewlett-Packard 5830A gas chromatograph equipped with a Hewlett-Packard 18850 ACG terminal/programmer. A glass column (1.83 m × 2 mm i.d.) packed with 3% OV-225 on 80–100 Supelcoport (Supelco, Inc., Bellfont, PA) was used for analysis. The column temperature was maintained at 250 °C, the FID port temperature at 225 °C, and the injection port temperature at 225 °C. Nitrogen was used as a carrier gas, and its flow was maintained at 20 mL/min.

Other Analytical Techniques. The methoxyl content of unfractionated pectin as well as various pectic polysaccharides obtained folowing ion-exchange chromatography of pectin was determined by the method of Wood and Siddiqui (1971). The total carbohydrate content of various fractions was estimated with α-naphthol reagent by using galactose as the standard (Dische, 1962). Reducing sugars were estimated by using ferricyanide reagent (Ashwell, 1957), with galactose as the standard, and pentose was estimated by using orcinol reagent (Brown, 1946), with arabinose as the standard. The uronic acid content of samples was assayed according to the methodology described by Bitter and Muir (1962).

RESULTS AND DISCUSSION

The use of ion-exchange resins to resolve molecules of biological origin is a classical technique employed by chemists. Shown in Figure 1 is the elution profile of various pectic polysaccharides when pectin was subjected to DE-52-cellulose ion-exchange chromatography. A total of four pectic polysaccharide fractions was resolved. Washing of the column with 0.05 M sodium phosphate did not cause the elution of any polysaccharides. In addition, washing of the column with 0.5 M sodium phosphate resulted in the complete elution of acidic polysaccharide held onto column as revealed by the absence of carbazol- or α-naphthol-reacting material in 1.0 M sodium phosphate eluate.

Table I shows the relative proportion of various isolated pectic polysaccharides found in unfractionated grapefruit pectin. It appears that fraction III, eluted with 0.25 M sodium phosphate buffer, represents the major component whereas fraction II, eluted with 0.1 M sodium phosphate buffer, represents a minor component of grapefruit pectin.

Table II summarizes the results obtained when unfractionated pectin and various fractions of pectin were analyzed for their methoxy content. The methoxy contents of unfractionated pectin and fraction I, which represents material not exchanged by the ion-exchange column under conditions described earlier, were found to be similar. Observed was the progressive decrease in the methoxy content of fractions eluted with increasing salt concentration. These results suggest that anion ion-exchange chromatography of pectin results in the resolution of pectic polysaccharides according to differences in the charge characteristics.

Summarized in Table III is the sugar composition of pectin and pectic polysaccharides fractionated thereof. As could be seen, no striking differences were observed in the

Table III. Chemical Composition of Various Fractions from the DE-52-cellulose Column

	galacturonic acid, %	rhamnose, %	arabinose, %	xylose, %	mannose, %	galactose, %	glucose, %
pectin (unfractionated)	76.00	7.13	2.65	0.75	0.42	11.50	1.51
fraction I	69.00	12.68	5.92	2.61	0.72	7.23	1.69
fraction II	74.05	2.61	3.36	1.11	0.30	13.23	5.55
fraction III	80.75	7.10	2.18	0.79	nil	7.00	2.15
fraction IV	67.56	20.27	1.47	1.44	nil	4.98	4.81

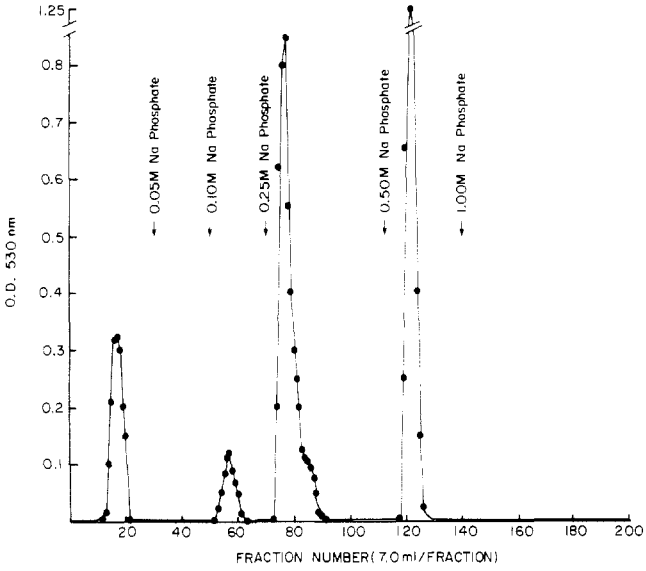

Figure 1. DE-52 ion-exchange chromatography of pectin. For details, see the text.

galacturonic acid content of these fractions. However, these fractions differ from each other in their neutral sugar composition. Of interest is the neutral sugar composition of fraction I (eluted with 0.025 M sodium phosphate) and fraction IV (eluted with 0.5 M sodium phosphate). These fractions were found to be rich in their rhamnose and poor in their galactose content. The sugar composition of unfractionated pectin was found to be similar to the sugar composition reported earlier for citrus pectin (Aspinall et al., 1968). It is important to point out at this juncture that the chemistry of the neutral sugar components of the commercially available pectin described here is remarkably different from the chemistry of pectin isolated by employing gentle extraction procedures (Baig et al., 1980). As noted earlier by several investigators (Aspinall, 1970), this difference appears to be caused by differences in the methodology used to extract pectin from grapefruit.

The results obtained from these studies clearly affirm the heterogeneous nature of commerically available citrus pectin and conclusively indicate that the grapefruit pectin is composed of a number of pectic polysaccharides differing in their methoxy as well as neutral sugar composition. In view of this observed heterogeneity of dietary citrus pectin, it is apparent that investigative studies aimed at evaluating the dietary role of pectin in lowering cholesterol levels in man and laboratory animals must take into consideration the fact that pectin is not a single identity but a mixture of a number of pectic polysaccharides. In addition, since pectin represents the material found in the primary cell wall of plants, it is highly possible that the qualitative nature as well as quantity of various pectic polysaccharides found in pectin may vary with the degree of maturity/differentiation of the plant source from which dietary pectin is extracted. Meaningful evaluation of the dietary role of pectin could therefore be only achieved by using individual pectic polysaccharides of known physicochemical characteristics.

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Alfalfa Trypsin Inhibitor Inhibits Alfalfa Leaf Proteinase

The alfalfa (*Medicago sativa*) leaf trypsin inhibitor inhibits the alfalfa leaf proteinase. This inhibition is strictly dependent on ionic strength.

In a previous paper we brought up evidence suggesting that the alfalfa leaf neutral proteinase is a serine trypsin-like proteinase (Tozzi et al., 1981). This observation prompted us to investigate the inhibitory effect of the alfalfa leaf trypsin inhibitor described by Chang et al. (1978) on the endogenous proteinase. In the present communication we report that alfalfa trypsin inhibitor also inhibits alfalfa endogenous proteinase.

EXPERIMENTAL SECTION

Materials. The alfalfa trypsin inhibitor was purified according to Chang et al. (1978). The alfalfa proteinase was purified according to Tozzi et al. (1981). Trypsin, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA), and

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