Studies on the Role of Citrus in Health and Disease

M. MANSOOR BAIG and JAMES J. CERDA

Department of Medicine, Division of Digestive Disease and Nutrition, J-214
JHMHC, University of Florida College of Medicine, Gainesville, FL 32610

Since 1973, we at the University of Florida have been systematically evaluating the role of citrus fruits and their products in human nutrition. Our previous studies of citrus have yielded valuable information relating to the beneficial nutritional role of citrus in providing certain vitamins, replenishing lost electrolytes, and possibly inhibiting viral infections. Our ongoing research in the area of study of the chemistry and biological role of dietary citrus pectin in human nutrition has yielded results elucidating the complex chemical nature of pectin and the possible biochemical basis by which dietary pectin may cause lowering of serum and/or liver cholesterol. In addition, another series of ongoing experiments suggest that citrus pectin may influence the biological processes regulating the absorption of glucose in patients with postprandial hypoglycemia.

This article is a review of our major biomedical research efforts, previous and ongoing, on the role of citrus and citrus pectin in human nutrition. Its purpose is to focus attention, provide scientific evidence and evaluate the direction of future research on the beneficial role of citrus fruits in human nutrition.

NUTRITIONAL ROLE OF CITRUS BEVERAGES

Bioavailability of Water-soluble Vitamins

Nutritionally, the most important water-soluble vitamins in citrus fruits are ascorbic acid, folic acid and pyridoxine. Clinical studies on the bioavailability of these vitamins, as well as basic research on the absorption and chemistry of these vitamins, have yielded valuable information adding to our overall understanding of the nutritional quality and bioavailability of these vitamins found in citrus fruits.

Ascorbic Acid. Unlike a number of animal species, man and

0-8412-0595-7/80/47-143-025$05.00/0

other primates as well as the guinea pig depend on dietary sources for their vitamin C needs because these species lack the biochemical mechanism to synthesize this essential nutrient. Lack of vitamin C in dietary sources causes scurvy in adults which is characterized by sore spongy gums, impaired capillary integrity with subcutaneous hemorrhages and edema, joint pain, anorexia and anemia. In children, vitamin C deficiency causes tenderness and swelling of joints, arrested skeletal development, impaired wound healing, anemia and inadequate tooth development.

Citrus juice is rich in ascorbic acid and, therefore, is an important dietary source of this essential vitamin. Our studies were aimed at defining whether vitamin C from citrus sources is comparable with the synthetic vitamin in terms of bioavailability and intestinal absorption. These studies were conducted using human volunteers and guinea pig experimental models (1,2).

Prior to our work, absorption of vitamin C from intestine was believed to be passive, i.e., not involving active transport. Contrary to this belief, results from our in vivo perfusion studies on the saturation kinetics relating to the intestinal absorption of pharmacological doses of ascorbic acid in human volunteers and guinea pigs demonstrated that intestinal absorption of ascorbic acid is accomplished by an active transport mechanism, supporting the observations made previously by Stevenson (3). The observed active transport of ascorbic acid showed parameters characteristic of the phenomenon of saturability, i.e., as the dose of vitamin C presented to the intestine is increased, the relative proportion of ascorbic acid absorbed showed a decrease. The most efficient absorption of the vitamin was observed when amounts nearly identical to that present in orange juice were presented to the intestine. These observations were further corroborated by studies on the absorption of vitamin C in vitro on isolated jejunum and ileum segments of guinea pig with intact vascular supply using experimental guinea pig models. These studies, in addition to establishment of the active absorption of vitamin C by intestine, provided conclusive evidence that human volunteers perfused with pharmacological doses of vitamin C absorbed the vitamin less efficiently. Furthermore, it was shown that the amount of vitamin C present in orange juice is near optimum in terms of its efficient absorption by intestine, and the pharmacological doses of vitamin C supplied to human volunteers would appear to be of little or no additional nutritional value and may even be detrimental.

Folic Acid. Citrus juice both in frozen or fresh form is a rich and stable source of folate. The presence of vitamin C in orange juice protects it from oxidation and, unlike other nutritional sources of folate, the folate in orange juice is not subjected to destruction caused by cooking of foodstuff.

Research conducted in our laboratories has clearly established that, in addition to being rich in folate, citrus juice

contains N-5 methyl tetrafolate as the most predominant chemical form, which is metabolically active and most readily available. Its natural occurrence is unique in citrus fruits (4-6). Furthermore, by employing triple lumen perfusion techniques, it was shown that diphenylhydantoin does not influence the absorption of citrus folate in human volunteers. This may be clinically significant, since diphenylhydantoin in dosages sufficient to control epileptic seizures influences absorption of forms of folate present in other food sources.

Vitamin B-6. Comparative studies on the bioavailability of synthetic and naturally occurring forms of vitamin B-6 found in orange juice have demonstrated that the absorption of vitamin B-6 from orange juice in human volunteers is not efficient when compared with the synthetic forms of this vitamin (7). These observations are similar to the findings of others (8) that natural nutritional sources contain the B-6 largely in bound form and cooking of foodstuff enhances the bioavailability of this vitamin in animals. Naturally occurring vitamin B-6 is generally bound to proteins (9), however citrus fruit, which contains substantial amounts of bioavailable forms of vitamin B-6, is low in protein contents. Further investigations on the nature of the chemical component bound to vitamin B-6 found in citrus fruit have demonstrated that in citrus fruit vitamin B-6 is bound to a small (molecular weight:<3,500) heat-stable and non-proteinic molecule which exhibits binding to both pyridoxal and pyridoxine (10). These studies have contributed to our basic understanding of the bioavailability, absorption and chemistry of vitamin B-6 in general.

Electrolyte Balance

It has been previously demonstrated that considerable quantities of sodium and potassium are lost through sweating (11,12). Loss of potassium from skin has been estimated to be of the magnitude of 44% of the total potassium losses. Furthermore, loss of potassium caused by strenuous physical activity in hot climates may cause rhabdomyolysis and myoglobinuria.

Studies (13,14) were carried out to evaluate the role of an orange juice based "thirst-quencher" in replenishing the lost electrolyte balance caused by extreme exercise in a hot and humid climate, since orange juice thirst-quencher contains appreciable amounts of sodium and potassium. Members of the University of Florida Track Club were selected and studied for the sodium and potassium electrolyte balance changes caused by exercise, and monitored by employing whole body counting techniques using ^{40}K. These athletes ran 18 to 20 miles a day and were given three individual liquid electrolyte supplements, namely: Gatorade ^{R}, orange juice thirst-quencher and an electrolyte-sodium only solution. Results obtained from these studies demonstrated that only the

orange juice thirst-quencher with its high potassium content resulted in a positive potassium balance which even exceeded requirements recommended by the National Research Council for athletes. These findings lend additional support to the nutritive value of citrus beverages in maintaining the electrolyte balance in exercise, as well as in overall human health since potassium is essential for normal function of muscles, including the heart.

Resistance to Viral Infections

Although highly controversial, evidence has been presented in the literature which suggests that vitamin C could be involved in increasing tissue resistance to respiratory viruses (15). Orange juice is a natural source of vitamin C containing an average of 30 mg of vitamin C per 100 ml of juice. Studies (16) were carried out on the effect of ingestion of orange juice on Rubella virus infections in human volunteers who were infected with the virus either by nasal instillation or subcutaneous injection. Using this model, human volunteers infected intranasally develop respiratory symptoms whereas subcutaneous introduction of virus leads to development of systemic symptoms. The infected individuals were given a liter of orange juice a day whereas matched normal controls were instructed to eliminate citrus from their daily diet and not to take vitamin supplements. Results obtained from these preliminary experiments suggested that ingestion of orange juice might have an inhibitory influence on the development of only respiratory tract symptoms since no such influence on the development of systemic symptoms was observed in human volunteers infected subcutaneously. In addition, serum antibodies to Rubella virus appeared significantly earlier in individuals infected through the nose.

NUTRITIONAL ROLE OF DIETARY CITRUS PECTIN

Diseases such as atherosclerosis, colon cancer, constipation, gallstones and many other so-called "ailments of the Western world" are linked to a common etiology, i.e., deficiency of fiber in the diet. Atherosclerosis and coronary artery disease occur more frequently in populations showing a high incidence of hypercholesterolemia than in those showing hypocholesterolemia. Cholesterol is the major component of the atherosclerotic lesions which are characterized by intimal proliferation of smooth muscle cells accompanied by an accumulation of large amounts of connective tissue components such as collagen, elastin, glycosaminoglycans and deposition of extra- and intracellular lipid.

Elevated serum cholesterol levels are invariably associated with the etiology of atherosclerosis and coronary artery disease, and it has been shown by several investigators that pectin from a variety of sources when supplemented in the diet of a number of laboratory animals, as well as human volunteers, causes lowering

of liver and/or serum cholesterol levels (17). Furthermore, pectin has been shown to retard induced avian atherosclerosis (18).

Does Citrus Pectin Bind Bile Salts? A possible mechanism by which dietary pectin may cause lowering of cholesterol levels in rats has been reported (19). In these *in vitro* studies, pectin was found to inhibit the transport of taurocholic acid from everted sacs of rat intestine. The absorption of labelled cholesterol was depressed by the addition of 5% pectin to the diet as evidenced by increased excretion of labelled cholesterol and diminished cholesterol deposition in the liver. It was concluded from these studies that pectin lowers cholesterol levels in cholesterol-fed rats primarily by binding bile salts and, consequently, by impairing cholesterol absorption. Results similar to those obtained with dietary pectin and described have also been reported for other non-nutritive substances such as guar gum, psyllium seed colloid and seruglucan (20).

Dietary fibers in general have traditionally been tested for their binding of bile salts by employing centrifugation techniques. These analytical methods are not applicable to water-soluble mucilagenous fibers such as pectin. Therefore, in our attempts to extend and demonstrate similar binding of bile salts to citrus pectin, we have developed a methodology based on equilibrium dialysis to study interaction of bile salts with fibrous as well as mucilagenous dietary fiber. This method has been successfully utilized by us to study and compare fiber-bile salt binding and/or interaction for a variety of fibers, including pectin. In our studies we compared and studied the binding of bile salts by a variety of nonnutritive fibers by using both centrifugation and equilibrium dialysis techniques. These studies were carried out by utilizing ^{14}C sodium taurocholate. Centrifugation studies were performed according to established techniques. Equilibrium dialysis studies were performed utilizing Spectrapor 2 membrane and a Spectrum dialyzer. The percentage of binding was determined by assaying the radioactivity in the supernatant fluid obtained following centrifugation and in the dialysate solution opposite to the fiber-containing half cell. Comparable results were obtained by the two methods used to study the binding of bile salts, with an incubation mixture containing 2.5 mM of sodium taurocholate per mg of fiber. Bile salts binding was compared by these two methods using cholestyramine, a known resin which binds bile salts, ground alfalfa, miller's bran, cellulose, and citrus pectin and lignin. As summarized in Table I, results obtained from these studies clearly demonstrated the binding of alfalfa (8.4% by centrifugation vs. 14.4% by equilibrium), bran (1.7% by centrifugation vs. 8.5% by equilibrium) and lignin (2.4% by centrifugation vs. 1.4% by equilibrium) to sodium taurocholate. Binding of taurocholic acid to cholestyramine was found to be identical (84%) by the two methods. However, no binding of bile salts to pectin or cellulose was observed. Results obtained from these studies

Table I. *In vitro* interaction of bile salts with pectin and other dietary fibers

	Equilibrium dialysis (% bound)	Centrifugation (% bound)
alfalfa	14.4	8.4
bran	8.5	1.7
cellulose	nil	nil
pectin	nil	technical difficulties
lignin	1.4	2.4
cholestyramine	84.0	84.0

suggest that bran and alfalfa, although clinically less effective in lowering of serum and/or liver cholesterol levels, did bind small amounts of bile salt but only a fraction of that bound by cholestyramine resin, while pectin which is unique in causing lowering of cholesterol levels demonstrated no binding to bile salts. Although care must be taken in extrapolating these findings to *in vivo* processes which remain undefined, it was concluded that the phenomenon of binding of bile salts to dietary fiber, particularly pectin, is not sufficient to explain the biochemical basis by which dietary pectin causes lowering of cholesterol levels. It was, therefore, imperative to search for alternate biochemical and physiologic mechanisms to help explain the hypocholesterolemic effect of dietary pectin.

Interaction of Pectin with Serum Lipoproteins. Earlier in the course of epidemiologic studies showing a strong relationship between elevated levels of serum cholesterol and subsequent development of atherosclerosis, it was discovered that cholesterol found in plasma did not occur in the free state but was bound/carried in various lipoprotein fractions. Lipoproteins found in plasma are lipid-protein complexes of various sizes and densities. These complexes are constructed with the charged protein molecules on the surface and the nonpolar molecules, such as triglycerides and esters of cholesterol, on the inside. The lipoproteins function to transport cholesterol in a water-soluble form. Based on their densities, sizes and behavior upon preparative ultracentrifugation, four major kinds of lipoproteins are characterized to date. These are: chylomicrons which carry dietary triglycerides from intestine to nonhepatic tissues for utilization or storage, very low density lipoproteins (VLDL) containing triglycerides made primarily in the liver, the low density lipoproteins (LDL) and the high density lipoproteins (HDL). Of these lipoproteins, LDL are the major carriers of circulating cholesterol. Elevated levels of LDL in the serum contribute significantly to the coronary heart disease risk in persons older than age 50. On the other hand, elevated levels of HDL, which carry approximately one quarter of the cholesterol found in serum, has a beneficial influence on the overall health of individuals. In fact, increased levels of HDL in serum has been attributed with longevity (21). The role of VLDL in causing atherosclerosis remains unclear. It, therefore, follows that much of what has been learned in the past about the ill effects of high serum cholesterol can be attributed to the associated elevated levels of LDL or cholesterol carried in this lipoprotein fraction. Furthermore, results from recent studies (22) have shown that LDL, which are known to carry most of the cholesterol found in blood, play a key role in both the development of atherosclerotic lesions and in the regulation of cholesterol metabolism in a variety of cells. These studies suggest that specific binding sites for LDL present in normal cells are absent and/or defective in fibroblasts from subjects

with homozygous familial hypercholesterolemia. Interaction of LDL with a specific receptor at the cell surface of normal cells initiates a series of still poorly understood complex processes leading to endocytosis of LDL, the lysosomal degradation of internalized LDL and suppression of cholesterol synthesis in the cell. In individuals homozygous to familial hypercholesterolemia, a lack of binding sites at the surface of fibroblasts from these individuals lead to accumulation of large quantities of LDL in the blood stream and the development of severe, atherosclerotic disease. Results from these studies clearly suggest that serum cholesterol, most of which is carried in LDL, is causally involved in atherogenesis.

Pectin, a polyanionic heterogeneous mixture of complex polysaccharides of high molecular weights, is predominantly composed of linear (1-5) linked galactopyranosyl uronic acid residues (23). Like pectic polysaccharides, glycosaminoglycans are also polyanionic polysaccharides containing alternating uronic acid (L-iduronic acid and/or D-glucuronic acid) and hexosamine (D-glucosamine or D-galactosamine) residues and, exclusive of hyaluronic acid, all glycosaminoglycans are sulphated. In atherosclerosis, the interaction of glycosaminoglycans with lipoproteins has been suggested as being involved in the mechanism of sequestering LDL at the endothelias surface (24). Evidence for complex formation between LDL and glycosaminoglycans in human aorta intimal layer and demonstration of a correlation between the severity of atherosclerosis and the amount of LDL present in intima has been presented by several investigators (25). This interaction between polyanionic glycosaminoglycans and the cationic protein moiety of lipoproteins appears to be dependent upon the electrostatic forces between the two macromolecules (26).

Based on our knowledge of the facts that (a) lipoproteins are carriers of cholesterol in the blood stream, (b) they are involved in atherogenesis, (c) pectin when supplemented in diet causes lowering of serum and/or liver cholesterol in man as well as a number of laboratory animals and (d) polyanionic glycosaminoglycans interact with lipoproteins, it was of interest to us to investigate the interaction of polyanionic pectin with lipoproteins in order to explain the biochemical basis by which pectin may cause lowering of serum/liver cholesterol levels.

To test this hypothesis, very low density lipoprotein (VLDL, d<1.0 gm/ml), low density lipoprotein (LDL, d=1.02-1.063) and high density lipoprotein (HDL, d=1.09-1.21) were isolated from outdated human plasma by ultracentrifugation according to established procedures (27,28), using potassium bromide for density adjustments and stored at -20° C in the presence of 20% sucrose before use. The purity of individual lipoprotein fractions thus obtained was established by polyacrylamide gel electrophoresis in sodium dodecyl buffer system (29) and filtration through a Sepharose 6B column, equilibrated with 0.2 M potassium bromide in 0.1 M sodium phosphate buffer, pH 7.2. Protein (30) and cholesterol

contents of various lipoprotein fractions were estimated according to established procedures.

Commercially available grapefruit (Citrus paradisi) pectin, a gift from Lykes-Pasco Packing Company, Dade City FL, was reprecipitated three times using 70% ethanol prior to study of the interaction of pectin with serum lipoproteins. In addition, pectin from grapefruit albedo was also extracted in our laboratory according to established procedures of Thornber and Northcote (32). The chemical composition of the two pectins was found to be similar. These pectin preparations contained galacturonic acid which accounted for 76-78% by weight of the total pectin. The remainder was accounted for by neutral sugar components, primarily galactose and arabinose and trace amounts of rhamnose, xylose, mannose and glucose.

Interaction of pectin with various lipoprotein fractions was studied according to established analytical techniques successfully utilized to study interaction of glycosaminoglycans with serum lipoproteins as revealed by the formation of insoluble complexes (33). Pectin and lipoprotein preparations were dialysed against appropriate buffer solutions and the interaction was studied over a wide range of pectin:lipoprotein ratio, pH and a variety of experimental conditions. To test tubes, each containing 0.5 mg of lipoprotein in 0.2 ml of buffer, was added variable amounts of pectin solutions and the final volume of reaction mixture was brought to 4.0 ml with appropriate buffer. After incubation of reaction mixture for 15 min at room temperature, the formation of insoluble complexes was assayed by recording absorbance at 680 nm. Test tubes containing buffer and pectin, or lipoprotein alone, were also run in parallel, to serve as controls.

Results obtained from these studies revealed that, of all the lipoprotein fractions tested, the formation of insoluble complexes with pectin was limited specifically to LDL. The pH optimum of the observed interaction was found between pH 4.3 and 5.2. The interaction between pectin and LDL was found to be optimal in 0.05 M phosphate buffer, and increase in the molarity of buffer inhibited the formation of complexes. Furthermore, presence of divalent cations such as Mg, Ca and Mn in concentrations above 1 mM in the reaction mixture caused the inhibition of formation of complexes between pectin and LDL.

Although several investigators have previously demonstrated the *in vitro* interaction of serum lipoproteins with glycosaminoglycans, to our knowledge the interaction between serum lipoproteins and dietary pectin has never before been investigated. The results obtained from the *in vitro* studies described here clearly suggest that pectin interacts specifically with serum LDL and this interaction appears to be electrostatic in nature. In addition, the observed interaction appears to be of potential significance since LDL is the major biological carrier of cholesterol and the principal ingredient of atherosclerotic lesions found in diseased cardiovascular tissue. The observed interaction assumes

additional significance when one considers our unsuccessful attempts described earlier to demonstrate the binding of bile salts to pectin as the only suggested biochemical basis by which dietary pectin may cause the lowering of cholesterol levels.

The conclusion reached in our laboratory that pectin interacts primarily with LDL, although of significance, is at best only preliminary. A number of questions remain unanswered. These include (a) Is the in vitro interaction between LDL and pectin of any physiological importance? (b) Is there a formation of soluble complexes between LDL and pectin under physiological conditions? (c) Of a number of polysaccharides found in pectin, which unique polysaccharide is primarily involved in the observed interaction? and (d) Could this observed interaction have the merit to explain the biochemical basis by which dietary pectin may cause lowering of serum cholesterol levels? A prerequisite to answering these questions is the availability of individual and highly pure and structurally defined polysaccharides found in citrus pectin. In addition, availability of radiolabelled pectic polysaccharides would greatly enhance our understanding of the problems and questions posed earlier. We have been able to achieve fractionation as well as labelling of several polysaccharides found in citrus pectin. These studies are described as follows.

Fractionation and Chemistry of Citrus Pectic Polysaccharides. Pectic polysaccharides, commonly known as pectin, appear early in plant cell-wall formation. A series of complex biochemical steps results in the formation of cell plates followed first by its growth in area (primary cell wall) then in thickness (secondary cell wall). Exclusive of randomly oriented cellulose fibrils, primary cell wall is composed mainly of pectic polysaccharides (34). These pectic polysaccharides are rich in D-galacturonic acid, D-galactose and L-arabinose residues. With growth in thickness of cell wall (secondary cell wall), there appears to be a replacement of pectic polysaccharide deposition with polysaccharides rich in D-glucuronic acid or 4-O-methyl-D-glucuronic acid, D-xylose and D-glucose rich polysaccharides.

Earlier studies (35) led to the belief that pectic polysaccharides were a complex mixture of three groups of polysaccharides, pectic acid containing chains of 1-4 linked D-galacturonic acid, a galactan containing chains of 1-4 linked β-D-galactopyranose residues, and a highly branched araban containing 1-5 and 1-3 linked L-arabofuranose residues. Later studies (36) on the chemistry of pectic polysaccharides found in sisal plant pointed out that pectic acid-like substances are not polymers of D-galacturonic acid alone. Neutral sugars, especially D-galactose, L-arabinose and L-rhamonse, are integral components of acidic polysaccharides and linked to galacturonic acid chains. Further studies (37) on the structure and analysis of pectic polysaccharides from Lucerne have shown that the main chain of pectic acid

polysaccharides was composed of 1-4 linked D-galacturonic acid residues. However, the isolation of a partial hydrolysis product, the aldobiuronic acid, 2-O-(β-D-galacturonic acid) L-rhamnose provided evidence that the L-rhamnose residues were linked glycosidically to D-galacturonic acid and that L-rhamnose residues may act as branching points. Studies on the structure and analysis of pectic polysaccharides from soybean colyledon meal (38) and lemon peel pectin (23) revealed that L-arabinose and D-galactose residues were also integrated in the framework of D-galacturonic acid and the chain of 1-4 linked D-galacturonic acid was interrupted frequently by L-rhamnose residues. Traces of 2-O-methyl-D-xylose and L-fucose were also found in the pectic polysaccharides obtained from these tissues. In addition to these neutral sugar components found in pectic polysaccharides described above, D-apiose, a natural 5-carbon branched sugar, is also found in the pectic polysaccharides found in plants of the family, Zosteraceae (39). Furthermore, the cell wall polysaccharides from a number of plant sources appear to be covalently linked to a hydroxy-proline rich protein, extensin (40), adding further additional complexity to the chemistry of cell wall associated pectic polysaccharides.

Classic methods in use to fractionate plant cell wall polysaccharides are mainly based upon the differential solubilities of various cell wall polysaccharide constituents (32). To solubilize pectic polysaccharides, the cell wall material is extracted with water, ammonium oxalate or disodium EDTA. The solubilization of pectic polysaccharides could then be followed by solubilization of hemicelluloses, i.e., polysaccharides rich in D-glucuronic acid 4-O-methyl-D-glucuronic acid, D-xylose and D-glucose residues. The solubilization of hemicellulosic polysaccharides is accomplished by treatment of water, ammonium oxalate or EDTA insoluble material with a strong alkali.

We have recently achieved extraction of pectic polysaccharides from grapefruit primarily based on a procedure described by Thornber and Northcote (26). Grapefruit albedo was cut into small pieces and homogenized using a Vitris 45 homogenizer in hot 80% (v/v) ethanol. The ethanol-soluble material was removed from the insoluble residue by centrifugation of homogenate at 2000 x g for 30 min. This extraction with ethanol was repeated three times, following which the insoluble residue was recovered and extracted by continuous shaking in 2 volumes of chloroform:methanol (1:1 v/v) for 2 days at room temperature. At every 24 h interval chloroform:methanol soluble material was removed by centrifugation and fresh chloroform:methanol was added to continue extraction. The resulting delipidated, depigmented and dehydrated cell wall fraction was then air-dried and weighed. Pectic polysaccharides were then extracted from this dried residue with continuous shaking in 0.2 M disodium EDTA for 24 h and then solubilized pectic polysaccharides were removed by centrifugation. This extraction procedure was repeated and the EDTA soluble fractions thus

obtained were pooled and dialyzed extensively against running deionized distilled water. To the dialyzed EDTA soluble fraction representing pectic polysaccharides was added enough 95% ethanol to bring the final concentration of ethanol to 80% which resulted in the precipitation of these pectic polysaccharides. The precipitation was allowed to continue for two days at room temperature. The precipitated polysaccharides were recovered by centrifugation at 15,000 rpm for 1 h, dissolved in water and lyophilized. From a grapefruit weighing approximately 121 g, 2.5 g of pectic polysaccharides were recovered. Chemical analysis of the pectic polysaccharides thus obtained revealed the presence of only D-galacturonic acid and which accounted for 76% by weight of the total polysaccharides. The remaining 24% was accounted for by neutral sugar components: galactose, arabinose and trace amounts of rhamnose, xylose, mannose and glucose. This chemical composition is similar to the pectic polysaccharides isolated from lemon peel (23).

Labelling of Pectic Polysaccharides. We have successfully achieved the labelling of pectic polysaccharides by using two methods described below:

(a) Biosynthetic Labelling of Pectic Polysaccharides. Myo-inositol has been shown to be the precursor of oronosyl and pentysyl units of cell wall polysaccharides found in grapefruit (42). Labelled myo-inositol-2-^{3}H (Amersham Searle, Inc.) was supplied to ripening grapefruit (the size of a golf ball) by placing the cut surface of fruit stem in a small vial containing the label. After the labelled myo-inositol had been taken up, distilled water was added to keep the cut fruit stem submerged, and the grapefruit was allowed to metabolize the label for a period of four days. The labelled grapefruit was then successively extracted with hot 80% ethanol, chloroform:methanol (1:1v/v) and finally with 0.2 M EDTA to solubilize pectic polysaccharides. These extraction procedures are described above in detail. Chemical analysis of the pectic polysaccharides following hydrolysis by colorimetric and gas chromatographic techniques, demonstrated the presence of D-galacturonic acid as the major component, constituting approximately 76% of the total sugars found in the pectin. The remaining 24% was consisted of rhamnose, arabinose, xylose, mannose, galactose, glucose at 16, 33, 13, 3, 31 and 2%, respectively, of the total neutral sugar components. It should be pointed out at this point that the chemical nature of these pectic polysaccharides was found to be identical with the pectic polysaccharides obtained from mature grapefruit albedo as decribed above.

The specific radioactivity of the labelled pectic polysaccharides thus obtained was about 8000 cpm/mg of polysaccharides, counting efficiency of ^{3}H being 30%. Of the total radioactivity incorporated, almost 50% was localized in the galacturonic acid

residues whereas the remainder was accounted for by arabinose (21%), galactose (21%) and xylose (8%) residues. Results from these studies clearly demonstrated that labelled myo-inositol may successfully be used to incorporate the label in pectic polysaccharides found in grapefruit.

(b) Labelling of Pectic Polysaccharides Using Galactose Oxidase Tritiated Potassium Borohydride. Although we did achieve the labelling of citrus pectic polysaccharides by supplying labelled myo-inositol to ripening grapefruit as described above, the technique was expensive and time-consuming. Therefore, an alternate method for labelling of pectic polysaccharides was developed (43). The merit of this method lies in its rapidity, specificity and yield of labelled pectic polysaccharides with high specific radioactivity.

It has been established by several investigators and summarized in the background section of this proposal that, although pectic polysaccharides are primarily composed of polymers of galacturonic acid, neutral sugars such as galactose, arabinose and rhamnose also make up a considerable portion of these heterogeneous polysaccharides. The nature of glycosidic linkage appears to be 1-4 for galacturonans and galactans and 1-5 for arabans, suggesting therefore that, like the carboxyl group of galacturonic acid, the primary alcohol group of galactose is free and not involved in the formation of glycosidic linkages between various sugar residues found in pectic polysaccharides. This knowledge was utilized in the experiments described here.

Successful labelling of primary alcohol groups of galactose residues was achieved by first treating commercially available grapefruit pectin (Lykes-Pasco, Dade City FL) reprecipitated in 80% of ethanol in our laboratory before use, or grapefruit pectin prepared in our laboratory as described above, with galactose oxidase (Sigma Chemical Co.) in order to enzymatically modify the primary alcohol groups of galactose residues to an aldehyde group followed by the reduction of aldehyde group thus formed back to primary alcohol group with the concomitant introduction of ^{3}H by using tritiated potassium borohydride (Amersham Searle, Inc.) as a reducing agent. Examination of labelled pectic polysaccharides demonstrated the presence of label in the galactose residues. Although the successful labelling of primary alcohol group of galactose and/or galactosaminoglycans of animal and/or bacterial origin has been achieved by several investigators by this method originally described by Morell et al (44), to our knowledge, the present technique is the first to describe the use of Morell's method in labelling pectic polysaccharides.

Fractionation of Pectic Polysaccharides. A number of complex polysaccharides differing in their physical and chemical characteristics are found in pectin. This heterogeneous nature of pectin has been reviewed earlier. In order to characterize these

various polysaccharides, a combination of a number of fractionation procedures such as anion-exchange chromatography, molecular sieve chromatography and moving-boundary electrophoresis were employed to achieve the fractionaton of pectic polysaccharides.

(a) Fractionation of Labelled Pectic Polysaccharides. In our laboratory, we have recently attempted the fractionation of labelled pectic polysaccharides. These labelled polysaccharides were obtained following administration of labelled myo-inositol to ripening grapefruit through the cut fruit stem, as described earlier. DE-52 diethyl aminoethyl cellulose ion exchanger was poured in a column (1.5x24 cm) and equilibrated with 0.025 M sodium phosphate buffer, pH 6.0. Labelled pectic polysaccharides (50 mg) having approximately 400,000 cpm were dissolved in 30 ml of equilibrating buffer and then loaded onto the column. The column was washed with 200 ml of equilibrating buffer and then eluted with a linear gradient ranging from 0.025 M to 0.5 M sodium phosphate, pH 6.0, buffer, 500 ml each. At the end of the gradient run, the column was finally washed with 200 ml of 1 M sodium phosphate buffer, pH 6.0. Fractions were collected and an aliquot from each fraction was assayed for radioactivity. The examination of results obtained from these studies suggests the presence of label in at least eight partially or completely resolved pectic polysaccharides. More than 95% of the total radioactivity loaded onto the column was recovered in these eluted pectic polysaccharides having different ion-exchange properties (42),

(b Fractionation of Commercially Available Pectins. In another series of experiments we have attempted fractionation of commercially available citrus pectin by employing a stepwise elution method. To achieve this, grapefruit pectin was subjected to chromatography using DE-52 cellulose. Elution of the column with increasing salt concentrations resulted in the resolution of four chemically distinct pectic polysaccharides. These polysaccharides, eluted sequentially with 0.025, 0.1, 0.25 and 0.5 M sodium phosphate buffer, pH 6.0, composed 13, 5, 62 and 20%, respectively, of the total pectin subjected to chromatography. No striking differences in the galacturonic acid content, which ranged from 70-80% of these polysaccharides, were observed. However, polysaccharides eluted sequentially with increasing salt concentrations showed a noticeable decrease in the degree of methylation of their galacturonic acid residues. In addition, the neutral sugar composition of these individual pectic polysaccharides was different. Of interest was the neutral sugar composition of polysaccharides eluted with the highest salt concentration. This highly acidic polysaccharide was also high in rhamnose content. The results obtained from these studies affirm the heterogeneous nature of pectin, and suggest the presence of a unique, rhamnose-rich polysaccharide in grapefruit pectin.

Biochemical Basis of Observed Interaction Between Pectin and Lipoprotein. These studies await further research. A prerequisite to these studies is the establishment of the chemistry and structure of various polysaccharides found in citrus pectin. As described above, these studies are in progress and will eventually lead to the isolation of several pure citrus pectic polysaccharides of defined chemistry.

In our study, formation of isoluble complexes between pectin, a heterogeneous mixture of a number of neutral and acidic polysaccharides, and lipoprotein was studied. The basic limitation with the formation of insoluble complexes is that it is difficult to quantitate the said interaction. Furthermore, the observed interaction between pectic polysaccharides and lipoprotein is at a pH which is not physiological. We, therefore, are attempting to study this interaction under physiological conditions and by use of buffer systems which are devoid of cations, in order to facilitate formation of soluble complexes. In addition, by using labelled pectic polysaccharides, studies resulting in the elucidation of kinetics, specificity and nature of the interaction between labelled pectic polysaccharides and lipoprotein will be performed.

The specificity of the interaction will be determined with regard to a single labelled pectic polysaccharide by studying the competitive ability of other unlabelled pectic polysaccharides of known characteristics on the formation of labelled complexes. The data obtained from these studies will also be evaluated to determine the influence of change in molecular weight and/or structure of individual pectic polysaccharides on the binding to low density lipoprotein. Similar experiments will also be carried out to study the competitive inhibition of well established interactions between low density lipoprotein and various glycosaminoglycans caused by pectic polysaccharides. The observation of inhibition of interaction between lipoprotein and glycosaminoglycans by pectic polysaccharides may suggest, at least *in vitro*, the possibility of a basis by which dissolution of atherosclerotic plaques could be achieved by pectic polysaccharides.

Our preliminary experiments suggest that, like the interaction between glycosaminoglycans and lipoproteins, the interaction between pectin and lipoprotein is also caused by the electrostatic attraction between polyanionic pectin and cationic lipoprotein. To elucidate this hypothesis, the charge profile of lipoproteins will be altered chemically and the influence of these alterations on the interaction with pectic polysaccharides at optimum experimental conditions will be determined.

The significance of the observed interaction between pectin and LDL, and further biochemical elucidation of this interaction, has direct relevance to the etiology and/or cure of atherosclerosis. The role of dietary pectin in lowering of serum and liver cholesterol levels is well established. However, the biochemical basis by which dietary pectin, which is composed of

a number of complex polysaccharides, causes this lowering of cholesterol levels remains elusive. These studies will provide an answer to this problem. In addition, these studies may result in the identification of a unique and biologically active polysaccharide found in pectin, which may be solely responsible for lowering of cholesterol levels. Furthermore, the results obtained from these studies may provide us with clues to achieve dissolution of atherosclerotic plaques, since LDL is the major ingredient of these plaques and pectic polysaccharides interact with the LDL.

Acknowledgements

We are deeply grateful to the Florida Department of Citrus for grants supporting this research. The authors also wish to acknowledge the research efforts of Drs. Edward W. Nelson, Ralph T. Guild, T. J. Merimme, R.R. Streiff, R. Waldman and R. Ganguly. The expert technical assistance of Mr. Charles W. Burgin throughout the course of this research is deeply acknowledged and appreciated. Special mention should be made to Dr. L. E. Cluff, formerly, Professor and Chairman of the Department of Medicine, University of Florida College of Medicine. It was through his untiring efforts and initial direction that this research program was begun.

Literature Cited

1. Nelson, E.W.; Lane, H.; Fabri P.J.; Scott B. J. Clin. Pharmacol., 1978, 18, 325.
2. Nelson, E.W.; Streiff, R.R.; Cerda, J.J. Am. J. Clin. Nutr., 1975, 28, 1014.
3. Stevenson, N.R. Gastroenterology, 1974, 67, 952.
4. Streiff, R.R. Am. J. Clin. Nutr., 1971, 24, 1390.
5. Nelson, E.W.; Cerda, J.J.; Wilder, B.J.; Streiff, R.R. Am. J. Clin. Nutr., 1978, 31, 82.
6. Fabri, P.J.; Scott, B.; Nelson, E.W. Lab. Anim. Sci., 1977, 27, 687.
7. Nelson, E.W.; Lane, H.; Cerda, J.J. J. Nutr., 1976, 106, 1433.
8. Lantz, E. Bull. Agric. Exp.Sta.N.M.Coll.Agric., 1939, 263, 3.
9. Siegel, L.; Melnick, D.; Oser, B.L. J. Biol. Chem., 1943, 149, 361.
10. Nelson, E.W.; Burgin, C.W.; Cerda, J.J. J. Nutr., 1977, 107, 2128.
11. Consolazio, C.F.; Matoush, L.O.; Nelson, R.A.; Harding, R.S.; Canham, J.R. J. Nutr., 1963, 79, 407.
12. Schwartz, I.L.; Thaysen, J.H. J. Clin. Invest., 1956, 31, 114.
13. Lane, H.W.; Roessler, G.S.; Nelson, E.W.; Cerda, J.J. J. Nutr., 1978, 31, 838.
14. Lane, H.W.; Cerda, J.J. J. Am. Diet. Assoc., 1978, 73, 64.
15. Pauling, L. Am. J. Clin. Nutr., 1971, 24, 1294.
16. Ganguly, R.; Waldman, R.H. Ind. J. Med. Res., 1977, 66, 359.

17. Kay, R.M.; Truswell, A.S. Am. J. Clin. Nutr., 1977, 30, 171.
18. Fisher, H.; Griminger, P.; Weiss, H.S.; Siller, W.G. Science, 1973, 146, 1063.
19. Leveille, G.A.; Sauberlich, J. Nutr., 1966, 88, 209.
20. Kritchevsky, D.; Story, J.A. J. Nutr., 1974, 104, 458.
21. Tall, A.R.; Small, M.S. N. Engl. J. Med., 1979, 299, 1232.
22. Goldstein, J.L.; Brown, M.S. Curr. Top. Cell. Regul., 1976, 11, 147.
23. Aspinal, G.O.; Craig, J.W.T.; Whyte, J. Carbohydr. Res., 1968, 7, 442.
24. Day, C.E.; Powell, J.R.; Levy, R.S. Artery, 1975, 1, 126.
25. Srinivusan, S.R.; Radha Krishnamurthy, R.; Barenson, G.S. Arch. Biochem. Biophys., 1975, 170, 334.
26. Nakashima, Y.; DiFerrante, N.; Jackson, R.L.; Pownall, H.J.; J. Biol. Chem., 1975, 250, 5386.
27. Havel, R.J.; Eder, H.A.; Bragdon, J.H. J. Clin. Invest., 1955, 34, 1345.
28. Mahley, R.W., Weisgraber, K.H.; Innerarity, T. Biochemistry, 1974, 13, 1984.
29. Weber, K.; Osborn, M. J. Biol. Chem., 1969, 244, 4406.
30. Campbell, P.N.; Sargent, J.R. Tech. Prot. Biosyn., 1967, 1, 65.
31. McDougal, D.B.; Farmer, H.S. J. Lab. Clin. Med., 1957, 50, 485.
32. Tornber, J.P.; Northcote, D.H. Biochem. J., 1961, 81, 455.
33. Day, D.E.; Voet, R.L.; Levy, R.S. FEBS Letter, 1970, 7, 41.
34. Thornber, J.P.; Northcote, D.H. Biochem. J., 1961, 81, 449.
35. Hirst, E.L.; Jones, J.K.N. Adv. Carbohydr. Chem., 1946, 2, 235.
36. Aspinal, G.O.; Rodriquez, A.C. J. Chem. Soc., 1958, 4020.
37. Aspinal, G.O.; Franshawe, R.S. J. Chem. Soc., 1961, 4215.
38. Aspinal, G.O.; Hunt, K.; Morrison, I.M. J. Chem. Soc., 1967, 1080.
39. Ovadova, R.G.; Vaskovsky, V.E.; Ovadova, Y.S. Carbohydr. Res., 1967, 6, 328.
40. Lamport, D.T.A. Annu. Rev. Plant. Physiol., 1970, 21, 235.
41. Loewus, F.A. Ann. N. Y. Acad. Sci., 1969, 165, 577.
42. Baig, M.N., Burgin, C.W.; Cerda, J.J. Phytochemistry, 1980, (In Press)
43. Baig, M.M.; Cerda, J.J. Anal. Biochem., 1979, 98, 429.
44. Morell, A.G., Van Den Hamer, D.J.A.; Scheinberg, I.H.; Ashwell, G. J. Biol. Chem., 1966, 241, 3745.

RECEIVED May 22, 1980.