The Degree of Methylation Influences the Degradation of Pectin in the Intestinal Tract of Rats and In Vitro

Gerhard Dongowski,*2 Angelika Lorenz† and Jürgen Proll**

*Department of Food Chemistry and Preventive Nutrition, †Department of Gastrointestinal Microbiology, and **Department of Biochemistry and Physiology of Nutrition, German Institute of Human Nutrition Potsdam-Rehbrücke, D-14558 Bergholz-Rehbrücke, Germany

ABSTRACT We investigated the degradation, metabolism, fate, and selected effects of pectin in the intestinal tract of rats. Conventional and germfree rats were fed for 3 wk diets containing 6.5% pectin (degree of methylation 34.5, 70.8 and 92.6%, respectively) or pectin-free diets. Pectin passes the small intestine as a macromolecule. The molecular weight distribution of pectins isolated from intestinal contents of germfree rats were unaffected by diet. No or very little galacturonan was found in cecum, colon or feces of most of the conventional rats. In colon contents of some conventional rats, di- and trigalacturonic acid were present. Total anaerobic and Bacteroides counts were greater in groups fed pectin. The concentration of short-chain fatty acids (SCFA) was higher in cecum and feces in all pectin-fed groups. With increasing degree of methylation, the formation rate of SCFA decreased in the cecum of conventional rats. During in vitro fermentation of pectin with fecal flora from rats, unsaturated oligogalacturonic acids appeared as intermediate products. Low-methoxyl pectin was fermented faster than high-methoxyl pectins in vivo and in vitro. Pectin-fed rats had greater ileum, cecum and colon weights. We conclude that structural parameters of pectin influence its microbial degradation in the intestinal tract.

KEY WORDS: pectin • degree of methylation • rats • intestinal tract • fermentation • short-chain fatty acids

Pectin occurs in the human diet in different forms: as a constituent of plant cell walls (fruits, vegetables and products of them) or in isolated form (for instance in jams, jellies or milk products). In contrast to most of the other dietary fibers (DF), the homogalacturonan component of pectin consists of an acidic moiety, the galacturonic acid (GalA) joined in chains by α-(1→4) glycosidic linkages. The carboxyl groups of the GalA moieties are partly esterified by methanol. An important feature of homogalacturonans is the degree of methylation (DM), defined as the number of moles of methanol per 100 mol GalA. Many functional properties of pectin (behavioral gelation, binding or metal ions) are dependent on its structural parameters like molecular weight, DM or distribution of free and methoxylated carboxyl groups within the galacturonan chains. It was shown that some physiological effects like interactions with bile acids (1,2) or drugs in vitro (3) may be influenced by pectin structure.

In the small intestine, physiological effects of pectin (interactions with bile acids, lowering of serum cholesterol, effects on the postprandial lipemia, etc.) are closely related to its macromolecular status. Pectin is not depolymerized by intestinal enzymes; however, a partial degradation seems to be possible under the physicochemical conditions of the stomach and small intestine. Pectin is more or less completely fermented by the microflora in cecum and colon of conventional (normal) rats (4) or in the colon of humans (5). This degradation is a multistep process: depolymerization under formation of oligomeric and monomeric GalA, fermentation of the monomers preferentially via the Embden-Meyerhof-Parnas and the pentose phosphate pathways (6) and formation of the SCFA acetate, propionate, butyrate, and valerate as well as of different gases as end products. Among these, butyrate plays an important role in the energy metabolism and proliferation of colonocytes (7). As a result of fermentation of DF such as pectin, the qualitative and quantitative composition of the microflora (germ numbers and bacteria species) may be changed. Therefore, the determination of the production of SCFA and composition of microflora as well of the presence and disappearance of pectin gives information on its fate in the gastrointestinal tract. Furthermore, it is interesting to evaluate the intermediate formation of the oligogalacturonic acids (OligoGalA). The key enzyme in the degradation of pectin by the intestinal microflora is pectate lyase, which splits the α-(1→4) glycosidic bonds between the GalA units by a β-eliminative mechanism under generation of 4,5-double bonds in the nonreducing end of the cleaved substrate (unsaturated OligoGalA).

To be absorbed, pectin has to be degraded to GalA or its...
oligomers with a low degree of polymerization (DP). It is unclear whether OligoGalA appears as stable metabolites of pectin degradation in detectable concentrations in the colon of humans or cecum and colon of conventional rats. In contrast, intravenously applied or directly injected OligoGalA in the cecum was found in the urine of rats (8). It was shown that pectin and its oligomeric degradation products influence incorporation and excretion of lead in subchronic lead exposed rats (9). Rhannogalacturonan II a complex branched pectic polysaccharide can interact with lead ions in vivo (10). Furthermore, DF such as pectin stimulate intestinal microbial polyamine synthesis in rats (11). In contrast to conventional rats, intestinal microflora are absent in germfree rats. Therefore, microbial degradation of dietary fibers like pectin is not possible in this animal model. The combination of the two animal models allows a more detailed study of the fate of pectin in the ileum, cecum and colon.

In this study, the degradation, metabolism, and selected effects of pectin in the intestinal tract of conventional and germfree rats as well as in in vitro experiments were investigated. We especially studied the effect of the degree of methylation of pectin on these processes.

MATERIALS AND METHODS

Pectins. High- and low-methoxyl citrus pectins (H and L) without additives were obtained from Copenhagen Pectin A/S (Lille Skensved, Denmark) and purified by treatment with acidic aqueous ethanol. The DM of these macromolecular preparations was 70.8% and 34.5%, respectively. For preparation of the very highly methoxylated pectin V, the high-methoxyl pectin H was further methoxylated using initially 800 ml methanol/40 ml concentrated H2SO4 (6 d) per 100 g pectin and then 1 L methanol/37.5 ml concentrated H2SO4 (6 d) at 4°C. Finally, pectin V was washed repeatedly with 80% ethanol and dried.

Characterization of pectin. The galacturonan content of the pectin and OligoGalA preparations was determined by the m-hydroxybiphenyl method (12). Methyl ester groups were analyzed by the chromotropic acid method (13). The intrinsic viscosity [η], which is empirically related to the molecular weight by the Mark-Houwink relation, was determined in 0.155 mol/L NaCl (high-methoxyl pectins) or in 0.05 mol/L NaCl/0.005 mol/L sodium oxalate (low-methoxyl pectins) at 25.0°C and pH 6.0 using an Ubbelohde viscosimeter.

The molecular weight distribution of the pectins was determined by gel-permeation chromatography on a Shodex OHpak B 805 column (8 x 500 mm) from Showa Denko K.K. (Kawasaki, Japan; conditions: 3.3 mPa; 50°C; flow 0.4 ml/min; phosphate buffer, pH 6.5) using differential-refractometer/viscometer detection (Knauer, Neufahrn, Germany) in a concentration of 1% galacturonan. The refractive index (RI) detection corresponds to the concentration and the viscosity detection gives a measure of the molecular weight distribution of the pectins.

For calibration of the method, a pectin series (DM 72.0%) prepared mechanoanalytically by vibration milling (up to 100 h) was applied. This technique allows the variation of molecular weight of polysaccharides in dry state without variation in DM (14).

Animals. Male conventional rats (strain: Shoe:Wist) weighing 202 ± 11 g were obtained from Tierzucht Schönwalde GmbH (Schönwalde, Germany). The rats were housed individually in temperature and humidity controlled cages (22 ± 2°C and 55 ± 5%) on a normal light cycle (0600 to 1800 h, light; 1800 to 0600 h, dark).

Adult germfree male rats of the inbred strain AVN/Ipcw-Wistar (Rehbrücke) weighing ~270 g were obtained from the Germfree Animal Unit of the German Institute of Human Nutrition Potsdam-Rehbrücke. The animals were maintained in positive-pressure isolators (Metall und Plastik, Radolfzell, Germany) and housed individually in polycarbonate cages on irradiated wood chips (Altromin, Lage, Germany) at 22 ± 2°C and 55 ± 5% humidity on a normal light cycle (0600 to 1800 h, light; 1800 to 0600 h, dark).

TABLE 1

Composition of control and experimental diets

<table>
<thead>
<tr>
<th>Component</th>
<th>Diets C and C#</th>
<th>Diets L and L#</th>
<th>Diets H and H#</th>
<th>Diets V and V#</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein1</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Wheat starch2</td>
<td>830</td>
<td>546.6</td>
<td>530.8</td>
<td>542.6</td>
</tr>
<tr>
<td>Pectin3</td>
<td>0</td>
<td>83.4</td>
<td>99.2</td>
<td>87.4</td>
</tr>
<tr>
<td>Microcrystalline cellulose4</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Sunflower oil5</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin mixture6</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mineral mixture7</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

1 Dauermilchwerk Peiting GmbH, Landshut, Germany.
2 Keller und Strauss, Berlin, Germany.
3 Low-methoxyl (L) and high-methoxyl pectin (H) from Copenhagen Pectin A/S (Lille Skensved, Denmark). Very highly methoxylated pectin V was prepared from pectin H. Pectins L#, H#, and V# are γ-irradiated (20 kGy). Pectin concentration: 65 g galacturonan/kg diet.
4 Bettenmeier GmbH, Ellwangen, Germany.
5 Thomy GmbH, Karlsruhe, Germany.
6 Composition of the vitamin mixture (in mg/kg diet): retinol palmitate, 7; cholecalciferol, 0.02: α-tocopherol acetate, 240; menadione, 15; thiamine, 30; riboflavin, 30; pyridoxine, 22.5; cyanocobalamin, 0.05; niacin, 75; pantothenic acid, 75; folic acid, 15; biotin, 0.03; choline, 1500; L-aminooxyacetic acid, 150; myo-inositol, 150 (Altromin GmbH, Lage, Germany).
7 Composition of the mineral mixture (in mg/kg diet): Ca, 9300; P, 7300; K, 7100; Na, 4400; Cl, 3600; S, 1700; Mg, 800; Fe, 200; Mn, 100; Zn, 30; Cu, B; F, 4; I, 0.4; Se, 0.2; Co, 0.1 (Altromin GmbH).

Diets. The pelleted control Diet C and the experimental diets, H, and V prepared in our institute are characterized in Table 1. Diets for the experimental groups contained 6.5% pectin (galacturonan).

For the experiments with germfree rats, the diets C#, L#, H#, and V# prepared in our institute are characterized in Table 1. The rats were fed the control diets C or C# for 7 d after arrival. Then the rats were randomly divided into five groups (conventional) or four groups (germfree) and assigned to control diets or to the experimental pectin-containing diets. The rats had free access to food and water.

Sampling procedures. Rat growth and food intake were determined weekly. Complete feces were collected within two periods (A: d 8–10; B: d 18–20). At the end of the experiments, the contents of ileum, cecum and colon as well as the intestinal parts were prepared for analysis. A part of the intestinal contents and feces were immediately freeze-dried. These lyophilized materials were treated for inactivation of the enzymes for 20 min at 75–80°C in 5 ml of 96% ethanol. After addition of 5 ml of water, the mixtures were stirred for 30 min and centrifuged at 6000 x g for 30 min. In the supernatant, galacturonan was estimated by the m-hydroxybiphenyl method. Concentration and spectrum of OligoGalA were determined by high-performance thin-layer chromatography (HPTLC) (15) on silica gel after extraction with 50% aqueous ethanol. In the dried residues, the content of total galacturonan and the DM were estimated after extraction with 0.5% aqueous EDTA (pH 6.0). The extracted and purified pectins (0.5% galacturonan in phosphate buffer, pH 6.5) were characterized by gel-permeation chromatography.

Determination of SCFA. For estimation of SCFA using gas-chromatography (GC), suspensions prepared from fresh feces and intestinal contents were mixed with water (material/water 1:5; wt/v) and homogenized, and the dry matter was determined. After centrifugation, 50 μL of iso-butylate (internal standard), 280 μL of 0.36 mol/L perchloric acid solution and 270 μL of 1 mol/L potassium hydroxide solution were added to 100 μL of the supernatant. The freeze-dried samples were homogenized in a mixture of 200 μL of 5 mol/L formic acid and 800 μL of acetone. One microliter of the supernatant after centrifugation was assayed on a 25 m x 0.32 mm (i.d.) Carbowax 20M column using a temperature program. The GC system (Hewlett-Packard, Waldbronn, Germany) consisted of a HP
Microbiology. The numbers of viable cells were determined using 0.5-g samples of fresh feces for serial dilution and subsequent plating on selective media and Columbia blood agar followed by aerobically or anaerobically incubation at 37°C. Plates were incubated anaerobically in an atmosphere with 10% CO₂, 10% H₂, and 80% N₂. The following media and incubation models were applied: Endo agar (Merck) aerobically for Enterococci and anaerobically for Escherichia coli and Enterobacteriaceae, MRS agar (Merck) aerobically for Lactobacillus spp., Columbia blood agar (Merck) aerobically for aerobic bacteria and Columbia blood agar for anaerobes, Columbia blood agar (Merck) with thioglycolate broth (Merck) at 37°C for 6 d. Sterility was proved by visual test and plating on Columbia blood agar (Merck) aerobically and anaerobically.

In vitro fermentations. Pectin media (150 mL; 0.5% galacturonan) were incubated with 4 g rat fecal flora at 37°C without aeration. The contents of macromolecular pectin and OligoGalA as well as of SCFA were estimated in the culture after different periods (16). OligoGalA were determined using the HPTLC system from Camag (Muttenz, Switzerland) on silica gel 60 developed with n-propanol-water (7 + 3.75) and (7 + 2.75). The spots were detected by measuring at 235 nm and after dipping in the m-hydroxybiphenyl reagent at 525 nm. The system was calibrated with a mixture of OligoGalA (DP 2–15) prepared from pectic acid using pectate lyase from Erwinia carotovora (15).

Statistical analysis. Results are expressed as mean values and SD. Statistical significance was determined using one-way ANOVA followed by Student’s t-test. Differences of P < 0.05 were considered significant.

Ethical considerations. The experimental protocol was performed according to international and national guidelines. All treatments and diets were formally approved by the Animal Welfare Committee of the State Brandenburg (Ministry of Nutrition, Agriculture, and Forestry, Germany).

RESULTS

Pectin preparations. The original pectin preparations used are characterized in Table 2. Chemical esterification (methylation) of pectin H resulted in an increase of DM to 92.6% (pectin V). The intrinsic viscosities [η] of the pectins L, H, and V were 395, 692, and 421 mL/g galacturonan, respectively.

For calibration of the gel-permeation chromatography, pectins from a vibration milling series with known intrinsic viscosities [η] were used. With decreasing [η] (i.e., with decreasing molecular weight), the maximum of the viscosity distribution shifted toward higher retention volumes with decreasing specific viscosity. Because of the very high viscosity of the pectins with the highest molecular weights within the calibration series used, it was necessary to reduce the concentration of galacturonan during the chromatographic measurements from the normally applied 0.5% galacturonan to 0.3% (pectin with [η] 453 mL/g), 0.15% (pectin with [η] 720 mL/g) or 0.1% (pectin with [η] 1178 mL/g) galacturonan, respectively (Fig. 1). Galacturonic acid gave a very low viscosity peak at a relatively high retention volume. The corresponding concentration chromatograms (0.5% galacturonan) showed an increasing signal at a retention volume of ~15.5 mL with decreasing molecular weight of the galacturonan, whereas the concentration in the range of retention volume between 9 and 13 mL was diminished continuously.

The viscosity distributions and concentration chromato-
grams of the original pectin L, H and V, used in the diets are shown in Figure 2. Peak maxima (Table 2) and areas under the curves of the viscosity distributions (not shown) corresponded closely to the intrinsic viscosities $[\eta]$ of the original pectins.

**Influence of irradiation on pectin.** It is necessary to use sterile diets for the germfree rats. Heating at temperatures $\geq 120^\circ$C resulted in a strong depolymerization and destruction of pectin. Therefore, $\gamma$-irradiation was used to sterilize the diets. In a model experiment, the effects of the irradiation dose (9–24 kGy) on properties of pectins and the microbial counts were evaluated. With increasing irradiation dose, the intrinsic viscosities $[\eta]$ (and, therefore, the molecular weights) of the pectins were reduced (for instance from 395 to 173 mL/g galacturonan; low-methoxyl pectin). This effect was accompanied by a decrease in the viscosity of the aqueous solutions of the pectins. In contrast, the DM of the pectin preparations was not altered. After irradiation with 9 kGy, microbial growth was found in only 1 of 15 samples. No counts were detected in all pectin samples irradiated with $\geq 12$ kGy (results not shown). We, therefore, decided to irradiate the diets for germfree rats in the following experiments with 20 kGy. This treatment resulted in a partial depolymerization of pectin. The intrinsic viscosities $[\eta]$ of the pectins L#, H# and V# isolated from the irradiated diets were 284, 446 and 267 mL/g galacturonan, respectively (Table 2). Irradiation of pectin alone or as a component of the diet resulted in very similar effects on the molecular weight.

**Food intake, weight gain, and behavior of the rats during the experiments.** All diets were well accepted by the rats. Treatment related changes in appearance or behavior of the rats were not observed. The rats appeared to be healthy during the experiment. No mortality was caused by the administration of pectin preparations.

The weight gain and the food intake per day of conventional rats were not different in groups fed the pectin-containing diets and the control diet throughout the 21-d experiment (Table 3). Furthermore, the feed efficiencies (g gain/g feed) did not differ among groups. Overall, after an adaptation period of 7 d (to the control diet), partial substitution of wheat starch by the pectins did not affect body weight or feed efficiency.

The body weight of the germfree rats increased by $\sim 15$ g during the experiment without differences among groups. In wk 1, the food intake was $9.1 \pm 1.9$ g/d in the control and $11.9 \pm 0.7$ to 12.0 $\pm 0.8$ g/d in the pectin-fed groups. In wk 3, the food intake was higher: $12.9 \pm 3.0$ g/d in the controls and $12.9 \pm 1.1$ to $13.9 \pm 2.8$ g/d in the pectin-fed groups. In summary, the food consumption tended to be higher in germfree rats when the diets were enriched with pectin, perhaps a result of the reduced amount of available energy in these diets.

**Pectin in the intestinal tract of conventional rats.** Pectin was isolated from the intestinal contents (ileum, cecum, and colon) and feces. Beside estimation of contents of galacturonic acid and DM, the molecular weight distribution of the extracted pectins was determined.

In the ileum contents, approximately 30 mg galacturonan (average values) were found in the pectin-fed groups (Table 4). Additionally, up to 7% low-molecular galacturonan was present in the fraction soluble in 50% ethanol. The DM was not different from the original pectins (Table 2). The following DM were found in the pectins extracted from ileum contents: 34.1% (pectin L), 70.6% (pectin H), and 91.6% (pectin V). The isolated pectins were macromolecular (Fig. 3). The maximum of the viscosity distribution appeared at a retention volume of $\sim 13$ mL. This showed that the depolymerization of the pectins was relatively low in ileum. OligoGalA were not present in the ileum contents. In cecum and especially in colon contents, no or only very little galacturonan was found in most of the conventional rats (Table 4). Surprisingly, in one or two rats of each group, galacturonan was present even in the lower parts of gastrointestinal tract.

In both sampling periods, no galacturonan was found in the 24-h fecal samples (Figure 4, curves 1 and 2). In some fecal samples, small amounts of highly degraded pectin were present, especially in the group fed the highest methoxylated pectin (curve 3). It should be taken into consideration that in contrast to all other gel-permeation chromatographic determinations of intestinal contents and feces materials, 1% dry matter was applied here for analysis (instead of 1% galacturonan).

**TABLE 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial weight</th>
<th>Weight gain$^2$</th>
<th>Food intake$^2$</th>
<th>Feed efficiency$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$g$</td>
<td>$g/d$</td>
<td>$g$ gain/g feed</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>266.5 $\pm$ 6.0</td>
<td>3.49 $\pm$ 0.80</td>
<td>16.48 $\pm$ 1.60</td>
<td>0.21 $\pm$ 0.03</td>
</tr>
<tr>
<td>L</td>
<td>272.3 $\pm$ 7.6</td>
<td>3.00 $\pm$ 0.89</td>
<td>15.53 $\pm$ 2.07</td>
<td>0.19 $\pm$ 0.04</td>
</tr>
<tr>
<td>H</td>
<td>270.3 $\pm$ 11.1</td>
<td>3.03 $\pm$ 0.42</td>
<td>16.18 $\pm$ 1.29</td>
<td>0.19 $\pm$ 0.02</td>
</tr>
<tr>
<td>V</td>
<td>268.3 $\pm$ 12.7</td>
<td>2.96 $\pm$ 0.77</td>
<td>16.12 $\pm$ 2.03</td>
<td>0.18 $\pm$ 0.04</td>
</tr>
<tr>
<td>H#</td>
<td>275.0 $\pm$ 12.3</td>
<td>2.43 $\pm$ 0.93</td>
<td>15.71 $\pm$ 2.55</td>
<td>0.16 $\pm$ 0.04</td>
</tr>
</tbody>
</table>

$^1$ Values are means $\pm$ SD, $n = 8$–10.

$^2$ During the 21-d experiment.

$^3$ Pectin concentration: 65 g galacturonan/kg diet.
OligoGalA was not detected in any ileum and cecum contents nor in any fecal samples of conventional rats. However, unsaturated di- and trigalacturonic acids were found in the colon contents of three rats using HPTLC. In Figure 5, a typical chromatogram is shown in comparison to an OligoGalA standard prepared from pectic acid by action of pectate lyase from *E. carotovora*.

**Pectin in the intestinal tract of germfree rats.** The irradiated pectin preparations L#, H# and V# showed their viscosity maxima at a retention volume between 12.5 and 13.6 mL (Table 2).

The DM of pectin was not changed in the upper part of gastrointestinal tract or in the cecum or colon of germfree rats. For instance, the following DE were found in the pectins isolated from the colon contents (n = 4): 34.6 ± 0.3% (pectin L#), 70.7 ± 0.6% (pectin H#), and 89.8 ± 0.3% (pectin V#). In all intestinal contents and additionally in the feces of the germfree rats, high amounts of galacturonan were present, especially when the very highly methoxylated pectin was fed. In cecum, significantly higher concentrations of pectin were found when the very highly methoxylated pectin V# was present in the diet (Table 4). The reason for this effect is not yet clear.

The pectins isolated from the intestinal contents or feces essentially were not depolymerized. Therefore, the molecular weight distribution of the pectins extracted from ileum or from cecum were relatively unaffected by diet. In Figure 6, the

### TABLE 4

<table>
<thead>
<tr>
<th>Pectin3</th>
<th>Conventional rats</th>
<th>Germ-free rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ileum</td>
<td>Cecum</td>
</tr>
<tr>
<td>L or L#</td>
<td>33.4 ± 5.3</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>H or H#</td>
<td>29.0 ± 4.7</td>
<td>13.2 ± 22.3</td>
</tr>
<tr>
<td>V or V#</td>
<td>28.8 ± 10.8</td>
<td>34.4 ± 35.0</td>
</tr>
</tbody>
</table>

---

1. Values are means ± SD, n = 4–10 (conventional rats) or n = 4–5 (germ-free rats). Means in a column without a common letter differ, *P* < 0.05.
2. After the 21 d experiment.
3. Pectin concentration: 65 g galacturonan/kg diet.
4. A, d 8–10; B, d 18–20.
viscosity distributions and concentration chromatograms of the pectins isolated from the ileum, cecum, colon and feces of one germfree rat fed the very highly methoxylated pectin are combined. Small differences appeared only in the low-molecular weight portion of the concentration chromatogram.

**Formation of SCFA.** In cecum contents of the control group, total SCFA concentration was 38.6 μmol/g dry matter. In rats fed pectin, 88.7–112.4 μmol SCFA/g dry matter were found (Table 5). Although differences in concentration of the SCFA formed were not always significant, more SCFA generally were produced from low-methoxyl pectin as substrates. During the fermentation period, the amounts of macromolecular pectin and of total pectin diminished continuously. In contrast, the low-molecular pectin fraction consisting of OligoGalA was increased at first and decreased later (Table 7). A relatively broad spectrum of OligoGalA (DP 2–8) appeared (Table 8). The amount and composition of the oligomers changed continuously. Only di-, tri- and tetragalacturonic acids were present after 24 h incubation. All OligoGalA found were unsaturated, meaning that they had double bond on the nonreducing end of their molecules. This was shown by a detection method that is specific for double bonds in galacturonans. The spectrum of unsaturated OligoGalA formed from pectin is typically for action of pectate lyase (EC 4.2.2.2). These results are in good agreement with in vitro fermentation experiments using human fecal flora and pectins with different DM (15).

As expected, only very small concentrations of acetate were detected in intestinal contents and feces of germfree rats.

**Germ numbers.** Microbial counts were estimated on d 11 and 21 of the experiment using fresh fecal flora from five rats of each group. Results are summarized in Table 6 (in log colony-forming units/g wet feces). In all experimental groups fed diets containing macromolecular pectins, significantly higher counts of total anaerobes were found. This effect was especially true for the increase in *Bacteroides* spp., but there were no differences among rats fed the various pectin preparations. For *Coliforms*, *Enterococcus* and *Lactobacillus* numbers, there were no or minimal effects of the pectins.

**In vitro fermentation.** Insights into the mechanisms occurring during fermentation of pectin by the intestinal microflora can be obtained by in vitro techniques. We used fresh fecal flora from rats of the control group and those fed low-methoxyl pectin as substrates. During the fermentation period, the amounts of macromolecular pectin and of total pectin diminished continuously. In contrast, the low-molecular pectin fraction consisting of OligoGalA was increased at first and decreased later (Table 7). A relatively broad spectrum of OligoGalA (DP 2–8) appeared (Table 8). The amount and composition of the oligomers changed continuously. Only di-, tri- and tetragalacturonic acids were present after 24 h incubation. All OligoGalA found were unsaturated, meaning that they had double bond on the nonreducing end of their molecules. This was shown by a detection method that is specific for double bonds in galacturonans. The spectrum of unsaturated OligoGalA formed from pectin is typically for action of pectate lyase (EC 4.2.2.2). These results are in good agreement with in vitro fermentation experiments using human fecal flora and pectins with different DM (15).
The content of SCFA which are typical end products of fermentation of dietary fibers increased continuously during the fermentation period (Table 7). Acetate was also the predominant SCFA in the in vitro fermentation (≥80%). Propionate was present at 10–13 mol/100 mol. The proportion of butyrate increased from 3.2 to 8.5 mol/100 mol during the fermentation period.

Weights of intestines and intestinal contents. The presence of more viscous DF in the intestine resulted not only in effects on the microflora but may also in direct and indirect effects on the intestines itself. As an example for such effects, the weights of the intestines were determined in the rats at the end of the experiment. The walls of the ileum and colon of conventional rats were heavier when pectin-containing diets were fed (Table 9). However, no influence of DM was found. In contrast, there was a correlation between the degree of methylation and the weight of cecum (in trend). This variable increased with the DM of the pectins fed.

In germfree rats, including pectin in the diets resulted in significantly higher weights of ileum, cecum and colon. Generally, the weights of the intestines were greater in germfree rats than in conventional rats.

With one exception, weights of intestinal contents were higher groups fed the pectin-containing diets compared with the control groups (Table 9).

### DISCUSSION

Zhang and Lupton (17) reported that 6% fiber in the diet of rats corresponds to an intake of ~30 g DF/d in humans. Therefore, the use of 6.5% galacturonan in our diets seems to be a reasonable concentration. A daily intake of at least 30 g DF for adults is also generally recommended (18).

It was necessary to sterilize the diets for the germfree animals. The γ-irradiation partially depolymerized the pectins. In the experiments with conventional rats, we used additionally the irradiated pectin H#. Therefore, it was possible to study the influence of the molecular weight of pectin on its actions in intestinal tract and on its microbial degradation. It has been shown that irradiation can also induce different effects in pectin molecules: decrease in molecular weight and DM, loss in viscosity and/or in functional properties such as gelation. Normally, these effects are stronger when pectin is displayed in a table format.

### TABLE 6

**Microbial counts in fresh feces of the rats after 11-d and 21-d feeding control diet (C) or diets containing low-methoxyl pectin (L), high-methoxyl pectin (H), very highly methoxylated pectin (V) or irradiated high-methoxyl pectin (H#)1,2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total anaerobes</th>
<th>Bacteroides</th>
<th>Clostrids</th>
<th>Enterococcus</th>
<th>Lactobacillus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>11</td>
<td>21</td>
<td>11</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>7.76 ± 0.54b</td>
<td>7.60 ± 0.14b</td>
<td>6.62 ± 0.40b</td>
<td>6.80 ± 0.56b</td>
<td>5.86 ± 0.84b</td>
</tr>
<tr>
<td>L</td>
<td>8.42 ± 0.33a</td>
<td>8.50 ± 0.25a</td>
<td>8.18 ± 0.26a</td>
<td>7.98 ± 0.29a</td>
<td>6.36 ± 0.11a</td>
</tr>
<tr>
<td>H</td>
<td>8.94 ± 0.53a</td>
<td>9.16 ± 0.47a</td>
<td>8.60 ± 0.73a</td>
<td>8.78 ± 0.78a</td>
<td>7.44 ± 0.91</td>
</tr>
<tr>
<td>V</td>
<td>9.12 ± 0.83a</td>
<td>7.88 ± 0.58a</td>
<td>9.00 ± 0.99a</td>
<td>8.22 ± 1.09a</td>
<td>7.08 ± 1.69</td>
</tr>
<tr>
<td>H#</td>
<td>8.36 ± 0.53b</td>
<td>8.30 ± 0.07a</td>
<td>8.10 ± 0.87a</td>
<td>7.86 ± 0.53a</td>
<td>5.94 ± 0.77</td>
</tr>
</tbody>
</table>

1 Values are means ± sd, n = 5. Means in a column without a common letter differ, P < 0.05.
2 Numbers of viable cells were determined using 0.5 g of fresh feces for serial dilution and subsequent plating on selective media and columbia blood agar followed aerobically or anaerobically incubation at 37°C.
irradiated in solution compared with irradiation in dry state (19–22).

Pectin-depolymerizing enzymes are not present in the upper part of the gastrointestinal tract. Therefore, pectin was not distinctly depolymerized or demethoxylated under the physicochemical conditions in the stomach and small intestine. Furthermore, relatively small alterations of the macromolecular status of pectin and of its DM occurred in cecum and colon of germfree rats. In contrast to this, no or very little galacturonan was found in the contents of colon and in feces of most of the conventional rats as a result of the more or less intense enzymatic degradation and fermentation of pectin by the microflora. Surprisingly, even macromolecular galacturonans appeared in some of the feces. Likewise, Nyman and Asp (23) reported that 19 ± 12% or 25 ± 20% galacturonan (low-methoxyl or high-methoxyl pectin, respectively) were excreted by rats fed diets with ~9% pectin. In contrast, Cummings et al. (5) found no increase in fecal excretion of pectin by men after intake of 36 g pectin/d for 6 wk. In an interlab-

### TABLE 7

<table>
<thead>
<tr>
<th>Incubation time, h</th>
<th>pH</th>
<th>Macro</th>
<th>Low</th>
<th>Sum³</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/L</td>
<td></td>
<td></td>
<td></td>
<td>mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.15</td>
<td>0.072</td>
<td>4.862</td>
<td>4.934</td>
<td>1.06 ± 0.13⁶</td>
<td>0.13 ± 0.04⁶</td>
<td>0.04 ± 0.02⁶</td>
<td>1.23</td>
</tr>
<tr>
<td>2</td>
<td>7.04</td>
<td>0.522</td>
<td>3.843</td>
<td>4.365</td>
<td>2.16 ± 0.22⁵</td>
<td>0.32 ± 0.12⁵</td>
<td>0.13 ± 0.02⁵</td>
<td>2.62</td>
</tr>
<tr>
<td>4</td>
<td>6.92</td>
<td>1.386</td>
<td>2.222</td>
<td>3.610</td>
<td>5.14 ± 0.34³</td>
<td>0.82 ± 0.17³</td>
<td>0.42 ± 0.05³</td>
<td>6.38</td>
</tr>
<tr>
<td>8</td>
<td>6.90</td>
<td>2.462</td>
<td>0.579</td>
<td>3.041</td>
<td>14.97 ± 0.88³</td>
<td>2.43 ± 0.56³</td>
<td>1.03 ± 0.11³</td>
<td>18.43</td>
</tr>
<tr>
<td>12</td>
<td>6.78</td>
<td>1.613</td>
<td>0.241</td>
<td>1.854</td>
<td>20.41 ± 1.12³</td>
<td>3.14 ± 0.41³</td>
<td>1.77 ± 0.18³</td>
<td>25.32</td>
</tr>
<tr>
<td>24</td>
<td>6.23</td>
<td>0.180</td>
<td>0.120</td>
<td>0.300</td>
<td>33.13 ± 2.30³</td>
<td>4.95 ± 0.44³</td>
<td>3.54 ± 0.44³</td>
<td>41.62</td>
</tr>
</tbody>
</table>

1 Values are means ± so, n = 3. Means in a column without a common letter differ, P < 0.05.

2 Incubation of 150 mL pectin media (0.5% galacturonan) with 4 g rat fecal flora at 37°C; uptake of samples after 0–24 h; determination of pectin fractions by m-hydroxybiphenyl method and of short-chain fatty acids by gas-chromatography.

3 Maximum concentration: 5 mg galacturonan/mL incubation solution.

**Macro**, macromolecular galacturonan fraction; **Low**, low molecular galacturonan fraction.

### TABLE 8

<table>
<thead>
<tr>
<th>Incubation time, h</th>
<th>Di</th>
<th>Tri</th>
<th>Tetra</th>
<th>Penta</th>
<th>Hexa</th>
<th>Hepta</th>
<th>Octa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.072 ± 0.003</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.025 ± 0.006³</td>
<td>0.309 ± 0.018³</td>
<td>0.118 ± 0.021³</td>
<td>0.052 ± 0.012³</td>
<td>0.017 ± 0.005³</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.207 ± 0.011³</td>
<td>0.622 ± 0.028³</td>
<td>0.300 ± 0.021³</td>
<td>0.165 ± 0.014³</td>
<td>0.083 ± 0.011³</td>
<td>0.011 ± 0.006³</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0.518 ± 0.025³</td>
<td>1.042 ± 0.062³</td>
<td>0.529 ± 0.033³</td>
<td>0.239 ± 0.015³</td>
<td>0.098 ± 0.010³</td>
<td>0.025 ± 0.002³</td>
<td>0.012 ± 0.010³</td>
</tr>
<tr>
<td>12</td>
<td>0.458 ± 0.018³</td>
<td>0.703 ± 0.041³</td>
<td>0.297 ± 0.023³</td>
<td>0.099 ± 0.008³</td>
<td>0.034 ± 0.007³</td>
<td>0.021 ± 0.008³</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0.063 ± 0.012³</td>
<td>0.045 ± 0.010³</td>
<td>0.072 ± 0.008³</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Values are means ± so, n = 6 (three repetitions; two replicates). Means in a column without a common letter differ, P < 0.05.

2 Incubation of 150 mL pectin media (0.5% galacturonan) with 4 g rat fecal flora at 37°C; uptake of samples after 0–24 h; determination of oligogalacturonic acids by HPTLC.
Likewise, Demigné conventional and germfree rats fed pectin-containing diets. Therefore, the intestine has to trans-
colon of germfree rats, higher viscosities are present in the
(butyrate) concentrations in the distal colon and feces.
resistant starch was partly shifted from the cecum to the distal
the distal colon and feces. Recently, Morita et al. (39) de-
conjugated pectins, higher concentrations of SCFA appear in
excretion.
cholesterol level and cecal propionate production and bile acid
static cultures of pectin with cecal leavings from a rat. Fur-
morphometric measurements by computerized image analysis that the applied
pectin in vitro (16) and in vivo may be the reason for the greater
weights of intestines of conventional rats given pectin V. The
technical and physiological actions.

LITERATURE CITED


