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CHEMICAL AND IMMUNOCHEMICAL STUDIES ON THE STRUCTURE OF FOUR SNAIL GALACTANS*

HAGEN BRETTING,
Zoologische Institut der Universität Hamburg (Federal Republic of Germany)

NOEL F. WHITTAKER,
National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205 (U.S.A.)

ELVIN A. KABAT,
National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205; Departments of Microbiology, Human Genetics and Development, and Neurology, and the Cancer Center/Institute of Cancer Research, Columbia University College of Physicians and Surgeons, New York, NY 10032 (U.S.A.)

KATHARINA KÖNIGSMANN-LANGE,
Zoologische Institut der Universität Hamburg (Federal Republic of Germany)

AND HANS-JOACHIM THIEM
Organisch-chemisches Institut der Universität Hamburg (Federal Republic of Germany)

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ABSTRACT

The four snail galactans studied are polysaccharides of high molecular weight that are composed entirely of D- and L-galactosyl residues. AaG and CnG, which had not previously been studied, are highly branched galactans composed mainly of (1→3)- and (1→6)-linked galactosyl residues, as shown by the results of periodate oxidation and permethylation studies. On methylation, HpG, CnG, and AaG yielded ~40% of 2,3,4,6-tetra-, 40% of 2,4-di-, 7–14% of 3,4,6-tri-, and 8–12% of 2,4,6-tri-*O*-methylGal derivatives. BgG gave equal amounts of tetra- and di-*O*-methyl derivatives, and 8.5% of 2,4,6-tri-*O*-methylGal, and 10% was unmethylated Gal, indicating 1, 2, 3, 4, and 6 substitution not previously reported in nature. Antisera to the four galactans showed various degrees of cross-reactivity, indicating structural differences ascribable partially to determinants involving a galactose phosphate and, probably, to the linkage and the position of L-Gal in the molecule. The galactans differed in susceptibility to D-galactose oxidase, and some of the immunochemical observations are most probably attributable to species-specific differences in distribution of linear stretches and branches. The first stages of Smith degradation of HpG and AaG showed a substantial increase in unsubstituted (1→3)- and (1→6)-linked

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residues. These results, and the appearance of linear stretches within the native galactans preclude the strictly dichotomously-branched structure proposed earlier.

INTRODUCTION

Snail galactans, first recognized by Hammarsten[1], are branched polysaccharides, composed of D- or of D- and L-galactose[2-5], that have molecular weights of several million, and are synthesized in the albumen glands of pulmonate snails, and secreted as part of the perivitelline fluid embedding their eggs. These polysaccharides provide nutritive substrates for the developing embryos or the freshly hatched snails[6].

Although galactans have been isolated from a variety of different snails[4,7-10], the structures of only two of the four galactans herein described had been studied earlier. One is the *Helix pomatia* galactan (HpG)**, reported[11] to have 85% of D-Gal and 15% of L-Gal residues[5] glycosidically linked (1→3) and (1→6). O'Colla[12] applied the Barry-degradation procedure, and proposed a dichotomously branched structure for this polymer, to replace Bell and Baldwin's proposal[5] of a linear polymer bearing one galactosyl group as a side chain for every D-Gal unit in the main chain. From the optical rotation of the polysaccharide ($[\alpha]_D^{20}$ −16.1 to −23°])[11-13] and of oligosaccharides obtained by acid hydrolysis, May and Weiniand[14,15] concluded that the terminal, nonreducing D-Gal groups are linked β, and the L-Gal residues are linked α.

The other, *Biomphalaria glabrata* galactan (BgG), has not been so clearly defined. Corrêa *et al.*[16] established that BgG is also composed of D- and L-Gal, and inferred from periodate oxidation studies on KOH-extracted and Barry- or Smith-degraded material that the Gal residues are linked (1→3) and (1→6). As the optical rotation was low ($[\alpha]_D^{20}$ +19.5°), it was assumed that D- and L-Gal residues are linked β. The location of the L-Gal remained uncertain, and the ratio of 36% of L- to 64% of D-Gal was deduced only from enzymic studies with D-galactose oxidase; hydrolysis was only carried out to 40%, leaving a product having $[\alpha]_D^{20}$ +56°.

Other snail galactans, those of *Lymnea stagnalis*[4] and *Strophocheilus oblongus*[17], do not contain L-Gal. The galactan of *Ampullarius* sp. contains[18] 98% of D-Gal and 2% of L-Fuc. In *Ampullarius* and *Strophocheilus*, the D-Gal residues are mainly linked (1→3) and (1→6), but, after permethylation and hydrolysis, both galactans yielded trimethyl derivatives, indicating unbranched, internal residues, presumed[12,16] not to occur in the dichotomously branched structures of HpG and BgG.

A sponge lectin from *Geodia cynodium*[19], and two human IgM myeloma proteins[20] specific for pyruvylated D-Gal, were found to react quite differently in quantitative precipitin tests to the four galactans (HpG, EgG, CnG, and AaG), indicating structural differences among them. HpG and BgG were obtained in highly purified form by affinity chromatography on the D-Gal-specific *Axinella polypoides* lectin coupled to Sepharose 4B.

These galactans were immunogenic in rabbits, and the high-titer antisera exhibited strong specificity for the galactan with which the rabbit had been immunized,

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**Abbreviations used: Hp, *Helix pomatia*; Bg, *Biomphalaria glabrata*; Aa, *Arianta arbustorum*; Cn, *Cepaea nemoralis*; G, galactan; and Galol, galactitol.

cross-reactions with the other galactans ranging from strong to almost negative. The immunochemical findings showed the need to reinvestigate the structures of these four galactans by the techniques of gas–liquid chromatography (g.l.c.) and mass spectrometry (m.s.).

EXPERIMENTAL

Galactans. — The Aa and Cn snails were collected near Nürnberg; Hp snails were purchased from R. Stein (Esslingen). The albumen glands were removed, and the galactans were prepared[3] as follows. Freshly dissected albumen glands (20 g) were homogenized, extracted with 0.9% saline containing 40mM $CaCl_2$ (200 mL) for 14 h at 4°, and centrifuged at 150,000*g* for 2.5 h. The surface of the translucent pellet was rinsed twice, to remove precipitated material, and the sediment was redissolved in saline. Ultracentrifugation was repeated twice. The sediment was dialyzed against distilled water, and lyophilized.

Bg snails were kept in the Institute; the spawn was collected, and treated similarly to the albumen glands for preparation of the galactan.

The HpG was obtained[3] by boiling the first sediments of the galactan extracts in 30% potassium hydroxide solution, and subsequently precipitating with ethanol at 2°.

HpG and BgG were also purified by affinity chromatography. The D-Gal-specific lectins of the sponge *Axinella polypoides*, purified as described[21], were coupled to Sepharose 4B by the cyanogen bromide technique[22,23] as described by Bretting *et al.*[24]. A column (1.5 × 30 cm) filled with *Axinella* lectin–Sepharose 4B was equilibrated with 40mM $CaCl_2$ solution in 0.9% saline, and the galactans (20–40 mg) were applied. Effluents and eluates from the column were monitored at 280 nm. The bound material was eluted with D-Gal (1.8 mg/mL). Materials in the peaks were pooled, and dialyzed extensively against saline.

Galactan preparations obtained after ultracentrifugation and the D-Gal eluates from affinity chromatography are referred to as native galactans, as distinct from material treated with KOH, hydrazine, or acid. Hydrazinolysis of the native galactans was performed with anhydrous hydrazine, as described by Krotkiewski and Lisowska[25].

Immunology. — Antisera to galactans were raised in rabbits immunized once a week for three weeks with the galactan (250 μg) in Freund's complete adjuvant (0.5 mL). After a rest for two weeks, the immunizing schedule was repeated. The D-Gal eluates from the *Axinella* column were used to prepare the anti-HpG and anti-BgG antisera. Thrice ultracentrifuged AaG, and the first Smith-degraded HpG, were used for inducing production of antibody to these polysaccharides.

Quantitative, precipitin analysis in a total volume of 250 to 300 μL was performed by a microprecipitin technique[26,27]. Total nitrogen in washed precipitates was determined by the ninhydrin procedure[28]. To determine the amount of galactan in the specific precipitates, determinations were set up at the beginning of the equiv-

alence zone, using larger volumes of antiserum (150, 100, 150, and 400 μL) and of antigen (300, 286, 297, and 230 μg of HpG, AaG, BgG, and HpG (1st Smith-degraded), respectively. After being washed, the precipitates were dissolved in dilute H_2SO_4 (500 μL), the protein precipitated with 15% trichloroacetic acid[29], and the supernatant liquor analyzed for galactose by the orcinol method[26]. Immunodiffusion was conducted by the Ouchterlony method[30], in 1.5% agar in 0.05M sodium barbital (pH 8.3). Immunoelectrophoresis was performed at 220 V for 2 h according to the technique of Grabar and Williams[31].

Determination of sugars. — 6-Deoxyhexose, hexosamine, *N*-acetylhexosamine, and hexose were determined by colorimetric methods[27]. Uronic acid was determined by the method of Dische[32] as described[33], using 50–100-μg samples. For thin-layer chromatography (t.l.c.), samples were hydrolyzed in 15mM sulfuric acid for 18 h at 100°, and the acid was neutralized with $BaCO_3$. T.l.c. was performed on silica gel 60 F 254 foil (Merck, Darmstadt) with 5:4:1 1-butanol–acetone–aq. sodium dihydrogenphosphate (0.16%). D-Gal, D-Glc, GlcA, GalA, Rha, and Ara were used as standards. After seven developments, the plates were first sprayed with 0.3% 1,2-naphthalenediol in ethanol and then with concentrated sulfuric acid. On heating at 150°, colored spots appeared, and their R_F values were determined.

Identification and quantitative determination of individual sugars were performed by gas–liquid chromatography (g.l.c.) in two different ways. (*1*). The samples were methanolyzed in 3.3% methanolic HCl for 16 h at 65°, and the products *O*-acetylated with 1:1 acetic anhydride–pyridine. The methyl α- and β-glycosides were separated, on Chromosorb Q (80–100 mesh) coated with 3% of ECNSS-M, in a steel column (1.83 m × 6.4 mm i.d.) in a Perkin–Elmer 800 gas chromatograph at 160°. (*2*) Individual sugars were also identified as alditol acetates as described by Sloneker[34]. After hydrolysis in 15mM H_2SO_4 for 18 h, and neutralization of the acid with $BaCO_3$, separation was achieved isothermally at 180° in a Packard 419 gas chromatograph having a glass column filled with the aforementioned packing. Inositol, added before the hydrolysis, was used as the internal standard in both cases.

Permethylated galactans were treated with 90% formic acid for 1 h at 100°, and were hydrolyzed, in 65mM H_2SO_4, in a boiling-water bath for 20 h, made neutral with $BaCO_3$, the sugars reduced with $NaBH_4$, and the alditols *O*-acetylated according to Parolis and McGarvie[35]. The partially methylated alditol acetates obtained were separated by g.l.c. in glass columns (2.5 m × 4 mm) of Chromosorb Q (80–100 mesh) coated[36,37] with 15% of Apiezon T.

Quantitative analysis of the partially methylated alditol acetates was also accomplished with a Hewlett–Packard 3380 A integrator coupled to a Shimadzu Mini-2 g.l.c. apparatus having a 25-m Quadrex-glass, capillary column (coated with OV-225) at 200°; g.l.c.–m.s. was performed with a Perkin–Elmer Sigma 3 gas–liquid chromatograph having a glass column (180 × 0.2 cm i.d.) packed with 3% of OV-225 on Gas-Chrom Q (100–120 mesh), at 200°, A V. G. Micromass 7070 F mass spectrometer operated at 70 eV, with a DS 2050 data system, was used to analyze the g.l.c. effluent[36,37].

The relative, molar concentration was calculated from the peak area of the flame-ionization detector, and corrected for the number of carbon atoms present in the derivative[38].

Other determinations. — For amino acid analysis, salt-free samples were hydrolyzed in constant-boiling HCl, in sealed, evacuated Pyrex tubes, for 24 h at 110°. Quantitative analysis was performed by Dr. Müller (Allgemeines Krankenhaus, Barmbek) according to the technique of Spackman *et al.*[39], using a Multichrom M amino acid analyzer (Beckman Instruments).

Phosphate determination was conducted according to Ames[40]. Optical rotations were measured with a Perkin-Elmer polarimeter MC 241 employing aqueous solutions of carbohydrates (5–10 mg/mL).

Infrared spectra were recorded, for KBr discs, with a Perkin–Elmer spectrophotometer 399.

Oxidation of D-Gal residues with D-galactose oxidase was performed in a total volume of 0.8 mL. The sugar (50–600 μg) was dissolved in 0.1M sodium phosphate buffer (pH 7.0) to which *o*-dianisidine (85 μg/mL) and 25 μL of peroxidase (50 μg/mL) were added. The reaction was started by adding D-galactose oxidase (40 μg). The extinction at 450 nm was measured[41] after 10 and 60 min. The enzymes and *o*-dianisidine, in their purest form, were from Sigma (München).

Periodate oxidation was performed with 9mM sodium metaperiodate in the dark for 92 h at 0°. The galactan concentrations were ~2 mg/mL. To compare the results of these experiments with those from previous studies on HpG, periodate oxidation of HpG was also performed at room temperature and with higher concentrations of galactan and metaperiodate. The concentration of galactan was 24 mg/mL, and 121mM sodium periodate was used; these conditions are comparable to those of O'Colla[12]. Aliquots were taken several times for determination of consumption of periodate and liberation of formic acid and formaldehyde. Periodate uptake was analyzed iodometrically by a microtitration method described by Schiffman *et al.*[42]. Formic acid was determined by adding an excess of 0.10M NaOH solution to the sample and back-titrating with 0.10M HCl, using Methyl Red as the indicator. Ten minutes prior to titration, the excess of periodate was decomposed by adding a 10% solution of ethylene glycol. Formaldehyde was determined by the chromotropic acid method[26].

For Smith degradation, HpG (972 mg) and AaG (509 mg) were oxidized with 0.04M sodium metaperiodate for 162 h at 0°. The total volumes were 210 and 105 mL, respectively. Periodate uptake and liberation of formic acid and formaldehyde were determined at intervals. The excess of the oxidant was decomposed with 7.5 and 3.7 mL of ethylene glycol solution, respectively. The solutions were dialyzed against distilled water for 7 days. Sodium borohydride (1.5 and 0.75 g, respectively) was added; after 24 h at room temperature, the excess of $NaBH_4$ was decomposed with acetic acid. The solution was hydrolyzed with 0.5M HCl for 24 h at room temperature, and dialyzed for 7 days at 4° (see refs. 43 and 44).

Attempted permethylation of the galactans by the Kuhn method, with methyl iodide in *N*,*N*-dimethylformamide, and BaO as the catalyst[45], was ineffective, even

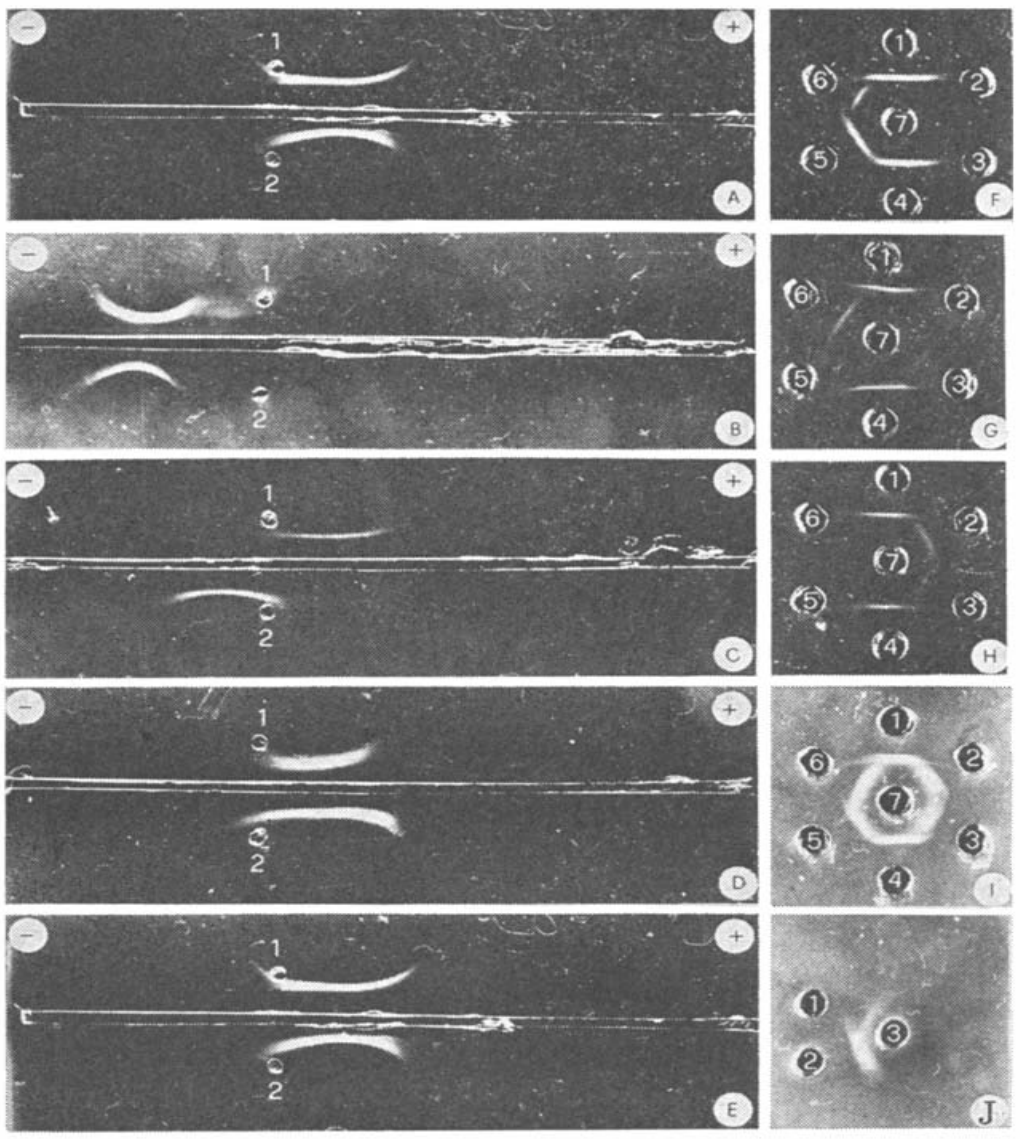


Fig. 1. Immunoelectrophoretic (A–E) and immunodiffusion (F–J) patterns of the different snail galactans. (A) 1, HpG (5.3 mg/mL), and 2, HpG after hydrazinolysis (5.6 mg/mL); (B) 1, AaG (5.1 mg/mL) and 2, AaG after hydrazinolysis (6.0 mg/mL); (C) 1, HpG after one cycle of Smith degradation (4.79 mg/mL) and 2, BgG (5.2 mg/mL); (D) 1, CnG (4.7 mg/mL) and 2, CnG after hydrazinolysis (5.3 mg/mL); (E) 1, HpG after KOH treatment (6.8 mg/mL) and 2, HpG after 20 min of acid hydrolysis (3.3 mg/mL); the troughs in A, D, and E contained anti-HpG antiserum, the trough in B, anti-AaG serum, and in C, anti-BgG serum; (F) 1 and 4, HpG, 2, HpG after one cycle of Smith degradation, 3, AaG, 5, CnG, 6, BgG, and 7, anti-HpG serum; (G) 1 and 4, AaG, 2, AaG after one cycle of Smith degradation (5.1 mg/mL), 3, BgG, 5, HpG after one cycle of Smith degradation, 6, CnG, and 7, anti-AaG serum; (H) 1 and 4, BgG, 2, HpG, 3, CnG, 5, HpG after one cycle of Smith degradation, 6, AaG, and 7, anti-BgG serum; (I) 1 and 4, HpG, 2, HpG after KOH treatment, 3, HpG after hydrolysis in 0.03M HCl for 20 min at 100°, 5, HpG after hydrazinolysis, 6, CnG after hydrazinolysis, and 7, anti-HpG serum; (J) 1, HpG after hydrazinolysis (16 mg/mL), 2, AaG (6.0 mg/mL), and 3, anti-AaG serum. The concentrations of the galactan solutions in the Ouchterlony test were identical to those used in immunoelectrophoresis, if not stated otherwise.

after the methylation procedure had been repeated four times. Permethylation of the galactans was successfully and readily accomplished by the Hakomori method as described by Hakomori[46] and by Sandford and Conrad[47]. This procedure was used with 10–20 mg of polysaccharide. The galactans were soluble in dimethyl sulfoxide at 0.5 mg/mL. After ultrasonication for ~1 h, the polysaccharides were incubated overnight with methylsulfinyl anion[48], and methylation with methyl iodide was conducted for 16 h. After dialysis and lyophilization, the procedure was repeated. A subsequent Purdie methylation[49] resulted in a product free from hydroxyl groups, as checked by i.r. spectroscopy.

Analysis for short-chain carboxylic acid esters, lactones, and anhydrides was performed by the Hestrin method, according to Downs and Pigman[50].

Glycerol[51] (after Smith degradation), pyruvate[52], and lactate[53] (after hydrolysis of the galactans) were determined enzymically by use of the appropriate test-kits (Boehringer, Mannheim).

RESULTS

Purity and cross-reactivities of the galactans. — The four galactans, purified by repeated sedimentation in an ultracentrifuge, were examined by electrophoresis in 7.5 and 12% poly(acrylamide) gels. The galactans did not enter the separating gel, because of their size, and contaminating proteins could not be detected. On Sepharose 4B, the single peak monitored by u.v. absorption corresponded to the galactans as detected by the orcinol method.

Horstmann[3] had determined the molecular weight of HpG to be 4.0 million by sedimentation velocity. In gel filtration on Sepharose 4B, the four native galactans were eluted in identical volumes. HpG, and AaG after one cycle of Smith degradation, were eluted later, and molecular weights of 1.5 to 2.0 million were calculated, using the HpG and arabinogalactan (mol. wt. 50,000) as standards. AaG and CnG formed one precipitin line with the antisera raised against albumen-gland extracts or purified galactans of Aa and Hp (see Figs. 1B and 1D), respectively. HpG showed a strong, main band and a second, faint, diffuse, precipitin line.

The anti-BgG serum revealed three components in the BgG preparation. The HpG and BgG were further purified by affinity chromatography[24] on *Axinella polypoides* lectin coupled to Sepharose 4B, and elution with 0.01M Gal, and then displayed only one band (see Figs. 1A and 1C) in immunoelectrophoresis. For all further studies on the BgG, only the D-Gal eluate was used. Although the third sediment of HpG contained a small second component, it was used for most of the studies, because of the ease of obtaining large quantities of it. However, methylation studies were conducted with both the third sediment and the dialyzed D-Gal eluate. The third sediments of AaG and CnG were employed exclusively.

The purified polysaccharides showed pronounced differences in electrophoretic mobility. In agar, the AaG (as already demonstrated[10]) and BgG moved towards the cathode (see Figs. 1B and 1C), whereas HpG, CnG, and the HpG (after one cycle of Smith degradation) appeared to migrate towards the anode (see Figs. 1A, 1D, and 1C), but, in agarose, in which endosmotic effects are lessened, AaG and BgG did not move, whereas HpG and CnG moved to the anode. Thus, the AaG and BgG are essentially uncharged, whereas HpG and CnG are negatively charged. Material treated for 20 min with hydrazine, KOH, or acid did not show substantial differences in mobility (see Figs. 1A, 1B, 1D, and 1E). To ascertain whether the differences in migration reflected structural differences in the galactans, Ouchterlony tests were performed (see Figs. 1F–1J). As may be seen in Fig. 1F, the antiserum raised against HpG (D-Gal eluate) reacted well with HpG and CnG, and their

precipitin lines fused completely. However, this antiserum was only partly precipitated by BgG, creating a spur with the HpG and CnG, whereas AaG did not react. The antiserum to the BgG D-Gal eluate (see Fig. 1H) reacted well with the BgG, only partly with HpG and CnG (forming spurs), weakly with HpG after one cycle of Smith degradation, and did not react with AaG. A corresponding result was found with the anti-Aag antiserum (see Fig. 1G), which did not react with BgG, and reacted partially with HpG and CnG, but well with AaG. Ouchterlony tests with the antiserum to HpG after one cycle of Smith degradation were not conducted, because of the low antibody titer. It is remarkable that, after hydrazinolysis, HpG and AaG reacted well with the anti-AaG antiserum, but spur formation was seen on the AaG side, demonstrating that the carbohydrate structure of both galactans had to be different, at least in some determinants (see Fig. 1J).

Carbohydrate analysis. — Colorimetric methods for the determination of sugars in the polysaccharides showed that 6-deoxyhexoses and *N*-acetylhexosamines were absent, and that the polysaccharides were entirely composed of hexoses. Separation of the methanolized galactans, after *O*-acetylation, by g.l.c. using Chromosorb Q coated with 3% of ECNSS-M, revealed that galactose was the only hexose present in the polysaccharide, except for a trace of pentose. The identical carbohydrate composition was found when the hydrolyzed galactans were reduced, and monosaccharides identified as alditol acetates by g.l.c. Galactose and a trace of arabinose were the only sugars detected.

Although Vaith *et al.*[54] had reported that HpG contains 16.7% of hexuronic acids, the i.r. spectra of native and hydrolyzed HpG showed no band at 1750 cm^{-1}, whereas a mixture containing 85% of D-Gal and 15% of GalA or GlcA gave a peak in this region, as would be expected for the carboxyl group of a hexuronic acid. On hydrolysis of HpG, no GlcA or GalA could be detected by t.l.c., or colorimetrically by the Dische method[26,32]. Thus, our HpG preparations, like those of Bell and Baldwin[5] and May[2], contained only D- and L-Gal.

The specific optical rotations of the native polysaccharides and their hydrolysis products are given in Table I. They were negative ($[\alpha]_D^{20}$ −13.9 to −15.6°) for HpG, CnG, and AaG, but positive for BgG and for both Smith-degraded galactans ($[\alpha]_D^{20}$ +15.5 to +17.4°). Almost identical values of $[\alpha]_D^{20}$ (+56.0 to +60.9°) were observed for the hydrolyzates of all four native polysaccharides. These differed substantially from the value expected for D-Gal after mutarotation, namely, $[\alpha]_D^{20}$ +80.5°.

As galactose was the only monosaccharide detected, it was assumed that the hydrolyzates contained a mixture of D- and L-Gal, as established by Bell and Baldwin[5] for the *Helix pomatia* galactan. A content of ~15% of L-Gal in the galactans would account for the $[\alpha]_D^{20}$ values found. To confirm this inference, the galactans were hydrolyzed in 0.03M HCl, the acid neutralized, and the contents oxidized with D-galactose oxidase, which acts on D-Gal, but not[41] on L-Gal. Some 20–30% less hexose was obtained by this method than was found colorimetrically, indicating the presence of L-Gal (see Table I).

TABLE I

COMPOSITION AND PROPERTIES OF THE SNAIL GALACTANS AND THEIR SMITH-DEGRADED PRODUCTS

Galactan studied	*Glycerol (%)*	*Phosphorus (%)*	*Nitrogen (%)*	$[\alpha]_D^{20}$ *(degrees)*		*Oxidized by D-galactose oxidase (%)*	L-*Gal (%)*[a]
				Before hydrolysis	*After hydrolysis*		
HpG, native	none	0.61	0.9	−15.6	+59.6	74.4	13.0
HpG treated with hydrazine	n.d.[b]	0.8	none	−16.1	n.d.	n.d.	n.d.
HpG treated with acid, 20 min	n.d.	0.62	0.74	−9.4	n.d.	n.d.	n.d.
150 min	n.d.	0.87	0.6	+5.1	n.d.	n.d.	n.d.
480 min	n.d.	2.8	0.6	+14.6	n.d.	n.d.	n.d.
AaG, native	none	none	0.56	−15.4	+56.0	73.2	15.2
CnG, native	none	0.56	0.6	−13.9	+56.9	68.0	14.7
BgG, native	none	0.84	0.56	+15.5	+60.9	79.0	12.2
HpG after 1st IO_4^- oxidation	5.1	0.4	0.7	+17.4	+86.8	94.0	0.0
AaG after 1st IO_4^- oxidation	5.2	none	0.3	+16.7	+79.0	98.0	0.0

[a]Calculated from $[\alpha]_D^{20}$. [b]Not determined.

Non-carbohydrate components. — HpG was reported[3] to contain ~0.53% of phosphorus, and this could account for the migration of the polysaccharide towards the anode in immunoelectrophoresis. However, BgG, which appeared to move in the opposite direction, contained ~40% more phosphorus than HpG. CnG had a phosphorus content similar to that of HpG, whereas AaG had the same electrophoretic mobility as BgG, and was essentially free from phosphorus. These results are summarized in Table I.

No substantial differences in the nitrogen contents of the four galactans were found. They ranged from 0.9% in HpG to 0.6% in AaG (see Table I). By amino acid analysis, ~70 to 100% of the nitrogen was amino acid nitrogen. The amino acid composition was similar for all four galactans.

As the galactans had different electrophoretic mobilities, they were screened by the Hestrin method[50] for short-chain carboxylic esters, lactones, and anhydrides. Hydrolyzed and nonhydrolyzed native galactans did not show even traces of these components. Sulfate, pyruvate, and lactate could not be detected.

After complete hydrolysis, 5% of glycerol was detected in the Smith-degraded HpG and AaG, whereas glycerol was absent from all of the native galactans; this probably resulted from the Smith degradation, but it had not been split off by the mild hydrolysis step.

Immunochemical studies. — To evaluate specificity relationships, quantitative precipitin reactions were performed with the antisera to the purified galactans. As may be seen in Fig. 2a, 32 μg of HpG precipitated the maximum (5.8 μg) of N from 15 μL of anti-HpG serum, and 10 μg of HpG were required for 50% precipitation; 30 μg of CnG and BgG precipitated 3.7 and 3.4 μg of N, respectively. HpG, after one cycle of Smith degradation, gave only 1.8 μg of N at equivalence; AaG and its first Smith-degradation product did not react. However, with anti-AaG serum (see Fig. 2b), HpG precipitated ~50% of the 9.0 μg of nitrogen that was precipitated with AaG. CnG reacted like HpG. BgG precipitated only 2.9 μg of N, and HpG and AaG after one Smith degradation reacted poorly, giving 1 and 1.8 μg of N, respectively. AaG treated with D-galactose oxidase reacted only slightly less well than the original material.

With anti-BgG serum, similar patterns were seen (see Fig. 2d). BgG reacted best, giving 9.8 μg of precipitate N with 86 μg of galactan; 50% of the maximally precipitable N was obtained with 20 μg of the polysaccharide. HpG precipitated 6.5 μg of N at the maximum; CnG precipitated 5.0 μg of N; and AaG, only 1.6 μg of N. HpG, after one cycle of Smith degradation, was almost as active as CnG, precipitating ~50% of the N found with BgG. AaG, after the first Smith degradation, was almost completely inactive. A usable antiserum was obtained only to the Smith-degraded HpG, but the antibody content was substantially lower than those of the other antisera, necessitating the use of 200-μL samples, as compared to 15 to 50 μL. The results are given in Fig. 2c. With Smith-degraded HpG, ~6 μg of N was obtained, whereas native HpG precipitated ~3.7 μg of N. CnG was almost as active as HpG,

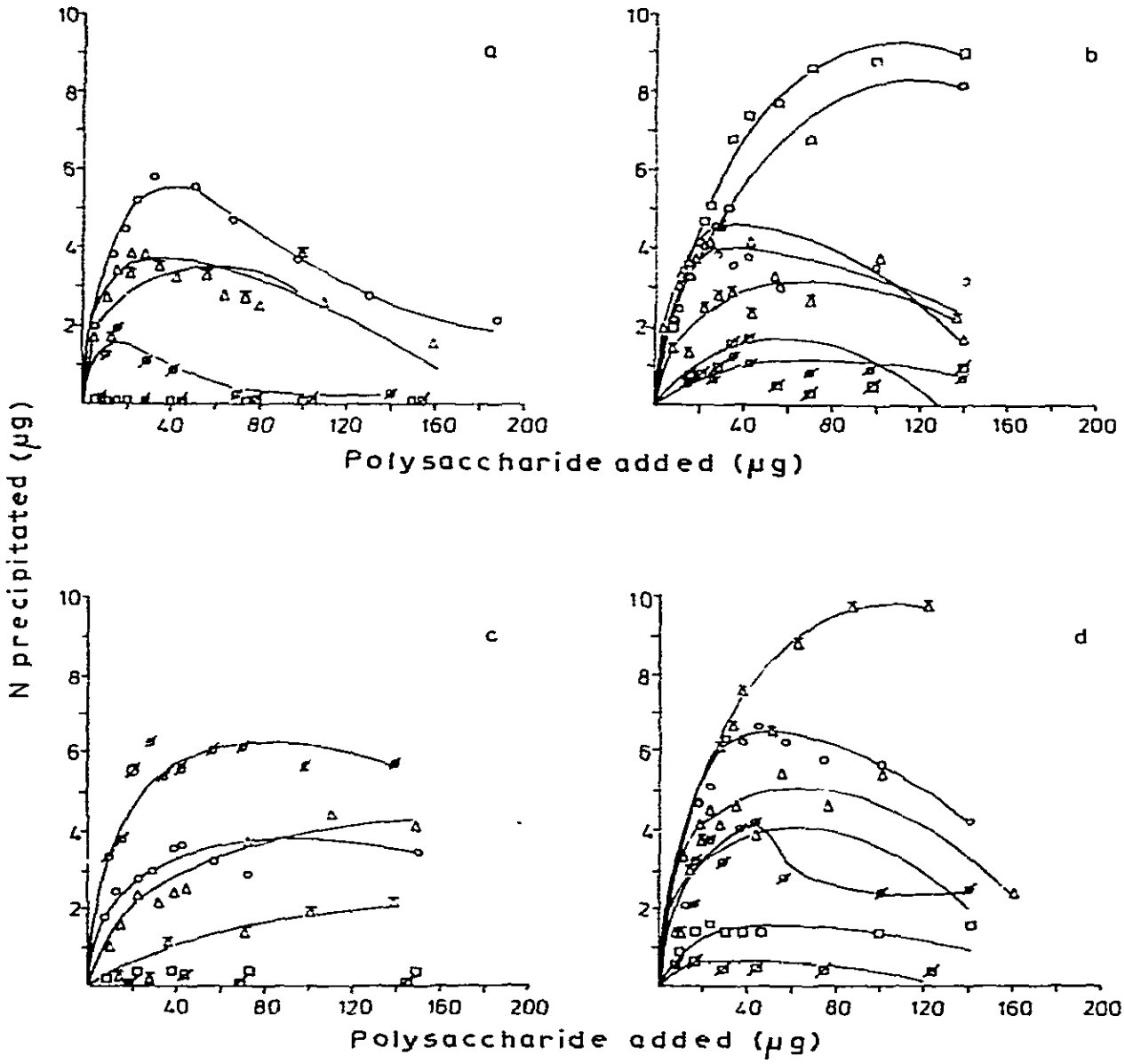
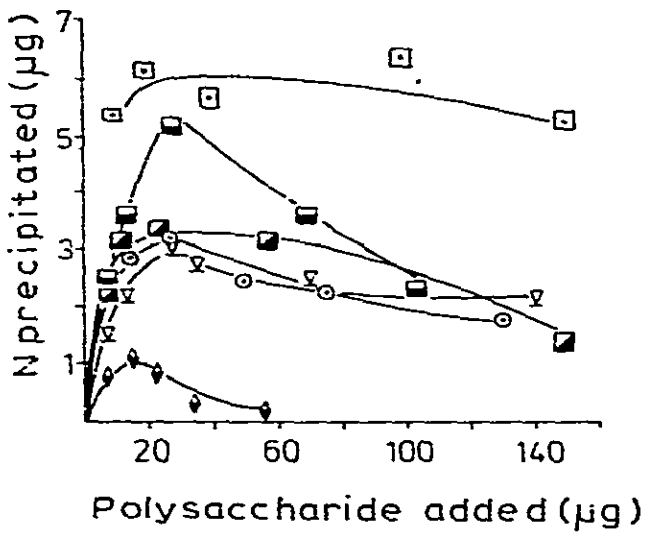


Fig. 2. Quantitative precipitin curves of (a) anti-HpG serum (15 μL), (b) anti-AaG serum (30 μL), (c) anti-HpG after one cycle of Smith degradation antiserum (200 μL), and (d) anti-BgG serum (50 μL) with the different galactans used in this study. [Total vol.: a, b, and d, 250 μL; c, 300 μL. Key: ○ *Helix pomatia* galactan; Ø *Helix pomatia* galactan 1st $IO_4^-BH_4^-$; □ *Arianta arbustorum* galactan; ⊠ *Arianta arbustorum* galactan 1st $IO_4^-BH_4^-$; △ *Cepaea nemoralis* galactan; ⌓ *Arianta arbustorum* D-Gal oxidase-treated; and Δ̄ *Biomphalaria glabrata* galactan.]

whereas BgG reacted poorly, precipitating only 1.5 μg of N; AaG and its first Smith-degradation product were completely inactive.

The low nitrogen content of the galactans made it unnecessary to correct the value of the total N precipitated for any antigen N in the precipitates. Essentially all of the nitrogen in the galactans was removed by treatment with anhydrous hydrazine. The hydrazine-treated samples reacted with the antiserum, and showed the same specificity relationships (data not shown) as the untreated galactans. However, less N was precipitable by the hydrazinolyzed samples, probably because of some depolymerization of the galactans by the hydrazine. HpG treated with potassium hydroxide, or hydrolyzed with 0.03M HCl for 20 min at 100° and separated on Biogel-P2 from the components of low molecular weight also gave only 50% of the N precipitated with native HpG (see Fig. 3). Absorption of the antisera with KOH-treated HpG, and subsequent incubation with native galactan yielded only 1 μg of N, one-third of the amount expected from the difference between the precipitin curves of native and KOH-treated polysaccharides.



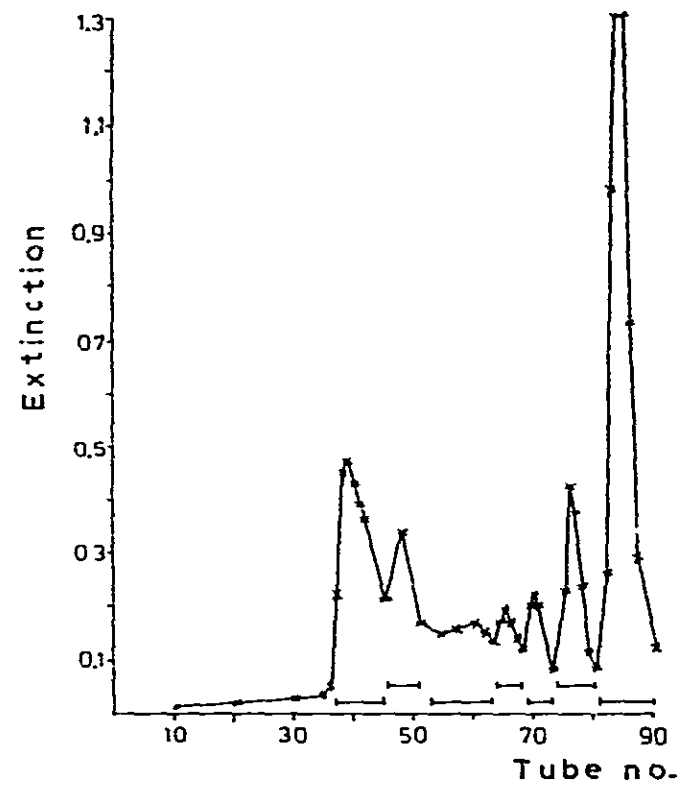


Fig. 3. Quantitative precipitin curves of anti-HpG serum and HpG after various treatments as indicated. [Key: ▽ HpG, KOH treated; ◪ HpG, 20-min acid-treated; ⊙ simultaneous incubation with KOH and acid-treated HpG in equal proportions; ⊡ simultaneous incubation of 30 µg of HpG and various amounts of AaG; ◆ anti-HpG antiserum absorbed with HpG, 20-min acid-treated, and incubation of various amounts of HpG; ▬ HpG, D-galactose oxidase-treated. Anti-*Helix pomatia* galactan, 15 µL; total vol., 250 µL.]

Fig. 4. Separation, on Biogel P-2, of HpG treated with 0.03M HCl for 480 min at 100°. (Detection of the galactose-containing material was performed by the orcinol method. Seven fractions were pooled, as indicated by bars.)

A similar result was obtained when the anti-HpG antiserum was absorbed with HpG that had been treated with 0.03M HCl for 20 min at 100° (see Fig. 3). Simultaneous incubation of KOH-treated and partially acid-hydrolyzed HpG did not precipitate more nitrogen; were the two reacting with different populations of antibody, 6 µg of N (the sum of the separate reactions) should have been obtained (see Fig. 3). Addition of AaG in various amounts to 30 µg of HpG did not inhibit precipitation.

Analysis of washed, specific precipitates showed that 95, 90, 75, and 50% of the added HpG, BgG, AaG, and HpG (1st Smith-degradation), respectively, were precipitated at the beginning of the equivalence zone, establishing that the various antibodies were specific for these galactans, and were not directed toward impurities in the preparations[26]. Prolonged hydrolysis of HpG in 0.03M HCl for 90, 150, 300, and 480 min gave a mixture of degradation products which, after neutralization with silver nitrate, could be partially separated into seven different fractions on Biogel P-2. The relative proportions of each fraction varied with the elapsed time of hydrolysis. Fig. 4 gives the results after hydrolysis for 480 min. Fractions were pooled as indicated, and used for precipitin and precipitin-inhibition studies. Fraction I, comprising tubes 37–45, hydrolyzed for 90, 150, 300, and 480 min, did not precipitate with the anti-HpG serum, but completely inhibited the precipitin reaction between 30 µg of HpG and 15 µL of the anti-HpG antiserum (see Fig. 5), but did not inhibit the precipitin

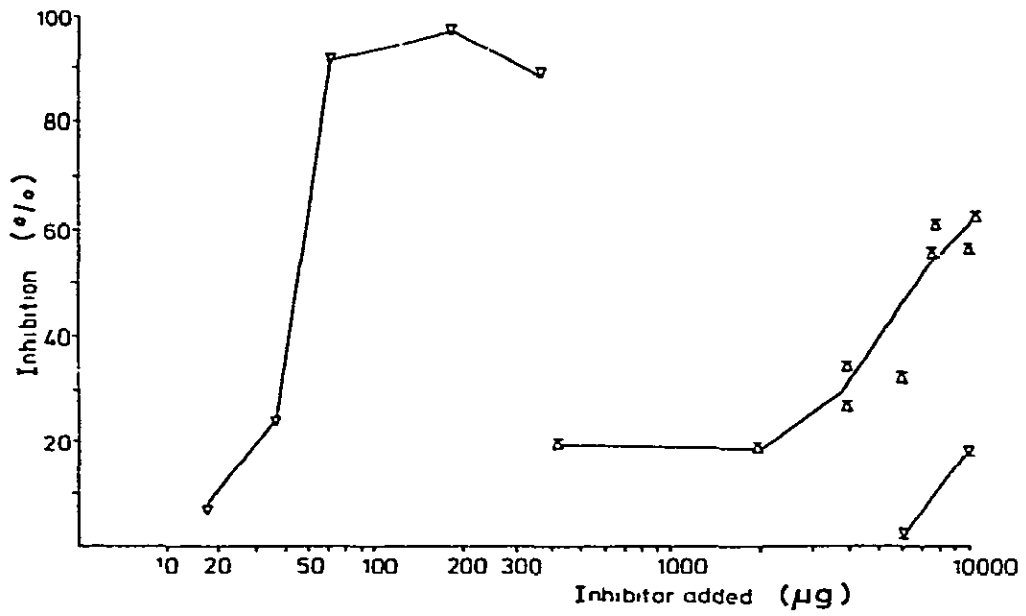


Fig. 5. Inhibition of precipitation of HpG (30 μg) and 15 μL of anti-HpG serum: with ▽, HpG after treatment with 0.03M HCl for 480 min at 100°, fraction I (tubes nos. 37–45) on Biogel P-2; Δ, D-Gal 6-P, ▽ D-Glc 6-P. [The following sugars were inactive at the highest quantities (μmol) tested: lactose (28.5), β-D-Gal-(1→4)-GlcNAc (2.9), β-D-Gal-(1→6)-Gal (0.65), β-D-Gal-(1→3)-D-GlcNAc (2.9), D-Gal-(1→3)-D-Gal (2.32), D-Gal (85.5), and L-Gal (61.1).]

reaction between 29.4 μg of AaG and 30 μL of anti-AaG serum, or between 29.6 μg of BgG and 50 μL of anti-BgG serum. The quantity, 60 μg, of Fraction I (tubes 37–45) giving almost complete inhibition of precipitation was only slightly higher than the amount of native galactan, 30 μg giving maximum precipitation. Fraction I thus shows only HpG specificity, and other determinants must be involved in the cross-reactions with anti-Aag and anti-HpG.

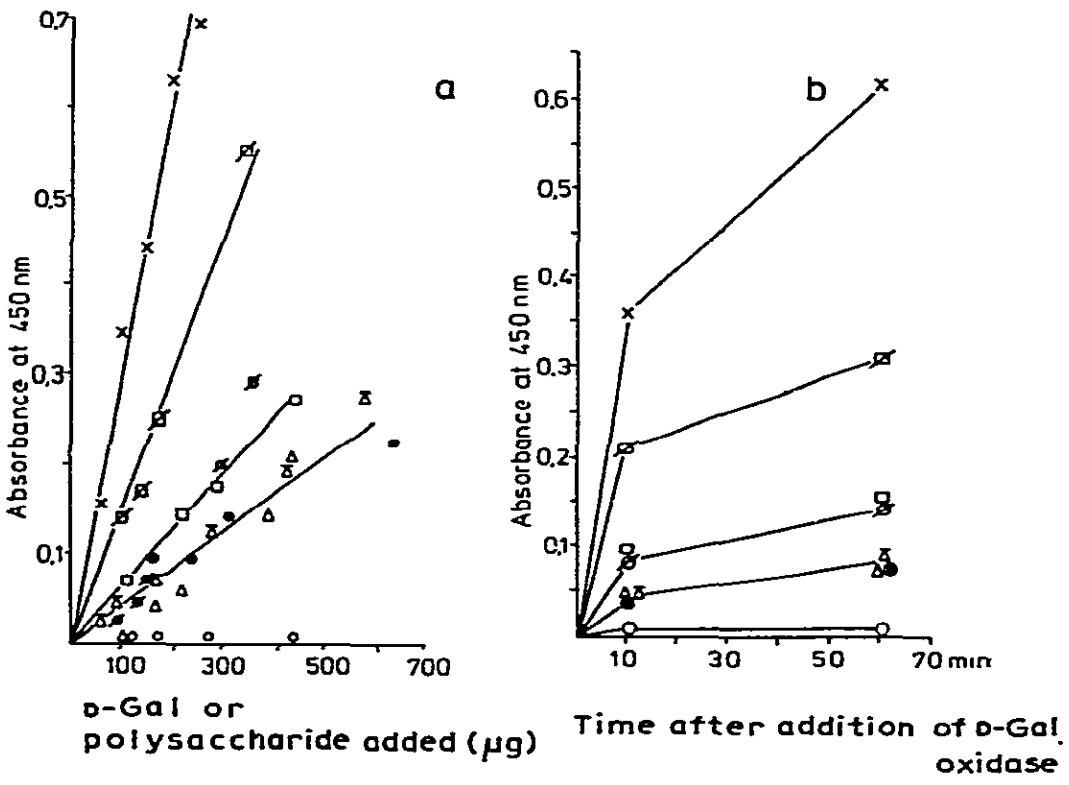


Fig. 6 (a) Oxidation of D-Gal and different galactans by D-galactose oxidase at various concentrations, 60 min after addition of the enzyme. (b) Time course of oxidation of D-Gal and different galactans by D-galactose oxidase. [Substrate (200 μg) was used for oxidation, which is expressed as the increase in absorbance at 450 nm. ×, D-Gal; ●, HpG after hydrazinolysis; other symbols as in Fig. 2.]

Of various other sugars tested (see Fig. 5), only D-Gal 6-phosphate inhibited the precipitin reaction between HpG and anti-HpG serum to ~60% on addition of 36 μmol, indicating that D-Gal 6-phosphate could be part of an antigenic determinant. No inhibition was found with 61.1 μmol of L-Gal.

Enzymic oxidation of the galactans. — The differences in structure that are found among galactans by precipitin studies were also evident from the oxidation of terminal D-Gal groups with D-galactose oxidase. This enzyme oxidizes D-Gal at C-6, with formation of D-*galacto*-hexodialdose[41], and it was found to act faster on polysaccharides than on D-Gal itself. Various amounts of the four native galactans and the two products after one cycle of Smith degradation, as well as HpG after hydrazinolysis, were oxidized enzymically. Fig. 6a demonstrates that oxidation, expressed as increasing extinction, had a linear dependency on the concentration of substrate over the range tested. Fig. 6b shows the variation in extinction with time of incubation, using 200 μg of each carbohydrate. Comparing AaG after one cycle of Smith degradation with D-Gal shows that about half of the hexose was oxidized.

Substantially less was converted into the dialdose for native AaG, and for the first Smith-degradation product of HpG. Both products gave ~25%. BgG, CnG, and HpG after hydrazinolysis gave only 12% of the values obtained with D-Gal. Native HpG was unaffected by D-galactose oxidase. HpG and AaG treated with D-galactose oxidase were unchanged in their precipitating potencies with the corresponding antisera (see Figs. 3 and 2b).

As the immunochemical findings clearly showed substantial differences among the various galactans, an attempt was made to elucidate the structural basis of these differences. O'Colla[12] and Corrêa *et al.*[16] had reported data on the periodate uptake of HpG and BgG, and we studied that of AaG and CnG, with HpG as the control; the results are given in Fig. 7. The native galactans consumed almost identical amounts of periodate (0.7–0.75 mol of periodate per mol of galactose residue) and liberated almost identical quantities of formic acid (0.25 mol/mol of galactose residue). Similar results were obtained for HpG and AaG after one cycle of Smith degradation. Both showed slightly increased periodate uptake, compared to the native galactans (0.75 and 0.9 mol/mol of galactose residue, respectively). Liberation of formic acid was almost as high as with the native galactans. These findings are compatible with (1→3)- and (1→6)-linkages in the galactans, as proposed[12] for HpG. Formation of formaldehyde from the native galactans could not be measured, because the absorption spectra of the developed color differed from that given by serine and erythritol. Formaldehyde formed by the Smith-degraded material was <0.1 mol/mol of galactose residue.

O'Colla[12] reported that KOH-extracted HpG consumed 1.16 mol of periodate, producing 0.55 mol of formic acid per mol of galactose residue, almost twice that found by us. His experiments had been performed at 12 times the concentration of periodate and probably at 20°, although the temperature was not mentioned. For better comparison, the periodate oxidation was repeated at 0° and 9 mmol, and at 20° and 120 mmol of periodate. At 20° and high concentration of periodate, con-

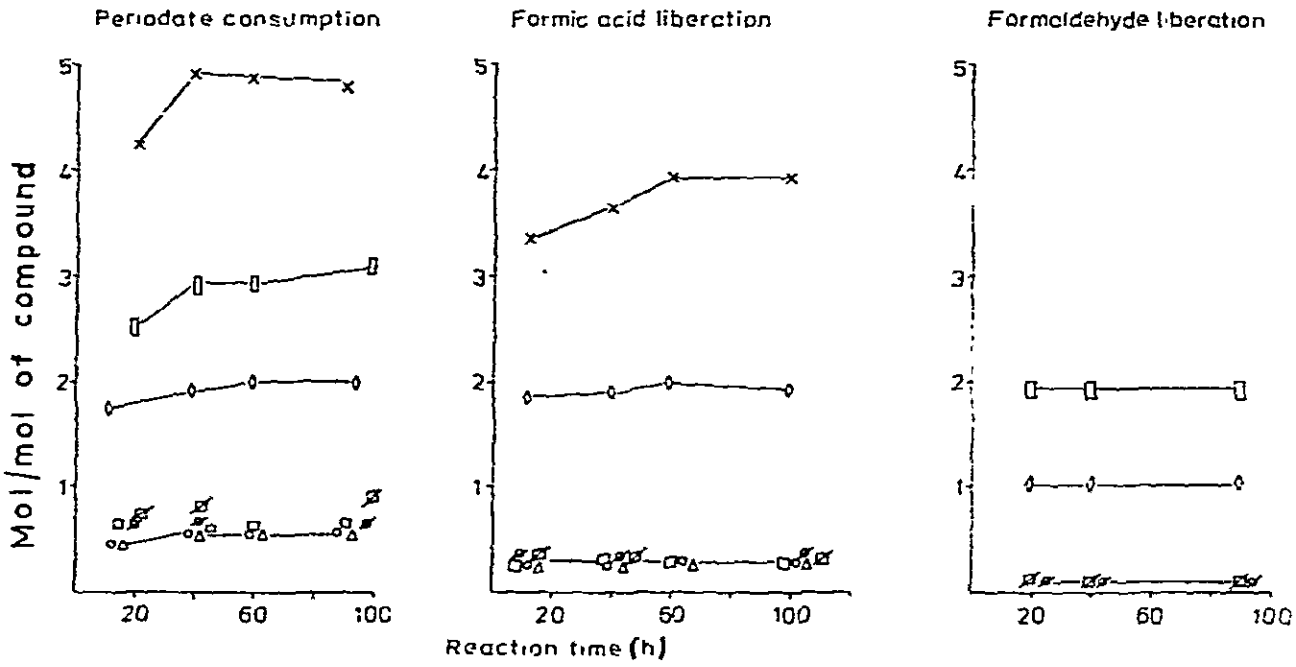


Fig. 7. Time course for periodate uptake, and liberation of formic acid and formaldehyde on periodate oxidation. [(top) Oxidation at 0°, with 9mM $NaIO_4$, by the various snail-galactans, and x, D-Gal; [] , erythritol; and ◇, serine. The symbols for the different galactan preparations are those used in Fig. 2. (bottom) Comparative periodate oxidation of HpG at 0° with 9mM $NaIO_4$, as well as at 20° with 120 mM $NaIO_4$. Periodate consumption (i) at 20° and 120mM $NaIO_4$, (ii) at 0° and 9mM $NaIO_4$; liberation of formic acid (iii) at 20° and 120mM $NaIO_4$, and (iv) at 0° and 9mM $NaIO_4$.]

sumption of $NaIO_4$ (1.0 mol/mol of galactose residue) and liberation of formic acid (0.4 mol/mol of galactose residue) was increased substantially compared with the results obtained at 0° and 9 mmol of periodate (see Fig. 7b).

Permethylation studies. — Permethylation of the galactans by the Kuhn method, with methyl iodide in *N,N*-dimethylformamide, and BaO as the catalyst[45], was unsuccessful, even after four attempted methylations. Complete methylation of the four native galactans, the Smith-degraded HpG and AaG, and the KOH-treated HpG, was achieved by two cycles of the Hakomori method[46], followed by a Purdie methylation[49]. Completeness of methylation was monitored by i.r. spectroscopy, as seen for AaG in Fig. 8.

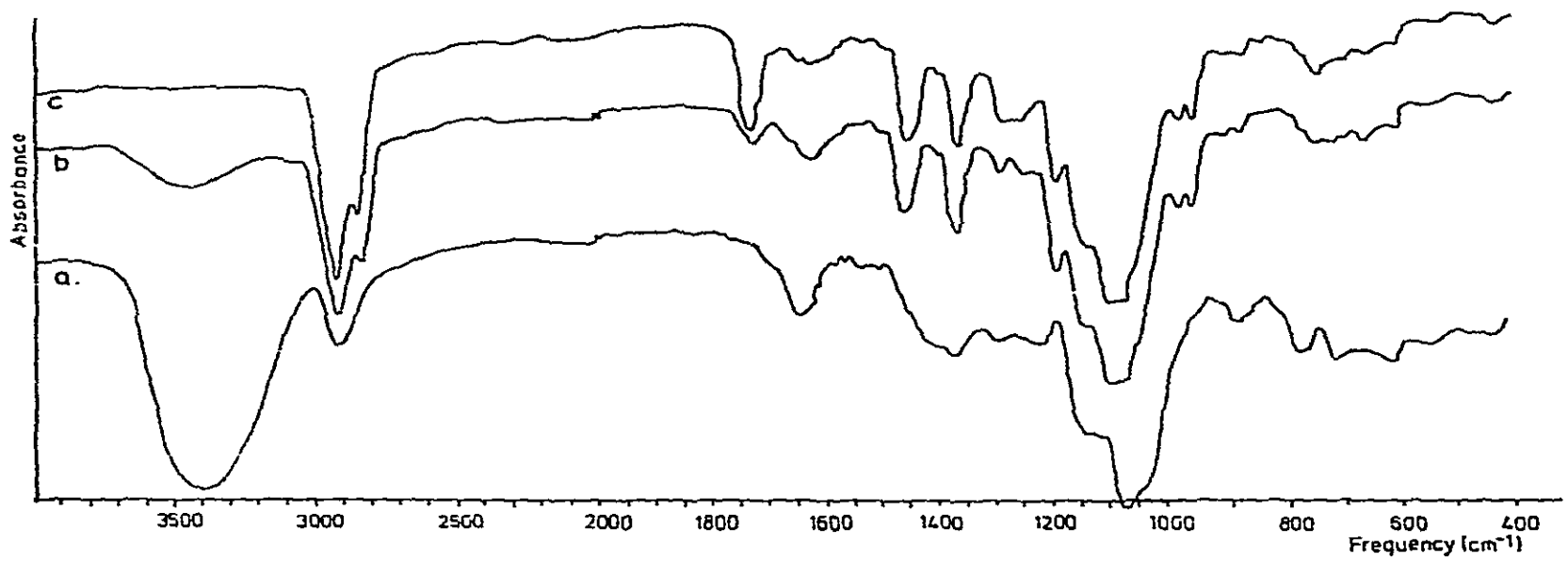


Fig. 8. Infrared spectra of native AaG, and the product at various stages during its permethylation. [(a) native AaG, (b) after the 2nd Hakomori methylation, and (c) after the final Purdie methylation.]

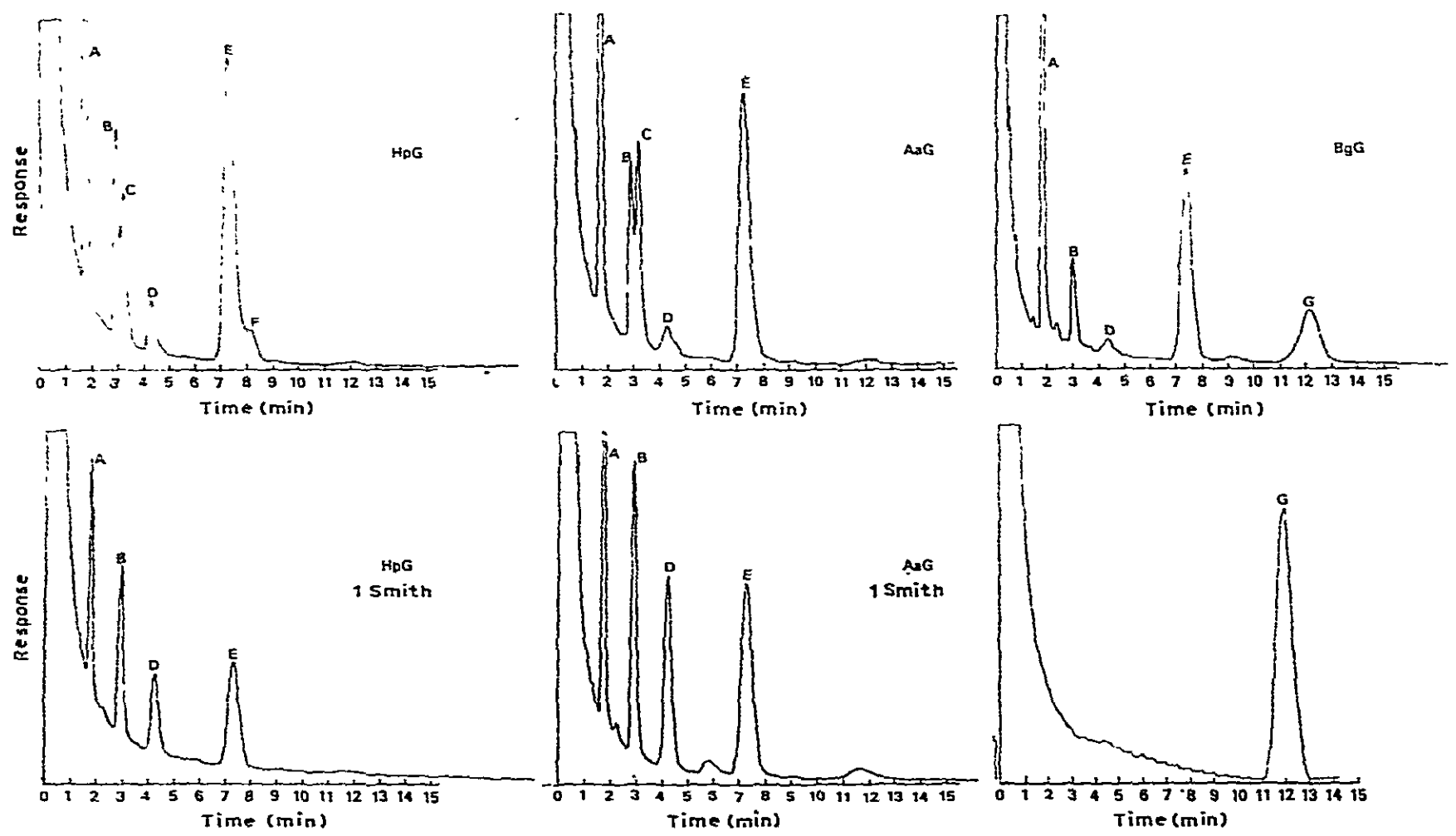


Fig. 9. G.l.c. patterns of the acetylated, reduced, hydrolyzed, permethylated galactans on 3% of SP-2340 on Supelcoport (100–120) mesh at 200°. [Key: A, 2,3,4,6-Me_4-Galol; B, 2,4,6-Me_3-Galol; C, 3,4,6-Me_3-Galol; D, 2,3,4-Me_3-Galol; E, 2,4-Me_2-Galol; F, 3,4-Me_2-Galol; and G, hexa-*O*-acetylGalol.]

Hydrolysis of the permethylated galactans, reduction, and per-*O*-acetylation resulted in various *O*-acetylated, methylated galactitol derivatives that were analyzed by g.l.c. and m.s.[36,37]. The components found are listed in Table II, and the g.l.c. patterns are shown in Fig. 9. HpG and CnG showed almost identical compositions

TABLE II

RETENTION TIMES (R_t)[a] AND RELATIVE MOLE RATIOS OF THE *O*-ACETYLATED AND *O*-METHYLATED GALACTITOL DERIVATIVES OBTAINED FROM THE VARIOUS SNAIL GALACTANS

Galactan studied	*2,3,4,6-Me_4-Gal*		*3,4,6-Me_3-Gal*		*2,4,6-Me_3-Gal*		*2,3,4-Me_3-Gal*		*2,4-Me_2-Gal*		*3,4-Me_2-Gal*		*Ac_6-Galol*	
	R_t	*Rel. mol. ratio*	R_t	*Rel. mol. ratio*	R_t	*Rel. mol. ratio*	R_t	*Rel. mol. ratio*	R_t	*Rel. mol. ratio*	R_t	*Rel. mol. ratio*	R_t	*Rel. mol. ratio*
	On 15% of Apiezon T, at 180°													
HpG native	1	39.1	1.32	9.1	1.39	10.8	1.73	3.2	2.37	37.8				
AaG native	1	38.2	1.31	14.1	1.38	11.2	—	—	2.36	36.5				
CnG native	1	42.3	1.32	7.3	1.39	11.7	1.73	1.1	2.37	37.6				
BgG native	1	40.4	—	—	1.39	8.5	—	—	2.37	40.6			3.77	10.5
HpG KOH-treated	1	42.5	1.32	11.7	1.39	8.6	1.73	2.1	2.37	35.1				
HpG 1st IO_4^-/BH_4^-	1	24.1	—	—	1.38	27.9	1.72	14.5	2.36	33.5				
AaG 1st IO_4^-/BH_4^-	1	24.3	—	—	1.39	24.0	1.73	19.4	2.37	32.3				
	On Quadrex glass, capillary column[b] coated with OV-225, at 200°													
HpG native	1	39.6	1.61	8.8	1.52	10.7	2.3	3.2	3.28	35.3	3.50	2.4		
AaG native	1	40.1	1.60	11.4	1.51	11.4	—	—	3.25	37.0	—	—		
BgG native	1	40.4	—	—	1.51	8.4	—	—	3.25	37.9	—	—	5.40	13.3
HpG 1st IO_4^-/BH_4^-	1	25.6	—	—	1.50	26.3	2.3	15.6	3.25	32.5	—	—		
AaG 1st IO_4^-/BH_4^-	1	24.8	—	—	1.50	23.8	8.3	19.3	3.25	32.1	—	—		
Corresponding structural feature									↓ 3		↓ 2		↓ ↓ ↓ 4 3 2	
	Gal-(1→		→2)-Gal-(1→		→3)-Gal-(1→		→6)-Gal-(1→		→6)-Gal-(1→		→6)-Gal-(1→		→6)-Gal-(1→	

[a]Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalol; 2,3,4,6-Me_4-Gal assignment by *mass spectrometry*. The 3,4,6-Me_3-Gal and 2,4,6-Me_3-Gal derivatives reverse their relative order of retention on the two different liquid phases. [b]The retention times for the 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and the 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl compounds are the reverse of those in the Apiezon column; they have been listed so that each column contains the same structural feature.

on Apiezon T, respectively producing 39 and 42 mol of 1,5-di-*O*-acetyl-2,3,4,6-tetra-, 9.1 and 7.3 mol of 1,2,5-tri-*O*-acetyl-3,4,6-tri-, 10.8 and 11.7 mol of 1,3,5-tri-*O*-acetyl-2,4,6-tri-, and 37.8 and 37.7 mol of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylGalol. Small proportions of 1,5,6-tri-*O*-acetyl-2,3,4-tri- and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methylGalol were found, but these were not used in computing a repeating unit. Comparable results were found with the OV-225 capillary column. The CnG peaks were identified only by g.l.c. (not by m.s.), and BgG showed a peak, identical in g.l.c. and in m.s., with that of hexa-*O*-acetylgalactitol.

AaG gave the identical derivatives, but the ratio of the two tri-*O*-methyl derivatives differed, compared to HpG and CnG. For native BgG, and for HpG and AaG after one cycle of Smith degradation, the 3,4,6-tri-*O*-methylGalol derivative was absent.

The percentage of 2,4,6-tri-*O*-methylGalol from the native galactans varied, but corresponded to ~8–11% of the total D-Gal residues. On a molar basis, the Smith-degraded HpG and AaG gave ~26.2 and 23.8% of 2,4,6-tri-*O*-methylGalol derivatives, and the 2,3,4-tri-*O*-methylGalol respectively increased from values corresponding to 3.2 and 1.1% to 15.6 and 19.2% of the carbohydrate content.

KOH-treated HpG afforded the same methylated compounds, in the same proportions, as those given by HpG.

DISCUSSION

The four galactans studied are polymers of high molecular weight composed entirely of D- and L-Gal in the ratio of ~6:1, similar to that estimated from the $[\alpha]_D$ value on hydrolysis, in good agreement with the values of Bell and Baldwin[5] and Weinland[15] for HpG, but differing substantially from the 36% of L-Gal reported for BgG by Corrêa *et al.*[16]. The value was based on oxidation of the D-Gal by D-galactose oxidase, and might be incomplete, as inferred by Fleitz and Horstmann[4] for the galactan of the snail *Lymnea stagnalis*. The L-Gal content calculated from the difference between the galactose content of the native galactans determined colorimetrically and enzymically with D-galactose oxidase was also somewhat greater than expected from the optical rotation, but this may also be due to incomplete oxidation.

For native HpG and AaG, $[\alpha]_D^{20}$ −15.6° and −15.4° changed, on Smith degradation, to $[\alpha]_D^{20}$ +15.5° and +16.7°, respectively. Oxidation with D-galactose oxidase, and the optical rotation of the hydrolyzed, Smith-degraded materials, indicated that both are free from L-Gal.

Phosphate was present in native and Smith-degraded HpG, CnG, and BgG, giving them a negative charge, but was absent from AaG and its Smith-degradation product. The four native galactans contained only 0.5 to 0.9% of N present as amino acids, and contained no sulfate, pyruvate, lactate, and short-chain carboxylic acids. Absence of uronic acids from HpG was reported by Bell and Baldwin[5,11], as well as by Grainger and Shillitoe[55]. No bands corresponding to uronic acids were seen in

the i.r. spectra of the four galactans before and after hydrolysis, nor were any such acids found colorimetrically[32] or by t.l.c. after hydrolysis.

The electrophoretic mobility differed strikingly among the galactans, and could not be correlated to their phosphate contents. In agar, the HpG, CnG, and HpG after one cycle of Smith degradation migrated to the anode, whereas AaG and BgG appeared to move towards the cathode (electroendosmosis) (see Fig. 1); hydrazinolysis did not change the direction of migration. BgG, having a higher phosphate content than HpG and CnG, migrated in the same direction as the phosphate-free AaG. In agarose, AaG and BgG did not move, whereas HpG, CnG, and HpG (1st Smith-degradation) moved towards the anode.

Permethylation with subsequent hydrolysis, and identification of the resulting products as partially *O*-acetylated, *O*-methylated alditols by g.l.c. and m.s., showed that the native HpG, CnG, and AaG contain the following structural elements:

$$\begin{array}{l} \text{L- and/or D-Gal-(1}\rightarrow\text{; }\rightarrow\text{2)-D-Gal-(1}\rightarrow\text{ and/or }\rightarrow\text{2)-L-Gal-(1}\rightarrow\text{; }\rightarrow\text{3)-D-Gal-(1}\rightarrow\text{ and} \\ \rightarrow\text{3)-D-Gal-(1}\rightarrow\text{.} \end{array}$$

$$\begin{array}{l} \text{6} \\ \downarrow \end{array}$$

In BgG, the position of L-Gal is uncertain, as Smith degradation was not performed and →2)-Gal-(1→ was not observed; the other elements are identical. The tetra- and di-*O*-methylgalactitol are found in about equal amounts, comprising ~80% of the galactose present in the undegraded polymer. The tri-*O*-methylgalactitol derivatives indicate that (1→3)- and (1→2)-linked galactose residues are present in the native galactans in comparable amounts, but the ratio varies somewhat, depending on the species studied. KOH-treated HpG exhibited the same structural elements in proportions very similar to those of the native galactan. Bell and Baldwin[5,11], in early permethylation studies, identified tetra- and di-*O*-methylgalactose, and also detected ~8% of a tri-*O*-methylgalactose which was not characterized. I.r. spectroscopy was not available at that time, and the product was considered to be the result of incomplete methylation.

Our methylation data elucidate certain structural elements of AaG and CnG not studied previously, and reveal features of the structures of HpG and BgG not previously known. Most important is the finding of ~1 mol of 2-linked galactose per ~10–12 mol in the intact galactan of Hp, Cn, and Aa. This galactose, as well as about four terminal galactose units in HpG and AaG are completely destroyed by one stage of Smith degradation, giving rise to an amount of terminal, 6-linked D-Gal equivalent to the 2-linked galactose (see Table II and Fig. 9). Thus, the following minimal, external structural-element could be present.

$$\begin{array}{l} \text{Gal-(1}\rightarrow\text{2)-Gal-(1}\rightarrow\text{3)-D-Gal-(1}\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \text{6} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \text{1} \\ \qquad\qquad\qquad\quad \text{Gal-(1}\rightarrow\text{3)-D-Gal} \end{array}$$

As all of the L-Gal is also destroyed by the Smith degradation, and if it is

assumed that these two circumstances are related, especially in view of the specificity of biosynthetic reactions involving sugars, it could be hypothesized either that (*a*) the L-Gal is terminal and linked (1→2) to the subterminal Gal, or (*b*) the subterminal Gal is also an L-Gal residue, or that both the terminal and the (1→2) subterminal residues are L-Gal, depending on the species studied; this was not suspected from the work of O'Colla[12], Baldwin and Bell[11], and others.

The rest of the structure cannot be defined without performing further stages of Smith degradation. During the first Smith degradation, a lower consumption of periodate (0.7–0.75 mol of IO_4^- per mol of Gal residue) was observed than would be expected from the permethylation data (0.9 mol of IO_4^- per mol of Gal residue). Periodate consumption increased with the temperature and with the concentration of periodate (see Fig. 7b); therefore, oxidation may have been incomplete under the conditions used. This could be due to hemicetal formation, as observed by Painter and Larsen[56], which would prevent oxidation of some terminal galactose residues, thus creating, after reduction and mild hydrolysis, ~7.5% of unsubstituted, internal Gal residues. Furthermore, 5% of glycerol was found in the first stage of Smith degradation (see Table I), and this was not split off during hydrolysis, accounting for 10% of all Gal units, and thus explaining the lower proportion of tetra-*O*-methyl-Galol detected. The two effects would give rise to ~17.5% of tri-*O*-methylGalol. Over 40% of tri-*O*-methylGalol derivatives was detected, and thus a substantial proportion of the unsubstituted internal Gal residues must have been generated *de novo*.

The presence of →3)-D-Gal-(1→ in the native galactan, and the substantial increase of tri-*O*-methylGalol derivatives in the Smith-degraded material, are inconsistent with a dichotomously branched structure[12,16]. O'Colla[12] and Corrêa *et al.*[16] implied that branching was symmetrical, so that each cycle of Smith degradation should result in a product containing equal proportions of terminal and doubly substituted galactose units, and neither unsubstituted (1→3) nor (1→6)-linked galactose residues could be generated.

However, a structure in which the branches differ in length and in the degree of branching could explain the increase of these in the first stage of Smith degradation, because they would be the remnants of branching points, where single-membered side-chains were split off during one cycle of Smith degradation. The 3.2 and 1.1 mol of →6)-Gal-(1→ found in the native HpG and CnG by methylation are ascribable to incomplete biosynthesis. From the content of phosphorus, there would be ~1 mol of galactose phosphate per 36.0 mol of galactose. As only ~20% was lost by Smith degradation, some of the phosphate must be linked to residues not susceptible to periodate oxidation.

Most unusual is the identification of hexa-*O*-acetylgalactitol (see Figs. 9 and 10), which constituted 13% of the methylated BgG on a molar basis. This compound could have arisen either from a 2,3,4,6-substituted D-Gal or from a hexasubstituted hexitol. Fig. 10 compares the mass spectrum of the compound with that of hexa-*O*-acetylgalactitol. Moreover, BgG showed no 6-linked or 2-linked D-Gal. As only Gal

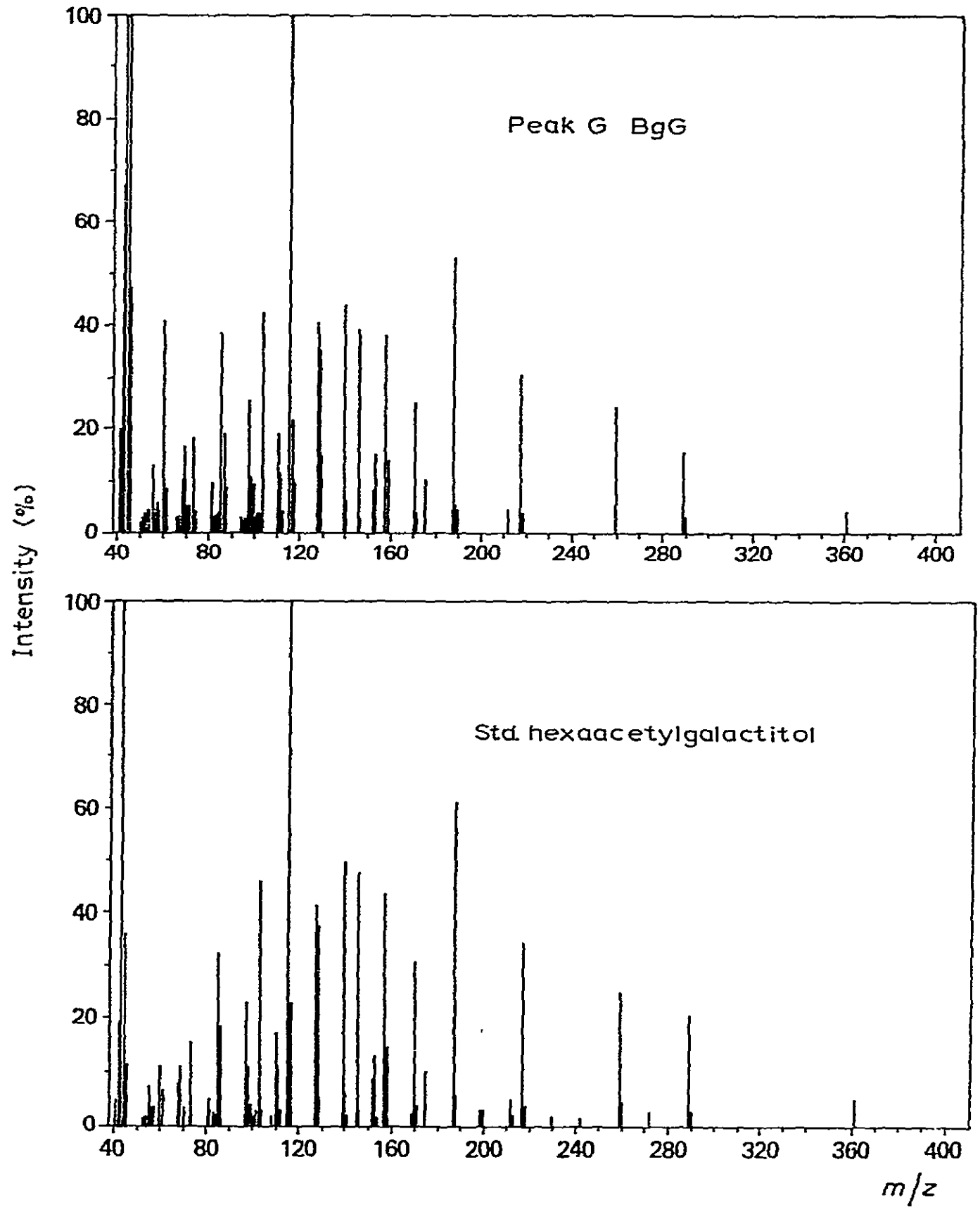


Fig. 10. Mass spectra of peak G of BgG (*top*) and of standard hexa-*O*-acetylgalactitol having the same retention-time and the following *m/z* values (intensity %): 85 (33), 97 (23), 103 (45), 115 (100), 127 (40), 128 (38), 139 (50), 145 (48), 157 (45), 170 (32), 187 (62), 217 (35), 259 (25), 289 (22), and 361 (8%).

was found after methanolysis and acetylation, hexitol is absent, and the methylation data establish a 1,2,3,4,6-linked Gal. Thus, BgG has structural features differing completely from those of the three other galactans, although it, too, contains L-Gal in similar proportions, and most of the Gal residues are (1→3)- and (1→6)-linked*.

*Drs. Bo Nilsson and David A. Zopf of the Laboratory of Pathology, National Cancer Institute, kindly methylated a sample of BgG and independently confirmed by g.l.c. and m.s. the presence of all peaks reported, including that of the hexa-*O*-acetylhexitol.

In the reduced hydrolysis-products from the methylation of other snail galactans, 2,4,6-tri-*O*-methylgalactitol was detected by Duarte and Jones[17] in that of *Strophocheilus oblongus* and, in addition, 3,4,6-tri-*O*-methylgalactitol was found in that of *Ampullarius* sp. by Feijo and Duarte[18], besides the main components, namely, 2,3,4,6-tetra- and 2,4-di-*O*-methylgalactitol. It thus seems that snail galactans are synthesized by following similar, general principles. It is surprising that even such closely related species as Hp and Aa (belonging to the same family of Helicidae) show important differences in their antigenic determinants. It is, therefore, of interest to attempt to correlate the structural and compositional data (see Table I) with the immunochemical findings shown in Figs. 1 and 2. Antisera to HpG cross-react substantially with CnG and BgG, but do not react with AaG; AaG is the only such galactan that contains no phosphate; thus, it might be expected that Gal 6-PO_4 is part of an antigenic determinant, and this is consistent with the finding that Gal 6-PO_4 was an inhibitor, although weak, of the HpG anti-HpG reaction (see Fig. 5). The antiserum to the Smith-degraded HpG cross-reacts similarly, indicating that the same antigenic determinants are involved, and the Smith-degraded HpG still contains 0.4% of phosphorus.

Anti-AaG serum also cross-reacts with HpG, BgG, and CnG; as AaG contains no phosphate, the cross-reactions must be due to other (probably uncharged) antigenic determinants of these three galactans. The substantially lower reactivity of the antiserum with Smith-degraded HpG and AaG (see Fig. 2b) indicates the loss of considerable numbers of antigenic determinants.

The findings are consistent with our studies on the cross-reaction of HpG and CnG with two human-myeloma antibodies[20] having specificity for 3,4- and 4,6-pyruvylated D-Gal, the cross-reaction being ascribable to the charged PO_4 group on the D-Gal, whereas AaG and BgG did not cross-react. L-Gal is not involved in the specificity of the two myeloma sera, because the periodate-oxidized HpG was a better precipitinogen than the native HpG. However, the differences in the antibody specificity are not ascribable only to such terminal residues as D-Gal 6-phosphate or L-Gal, but also to differences in the nonterminal structure. Otherwise, the lessened cross-reactivity of HpG, CnG, and BgG with the anti-AaG serum, for instance, as seen in quantitative precipitin assays (see Fig. 2b) and in the Ouchterlony test (see Figs. 2g,j), could not be explained.

Whether the penultimate sugar is, or is not, substituted, or whether the substituent is a monosaccharide or a short, branched, or unbranched, side chain must be of great importance for binding. This inference is supported by the observation that D-galactose oxidase showed striking differences in its attack on the different galactans (see Figs. 6a,b), indicating that the enzyme may be in contact with more than a single galactose residue in the galactan molecule during the oxidation. If the extent of the complementarity is insufficient, little or no oxidation takes place.

Further studies on the antigenic specificities of the snail galactans are clearly needed. The heterogeneity of the antibody populations revealed by the present studies, together with the unusual, structural differences among the galactans, strongly

indicates a need for preparation, and characterization of the specificities, of hybridoma antibodies to the galactans, so as to be able to characterize more precisely their antigenic specificities. In addition, oligosaccharides from the various snail galactans should be isolated and characterized.

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