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AN IMMUNOLOGICALLY ACTIVE ARABINOGALACTAN FROM *VISCUM ALBUM* 'BERRIES'

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Viscum album*; Loranthaceae; mistletoe berries; polysaccharide; arabinogalactan; immunological activity.

Abstract—From *Viscum album* 'berries', a complex acidic arabinogalactan (Fb) with mean M_r of 700 000 has been isolated by DEAE-Trisacryl M column chromatography and by affinity chromatography on Sephacryl S 1000 bound *Ricinus communis* lectin and separated into two arabinogalactan-fractions (RCA-N and RCA-G). Their structures were elucidated mainly by methylation analysis, partial acidic hydrolysis and ^{13}C-NMR spectroscopy. The acidic arabinogalactan Fb stimulates *in vitro* the alternative pathway of the human complement system.

INTRODUCTION

Various extracts of mistletoe (*Viscum album* L.) are used in adjuvant cancer therapy. As possible active principles of the drug, the typical mistletoe lectins and other proteins (Viscotoxins) are discussed [1]. Besides these constituents of the drug also polysaccharides with antitumour activity and general immunmodulating properties have been isolated [2–4]. Because there were only few data on the sugar composition available, [2, 5, 6], and in order to clarify the possible role of polysaccharides in the antitumour activity of the *Viscum* extracts, we have started a thorough chemical analysis of the polysaccharide fraction from stems, leaves and 'berries' of mistletoe. A preceding paper dealt with the isolation and characterisation of a highly esterified 1,4-galacturonan from stems and leaves [7]. Now we report on the isolation and structure elucidation of a water soluble immunologically active arabinogalactan from the 'berries'.

RESULTS AND DISCUSSION

Isolation and purification of the polysaccharide

Freeze dried 'berries' were defatted with petrol and methanol and the drug residue subsequently macerated with cold water. From this aqueous extract a crude polysaccharide fraction was obtained by precipitation with ethanol, which was further subjected to ultrafiltration to remove the major part of proteins. On DEAE Trisacryl M-chromatography the retentate revealed the presence of four optically active peaks (Fig. 1).

Most of the starting material was recovered in fraction Fb (61%) with minor amounts found in Fa (3%), Fc (3%) and Fd (0.7%). Fa and Fb with levorotation showed a similar high content of neutral sugars, whereas Fc was dextrorotatory and yielded a low concentration of neutral sugars. Treatment of Fc with polygalacturonase resulted in a complete depolymerisation, indicating that this fraction consisted mainly of a pectin with 1,4 linked D-galacturonic acid units. Fd was identified as ribonucleic acid as it was degraded by ribonuclease. The sugar composition of the main fraction Fb is shown in Table 1.

Because of its high content of arabinose and galactose, polysaccharide Fb can be classified as of the arabinogalactan type. On analysis by gel filtration on Sephacryl S-500 it displayed an unsymmetrical molecular-weight-distribution (M_r 700 000) with a tailing of the peak towards the exclusion volume. The sugar composition of the material in the leading edge and the main peak, however, showed no significant differences.

Orientating methylation analysis of Fb revealed the presence of terminal galactopyranosyl residues. Since this structural feature is a potential binding site for galactose-specific lectins, a further purification of Fb is possible by affinity chromatography, e.g. on immobilized *Ricinus communis* lectins (RCA). An isolation of an arabinogalactan by affinity chromatography on immobilized Tridacna-max. lectin has already been described by Gleeson *et al.* [8]. The usual method for testing the binding capacity of a lection to a polysaccharide is the double-diffusion technique in agarose gels [e.g. 9]. However when RCA and the arabinogalactan Fb were tested, no precipitation occurred. In contrast, after removal of the arabinose residues from Fb by partial hydrolysis (see below), a broad precipitation zone was obtained. An interaction of Fb and RCA, however, could be demonstrated by a gel filtration system (Fig. 2).

On account of their different molecular sizes Fb and RCA migrated differently when submitted to gel filtration on Sephacryl S-400, Fb appearing in the region of the exclusion volume and RCA near the total volume. When the two substances were incubated before chromatography, a part of the proteins (RCA) detected by their UV-absorption was eluted with the polysaccharide. This interaction was suppressed in the presences of the lectin inhibitor lactose. This indicated that at least one part of polysaccharide Fb was bound by RCA.

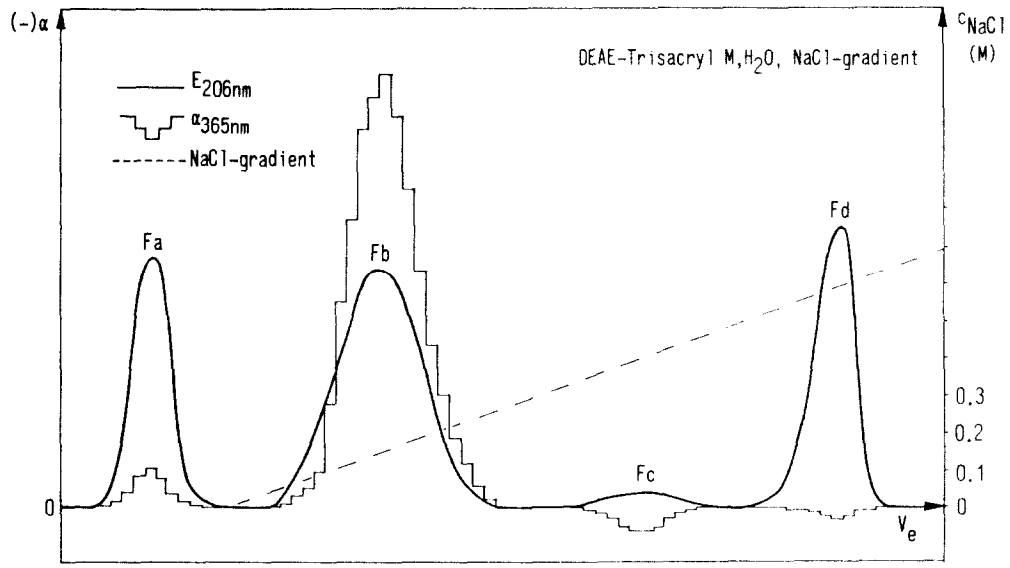

Fig. 1. Ion exchange chromatography–retentate after ultrafiltration.

Table 1. Analysis of the arabinogalactans Fb, RCA-N and RCA-G

	[weight %]		
	Fb	RCA-N	RCA-G
Neutral sugars (total)	58.0	57.9	63.3
Ara	33.3	37.3	31.1
Gal	22.0	19.3	27.2
Rha	1.7	2.0	2.0
Glc	1.0	tr.	3.0
Man	tr.	tr.	tr.
Xyl	tr.	tr.	tr.
Uronic acid	18.0	21.0	22.0
Protein	1.0	1.5	1.2

For preparative chromatography an appropriate affinity support was prepared by coupling RCA to Sephacryl S-1000 by the cyanogen bromide method [10]. When Fb was applied to a column of RCA-Sephacryl S-1000 about 65% of the material was not retained (fraction RCA-N). The bound fraction could be released by elution with 0.2 M lactose (fraction RCA-G). For analytical purposes the effluent of the affinity column was chromatographed further on a Sephadex G-25 column in order to separate the high molecular weight polysaccharide from lactose and detect it by a refractive index detector. For isolation on a preparative scale the Sephadex column was omitted. By overloading RCA-Sephacryl S-1000 with Fb the maximum capacity was determined to 2.8 mg RCA-G/10 ml support. The affinity column could be regenerated by flushing with lactose free buffer.

The M_r of both fractions was determined by gel filtration on Sephacryl S-500 as 180000 for RCA-N and as 900000 for RCA-G. The sugar composition of RCA-N and RCA-G is shown in Table 1.

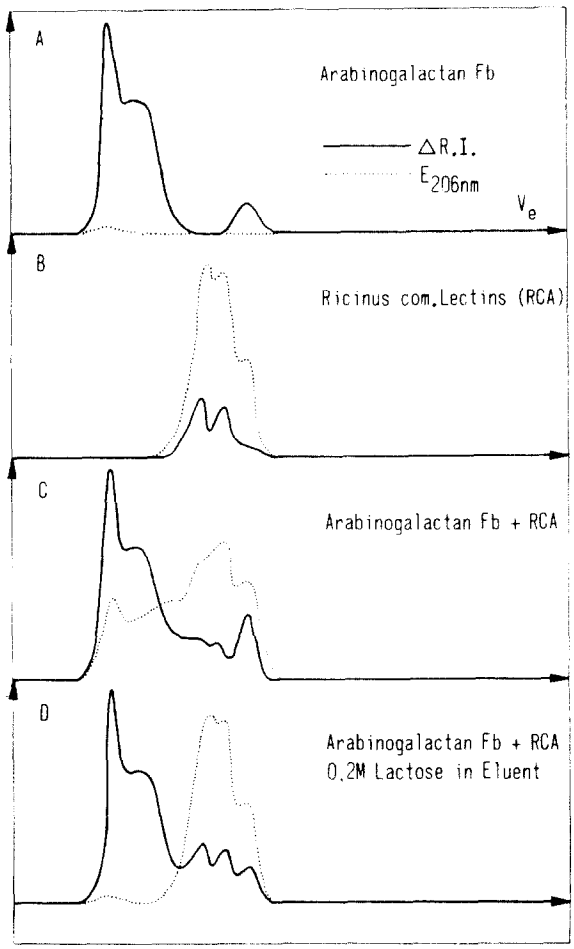

Fig. 2. Binding of *Ricinus com.* lectins (RCA) to the arabinogalactan Fb.

Structural analysis of partially hydrolysed arabinogalactan (Fbr)

Since for RCA-N and RCA-G similar backbone could be expected, we subjected polysaccharide fraction Fb to a partial hydrolysis. For the maximum release of arabinose a treatment with 0.05 M TFA/90°/2 hr was found to be necessary. Under these conditions also *ca* 20% of the total galactose content of Fb (mainly as oligogalactosides was lost) during subsequent dialysis against distilled water. In the undialysable polysaccharide part (Fbr), the sugars were determined as uronic acid (28%), galactose (48%), rhamnose (6%) and arabinose (2%). The uronic acid was released by treatment of Fbr with polygalacturonase and identified as D-galacturonic acid. By GPC-HPLC Fbr was resolved into two peaks (A: M_r 19 000–42 000, broad peak; B: M_r 3600).

On ion exchange chromatography on DEAE-Sepharose CL-6B, most of Fbr was retained on the column and *ca* 10–20% eluted in the neutral fraction. The acidic fraction was composed mainly of galactose and galacturonic acid, whereas in the neutral fraction only galactose was found. GPC-HPLC of the acidic fraction showed identity with peak A and correspondingly the neutral galactan with peak B of Fbr. Treatment of Fbr with 0.05 M TFA and GPC-HPLC analysis at different time intervals suggested that the neutral galactan (peak B) was released from the acidic galactan (peak A) under the conditions of partial hydrolysis. The acidic galactan presumably forms the galactose/galacturonic backbone in the arabinogalactan Fb. Because of the significant decrease of the molecular weight by acid treatment it is possible that acid labile linkages (e.g. to rhamnopyranosyl units) within this backbone were cleaved.

To ascertain that all the galacturonic acid found in Fbr was bound to galactose, affinity chromatography on RCA-sephacryl S-1000 was performed. Fbr was separable into a lectin-bound and -unbound fraction. Since both fractions did not differ in their galactose/galacturonic acid ratio, it can be assumed that in Fbr a covalent link exists between the galactose- and the galacturonic acid moiety. The low affinity of the lectin-unbound fraction was possibly due to the lower content of terminal galactopyranosyl residues in this polysaccharide, as confirmed by methylation analysis.

Methylation analysis of Fbr was performed according to Jansson *et al.* [11]. To achieve complete derivatisation acetylation of the polysaccharide [12] prior to the methylation step was necessary. One part of the methylated Fbr was carboxyl-reduced with $LiAlD_4$ [13]. The partially methylated alditol acetates detected by GC-MS analysis and identified by comparison with literature values are listed in Table 2. From the carboxyl reduced polysaccharide only two double deuterated galactose derivatives were obtained. The 2,3- di-*O*-methylgalactitol acetate indicated that galacturonic acid (= D-GalpA) must have been present in a 1,4-linkage. The second much smaller peak could be derived from a 1,2,4-linked D-GalpA, so that some of the galactan chains might be connected via O-2 to a 1,4-linked D-GalpA residue. The detection of derivatives of 1,2,4- and 1,4-linked rhamnopyranose suggests that in Fbr, like in other rhamnogalacturonans [14], small blocks of 1,4-linked D-GalpA are interspersed by rhamnopyranosyl units carrying most of the galactan chains via O-4. This model is supported by the fact that only monomeric D-GalpA and very small amounts of oligogalacturonides could be split off from Fbr by treatment with polygalacturonase. If longer uninterrupted D-GalpA chains had been present as characteristic for homogalacturonans, considerable amounts of di- and trigalacturonides would have been found.

Unlike other arabinogalactans (so called arabino-3,6-galactans) [15] the galactan core of Fbr is almost exclusively built up by 1,6 linked galactopyranosyl (= D-Galp) units. A few of them must have branches on position O-3 consisting of single D-Galp residues or other galactan chains. An alternative for these branching points, a 1,3-linked D-Galp with a substitution in position O-6, cannot be ruled out. The ^{13}C NMR spectrum of Fbr shows that the 1,6 linked D-Galp and the terminal D-Galp residues occur in the β-form.

Methylation analysis of polysaccharides RCA-N and RCA-G

Information on the types of linkages in the arabinose-moiety of the affinity-purified arabinogalactans RCA-N and RCA-G was obtained by methylation analysis (Table 2). From the deduced glycosyl linkage of RCA-N and RCA-G, we can assume that the complex arabinan side chains are connected with the GalpA/Galp-core.

Like arabinans from other sources [16, 17], these arabinan side chains are built up by 1,5 linked L-arabinofuranosyl residues (= L-Araf) with 1,3,5 and 1,2,5 linked

Table 2. Partially methylated alditol acetates and deduced glycosidic linkages of polysaccharides Fbr, RCA-G, RCA-N in [Mol %]

Derivative		Fbr	RCA-G	RCA-N	Deduced glycosidic linkage
2,3,5	OMe Ara*	—	0.37	0.36	term. Araf
3,4	OMe Rha	0.03	—	—	1,2 linked Rhap
2,3	OMe Ara	—	0.16	0.27	1,5 linked Araf
2,3,4,6	OMe Gal	0.18	0.02	0.02	term. Galp
3	OMe Rha	0.17	0.04	0.03	1,2,4 linked Rhap
2	OMe Ara	—	0.01	0.03	1,3,5 linked Araf
3	OMe Ara	—	0.06	0.09	1,2,5 linked Araf
2,3,4	OMe Gal	0.47	0.14	0.07	1,6 linked Galp
2,4	OMe Gal	0.08	0.20	0.14	1,3,6 linked Galp

*2,3,5-*O*-Methylarabinitolacetate.

L-Araf at the branching points. Because of the much lower content of 1,3,6 linked D-Galp in Fbr (= residue of partial hydrolysis) probably all arabinans or arabinosyl residues are connected via O-3 of the D-Galp branching points.

A relatively large number of terminal L-Araf residues is found specially in fraction RCA-G. This number exceeds the proportion which derives from the terminal groups of arabinan-side chains. Therefore, the occurrence of single L-Araf residues directly attached to the galactan-core can be assumed. Probably this type of substituent is also present in RCA-N. Because of the higher content of complex arabinans in this fraction (higher amount of L-Araf branching points), the occurrence of single L-Araf residues cannot be deduced from the result of the methylation analysis only. This could be achieved by recording the ^{13}C NMR spectra of both arabinogalactans.

^{13}C NMR spectroscopy

General structural features. The ^{13}C NMR spectrum of polysaccharide-fraction RCA-G with the assignment of the resonances for six different types of arabinose and galactose linkages is shown in Fig. 3. In the C-1-area of the spectrum (103-110 ppm) at least six signals could be observed indicating that at least six different linkage types occur in the arabinogalactan moiety of RCA-G. In Fig 4 the linkage types Aa, Ab, Ac, Ad, Ga, Gb are shown with the corresponding resonances given in Table 3.

The correct assignment of signals resulting from galactose residues could be confirmed by comparison with the ^{13}C NMR spectrum of partially hydrolysed arabinogalactan Fb, in which a set of six signals could be attributed to C-1–C-6 of 1,6-β linked D-Galp (linkage type Ga) and

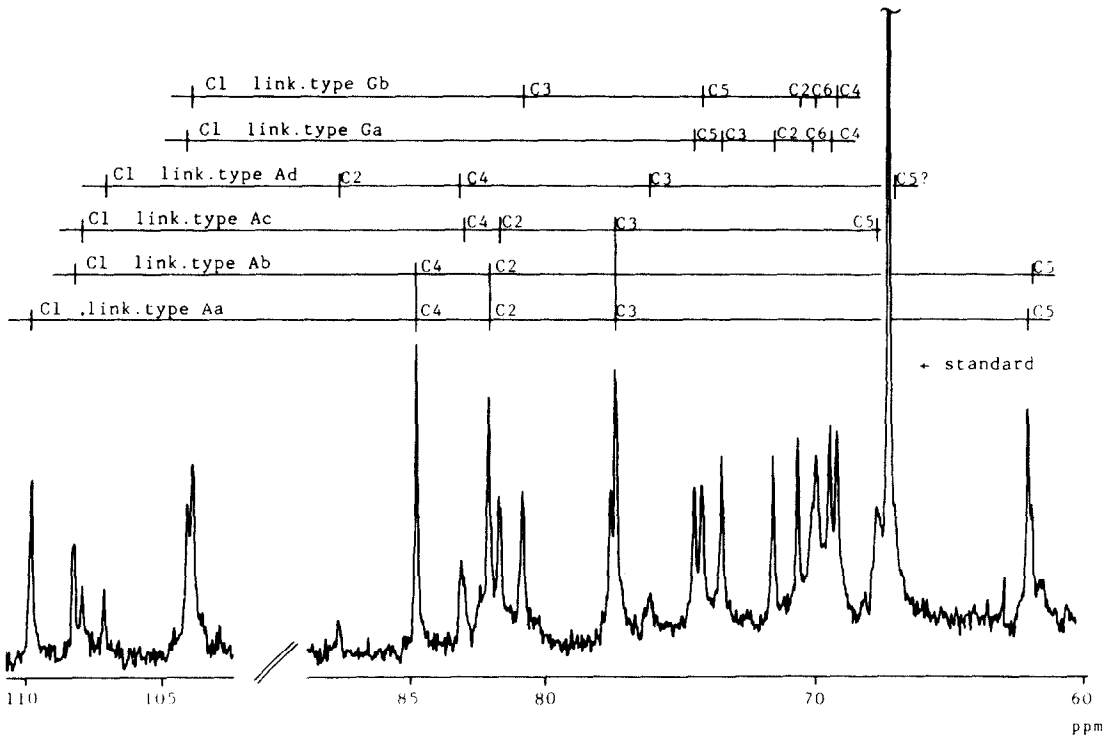

Fig. 3. Assignment of ^{13}C NMR signals of RCA-G, (Table 3) ^{13}C NMR data of RCA-G

Table 3. ^{13}C NMR data of RCA-G

Linkage type (see Fig. 4)	Chemical shifts (α) C-1	C-2	C-3	C-4	C-5	C-6
Aa: term. α-L-Araf	109.9	82.1	77.5	84.8	62.2	
Ab: term. α-L-Araf	108.3	82.1	77.5	84.8	62.0	
Ac: 1,5 linked α-Araf	108.0	81.7	77.5	83.0	67.7	
Ad: 1,2,5 linked α-Araf	107.2	87.7	76.1	83.1	*	
Ga: 1,6 linked β-D-Galp	104.2	71.6	73.5	69.5	74.5	70.1
Gb: 1,3,6 linked β-D-Galp	104.0	70.7	80.8	69.2	74.2	70.0

*Covered by internal standard 67.3 ppm.

the ^{13}C NMR spectrum of a 1,3,6-linked D-Galp containing tetrasaccharide [18] reported in the literature. In this linkage type C-1 resonates in a lower field than C-1 in 1,6-β linked D-Galp. Thus the signal located 0.2 ppm lower than C-1 of linkage type Ga should derive from linkage type Gb. Also the resonances of C-2 to C-6 (1,3,6 linked-β-D-Galp) in the above mentioned ^{13}C NMR study of the tetrasaccharide were in good agreement with those found for the equivalent unit in the *Viscum* polysaccharide Fb. As seen from the results of the methylation analysis, partial hydrolysis removes the substituents of O-3 in the D-Galp residues so that the acidlabile linkages to single L-Araf residues or to the arabinan chains can be placed at these positions in the genuine arabinogalactans.

Since after partial hydrolysis all signals above 105 ppm in the region of the C-1 resonances were missing these signals must derive from arabinose residues. Apart from a shift of 0.4 ppm, probably due to the recording of the spectra at different temperatures, the three signals at 108.3, 108.0 and 107.2 ppm appear at the same δ values as those found in the ^{13}C NMR spectra of arabinans like those of *Althaea officinalis* [16] and *Salix alba* [19]. According to the assignments given in [16] and [19], these resonances derive from the α-L Araf linkage types Ab, Ac and Ad. The signals for C-2–C-5 of each type can also be found in the spectrum of the *Viscum* arabinogalactan.

The C-1 signal of a terminal α-L-Araf residue which is bound to other arabinose units (e.g. in arabinans) is obviously located in a higher field than the C-1 resonance of a terminal α-L-Araf residue connected with a galactan. ^{13}C NMR data of the latter type were reported for arabinogalactans of *Nicotiana tabacum* [20], sugar cane [21] and *Echinacea purpurea* [22]. Methylation analysis of these polysaccharides revealed that most of the L-Araf units were attached directly to the galactan moiety. The resonance for C-1 of the pentose was observed at about 110 ppm. So in the spectrum of RCA-G the signal at 109.9 ppm derives from such an α-L-Araf residue (linkage type Aa). Considerable changes in the C-1 chemical shifts of a glycosylating residue dependent on conformational differences in the glycosylated residue were reported and discussed in detail by Shashkow [23]. For this reason it is not surprising that for linkage type Aa and linkage type Ab different resonances for C1 were observed.

Structural differences of the arabinogalactans RCA-N and RCA-G. To achieve maximum sensitivity while recording the ^{13}C NMR spectra of RCA-N and RCA-G, the NOE was not suppressed. Thus a quantitative estimation of the occurring linkage types (by integrating, e.g. of C-1 signals) within the same spectrum was not possible. Since, however, both spectra were recorded under identical conditions integrals of equivalent signals could be used to estimate the amount of the respective linkage type in either spectrum. Each signal found in the spectrum of RCA-G was also present in the spectrum of RCA-N. Both polysaccharides differ only in the relative proportions of the linkage types present.

The signals for the anomeric-C-atoms at 108.3, 108.0 and 107.2 ppm deriving from L-arabinan side chains are much more intense in RCA-N than in RCA-G. Whereas the predominating linkage type of arabinose in RCA-G (signal at 109.9 ppm) is Aa (see Fig. 4) indicating that mainly single α-L-Araf units are bound to the 1,6-β galactan chains.

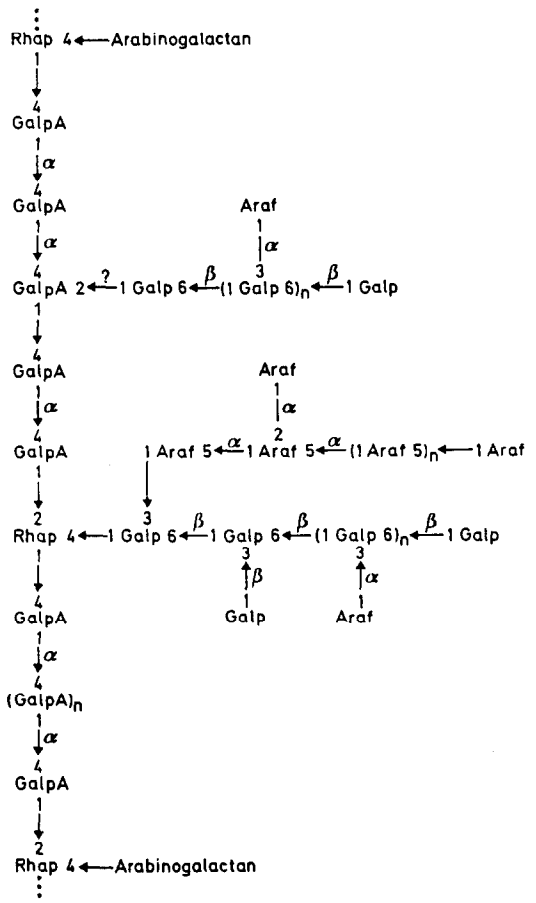

Fig. 4. Proposed structure for RCA-N and RCA-G.

Structure proposal for polysaccharides RCA-N and RCA-G

As seen from methylation analysis and ^{13}C NMR spectroscopy RCA-N and RCA-G do not represent different structure types but differ mainly in the relative proportions of single α-L-Araf residues and complex arabinans attached to the galactans. The structures of both fractions can be envisaged as in Fig. 4. The lower affinity of RCA-N to the immobilized *Ricinus* lectins is possibly due to a shielding of the potential lectin binding sites (terminal D-Galp) by the arabinan side chains which are more abundant in RCA-N than in RCA-G.

Immunological activity

The polysaccharide Fb was investigated in three immunological test systems. In the *in vitro* granulocyte test [24], which was performed with human granulocyte fractions and the *in vivo* carbon clearance test with mice [25], polysaccharide Fb did not enhance phagocytosis activity. In the human complement system, however, the polysaccharide displayed a pronounced effect. It triggers the alternative pathway and causes a 65–70% consumption of complement factors at a concentration of 1 mg/ml [26]. The cleavage of C-3 was demonstrated by immunoelectrophoresis.

EXPERIMENTAL

Analytical methods. Optical rotations were measured in aq. solution (*c* 0.2–0.3) at 15°. GC of alditol acetates was carried out on a Perkin–Elmer 900 GC using a glass column 180 cm × 2 mm) packed with GP 3% SP-2330 on 100/120 mesh Supelcoport, gas flow for Ar 30 ml/min. For partially methylated alditol acetates, a Perkin–Elmer Sigma 1B GC was used with fused silica capillary columns OV 225 (25 m × 0.23 mm i.d.) (Chrompack) 210 grd isothermal and a WCOT FS-OV 101 (25 m × 0.25 mm i.d.) (Machery–Nagel) temp. programmed 160–280°, 4 grd/min. GC for TMS (−)-2-butyl glycosides was done on a WCOT FS CP Sil 5CB (25m × 0.23 mm i.d.) (Chrompack). Carrier gas flow for capillary GC 1 ml/min Ar. All GCs were equipped with a flame-ionisation detector. GC-MS was carried out on a Hewlett Packard 5985A instrument using a fused silica column coated with OV 101 (temp. programmed 160–210°, 3 grd/min) interfaced to a Hewlett Packard 5985A spectrometer operating with an ionisation potential of 70 eV. ^{13}C NMR spectra were recorded in D_2O (int. standard 1,4-dioxan, δ 67.3 ppm) at 60°. A 5 us pulse width and 8000 Hz spectral width were used and a scan number of 25 000. For TLC silica gel 60 plates (Merck) were used. Sugars were detected by a spray-reagent (2 g aniline 2 g diphenylamine, 10 ml 85% *o*-phosphoric acid in 100 ml MeOH and heating at 120° for 15 min.

Plant material. Viscum album var. *album* (L.) growing on a single apple tree in Otterfing/Obb. F.R.G. was harvested in November 1982. The 'berries' were removed and washed briefly with cold deionized water followed by freeze-drying.

Isolation and purification. Extraction. The freeze-dried and ground 'berries' (136 g) were extracted first with petrol then with MeOH. The insoluble residue (52 g) was macerated overnight with 1.5 l deionized H_2O using a magnetic stirrer. After centrifugation, the supernatant (1.2 l) was filtered and 4.8 l of EtOH were added. The pptd material was redissolved in H_2O and freeze-dried (rawpolysaccharides F 11.2 g = 8% of the dried 'berries').

Ultrafiltration. A 250 ml ultrafiltration unit (Amicon) equipped with a membrane 3000T (Berghof GmbH) was used. 2.3 g of F were dissolved in 180 ml H_2O and concd to 60 ml. This procedure was repeated × 3. Recovery after freeze-drying: retentate 85%, filtrate 15%.

Ion exchange chromatography. 200–300 mg of $F > 300\,000$ were loaded on a column ($A = 5$ cm^2, $l = 14$ cm) of DEAE-Trisacryl M (LKB) in acetate form which was equilibrated with H_2O. Components on the column were eluted first with H_2O followed by gradient elution with NaCl (0–1 M in 500 ml H_2O). The effluent was monitored with a UV-detector at 206 nm (Uvicord S II, LKB) and a R. I. detector (R 403, Waters Ass.). 5 ml fractions were collected and the optical rotation (A_{365}) of each fraction was determined. Yields–see text.

Gel filtration. Gel filtration was performed with Sephacryl S-500 (Pharmacia) on a column ($A = 0.6$ cm^2, $l = 60$ cm) with 0.2 M NaCl as eluent. Detection UV 206 nm, R.I. A_{365}. An approximate M_r calibration curve was set up with the dextrans T 2000 (V_e of the first peak $= V_0$), T500, T110 (Pharmacia) and glucose ($V_e = V_t$).

Gel filtration to prove arabinogalactan–lectin interaction. A column ($A = 0.6$ cm^2, $l = 60$ cm) with Sephacryl S-400 (Pharmacia) with a buffer (0.05 M Na-Pi pH 7, 0.15 M NaCl) was used. 4 mg Fb or 0.4 mg lectins or a mixture of both (incubated for 30 min at room temp.) in 0.5 ml eluent were applied. To inhibit the lectin 0.2 M lactose was added to the buffer. Detection UV 206 nm, R.I., flow: 10 ml/hr.

Affinity chromatography. Isolation of *Ricinus* lectins (RCA): Seeds from *Ricinus communis* were purchased from Galke, F.R.G. The *Ricinus* lectins were isolated according to Nicolson *et al.* [28] using partially hydrolysed Sepharose 6B as described in ref. [26].

Coupling of RCA to Sephacryl S-1000 (cyanogen method, see ref. [30]: 20 g Preswollen Sephacryl S-1000 (Pharmacia) were stirred in 20 ml ice-cold H_2O. 500 mg BrCN were added in 10 ml H_2O and the pH of the solution was maintained at 11 by the addition of 2 M NaOH. After 20 min the activated gel was washed with 300 ml ice-cold H_2O and 250 ml ice-cold 0.7 M $NaHCO_3$. 98 mg RCA were dissolved in 100 ml H_2O, 1 g $NaHCO_3$ and the activated gel were added. The mixture was stirred for 2 days at 7°. Unbound proteins were removed by washing with H_2O and 0.05 M Na-Pi pH 7, 0.15 M NaCl. The amount of bound RCA (56%) was determined by comparing the protein content in the starting RCA-solution and the combined washing.

Affinity chromatography of polysaccharides on RCA-Sephacryl S-1000.

Analytical: the effluent of the column with RCA-Sephacryl S-1000 ($A = 0.6$ cm^2, $l = 10$ cm) was conducted on a Sephadex G-25 column ($A = 1.1$ cm^2, $l = 40$ cm). For elution 0.05 M Na-Pi pH 7, 0.15 M NaCl at a flow rate at 10 ml/hr was used. When unretained polysaccharides were washed from the column the desorption with 0.2 M lactose in the buffer was started. Detection: UV 206 nm, R.I., A_{365}. Preparative: only an affinity column ($A = 3.5$ cm^2, $l = 7.5$ cm) was used. In each cycle 15 mg Fb were applied, then the column was washed with 60 ml buffer (fraction RCA-N) and subsequently desorption with 40 ml 0.2 M lactose in the buffer occurred (fraction RCA-G). The affinity support was regenerated by washing with 70 ml lactose-free buffer. The equivalent fractions were concd under red. pres. and dialysed against deionized H_2O for 2 days. Residual lactose was removed by gel filtration on Sephacryl S-400 using 0.2 M NaCl as eluent. (Yields see text).

Analysis of sugar composition. Polysaccharides 1–5 mg were hydrolysed with 2 M TFA at 120° in a sealed tube. After the hydrolysis 1 mg myo-inositol was added as an int. standard. The solution was evapd under red. pres. The derivatization to alditol acetates was performed according to ref. [31] followed by GC as described above.

Identification and quantification of galacturonic acid: the uronic acid was identified in the partially hydrolysed arabinogalactan Fb (Fbr). Hydrolysis with 2 M TFA was performed as described above. Enzymatic hydrolysis by polygalacturonase from *Aspergillus niger* (E.C. 3.2.1.15) (Sigma) was done by incubating the aqueous solution of the polysaccharide (0.5%) together with 3–5 units of the enzyme for 3 hr at room temp. The uronic acids from the enzymatic and acid hydrolysate were enriched by ion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia) ($A = 0.2$ cm^2, $l = 5$ cm) equilibrated with H_2O. After removing unbound substances by elution with water the acid substances were desorbed by 0.2 M HOAc. The HOAc-fraction was concd under red. pres. and analysed by TLC. Solvent system *n*-butanol–Me_2CO–H_2O–HOAc (7:7:4:2). Besides galacturonic acid (R_f 0.25) another but much weaker coloured spot (R_f 0.25) was seen which could not be identified. Quantification of the uronic acid was done by the carbazole–sulphuric acid method according to ref. [32] using D-GalpA·H_2O as standard.

Methylation analysis: acetylation of polysaccharides was performed as described in ref. [33]. Briefly: 1–10 mg polysaccharide were dissolved in 0.4 ml formamide. This solution was mixed with 0.4 ml Ac_2O–pyridine (1:1) and after stirring for 3 hr at room temp., 10 ml of ice-cold H_2O were added. The acetylated polysaccharides pptd and were recovered by centrifugation. The

ppt. was washed twice with 1 ml of ice-cold H_2O, resuspended in water and freeze-dried. The methylation analysis of the acetylated polysaccharides was performed according to ref. [34]. Carboxyl-groups in the methylated polysaccharides were reduced with $LiAlD_4$ according to ref. [35]. GC and GC-MS were carried out as described above. Factors calculated from the ECR-theory [36] were used to correct the integrated peak areas.

Partial hydrolysis. By dilute acid: 140 mg arabinogalactan Fb were dissolved in 8 ml 0.05 M TFA. The solution was heated on a steam bath for 2 hr. After cooling it was transferred into a dialysis tubing and dialysed against dist. H_2O (3×100 ml). Undialysable residue (= Fbr) 45 mg. To an aliquot of the combined dialysates myo-inositol was added as an int. standard. The mixture was divided into two parts and concd. The mixture was divided into two parts and concd to dryness under red. pres. The sugar content was determined either directly after conversion to alditol acetates and after hydrolysis (2 M TFA/1 hr 120°).

By polygalacturonase: 2.5 mg partially hydrolysed Fb (Fbr) were dissolved in 1 ml H_2O. 0.14 units polygalacturonase were added and the mixture was dialysed against 250 ml of dist. H_2O for 5 hr at room temp. After this period dialysate and retentate were concd under red. pres. and assayed for oligogalacturonides by TLC in *n*-BuOH–Me_2CO–H_2O–HOAc (7:7:9:2). This test was paralleled by a degradation of polygalacturonate-Na (Sigma) where galacturonic acid, di- and trigalacturonides could be found in the dialysate.

Quantitative protein determination. Quantitative protein determination was performed according to ref. [37]. Bovine serum albumin (Merck) was used as a standard.

Determination of the absolute configuration of sugars. By the method of ref. [38] using (−)-2-butanol (Fluka Ag) and (±)-2-butanol (Merck) for butanolysis and TMSi-S reagent (Serva) for subsequent silylation it was ascertained that only following enantiomers occur: L-rhamnose, D-galactose, L-arabinose and D-galacturonic acid.

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