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Ribitol as a Major Component of Water-soluble Leachates from *Lobaria oregana*

Abstract. *Leachates from* Lobaria oregana *(Tuck.) Müll. Arg. were produced in the laboratory by misting thalli with rainwater. The leachate was separated into four fractions: a non-dialysable residue (10–15% of the total) containing protein, polysaccharide and probable polyphenolic materials; a dialysable acid-insoluble fraction thought to be tannin; an acid-insoluble, acetone-insoluble inorganic fraction composed largely of sodium, potassium, calcium and magnesium cations; and an organic fraction soluble in both aqueous acid and acetone consisting mainly of ribitol.* Lobaria *is presumed to constitute a major pool for polyols found in throughfall from the coniferous stands under study; as such, this lichen may be an important energy source for heterotrophic canopy microorganisms which subsist on leachates.*

Reports within the last twenty years have shown that a great variety of substances may be washed from plant surfaces in rainfall and that almost any plant surface may be susceptible to such leaching (Tukey, 1970). Studies in progress in this laboratory have implicated leaching as an important process for nutrient cycling within the coniferous forest canopy. The present study was undertaken to elucidate the chemical composition of leachates from the foliose cyanophilic lichen *Lobaria oregana* (Tuck.) Müll. Arg., an ubiquitous and abundant epiphyte in the canopy of old-growth Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) stands on the western slopes of the Oregon Cascades (Pike et al., 1975; Pike et al., 1977). *Lobaria oregana* is now known to fix nitrogen at high rates (Denison, 1973; Denison, Roose & Pike, unpublished) and to release large amounts of organic nitrogen during laboratory leaching experiments (G. Carroll, F. Carroll & J. Horstmann, unpublished). Since *Lobaria oregana* probably represents a major source of fixed nitrogen in the forest stands under study, our interest has focused on the organic components of *Lobaria* leachates as possible agents of nitrogen transfer from the lichen to other portions of the canopy and to the forest floor.

This report describes a chemical fractionation of *Lobaria* leachates, provides general characterizations for the various fractions and identifies a specific dominant organic compound.

Materials and Methods

Thalli from *Lobaria oregana* were collected during June and July 1976 from a single Douglas fir tree (1.9 m DBH, 77 m height) located in the H. J. Andrews Experimental Forest (44°10′N, 122°20′W) in the western Cascades approximately 70 km east of Eugene, Oregon. The tree occurs in a stand corresponding to the *Tsuga heterophylla/Rhododendron macrophyllum/ Berberis nervosa* community of the *Tsuga heterophylla* zone (Franklin & Dyrness, 1973). After collection, the samples were placed in a plastic bag, brought back to the laboratory, stored overnight at 4°C and subjected to misting the following day. Between misting episodes the entire leaching apparatus was placed in a 14°C room with fluorescent lights, and thalli were periodically sprinkled with distilled water to keep them moist.

Prior to leaching, thalli were placed in polypropylene funnels 17.7 cm in diameter whose necks had been machined to fit in the caps of 500 ml polypropylene bottles. The thalli were misted with rainwater which had been collected the previous winter and had been frozen until immediately prior to use. Typical leaching episodes lasted 2–4 h, after which the leachates in the bottles below the funnels were pooled and filtered through Nuclepore filters with a 0.2 μm

pore size. The filtered leachate was then lyophilized to dryness and stored at −20°C until chemical analyses could be carried out.

The sequence and descriptions of the various analytical procedures are included with the results below. Details on chemicals and instruments used can be summarized as follows: Water double-distilled in glass and reagent grade chemicals were used for all chemical procedures. Weights were obtained on Mettler balances. Optical rotations were obtained with a Perkin-Elmer 141 polarimeter in either chloroform or water. Infrared spectra were taken on a Beckman IR-7 spectrophotometer with chloroform solutions of leachates. Ultraviolet spectra were obtained from aqueous solutions on a Cary 15 spectrophotometer. Proton magnetic resonance and carbon-13 magnetic resonance spectra were obtained from solutions in deuterated dimethyl sulfoxide (DMSO-d_6), deuterated chloroform ($CDCl_3$) and deuterium oxide (D_2O) on a Varian XL-100 NMR spectrometer in conjunction with a Varian 620-i Fourier Transform Computer. Mass spectra were taken on a Consolidated Electronics Corporation Model 21-110A double focusing mass spectrometer. Combustion analyses were carried out on lyophilized samples of leachate with a Perkin-Elmer model 240 CHN Analyzer. Amino acid analyses were performed with a Technicon TMS system. Cation analyses were carried out on a Varian-Techtron AA-5 atomic absorption spectrophotometer. For vapor phase chromatography, a Hewlett Packard 700 chromatograph with a 1.83 m × .64 cm column of 10% DC-410 (silicone) on Firebrick with a helium pressure of 15 psi at 200–210°C was used.

Results and Discussion

The initial fractionation procedure involved dialysis of an aqueous solution of leachate followed by treatment (of the dialysate) with aqueous acid, then acetone. This procedure yielded 4 fractions: a high molecular weight fraction which would not pass through a dialysis membrane; a low molecular weight fraction insoluble in acid solutions; a low molecular weight acid-soluble, acetone-insoluble fraction; and a fraction soluble both in aqueous acid and acetone. Such fractionation was carried out several times, using different lots of leachate in each case. Because different lichen thalli which had experienced differing prior meteorological histories were used, the relative proportions of dry matter in each of the above fractions varied considerably from one fractionation experiment to the next. Consequently, relative proportions are expressed as a range of percentages in the discussion below.

For dialysis approximately 100 mg of lyophilized leachate was dissolved in 5 ml of water and dialyzed through regenerated cellulose dialysis tubing (average pore radius of 24 nm) into 2 × 100 ml of double distilled water. Following dialysis both dialysate and residue were evaporated to dryness under high vacuum. The residue generally accounted for 10–15% of the dry weight of the crude leachate. A portion of the total leachate was subjected to hydrolysis overnight in 6 N HCl at 100°C and was submitted for quantitative amino acid analysis. The results of this analysis showed that amino acids and their derivatives account for about 2% of the dry weight of the crude leachate. If most of the amino acids are present in proteins, about 2% of the crude leachate and 15–20% of the residue from dialysis should be detected as protein. These values are consistent with values derived independently from protein-dye binding assays (Bradford, 1976) conducted on similar leachates.

The crude lyophilized leachate was heated in 2 M trifluoroacetic acid at 100°C for 24 h; the solvent was evaporated under vacuum and the residue was soaked in an acetic anhydride/pyridine mixture at room temperature for 60 h. The solvent was again evaporated under vacuum and the residue was washed with chloroform. The acetylated hydrolysate was subjected to NMR analysis, and the presence of free sugars in the hydrolysates was inferred from the occurrence of multiple resonances around δ5 (anomeric hydrogen), δ4 (H-C-OAc) and δ2 (O-acetate) in the NMR spectra. When the low molecular weight dialysate was subjected to similar treatment, no evidence for

the presence of acetylated sugars could be found. From these data we conclude that an undetermined portion of the high molecular weight material consists of polysaccharides containing several sugars.

Tannins or other polyphenolic pigments may also have been present in the residue, as indicated by a high absorbance in the UV portion of the spectrum, increasing monotonically down to 200 nm; the extinction coefficient increased markedly with the addition of base.

The dialysate, representing 85–90% of the dry weight of the crude leachate, was treated with 10% aqueous hydrochloric acid at room temperature for 3 days under nitrogen. Five to ten percent of the total weight of the dialysate remained as a dark insoluble material after this time. This substance dissolved on subsequent treatment with base. Because of its pigmentation and solubility characteristics, we suspect this fraction to consist of low molecular weight acid non-hydrolyzable tannins. However, no further characterization has been carried out for this component.

The acid soluble low molecular weight fraction was evaporated to dryness *in vacuo*. Acetone was then added to the material (ca 1 ml/5–10 mg) and the solution was allowed to stand at room temperature under nitrogen for two days. The insoluble material which remained was filtered and dried; it constituted about 20% of the dry weight of the dialysate. This fraction did not reduce alkaline permanganate; combustion analyses revealed only a small amount of carbon and confirmed the largely inorganic nature of the fraction. Aqueous solutions gave a strong positive flame test for sodium and potassium; atomic absorption spectrophotometry showed the presence of large amounts of sodium and potassium as well as lesser quantities of magnesium and calcium. In total these ions were present in sufficient quantity to account for most of the total mass of the fraction as chloride salts. Since the cations were isolated as chlorides, no identification of the original associated anions was possible. Only traces of free orthophosphate and sulfate were detected in the crude lyophilized leachate.

The acetone soluble fraction, constituting 60–70% of the total dialysate, was then examined. Nuclear magnetic resonance studies revealed the presence of two major varieties of hydrogen which, on the basis of chemical shifts, were assigned as protons on carbon bonded to oxygen and as protons on isolated alkyl methyl groups. Carbon-13 NMR studies substantiated these assignments and further showed the presence of carbons bearing no hydrogen atoms, with chemical shifts consistent with their assignment as ketal type carbons. Infrared spectra revealed strong C-H, C-C and C-O spectral bands, with only very weak carbonyl and no hydroxide bands present. UV spectral analysis showed no chromophores, a finding consistent with NMR evidence suggesting no unsaturation. These data, coupled with previous NMR studies which showed the absence of sugars in the dialysate and with reports in the literature on the prevalence of polyols in lichens (Culberson, 1969; Lewis & Smith, 1969), led to the tentative identification of the major component in the acetone-treated fraction as the acetonide of one or several polyols. Presumably these polyols had been decomposed by the vigorous trifluoroacetic acid hydrolysis and thus were not detected in previous NMR studies as their acetates.

Attempts to separate and identify single acetonides using thin layer chromatography and vapor phase chromatography gave unsatisfactory results. Hydrolysis of the putative polyol acetonides in 6 N HCl at 100°C for 22 h and production of trimethylsilyl derivatives in trimethylamine with trimethylsilyl chloride was carried out. Separation of the derivatives by vapor phase chromatography was successful, but interpretation of the structural data on the four separated derivatives ultimately proved difficult and equivocal.

Consequently, a more direct isolated procedure for the low molecular weight components was carried out. One hundred mg of the crude *Lobaria* leachate was mixed under nitrogen with 2 ml of a 1:1 mixture of methylene chloride and pyridine, and 200 μl of trimethylsilyl chloride was added. The mixture was stirred overnight at room temperature or until formation of pyridine hydrochloride seemed complete. Water was added, and the organic phase separated, dried over anhydrous $MgSO_4$ and evaporated to dryness *in vacuo*. Chloroform soluble components of the trimethylsilylated crude leachate were then separated by vapor phase chromatography. This yielded a single major component with a retention time of 7.5 min and a minor component (ca. 10% of total) with a much longer retention time. Both components were collected and submitted for mass spectrometry. The minor component yielded only high mass decomposition peaks, and no further studies were conducted with it. The major component gave a parent ion with a molecular weight of 497.241 daltons, consistent with the $C_{19}H_{49}O_5Si_5$ fragment resulting from the loss of a methyl group from a pentakis-trimethylsilyl ether of a pentitol. Loss of a methyl group by the molecular ion is reported to be a characteristic mode of decomposition for alcohol trimethylsilyl ethers in the mass spectrometer (Waller, 1972). Nuclear magnetic resonance studies of the isolated derivative yielded spectra consistent with that of a polyol per-trimethylsilyl ether.

The crude lyophilized leachate had previously been found to be optically inactive. This also proved to be the case for isolated pentitol derivative. Since a racemic mixture would be highly unlikely in a natural product, it was concluded that either xylitol or ribitol (adonitol) must have been the parent compound in the crude leachate. Trimethylsilyl derivatives were synthesized from authentic samples of both sugar alcohols as outlined above. Both derivatives were subjected to vapor pressure chromatography and retention times on the column were noted. The xylitol trimethylsilyl ether showed a retention time of 9 mins; that for ribitol ether was 7.5 mins, a time identical with that for the *Lobaria* derivative. Co-injection of authentic pentakis-trimethylsilyl ribitol with the unknown trimethylsilyl ether also demonstrated their identity.

The discovery of ribitol in *Lobaria* leachates should come as no surprise. Ribitol has been implicated in transfer of fixed carbon from phycobiont to mycobiont in a variety of lichen species with *Trebouxia* or *Myrmecia* as the phycobiont (Richardson et al., 1967; Richardson, 1973); polyols are reported to accumulate in some lichens when they are subjected to periods of water stress (cf. Richardson et al., 1968). Continuing studies at the University of Oregon on leaching of various canopy components have shown that *Lobaria oregana* constitutes the major source of leached polyol in the coniferous canopy and that amounts released per gram thallus vary by as much as 2 orders of magnitude, depending on the prior hydration state of the thallus. Dry thalli leach far more ribitol than those which have been moist prior to leaching. Since ribitol constitutes the major constituent of *Lobaria* leachates, one should expect considerable variation in relative proportions of the various leachate components described here.

While we have been successful in identifying ribitol as the single dominant organic component in *Lobaria* leachates, we have had more difficulty in identifying specific nitrogenous components. Combustion analyses of the four fractions described above did not reveal significant concentration of nitrogenous components in any fraction. This suggests to us that nitrogen in *Lobaria* leachates does not occur in any single dominant compound but is rather localized in a number of separate compounds which occur individually in small quantities in the leachates and which collectively separate into all four of the fractions produced through successive dialysis and extraction in acetone.

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