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Moss Pigments

5. Studies of Phenolic Cell Wall Polymers in *Sphagnum nemoreum*

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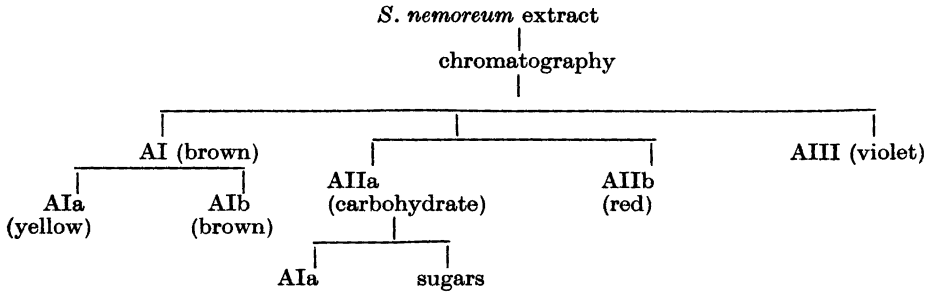
The alkali degradation and alkaline cupric hydroxide oxidation products of red and brown phenolic cell wall polymers from *Sphagnum nemoreum* Scop. have been studied. Alkali degradation gives several phenols of which phloroglucinol, orcinol, and resorcinol have been identified. Cupric hydroxide oxidation gives molar ratios (*p*-hydroxyphenyl/guaiacyl) 2:1 for the red polymer and 2.5:1 for the brown polymer. *p*-Hydroxyacetophenone, *p*-hydroxybenzaldehyde, acetovanillone, and vanillin have been separated by thin layer chromatography and determined by quantitative spectrophotometry. Some spectral properties are discussed and general aspects of the "*Sphagnum* lignin" given.

In paper 3 of this series we described the isolation of cell wall pigments in *Sphagnum* species and *S. nemoreum* in particular.[1] We established that at least the brown (AI) and the red (AIIb) chromatographically separated components are polymers. (Designations AI *etc.* refer to Scheme 1.) A polymeric carbohydrate fraction (AIIa) was shown to contain an unstable phenolic aglycone (AIa), which changes rapidly to a product with properties similar



Scheme 1. Pigment fractions in *S. nemoreum*.

to those of the brown polymer. The investigations on the third pigment fraction (AIII, violet fraction) will be presented in separate papers.

Spectral data of our polymers suggested that they are of the same type as Farmers "slightly altered lignin" from *S. cuspidatum*.[2,3] We therefore found it of interest to compare oxidative degradations of our products with investigations of "*Sphagnum* lignin" from other species, treated in a different way. We also wanted to reveal possible differences between the red and the brown polymers.

It should be pointed out, that the acid conditions used by us on isolation and separation of the components are necessary because of the instability of the red pigments in neutral or basic solutions. The acid conditions are, of course, not ideal for isolation of lignin-like substances.

The conventional Wiesner test for lignin (red coloration with phloroglucinol and concentrated hydrochloric acid) is of limited value for red colored moss species. However, *S. nemoreum* which has been thoroughly preextracted with methanol-HCl gives a positive reaction. Twenty other *Sphagnum* species, most of which are not reddish, have also been tested and all reacted positively.[4]

The IR spectra of our polymers (Fig. 1) are very similar to that of Farmer's and Morrison's *S. cuspidatum* "lignin".[3] Component AIa obviously corresponds to the "slightly altered lignin" from this species, obtained by acid hydrolysis of a phenol-carbohydrate complex. AIa is isolated by acid hydrolysis of the carbohydrate fraction (AIIa) and separated from the mixture of simple sugars by pentanol extraction. A part of the product is soluble in ether and can be precipitated with petroleum ether. The light yellow precipitate changes rapidly to a yellow-brown product, which is insoluble in ether. The IR spectrum of this component (AIa, Fig. 1) is somewhat better resolved than those of the brown (AI) and the red (AIIb) fractions but shows the same general features. The differences in intensity between OH- and CH-stretching bands

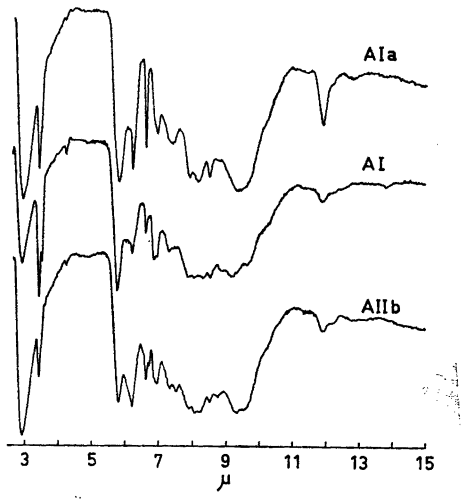


Fig. 1. Infrared spectra of cell wall polymers from *Sphagnum nemoreum* (KBr phase). AIa: aglycone from carbohydrate fraction; AI: brown polymer; AIIb: red polymer.

for AI and AIIb are not characteristic properties and may occur also in different batches of the same component.

The IR spectra of our polymers and those of coniferous lignin and dehydrogenation and acid polymerisation products of coniferyl alcohol and *p*-coumaryl alcohol are of the same general appearance. However, there are important differences, mainly in the carbonyl absorption region. The *Sphagnum* polymers have a very intense and broad band with maximum at 5.8 μ, whereas the corresponding band of coniferous lignin and its model compounds is of low intensity. Farmer and Morrison pointed out that some of this absorption is due to carboxylic acid groups.[3] The presence also of peripheral carbonyl groups is evident from reaction of our polymers with carbonyl reagent on chromatograms.

The UV spectra of AIa in ethanol and ethanolic potassium hydroxide are essentially identical with those published by Farmer and Morrison.[2,3] The neutral solution shows a very broad band with λ_{max} at about 290 mμ. In alkaline solution a broad band appears with λ_{max} 350—360 mμ, due to phenolate groups conjugated with carbonyl groups or unsaturated side chains. However, a considerable part of the absorption at 280—290 mμ is still present. This absorption may, at least in part, be ascribed to phenolate groups, unable to conjugate with the groups mentioned.

The visible spectra of the brown (AI) and the red (AIIb) polymers differ by a broad band for the latter with λ_{max} 525 mμ, which is not found in the spectrum of AI. In the UV region both polymers absorb strongly. Unlike AIa, they have no maximum at 290 mμ, only an inflexion appears on the absorption curve. Only tentative efforts have been made to use differential spectrophotometry in this region, but these have as yet not given any valuable information.

In alkaline solution the visible band of AIIb is shifted 40—50 mμ towards longer wavelengths and the chromophore is gradually destroyed. Polarography has been used in an attempt to reveal possible relationships between the chromophores of the red and the violet pigments. The violet pigment (AIIIa) is reduced polarographically with a well-defined half wave potential (methyl cellosolve: 0.1 M KCl, 9:1 v/v, pH 2).[5] The red polymer (AIIb) has only a very slight wave in the same region and the half wave potential can not be determined. Thus, evidence for a relationship between the chromophores is not established by polarography.

Simple flavonoid compounds do not seem to have been isolated from *Sphagnum*. Manskaya *et al.*, working with *S. magellanicum* drew the conclusion that the aromatic composition of *Sphagnum* cell walls depends on the presence of a phenolic glycoside and traces of vanillin, syringaldehyde, and *p*-hydroxybenzaldehyde.[6,7] The aglycone was preliminary characterised as a flavone derivative. As has already been pointed out,[3] the UV spectra and analysis data reported do not support this suggestion. Further evidence is required on this point.

The red coloration of some *Sphagnum* species has induced some authors to suggest that flavonoids are built into the cell walls.[8,9] We therefore considered it of some interest to study the alkali degradation of our polymers. A "normal" oxygenation pattern would be expected to give phloroglucinol derivatives

from flavonoid compounds. It was found, indeed, that both fractions give phloroglucinol as one of the main components of the neutral phenolic part. Resorcinol and orcinol are present in minor amounts. At least fourteen phenolic components are obtained, four of which also contain carbonyl groups.

The presence of phloroglucinol might possibly indicate, that acetate-derived units are present in the polymers, but the brown and red fractions do not seem to differ in this respect.

Previous investigations of nitrobenzene oxidation of *Sphagnum* material of varying origin have given slightly varying results (Table 1). Kratzl and

Table 1. Yields of phenolic aldehydes obtained on nitrobenzene oxidation of *Sphagnum* material.

Species	Yield of aldehyde % [a]			Total yield %	Ref.
	*p*OHB	V	Other ald.		
Species from *Cuspidata* sect.	100	—	—	0.82	14
S. cuspidatum	100	—	—	0.80	3
S. cuspidatum	100	—	—	5.5 [b]	2
S. cuspidatum	100	—	—	4 [c]	13
S. balticum *S. fuscum*	90	4	0.6 [d]; 0.1 [e]	0.6	11

[a] calculated on the total yield; [b] from isolated "slightly altered lignin"; [c] *p*-anisic acid obtained by permanganate oxidation of methylated moss; [d] syringaldehyde; [e] formylvanillin.

Eibl [10] (1951) could not detect any vanillin on oxidation of *Sphagnum* material and therefore assumed that "*Sphagnum* lignin" is of carbohydrate character and not aromatic. In 1952 Lindberg and Theander studied the oxidation products of *S. balticum* Russow and *S. fuscum* W. Klinggr.[11] *p*-Hydroxybenzaldehyde constituted 90 % of the total amount of aldehydes, vanillin 4 %, syringaldehyde 0.6 %, and formylvanillin 0.1 % (traces of other aldehydes). In an investigation of *S. cuspidatum* (1953), Farmer obtained only *p*-hydroxybenzaldehyde.[2] This paper seems to be the first investigation reported, performed on isolated "*Sphagnum* lignin" fractions. The total yield of aldehyde was 5.5 %, about ten times higher than that obtained from whole moss material by Lindberg and Theander. Farmer concluded that his "slightly altered lignin" is the source of the *p*-hydroxybenzaldehyde obtained by Lindberg and Theander. On spectroscopical grounds Farmer, however, did not accept the suggestion that "*Sphagnum* lignin" is formed from *p*-hydroxyphenyl building blocks. Recently Manskaya and Kodina suggested that "*Sphagnum* lignins" are derivatives of *p*-coumaryl alcohol, but they do not seem to have reported the isolation of *p*-coumaryl alcohol from *Sphagnum*.[12]

In 1955 Farmer and Morrison reported a yield of 4 % *p*-anisic acid on permanganate oxidation of methylated *S. cuspidatum* and could not detect any other simple aromatic acids.[13] They therefore assumed that vanillyl and syringyl groups, which are characteristic for the lignins of higher plants, are

Table 2. Spectrophotometrically determined concentrations of phenolic aldehydes and ketones from oxidation of the red (AIIb) and brown (AI) polymers.

Polymer	Yield (%) of compound [a]				Total yield [b] %	Molar ratio *p*-OHphenyl / guaiacyl
	*p*OHA	*p*OHB	AV	V		
AIIb	44	20	27	9	2.0	2:1
AIIb	42	19	30	9	1.6	1.9:1
AI	41	26	26	7	0.5	2.4:1
AI	49	18	24	9	0.8	2.6:1

[a] calculated on the total yield of aldehydes and ketones; [b] calculated on material used for oxidation.

absent in *Sphagnum*. In two later investigations on the same species Morrison [14] (1958) and Farmer and Morrison [3] (1964) isolated 0.82 and 0.80 %, respectively, of *p*-hydroxybenzaldehyde, which was the only aldehyde detected.

The results of our cupric hydroxide oxidation (Table 2) were somewhat unexpected because of the higher guaiacyl unit content of AI and AIIb, compared to the results of investigations of pre-extracted whole moss and the "slightly altered lignin" mentioned above (Table 1). The latter contained no guaiacyl units. Another striking result is the high contents of phenolic ketones. *p*-Hydroxyacetophenone (*p*OHA) constitutes 42—44 % of the total amount of phenolic aldehydes and ketones from AIIb and 41—49 % in AI, *i.e.* about twice the amount of *p*-hydroxybenzaldehyde (*p*OHB). The weight ratio acetovanillone (AV)/vanillin (V) is about 3:1 for each polymer. No syringaldehyde has been detected, but two other carbonyl-containing compounds (probably not phenolic) are present in minor quantities.

The results of Table 2 were obtained from two oxidations of each polymer where each value is the average from six measurements. The percentage of the various components varies to some degree, but the molar ratio (*p*-hydroxyphenyl/guaiacyl) remains fairly constant, being around 2:1 for the red polymer and 2.5:1 for the brown one. The total yield is of the same order as that reported by Lindberg and Theander. Acid treated coniferous lignin has been reported to give a low yield of aldehydes and our polymers are isolated under acid conditions. However, the nitrobenzene oxidation of whole *Sphagnum* moss (not acid treated) also gives low yields (Table 1). Another reason for the low yield could be that only the "core" of the polymers is oxidized. This does not seem to be the case, however, since only traces of unoxidized material remains. The main part, instead, is a complex mixture of acids, of which *p*-hydroxybenzoic acid and vanillic acid have been identified.

It is not known at the present to what extent the high proportion of ketones depends on the use of cupric hydroxide. This method of oxidation, however, has been reported to give somewhat larger quantities of acids than the nitrobenzene method.[15] In any case it should not be expected to be a cause for the higher guaiacyl unit content. A dependence on the isolation procedure is a probable explanation, but further investigations are required on this point.

Table 3. Analysis of mixtures of *p*-hydroxybenzaldehyde (*p*OHB) and acetovanillone (AV).

	Actual conc. mg/100 ml		Conc. calculated from spectral data mg/100 ml		
	AV	*p*OHB	Total	AV	*p*OHB
a *	0.40	0	0.402	0.402	0.000
b	0.32	0.08	0.402	0.318	0.084
c	0.24	0.16	0.402	0.236	0.166
d	0.16	0.24	0.402	0.152	0.250
e	0.08	0.32	0.402	0.075	0.327
f	0	0.40	0.402	0.001	0.401

* a–f refer to the curves of Fig. 2.

The small scale oxidation is analogous to that described by Towers and Maass.[15] Neutral phenolic oxidation products have in our investigation been separated on Avicel SF thin layer plates with butanol: 3% ammonium hydroxide (4:1 v/v) as solvent. The spectrophotometric determination is analogous to the method described by Lemon [16] and Stone and Blundell.[17] Standard curves were prepared from known concentrations of authentic compounds. *p*OHA and V could be determined directly after chromatography but *p*OHB and AV had to be determined in mixture in order to avoid the uncertainties introduced by two chromatographic separations. Details on the co-determination are given in the experimental part. The method requires that the substances have an isoabsorption wavelength.[16] The actual concentrations in a measurement series with known mixtures are compiled in Table 3 as well as the values calculated from spectral data. The agreement was found to be satisfying. The UV curves are shown in Fig. 2.

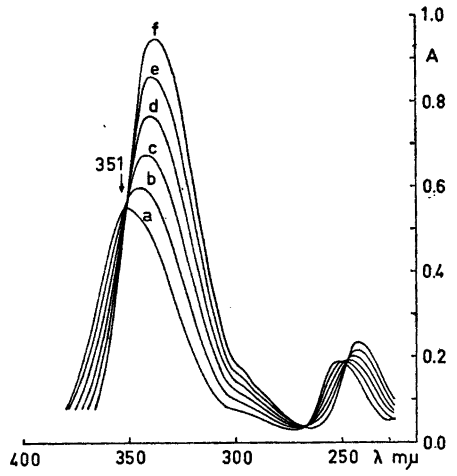


Fig. 2. Ultraviolet spectra of known mixtures of *p*-hydroxybenzaldehyde (*p*OHB) and acetovanillone (AV) in ethanolic potassium hydroxide. The curves a–f correspond to the concentrations listed in Table 3.

Elemental analysis data are given in the experimental part. The nitrogen content of both polymers is probably due to phenylalanine residues. Phenylalanine has been isolated in minor amounts from the oxidation mixture and tentatively characterized by its UV spectrum.

This investigation of the polymeric products obviously gives no direct evidence of the presence of flavonoid compounds or unambiguous evidence of a C_6C_3-unit of the *p*-coumaryl type as a precursor substance. Isolation (from uncolored species) of polymers by the milder methods known from lignin chemistry and enzymatic hydrolysis of the carbohydrate fraction is in progress and will be reported on later.

EXPERIMENTAL

Moss material. Sphagnum nemoreum was collected in the western Swedish mountains (provinces of Dalarna and Härjedalen).

Spectra. UV measurements were made with a Bausch & Lomb Spectronic 505 spectrophotometer and IR spectra were recorded on a Perkin-Elmer Model 157 spectrophotometer using the KBr disc technique.

Chromatography. Silica gel G (Merck) and Avicel SF cellulose (Kebo) for TLC were used. Phenols were detected with "Echtblausalz B" and phenolic aldehydes and ketones with 2,4-dinitrophenylhydrazine (2,4-DNPH). Solvent systems used were: BAW = butanol:acetic acid:water (6:1:2 by vol.), BDA = benzene:dioxan:acetic acid (90:25:4 by vol.), BMA = benzene:methanol:acetic acid (45:8:4 by vol.), BM1 = benzene:methanol (95:5 v/v), BM2 = benzene:methanol (5:3 v/v), BBW = butanol:benzene-water (1:19:29 by vol., upper phase), CMF = chloroform:methanol: 4 % formic acid (10:1:1 by vol., upper phase), BN = butanol:3 % ammonium hydroxide (4:1 v/v, upper phase).

Isolation of the polymers has been performed as described previously.[1] R_F-values (Avicel, BAW): AIa 0.95, AI 0.95, AIIb 0.75.

Elemental analyses. Found for AI: C 59.71; H 5.67; Cl 2.07; N 1.31. Found for AIIb: C 55.71; H 5.55; Cl 3.01; N 2.23.

Alkali degradation. 50—100 mg of polymer was treated with KOH at 200°. The degradation was performed either in an open platinum vessel in a nitrogen atmosphere or in a 10 ml stainless steel bomb with a screw on cap and teflon packings. Separation of the neutral phenolic fraction from carboxylic acids was performed with 5 % $NaHCO_3$ in the usual way.

Phenols from the alkali degradation were chromatographed on 0.25 mm Silica gel G plates with BDA or BMA as solvents. The spots with R_F-values corresponding to phloroglucinol, orcinol, and resorcinol were eluted with methanol and rechromatographed against authentic samples. R_F-values for phloroglucinol are: BDA 0.27; BMA 0.23; BM1 0.02; BM2 0.58. The component assumed to be phloroglucinol had the same R_F-values. R_F-values for resorcinol are: CMF 0.06; BDA 0.45. R_F-values for orcinol are: CMF 0.09; BDA 0.45. The spot corresponding to resorcinol-orcinol mixture in BDA was chromatographed on Whatman No. 1 paper (BBW) together with a mixture of the authentic substances. Elution out of the paper for 24 h gave good separation and the locations of the two spots from the unknown mixture were found to correspond to those of the reference substances.

Two-dimensional chromatography on Silica gel G in CMF and BDA gave fourteen phenolic components, four of which also reacted with 2,4-DNPH.

Phenolic acids from the alkali degradation were chromatographed two-dimensionally (Avicel, CMF and BDA). Several trailing components of phenolic character were detected. The mixture was not further worked up.

Cupric hydroxide oxidation. The oxidation was performed in a 10 ml stainless steel bomb with 1.3 g $Cu(OH)_2$ and 5.0 ml of 2 M NaOH for 50 mg of polymer. The temperature was kept at 175° during 1—2 h.

Isolation of phenolic aldehydes and ketones. For identification of the aldehydes and ketones 450 mg of polymer were oxidized. Preparative chromatography on Whatman

No. 3 MM paper with BN as solvent gave three bands: *p*OHA with the highest R_F-value, a mixture of *p*OHB and AV in an intermediate band, and V with the lowest R_F-value. The bands were eluted with 99.5 % EtOH. *p*OHB and AV were separated on a preparative scale on paper with water as solvent. The components were identified by co-chromatography with reference substances in several solvent systems and by UV spectra in hexane, 99.5 % EtOH, and EtOH containing 7.0 ml of 0.2 % KOH in EtOH/100 ml solution. In the case of *p*OHA and *p*OHB the IR spectra were also recorded. R_F-values are: (Avicel, BN) *p*OHA 0.60, *p*OHB 0.52, AV 0.52, V 0.44; (Avicel, H_2O) *p*OHB 0.53, AV 0.63.

Standard curves. The standard curves were prepared according to Lemon.[16] A good linear relationship between concentration and absorbance was obtained in each case. λ_{max} for the phenolate ions in EtOH-KOH are: *p*OHA 332 mμ; *p*OHB 338 mμ; AV 350 mμ; V 356 mμ.

Chromatographic recovery. The recovery was determined for each component at different concentrations. Known amounts in 10—20 μl of solution were placed as spots on Avicel plates. The solvent (BN) was allowed to run 14—16 cm, the plates were dried at room temp., and a marker strip was developed with 2,4-DNPH. The corresponding test spots were eluted with measured volumes of the EtOH—KOH solvent during 0.5 h. UV measurements were made against blank eluent and the concentrations read from the standard curves. The recoveries were found to vary within ± 2 % from the following average values: *p*OHA 82 %; *p*OHB 83 %; AV 79 %; V 79 %. In the calculations of Table 2 the value 80 % has been used for all components.

Quantitative determination. 50—60 mg of polymer was oxidized. The phenolic part was dissolved in 500 μl 99.5 % EtOH. For AI 20 and 30 μl was used for chromatography and for AIIb 10 or 15 μl, because of the higher yield. The solvent was allowed to run 14—16 cm, the plates dried and the spots corresponding to *p*OHA, a mixture of *p*OHB AV, and V, were eluted with known volumes of EtOH—KOH (EtOH + 7.0 ml of 0.2 % KOH in EtOH/100 ml solution). After 0.5 h the UV spectra were measured against blank eluent. For *p*OHA and V the concentrations could be calculated directly from the standard curves.

Determination of pOHB and AV in mixture. Pure samples of *p*OHB and AV were dissolved in EtOH—KOH (0.40 mg/100 ml). The solutions were mixed in various proportions, always to make the total conc. 0.40 mg/100 ml. The UV spectra were recorded and the isoabsorption wavelength found was 351 mμ (Fig. 2 and Table 3). The extinction coefficient at 351 mμ was calculated from eqn. 1. Eqn. 2 was used in order to control the validity of the data obtained (Table 3). The non-coincident wavelength used in the calculations (338 mμ) is the λ_{max} for *p*OHB in EtOH—KOH. The coefficients ε_{AV}^{338} and ε_{pOHB}^{338} were obtained from standard curves.

$$\varepsilon_{351} = \frac{A_{351}}{C} \qquad \text{(Eqn. 1)}$$

$$c_1 = \frac{A_{338} - \varepsilon_2^{338} \times C}{\varepsilon_1^{338} - \varepsilon_2^{338}} \qquad \text{(Eqn. 2)}$$

ε_{351} = 1.390 (calculated from spectra, Fig. 2)
ε_i^{338} = extinction coeff. for component i at 338 mμ.
ε_{pOHB}^{338} = 2.353 (conc. in mg/100 ml); ε_{AV}^{338} = 1.163
C = total conc. in mg/100 ml
c_1 = conc. of component 1 in mg/100 ml

The total conc. of the unknown mixture is thus calculated from eqn. 1 and the conc. of *p*OHB and AV obtained by use of eqn. 2.

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