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Moss Pigments

7. Preliminary Investigations of the Violet Pigments in *Sphagnum nemoreum*

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Two violet pigments of oxonium type are isolated from *Sphagnum nemoreum* Scop. Acid hydrolysis of one pigment gives the other one and an unstable acid. IR and UV data are compared with those of flavylium compounds. Transformations of the non-esterified pigment at pH 0.5–6.0 are studied by UV spectroscopy and compared with those of luteolinidin. The pK-values for the equilibria "violet acid" ⇌ "yellow base", and luteolinidin ⇌ 2,3′,4,4′,6-pentahydroxychalcone, for the violet pigment and luteolinidin, respectively, are determined. Polarographic reduction occurs at the same potential as that of two flavylium compounds studied. Some elemental analyses and chromatographic data are given.

The first suggestion concerning the chemical nature of the reddish-violet pigments in *Sphagnum* species was put forward by Paul, who proposed that they are related to anthocyanins.[1] Paul also suggested that the pigments are bound to the cell wall and this has later been confirmed by Herzfelder [2] and by Rothe.[3]

Paton and Goodman suggested that the violet pigment in *Sphagnum nemoreum* is an anthocyanin,[4,5] but Bendz, Mårtensson and Terenius [6] and, somewhat later, Rothe [3] and Rudolph [7] pointed out that the pigments of *S. nemoreum* and *S. magellanicum* are not glycosides.

In paper 3 of this series Bendz, Mårtensson and the present author described the isolation of the violet pigment fraction from *S. nemoreum*.[8] We also succeeded in the separation of the violet fraction into two components, AIIIa and AIIIb. AIIIb on acid hydrolysis gave an unstable acid and a pigment part with R_F-values, UV and IR spectra identical with those of AIIIa. Thus, in order to characterize the chromophore, the investigations have been concentrated on AIIIa.

The aim of the present investigation was to establish an eventual relationship between the violet pigment and anthocyanidins by comparison of their spectral and some other properties.

As has been described earlier the two violet pigments have been separated on cellulose columns, using methyl cellosolve:methanol (1:1, 1 % conc. HCl) as solvent.[8]

Repeated chromatography and re-precipitations from acid methyl cellosolve or methanol solutions finally gives chromatographically homogeneous pigments. The isolation is complicated by a bleaching of the color, sometimes occurring, and by the small yield — around 2 mg per 100 g of dried moss material used. Both pigments, unless carefully purified, contain up to 3 % of nitrogen due to a contamination. However, both pigments can now be obtained free from nitrogen if the above procedure is used and the amphoteric properties are probably due to an oxygen function.

The analyses of simple flavylium salts and anthocyanidins are often complicated by their ability to retain hydroxylic solvents. Similarly, the violet pigments (although almost insoluble in water) are difficult to obtain free from water, methanol, and methyl cellosolve. Thus, calculations of molecular formulas from elemental analyses are necessarily uncertain. Two ultramicro elemental analyses of pigment AIIIa gave the following results: C 60.9; H 5.0; Cl 5.8—6.0 % (4.1 % H_2O) and: C 60.1; H 5.3; Cl 7.0 % (2.7 % H_2O). The component AIIIb analyzed for: C 52.3; H 5.5—5.7; Cl 5.5 % (1.5 % H_2O).

It has not been possible to determine the molecular weights of the salts. Mass spectrometric determination is not possible because of the instability at higher temperatures and the low vapour pressure at moderate temperatures. The largest fragment obtained from AIIIa is m/e 148, and many smaller fragments with $m/e < 100$ are obtained. At the present state these do not give much valuable information.

R_F-Values for the violet pigments are lower than that for any known anthocyanidin in all systems tested. For instance, in the Forestal solvent the

Table 1. R_F-values of the violet pigments in *S. nemoreum*.

I. TLC on Avicel cellulose plates

Solvent	$R_F \times 100$	
	AIIIa	AIIIb
BAW (6:1:2)	6	8
MeOH-MeC (1:1)[a]	17	14
HCOOH-MeOH (8:5)	14	16

[a] Methanol-methyl cellosolve (1 % conc. HCl)

II. Descending paper chromatography

Solvent	$R_F \times 100$	
	AIIIa	AIIIb
Forestal	16	14
BuOH-2 M HCl(1:1)	4	6

R_F-value (0.3) for delphinidin (the most highly hydroxylated anthocyanidin) is twice that of the *S. nemoreum* pigments (0.14). Some chromatographic data are compiled in Table 1.

Fig. 1 shows the UV and visible spectra of AIIIa in methanol-0.01 % conc. HCl and in the same solution with some aluminium chloride present. The violet pigment has two bands in the UV region with maxima at 245 nm and 296 nm. Generally, anthocyanidins have only one band in the UV region with λ_{max} around 280 nm. However, luteolinidin has a shoulder at 240 nm (Fig. 5) and in the spectra of flavylium salts two bands below 300 nm are often observed. The intensity of the visible band of AIIIa relative to the bands in the UV region is lower than the corresponding ratio for luteolinidin (Fig. 1 and Fig. 5). With aluminium chloride present the visible band of AIIIa is shifted bathochromically (Fig. 1, curve 2). The bathochromic shift with aluminium chloride, caused by chelation, is a frequently used criterion for the presence of vicinal phenolic hydroxyls in anthocyanidins and for luteolinidin it is unusually large (Fig. 5, curve 2).

The violet pigment has a shoulder to the 296 nm band at about 325 nm, and to the visible band in the region 400—450 nm. Similarly, anthocyanidins with the 5-hydroxyl group free have a shoulder to the visible band at about 400 nm.[9] The spectrum of delphinidin shows a shoulder to the 280 nm band at *ca.* 300 nm. Thus, there are certain similarities between the spectra of AIIIa and anthocyanidins in methanol-HCl solutions.

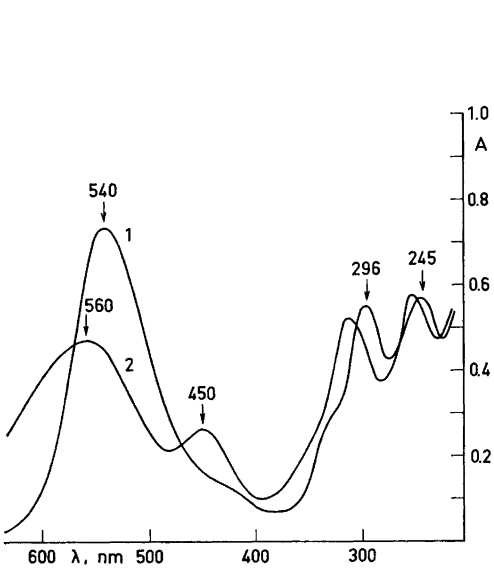


Fig. 1. Spectra of the pigment AIIIa (AIIIb essentially identical) in methanol-0.01 % conc. HCl (1); with aluminium chloride present (2).

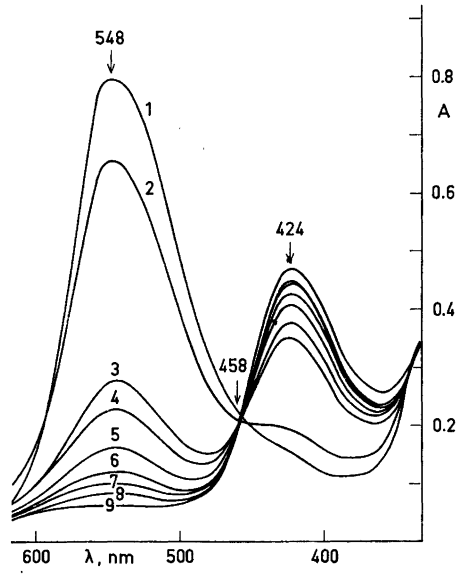


Fig. 2. Visible spectra of AIIIa in buffered methyl cellosolve solutions at pH 0.92 (1); pH 2.62 (2); pH 3.76 (3); pH 3.96 (4); pH 4.23 (5); pH 4.50 (6); pH 4.71 (7); pH 4.86 (8); pH 5.92 (9). Spectra recorded after 20 h.

As mentioned above, the color sometimes bleaches out during the isolation. In order to obtain optimal conditions for the isolation it was necessary to study the pH-dependence of the chromophore. Furthermore, such a study might give useful information concerning the transformations of the pigment at various pH's. In this investigation a parallel investigation was performed on luteolinidin. The method used is essentially that of Jurd and Geissman,[10,11] but because of the low water solubility of the pigment (AIIIa), the measurements were performed in methyl cellosolve with 10 % aqueous buffer present.

The visible spectra of AIIIa at various pH's are shown in Fig. 2. For the equilibrium "violet acid" ⇌ "yellow base" a well-defined isosbestic point is found at 458 nm. The pK's for the equilibrium calculated from the curves of Fig. 2 are compiled in Table 2. No correction for the ionic strength has

Table 2. pK of "violet acid" (AIIIa) ⇌ "yellow base" equilibrium.[a]

pH	Abs. at λ_{max}, 548 nm	AIIIa %	pK[c]
0.92	0.796[b]	100	
2.62	0.657	81.1	3.23
3.76	0.280	29.9	3.39
3.96	0.229	23.0	3.44
4.23	0.164	14.1	3.45
4.50	0.123	8.6	3.48
4.71	0.102	5.7	3.50
4.86	0.085	3.4	3.41
5.92	0.060	0	

[a] Rounded off average pK = 3.4 ± 0.2. [b] The same absorbance at pH 0.65. [c] Ionic strength term omitted.

been made. The pK found for AIIIa (3.4) is the same as that found for luteolinidin in a corresponding measurement series (Fig. 6 and Table 3). Jurd has reported the pK 3.17 for 3'-methoxy-4',7-dihydroxyflavylium chloride,[10] and

Table 3. pK of luteolinidin ⇌ 2,3',4,4',6-pentahydroxychalcone equilibrium.[a]

pH	Abs. at λ_{max}, 506 nm	luteolinidin %	pK[b]
0.65	0.655	100	
2.19	0.605	91.6	3.23
2.60	0.556	83.4	3.30
3.22	0.434	64.5	3.48
4.04	0.237	29.8	3.65
4.52	0.102	7.0	3.40
6.08	0.060	0	

[a] Rounded off average pK = 3.4 ± 0.3. [b] Ionic strength term omitted.

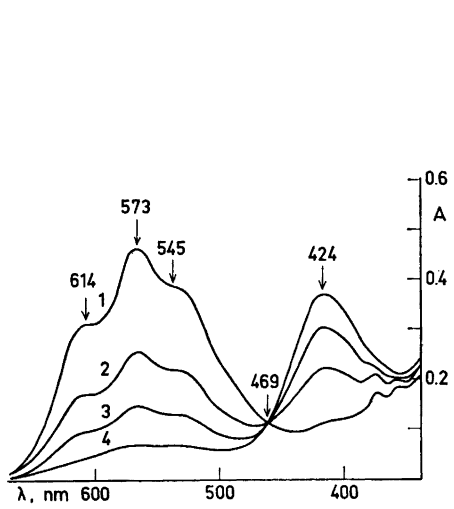


Fig. 3. Visible spectra of AIIIa in buffered methyl cellosolve (pH 6.0) after: 2 min (1); 20 min (2); 1 h (3); 2 h (4).

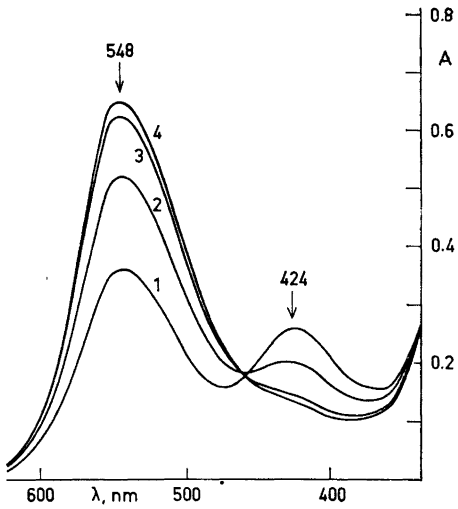


Fig. 4. Visible spectra of AIIIa after acidification to pH 0.5 of the solution of "yellow base" (Fig. 3 (4)): after 5 min (1); 20 min (2); 45 min (3); 1 h (4).

Albert gave the approximate value pK 4 for anthocyanins,[12] that is, of the same order as the pK obtained here.

In the violet → yellow transformation of AIIIa it was found that there appeared a blue intermediate. This "blue base" was converted to the "yellow base" within 20 min at pH 5.5, whereas at pH 6.0 (Fig. 3) this conversion was complete only after 2.5 h. When the solution of the "yellow base" at pH 6.0 was acidified to pH 0.5 and spectra recorded at different time intervals, the curves of Fig. 4 were obtained. As can be seen the original "violet acid" was completely reformed within one hour, and the "blue base" could not be detected in the reverse reaction. If it were formed, it was obviously converted to the violet form too rapidly to be detected at pH 0.5.

At pH < 0.2 the pigment was completely decolorized, and the chromophore could not be reformed at higher pH — an indication of a drastic change of the molecule. Thus it is necessary to perform all isolation work at pH 1—2, and extreme pH's have to be avoided.

The similarity in the behaviour of AIIIa and luteolinidin in the transformation studies is striking. At pH 6.0 luteolinidin exists as a red anhydro-base (one of three possible tautomers, Scheme 1). This base was converted to the chalcone within 24 h (Fig. 7), and when the solution was acidified to pH 0.5, the anthocyanidin was reformed in 1.5 h, without the appearance of anhydro-base. The pentahydroxychalcone from luteolinidin does not seem to have been synthesized, but according to Jurd's studies,[10] there is little doubt that the yellow product with λ_{max} 382 nm is the chalcone (Scheme 1). It has not been established whether it is the *cis-* or the *trans* form of the chalcone,

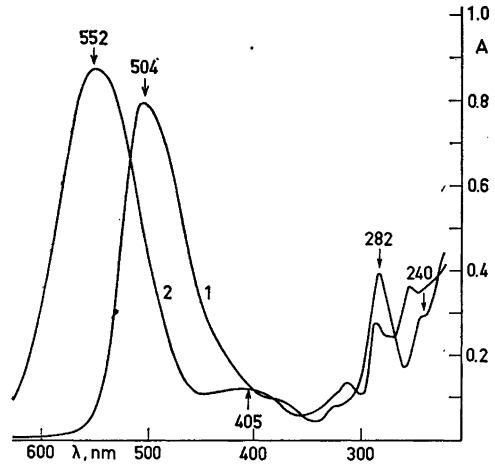


Fig. 5. Spectra of luteolinidin in methanol-0.01 % conc. HCl (1); with aluminium chloride present (2).

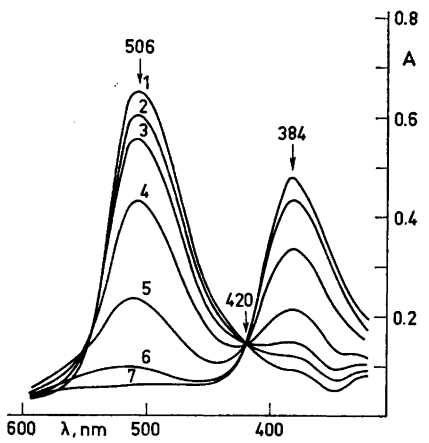
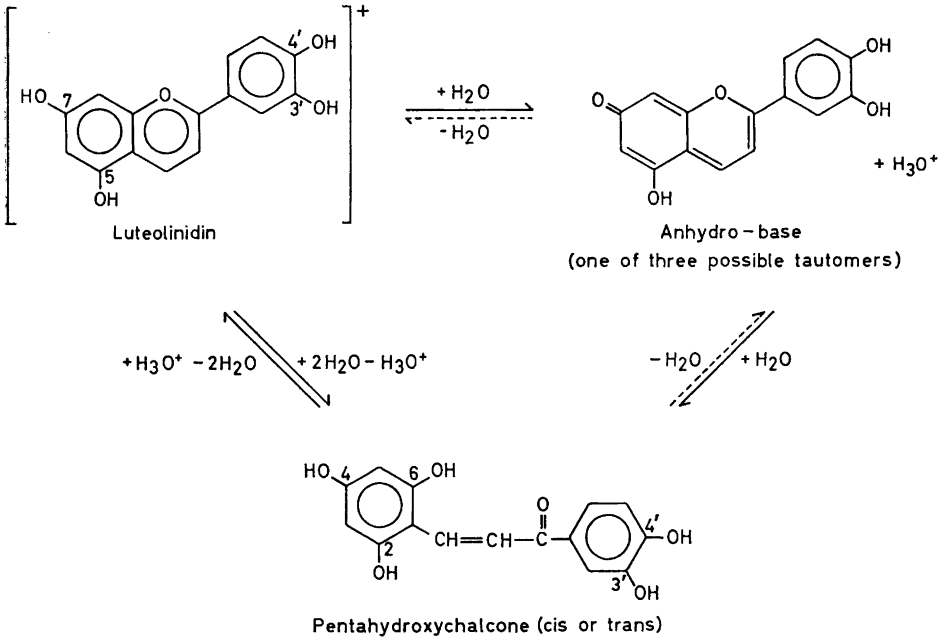


Fig. 6. Visible spectra of luteolinidin in buffered methyl cellosolve solutions: at pH 0.65 (1); pH 2.19 (2); pH 2.60 (3); pH 3.22 (4); pH 4.04 (5); pH 4.52 (6); pH 6.08 (7). Spectra recorded after 45 h.



Scheme 1. Transformations of luteolinidin at pH 1—6.

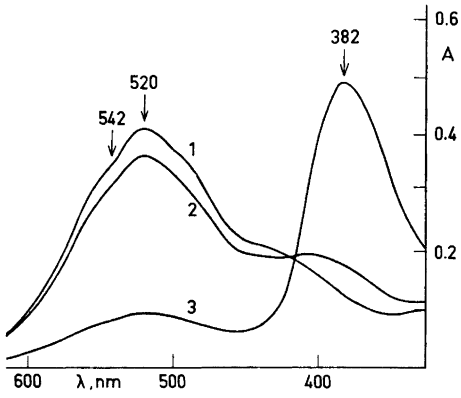


Fig. 7. Visible spectra of luteolinidin in buffered methyl cellosolve (pH 6.0) after: 2 min (1); 50 min (2); 20 h (3).

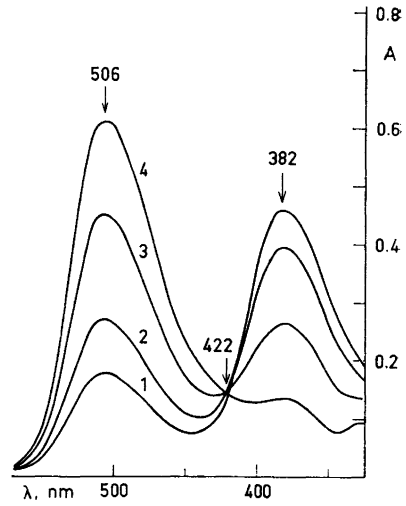


Fig. 8. Visible spectra of luteolinidin after acidification to pH 0.5 of the solution of its corresponding chalcone (Fig. 7 (3)): after 2 min (1); 15 min (2); 50 min (3); 1 h, 20 min (4).

but the rapid transformation to the flavylium salt indicates that it might be the *cis*-chalcone.[10]

The transformations shown in Scheme 1 for luteolinidin are characteristic for 3-desoxyanthocyanidins. The anhydro-bases are the true conjugate bases of the oxonium compounds but in the pH-range 2—5 they react rapidly with water to give chalcones (possibly *via* flavenols). Therefore, the equilibrium flavylium ion $\rightleftharpoons$ chalcone is generally studied. Jurd and Geissman, studying the transformations of 4′- and/or 7-hydroxylated flavylium salts, observed that the transformation anhydro-base → chalcone is very rapid at pH 5.6, much slower at pH 6—7, and very slow for the ionized compounds at higher pH.[11] Although it is to early at this time to conclude that the "yellow base" of AIIIa is a chalcone, the similarity with the behaviour of 3-desoxyanthocyanidins is striking.

Somewhat astonishingly luteolinidin on standing at pH < 0.2 was completely decolorized. The color could not be regenerated at higher pH, and thus the molecule may be split, as yet in an unknown way. This was a further and unexpected similarity, since the destruction of 3-desoxyanthocyanidins at low pH does not seem to have been reported.

In a recent investigation of flavylium salts in this laboratory, it was found that most of them absorb in the infrared at 1620—1640 cm^{-1}, a band ascribed to the heterocyclic oxygen.[13] Both violet pigments possess a band at 1622 cm^{-1} (Fig. 9) although of lower intensity than generally is the case for flavylium salts. Two band groups, each with 3—4 bands centered at 1600 cm^{-1} and

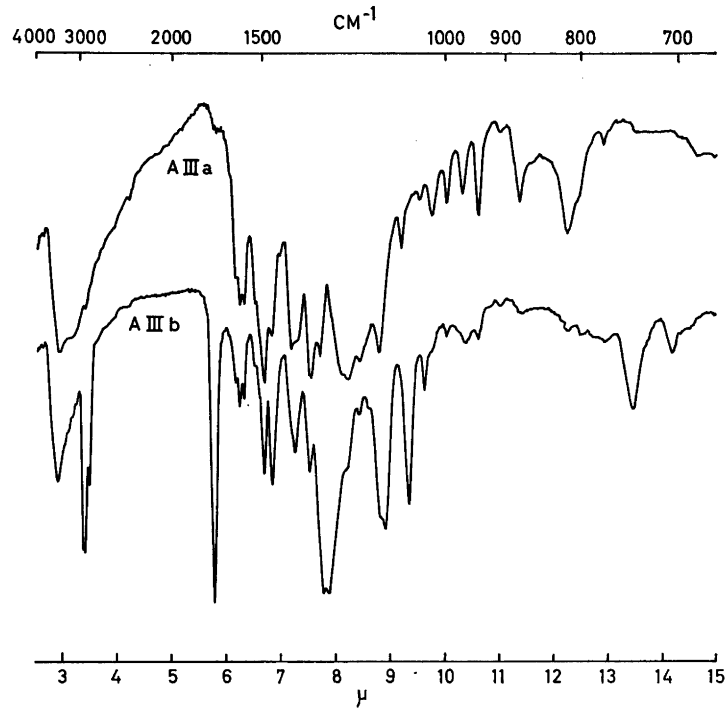


Fig. 9. Infrared spectra of the violet pigments from *S. nemoreum* (KBr phase).

1500 cm^{-1} often appear in the spectra of flavylium salts, and this is true also for the violet pigments. Another common characteristic is the high intensity of the absorption in the 1500—1000 cm^{-1} region and the relatively low intensity of the bands in the aromatic substitution region. The ester carbonyl absorption of AIIIb appears at 1735 cm^{-1} and there is also intense C—H stretching of the aliphatic type.

An effort to study the NMR spectrum of AIIIa failed because of the low solubility in trifluoroacetic acid. Only qualitative evidence for the presence of aromatic, aliphatic, and phenolic protons was obtained.

The polarographic reduction curves of AIIIa and two flavylium compounds are shown in Fig. 10. AIIIa (saturated solution in methyl cellosolve:1 M KCl, 9:1, pH 1.8) (curve 1, Fig. 10) has a half-wave potential at −0.3 V against the saturated calomel electrode, and the two flavylium salts are reduced at the same potential. Zuman has studied some known anthocyanidins polarographically and found the potential −0.4 V relative to the Tl^+ ion.[14]

AIIIa can be reduced with Adams' catalyst and hydrogen at atmospheric pressure, a reaction which has also been reported for some flavylium salts.[15] This reduction product and the "yellow base" now seem to offer opportunities for a molecular weight determination and are under investigation.

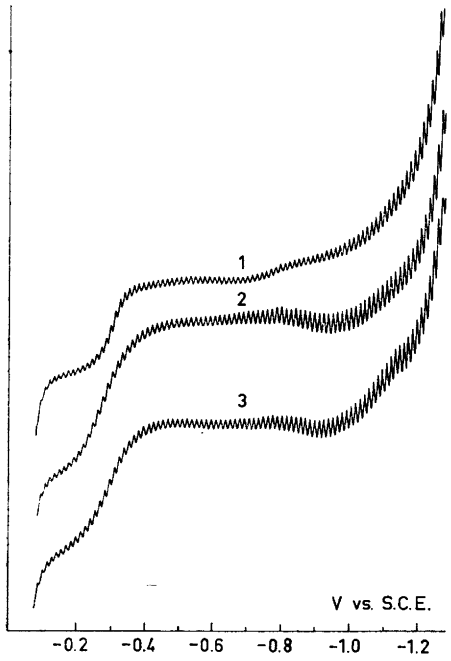


Fig. 10. Polarograms of the pigments AIIIa (1), 3,4′,7-trihydroxyflavylium chloride (2), and 3′-methoxy-4′,5,7-trihydroxyflavylium chloride (3). Solvent: methyl cellosolve:1 M KCl (aq.) (9:1, pH 1.8). Concentrations unknown — for AIIIa saturated solution. Half-wave potentials: (1) −0.32 V; (2) −0.32 V; (3) −0.30 V.

A small scale alkali degradation of AIIIa gave one phenol (λ_{max} 293, 322 (i) nm), one phenolic ketone (possibly acetovanillone), and four phenolic acids.

The general spectral properties, transformations at different pH, pK, polarographic reduction potential, the amphoteric properties due to an oxygen function (by elemental analyses), the reducibility — all these properties can in some way or other be correlated with the properties of anthocyanidins and seem to be a good collective evidence of the anthocyanidin character of the chromophore of the violet *Sphagnum* pigments. The structural studies will be continued.

EXPERIMENTAL

Moss material. Reddish-violet *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 recorded on a Perkin-Elmer Model 157 spectrophotometer using the KBr disc technique.

Polarography. The polarographic measurements were made with a Radiometer type PO 4c polarograph.

Chromatography. Avicel SF cellulose (Kebo) was used for TLC, and Whatman No. 1 paper for PC. Solvents: BAW = butanol:acetic acid:water (6:1:2, by vol.), MeOH:MeC = methanol:methyl cellosolve (1:1, v/v, 1 % conc. HCl), formic acid:methanol (8:5, v/v), Forestal = acetic acid:conc. HCl:water (30:3:10, by vol.), butanol:2 M HCl (1:1, v/v, upper phase).

pK determination. Solutions of the pigments in methyl cellosolve-0.01 % conc. HCl were prepared. Aliquots (9 ml) were made up to 10 ml with HCl-KCl buffer for pH < 1 and phosphate-citrate buffer for higher pH-values. The solutions were allowed to stand in the dark at room temp. until equilibrium was reached. Measurements were made against blanks of the same pH and ionic strength as the pigment solutions.

Luteolinidin. A synthetic sample, tested for purity with chromatography, IR and UV spectra, was used.

Hydrolysis of AIIIb. The hydrolysis was performed at 90° in a nitrogen atmosphere in methyl cellosolve-HCl. The mixture was poured into water and extracted with ether. The ether phase contained resinous acidic material and an unstable ketonic product. The precipitated pigment had identical R_F-values, IR and UV spectra with those of AIIIa.

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