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Review Paper

Lindow Man, Tollund Man and Other Peat-Bog Bodies: The Preservative and Antimicrobial Action of Sphagnan, a Reactive Glycuronoglycan with Tanning and Sequestering Properties

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ABSTRACT

The tanning reaction that contributes to the preservation of animal tissues by peat consists of a Maillard reaction between the free amino-groups of collagen and reactive carbonyl groups in a soluble glycuronoglycan ('sphagnan') containing residues of D-lyxo-*5-hexosulopyranuronic acid. Sphagnan is a complex, pectin-like material which is covalently linked to cellulosic and amyloid-like chains in living* Sphagnum *moss, but slowly liberated by autohydrolysis into the ambient water as the dead moss is converted into peat. It is a precursor of aquatic humus from* Sphagnum *peat, and the tanning of adventitious collagen in animal remains is only one manifestation of the continuous incorporation of ammonia, amino-acids and polypeptides from a wide variety of sources into the structure of the humic acid molecule. Sphagnan can also suppress microbial activity by reacting with exo-enzymes and sequestering essential, multivalent metal cations.*

INTRODUCTION

The discovery in August 1984 of exceptionally well-preserved human remains ('Lindow Man'), about 2000 years old, in a peat bog near Manchester (Stead *et al.*, 1986) has revived interest in the preservative, and putatively antimicrobial, properties of peat. A similar discovery

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('Tollund Man') had been made near Aarhus (Denmark) in May 1950 (Glob, 1971). These, however, were only the most intact of about 1400 similar discoveries made throughout northern Europe (Stead *et al.*, 1986; Glob, 1971; Ross & Robins, 1989; Coles & Coles, 1989). Together with numerous discoveries of the remains of animals and of woollen and wooden artefacts (Stead *et al.*, 1986; Coles & Coles, 1989), they leave no doubt as to the generality of the preservation phenomenon.

Whereas the bodies themselves are of archeological interest only, the mechanism of preservation is of ecological significance, and it could suggest applications in many areas, such as pharmaceuticals, surgical dressings, sanitary engineering and waterworks technology. Since preservation represents a *cul-de-sac* in the nitrogen cycle, it is also of fundamental significance for horticulture and agriculture in peaty soils.

Current ideas about the mechanism are mostly speculative, but the British Museum team nonetheless made a key observation on Lindow Man: the best-preserved parts were the connective tissues, in which the collagen fibres appeared to be *tanned*, as in leather (Connolly *et al.*, 1986). This prompted us to look for tanning agents in *Spagnum* moss and in peat-bog water, and we now report that one has been found. Contrary to expectations, however, it was not a polyphenol but a pure polysaccharide for which we propose the name *sphagnan*.

Polysaccharides do not normally tan, but sphagnan is almost unique in that its chains contain many reactive carbonyl groups in addition to the usual reducing end-group. These are located in residues of D-*lyxo*-5-hexosulopyranuronic acid (5-keto-D-mannuronic acid), which comprise ~25% of the polymer (Painter, 1983*a*). In its reactivity with phenylhydrazine, other carbonyl reagents, and all primary amines, sphagnan resembles periodate-oxidised polysaccharides, whose tanning properties are well known (Nayudamma, 1975; Clark & Courts, 1977). Only one other naturally occurring polysaccharide with these properties has so far been reported, namely, the capsular polysaccharide of *Streptococcus pneumoniae*, Type 5 (Jansson *et al.*, 1985).

Since factors other than tanning could also contribute to preservation, alternative explanations will be discussed fully, in an attempt to provide a balanced account. The most credible alternatives are, however, ultimately dependent upon the properties of sphagnan, or of the aquatic humus of which it is a precursor (Painter, 1983*b*).

THE SPHAGNOL HYPOTHESIS

This hypothesis has become so widely accepted that it is reported as a fact by the Encyclopaedia Britannica (1975). It postulates that preserva-

tion is due to the presence in *Sphagnum* moss of an antimicrobial compound called *sphagnol.* It is based upon a misunderstanding of statements made in a somewhat inaccessible paper and a review by Czapek (1899, 1913). The name *sphagnol* was first given by Czapek to an unidentified, crystalline compound obtained by boiling extractive-free *Sphagnum* moss with 0·25 M sodium hydroxide. It gave a red colour with Millon's reagent for phenols, and a reddish brown colour with aqueous ferric chloride. It had bacteriostatic and fungistatic properties, and was 'fairly strongly toxic' to *Daphnia* (a freshwater crustacean). *Czapek made it clear that sphagnol was not present in the free state in the living moss.* He considered that it was a fragment of a cell-wall polymer. The polymer was later recognised as a special kind of lignin with a low methoxyl content (Hegnauer, 1962).

The identity of sphagnol may never be known with certainty. Rudolph and Engmann (1967) repeated Czapek's experiment, and obtained five Millon-positive products, among which *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were identified. Very probably, however, sphagnol was the component that crystallised most easily from the mixture, and in our hands this was *p*-hydroxybenzoic acid. Since oxygen was not excluded during boiling with alkali, it is probably an oxidation product of moss lignin. Lindberg and Theander (1952) found that controlled oxidation of solvent-extracted *Sphagnum* with nitrobenzene yielded mainly *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic acid (together with *p*-hydroxybenzyl alcohol) could have been formed from this, under alkaline conditions, by a Cannizzaro reaction. Farmer and Morrison (1955) later showed that methylation of solvent-extracted *Sphagnum*, followed by oxidation with permanganate, yielded *p*-methoxybenzoic acid.

The pH in a highmoor *Sphagnum* peat bog is typically 3·2–4·5 (Clymo, 1984), and beneath the surface carpet of living moss the oxygen tension falls off sharply with increasing depth (Given & Dickenson, 1975). These conditions are so different from those used to isolate sphagnol that the popularity of the sphagnol hypothesis is surprising. The sense of the question nonetheless remains: does *Sphagnum* moss contain, or liberate upon its death and decay into peat, a substance with antimicrobial activity?

With the preconception that any such compound would probably be a polyphenol with some tanning activity, we made a systematic search of different extracts of the fresh moss and of peat, prepared both before and after treatment with dilute acid. The extracts were fractionated by column chromatography, and the antimicrobial activities of the fractions were measured as *phenol coefficients* (Croshaw, 1981). The phenol coefficient of the total phenolic fraction, which represented $< 2\%$ of the dry weight of the moss, was only 0·03, and that of the most active fraction,

which represented 0·1% of the moss, was $< 0{\cdot}5$. Because of the ubiquity of phenolic compounds in vascular plants, which undergo rapid microbial breakdown under both aerobic and anaerobic conditions, this must be considered a very commonplace result, which cannot explain the preservative properties of peat.

The likely properties of any antimicrobial substances in peat can be inferred from the successful cultivation, on an industrial scale, of yeasts, fungi and bacteria on acid hydrolysates of peat (Fuchsman, 1980). Most phenols and phenolic acids are soluble and stable in dilute mineral acids, and should therefore be present in these hydrolysates, whereas lignin and humic acids are insoluble in acid, and in the industrial process they are filtered off. Any antimicrobial substances must therefore be either insoluble in hot, dilute mineral acid, or decomposed by it.

OXYGEN DEPRIVATION AND LOW pH

Waksman (1930) strongly contested the idea that peat possessed antimicrobial properties, after finding 10^4–10^6 bacterial cells per gram in the anoxic region (Waksman & Stevens, 1929). These counts were low compared to those found in the oxygenated, surface region (10^8 cells/g), and very low compared to those found, for example, in the rotting roots of grass and lucerne (10^{10} cells/g), but they disproved earlier contentions that the anoxic region of peat is sterile.

Waksman pointed out that the anoxic conditions and low pH would severely restrict the *scope* of microbial activity in peat. He did not state that they were also responsible for its low *intensity*, but his data are widely interpreted as showing this, and the extreme view is sometimes expressed that they account completely for the preservative properties of peat.

In discussions of this topic, the acidity of peat-bog water is often exaggerated by the use of vague words such as 'very' and 'highly'. In fact, the pH of lowmoor peat (in which Lindow Man and Tollund Man were both found) is usually 5·5–6·5 (Waksman, 1930). In highmoor peat the pH range is 3·2–4·5 (Waksman, 1930; Clymo, 1984), but this is not lower than the pH at which vigorous anaerobic fermentation occurs in the production of many wines and ciders, and some yoghourts.

In numerous horticultural applications, peat is neutralised with lime and exposed to the atmosphere, but even under these conditions the loss of organic material (by conversion into carbon dioxide and ammonia) is

much slower than it is from humus originating from vascular plants (Puustjärvi, 1976–77).

DEPRIVATION OF ESSENTIAL METAL CATIONS

The high cation-exchange capacities and selectivities of *Sphagnum* holocellulose, the soluble fragment (sphagnan) liberated from it by auto-hydrolysis, and the aquatic humus formed from sphagnan by dehydration and partial decarboxylation (Painter, 1983*b*), have been reported elsewhere (Smidsrød & Painter, 1984; Andresen *et al.*, 1987). Peat-bog bodies are permanently immersed in a solution of sphagnan in various stages of conversion into aquatic humus, and it is therefore not surprising that the bones, teeth, hair and nails of Lindow Man were found to be almost completely decalcified (Connolly *et al.*, 1986). Decalcification has previously been ascribed to the acidity of peat-bog water, but it would occur at least as easily, by complex formation, at pH 7.

To grow in peat, therefore, micro-organisms have to compete for essential metal cations with a massive excess of a powerful chelating agent. This will clearly limit not only the *scope* but also the *intensity* of microbial activity. To illustrate this, we selected a strain of *Azotobacter vinelandii* with a known requirement for calcium ions, and measured the effects of sphagnan, aquatic humus, ethylenediamine tetra-acetic acid (EDTA) and phenol upon its growth rate in a standard medium.

Upon a weight basis, the test samples and EDTA were about equal in inhibitory activity, and by increasing their concentration relative to that of Ca^{2+} in the medium, it was easy to obtain apparent 'phenol coefficients' greater than 10. When an excess of Ca^{2+} was added to the medium, however, the bacteria grew at the same rate in the presence of the test materials as in the control experiments.

This result makes it relevant to enquire whether peat, in its natural state, ever becomes so saturated with essential metal cations that this kind of antimicrobial activity ever ceases to operate. Bodies completely reduced to skeletons have been found in peat, but it is impossible to tell how soon after death they became submerged in bog water (Ross & Robins, 1989; Coles & Coles, 1989). It is, however, possible to state that the sort of peat in which preserved bodies have been found does not normally accumulate on fertile terrain. It is usually found in basins in solute-poor, erosion-resistant, Precambrian rocks such as the Laurentian Shield in Canada, the Fennoscandian Shield in Finland, Sweden, Northern Denmark and Eastern Norway, and the Caledonian Geosyncline in Norway, Scotland, Wales and Ireland. Moreover, the growth

of most *Sphagnum* species is actually inhibited by even moderate concentrations of calcium ions (Clymo & Hayward, 1982).

NITROGEN DEPRIVATION

The nitrogen content of *Sphagnum* moss is unusually low, ~ 0·5%. Even if all this nitrogen became freely available to bacterial and fungal saprophytes after the death of the moss, their growth would probably be nitrogen-limited. In the event, this is certainly so; a positive correlation between decay rate and nitrogen content has been observed, and it has been shown that increasing the nitrogen content of peat-forming vegetation by fertilisation increases its rate of decay when it dies (Coulsen & Butterfield, 1978).

The nitrogen content of peat is, on average, significantly higher (0·6–1·9%) than that of the living moss (Mattson & Koutler-Andersson, 1955; Smith *et al.*, 1958). Some of the additional nitrogen may have been absorbed directly from the atmosphere (as ammonia), but most of it probably originated from amino-acids and polypeptides synthesised by surface flora (bacteria, fungi and higher plants) and fauna (mostly arthropods) other than *Sphagnum* (Mattson & Koutler-Andersson, 1955). Support for this idea is provided by the regular presence in peat hydrolysates of glucosamine, which is not found in *Sphagnum* (Parsons & Tinsley, 1961; Schnitzer & Khan, 1972).

The nitrogen is concentrated in the humic acid fractions of the peat. These include the 'aquatic humus' that is soluble at the pH of the bog (Gjessing, 1976; Painter, 1983*b*; Smidsrød & Painter, 1984), and fractions extracted from the insoluble part with neutral buffers or alkali (Smith *et al.*, 1958; Smith & Lorimer, 1964). Nitrogen contents ranging from 1·5% to 10% are reported, but most values cluster in the region of 2·5–3·5% N (Breng, 1976).

Almost all of this nitrogen seems to have become incorporated into the structure of the humic acid, by a variety of covalent bonds which differ markedly in their stability to acid. Boiling with dilute mineral acid normally releases some ammonia and amino-acids, and prolonged boiling with 6 M hydrochloric acid then releases additional amino-acids from intact polypeptide chains. Up to 50% of the nitrogen, however, remains firmly bound to the dark brown, aromatic chromophore (Bremner, 1967; Schnitzer & Khan, 1972; Breng, 1976).

It is questionable whether any of this nitrogen can be utilised by micro-organisms under anoxic conditions (Campbell & Lees, 1967). The active decomposition of humic acids by some fungi has been observed,

but only in the presence of molecular oxygen and another source of carbon (Hurst & Burges, 1967; Schnitzer & Khan, 1972). Even in well-aerated soil, only about 1–3% of this kind of nitrogen is utilised in a growing season (Bremner, 1967). The extreme resistance to biodegradation of any adventitious protein that becomes incorporated into the humic-acid complexes implies that the coupling reaction may be regarded as a kind of 'tanning'. It is reasonable to expect that any animal remains in the peat would be 'tanned' by the same kind of reaction, and that any exocellular proteases and other enzymes secreted by putrefactive bacteria could also be inactivated by it.

CRITERIA OF TANNING

Biochemists usually think of tanning as the formation of a hydrogen-bonded complex between any protein and any polyphenol. In fact, chromic salts and formaldehyde are the two most important industrial tanning agents, and only two classes of naturally occurring polyphenols (polyesters of gallic acid and polymeric proanthocyanidins) are ever used to manufacture leather (Gustavson, 1956). The latter occur mostly in the leaves and bark of woody dicotyledons, and not at all in mosses (Bendz *et al.*, 1966). Most other polyphenols are useless for making leather, because the complexes are too soluble in water. Gustavson (1956) gives three criteria of tanning:

(1) Tanning consists of the cross-linking of collagen fibres in such a way as to expel water from the interstices and to prevent rehydration upon subsequent heating in water.
(2) Tanning must confer resistance to microbial attack. Since collagen contains numerous lysine and arginine residues (Eastoe & Leach, 1977), trypsin is used as a standard enzyme to monitor the decrease in digestibility.
(3) A solution may be said to have tanning properties if it precipitates gelatin from aqueous solution, and loses this property after filtration through hide powder.

The most important carbonyl compounds that are used in tanning are formaldehyde, glyoxal, pyruvaldehyde, acrolein and glutaraldehyde, and their most important function is to form, in the presence of an acidic catalyst, polyacetal bridges between the amino-groups on one collagen molecule and those on another. The most significant amino-groups are

the ε-amino groups of lysine residues and those comprising the guanido-groups of arginine residues, but the amido-groups of asparagine and glutamine also react (Gustavson, 1956).

Formaldehyde is particularly effective because of its strong tendency to form polyoxymethylene chains. The fact that these may vary in length increases the possibilities for cross-linking, and, hence, the efficiency of the tanning. Although simple reducing sugars and the reducing end-groups of polysaccharides may form glycosylamines with the amino-groups on collagen, they cannot tan, because they do not cross-link the chains. Periodate-oxidised oligo- and polysaccharides are, on the other hand, good tanning agents, because the multiplicity of aldehydic groups in these polymers offers broad scope for cross-linking (Nayudamma, 1975; Clark & Courts, 1977).

QUEST FOR TANNING AGENTS IN NATURAL PEAT-BOG WATER

Aquatic humus comprises >95% of the organic matter in bog water (Smidsrød & Painter, 1984). It is liberated continuously into the ambient water almost from the moment when the new moss dies on the surface until it has been converted into a dark brown, coal-like material 3000–7000 years later. Systematic analyses of the residual (insoluble) peat have shown that the lignin-to-carbohydrate ratio increases with age (depth below the surface). This is due mainly to losses in the easily hydrolysable fraction described as 'hemicellulose' (Waksman, 1930; Mattson & Koutler-Andersson, 1955). This fraction would consist mainly of the material now described as 'sphagnan'.

We have investigated a sample of aquatic humus isolated from water that had collected in a hole, 1·5 m deep, dug in a peat-bog (Painter, 1983*b*; Smidsrød & Painter, 1984). This would have been leached by rainwater from peat up to ~1000 years old. The humus was a complex glycoconjugate, containing a glycuronoglycan moiety (46%), a dark brown chromophore (38%), and nitrogen corresponding to 16% of polypeptide. It had only weak tanning properties, imparting only a faint turbidity to gelatin solutions. When, however, the glycuronoglycan moiety was isolated after selective, chlorous-acid oxidation of the chromophore, it gave a dense precipitate with aqueous gelatin. This suggested that the glycuronoglycan was the original tanning agent, that it had already 'tanned' the polypeptide part of the glycoconjugate, and that it had then lost most of its surplus tanning capacity during the subsequent humification process.

LABORATORY SIMULATION OF *SPHAGNUM* DIAGENESIS†

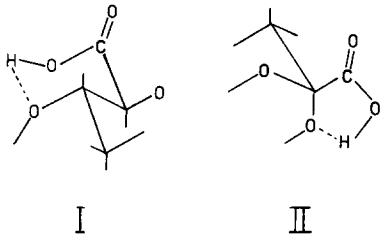
The disadvantage in working with natural samples of aquatic humus is that too little is known about their previous history: the natural process of humification occurs too slowly to be followed closely. There is also a variety of biological starting materials; perhaps as many as six different *Sphagnum* species, other mosses such as *Hypnum*, and vascular plants such as cottongrass (*Eriophorum*), heath plants (*Ericales*), reeds (*Phragmites*), sedges (*Carex*) and rushes (*Scheuchzeria*). In addition, only vague generalisations can be made about the most significant sources of nitrogen, and the multitude of microflora and fauna through which these are mediated. The mechanism of humification and of any concomitant tanning can therefore be studied only by laboratory simulation.

It has long been suspected that the slow process of humification that takes place in the anoxic peat below the surface layer of a peat bog is chemical rather than microbiological (Waksman, 1930; Kononova, 1961). Although regions of significant anaerobic bacterial activity have been discovered (Waksman & Stevens, 1929; Waksman, 1930), humification seems to occur just as readily in other regions where it is very low (Given & Dickenson, 1975). The resistance of *Sphagnum* holocellulose to enzymic digestion (Andresen *et al.*, 1987) likewise argues against a microbiological mechanism.

Even where a microbiological *contribution* to diagenesis is demonstrable, however, the chemical contribution would still be large under the acidic conditions in the bog. This is because hexuronic acid residues, within precisely the relevant pH range (2·0–4·5), have the ability selectively to hydrolyse adjacent glycosidic linkages in glycuronoglycan chains by intramolecular autocatalysis (Smidsrød *et al.*, 1969). The mechanism is a special case of general acid catalysis, and entails the direct donation, by the un-ionised carboxyl group, of a proton to a neighbouring glycosidic oxygen atom (Fig. 1). For (1→4)-linked glycuronans such as alginates and pectates, the intermediate hydrogen-bonded ring is six-membered, as in (I), and at pH 3·5 the resultant rate of hydrolysis is two to three orders of magnitude greater than would be expected for ordinary, proton-catalysed hydrolysis.

In 2-linked 2-aldulopyranosonic acid residues such as those of 3-deoxy-D-*manno*-2-octulopyranosonic acid (KDO) and 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-noulopyranosonic acid (*N*-acetylneuraminic acid), and also in 5-linked D-*lyxo*-5-hexosulopyranuronic acid, the intermediate would be five membered, as in (II). In these cases,

†*Editor's note*: Additional experimental details are given at the end of this review paper.



I II

Fig. 1. Intramolecular autocatalysis of acid hydrolysis in *Sphagnum* holocellulose through neighbour-group participation by the carboxyl groups in residues of D-*lyxo*-5-hexosulopyranuronic acid.

precise kinetic data are lacking, but the extreme acid-sensitivity of KDO linkages in bacterial lipopolysaccharides, and of *N*-acetyl-neuraminidic linkages in mammalian glycoproteins, is well known.

Activation energies for the acid-hydrolysis of glycopyranosidic linkages are high (typically 33 ± 2 Kcal/mol), corresponding to a temperature coefficient for the rate constant of ~15% per degree in the range from 20°C–100°C (BeMiller, 1967; Painter, 1973; Painter, 1980). This suggests that it should be possible to compress a thousand years of peat-bog history at 20°C into about five days by increasing the temperature to 100°C. The simulation would not be wholly realistic, however, unless all the reactions had the same activation energy; this is unlikely, and some reactions are probably speeded up more than others.

In a typical simulation experiment, extractive-free *Sphagnum acutifolium* was converted fully into its free-acid form by an acid wash, suspended in distilled water at pH 6, and heated under reflux at 98°C in a stream of nitrogen. At daily intervals for 10 days, the soluble material liberated by autohydrolysis was collected, analysed, and tested for tanning properties.

After the first day, the autolysate contained some sphagnan, together with a complex misture of polyphenols representing ~1% of the dry weight of the moss. The polyphenols appeared from their nuclear magnetic resonance spectra and behaviour in thin layer chromatography (t.l.c.) systems to be oligomeric or polymeric, and they may therefore have been fragments of lignin. They did not precipitate gelatin from aqueous solution. On subsequent days the solubilised material consisted of almost pure sphagnan, apparently identical with that prepared from the chlorite holocellulose of the moss (Smidsrød & Painter, 1984), except for the presence of further traces of polyphenols. These fractions gave dense precipitates with aqueous gelatin, and were sorbed on to hide powder.

After 10 days, ~23% of the dry weight of the extractive-free moss had been solubilised as sphagnan, and ~6% as monosaccharides. In the natural situation, the latter would be readily assimilated by bacteria, and

hence the discovery of bacteria in peat does not prove that they are a causative agent of diagenesis. The insoluble residue remaining after autohydrolysis was a dark brown, fibrous material, fairly similar in appearance to solvent-extracted peat, 2000 years old.

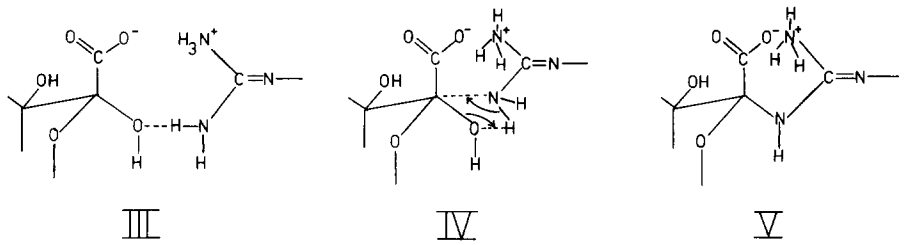
THE FIRST STEPS IN TANNING BY SPHAGNAN

The precipitate that forms immediately upon mixing aqueous solutions of sphagnan and gelatin must be a macromolecular salt, because its formation is inhibited by high (> 0·5 M) concentrations of sodium chloride. It is, however, much more dense than the coacervates that gelatin forms with other acidic polysaccharides (Doyle *et al.*, 1967; Woodside *et al.*, 1968).

The residues of D-*lyxo*-5-hexosulopyranuronic acid must play a special role in stabilising the ionic interaction, because reduction of the carbonyl groups with 20% aqueous sodium borohydride (Painter, 1983*a*) eliminates the ability of sphagnan to precipitate gelatin.

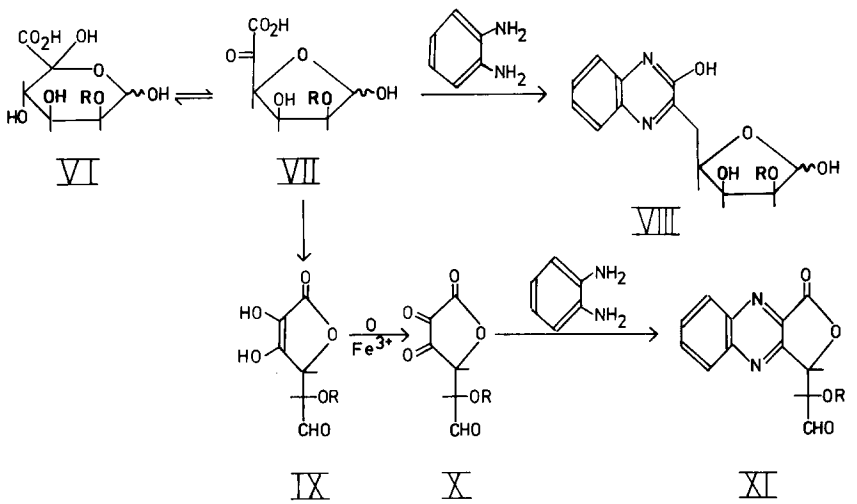
All amino-acids inhibited the precipitation at high concentrations (> 100 mM), but lysine, polylysine and urea were effective at a concentration of 20 mM, and arginine and guanidine hydrochlorides at 5 mM. It therefore seemed likely that the most favourable kind of structure for complex formation would be one with two amino groups, one of which could form an ionic bond with the carboxyl group, while the other could form a hydrogen bond with the hemiketal hydroxyl group, of a D-*lyxo*-5-hexosulopyranuronic acid residue (III, Fig. 2). Elimination of water, as in IV, with formation of a glycosyamine (V), should then follow spontaneously.

To test these ideas, *o*-phenylenediamine was chosen as a model substance, because its aromatic ring would supply an easily identifiable chromophore, and because its reaction with α-keto-acids to give quinoxalinol derivatives is already well documented (Hockenhull & Floodgate, 1952). In dilute aqueous solution at room temperature, it was rapidly taken up by the free-acid forms of whole, extractive-free *Sphagnum* and its chlorite holocellulose, as it would be by any cation exchanger. During the ensuing 12 h there was slow development of a saffron-yellow colour. Under the ultraviolet light microscope this appeared as a brilliant yellow fluorescence in the hyaline cell walls. With samples of sphagnan prepared either from the extractive-free moss or its holocellulose, the derivative was soluble in water, and showed a broad absorption band at 400–480 nm. It probably contained more than one kind of quinoxaline derivative, but the quinoxalinol structure VIII (Fig.



III IV V

Fig. 2. Glycosylamine formation between position 5 of residues of D-*lyxo*-5-hexosulopyranuronic acid in sphagnan and the guanido-groups of arginine residues in collagen. The electrostatic interaction is expected to promote glycosylamine formation by bringing about a high localised concentration of reactive amino-groups in the vicinity of the polyanion.



VI VII VIII IX X XI

Fig. 3. Formation of quinoxaline derivatives by the reaction of *o*-phenylenediamine with terminal units of D-*lyxo*-5-hexosulopyranuronic acid in sphagnan.

3), formed from end-groups of D-*lyxo*-5-hexosulopyranuronic acid (VI) after isomerisation to D-*lyxo*-5-hexosulofuranuronic acid (VII) is one possibility.

There is, however, an alternative first step which may be prominent whenever proteins are tanned by sphagnan in the presence of oxygen. Since most of the nitrogen in peat is supplied at the surface, in the oxygenated region, this mechanism is very relevant. It was suggested by an observation that, when sphagnan or *Sphagnum* holocellulose were stored dry in their free-acid forms, they slowly developed a capacity to

decolorise 2,6-dichlorophenol-indophenol, a standard reagent for estimating vitamin C (Roe, 1954).

Terminal units of D-*lyxo*-5-hexosulofuranuronic acid (VII, Fig. 3), in analogy with 2-keto-aldonic acids, would be expected to enolise and lactonise spontaneously (Green, 1957) with formation of the ascorbic acid analogue (IX), under mildly acidic conditions. In the presence of oxygen and catalytic amounts of ferric ions, IX would undergo oxidation to give the reactive analogue (X) of dehydroascorbic acid. This structure resembles that of ninhydrin, and would be expected to react readily with proteins and all primary amines. Dehydroascorbic acid, generated *in situ* by atmospheric oxidation of ascorbic acid, has been used to derivatise chitosan in aqueous solution (Muzzarelli *et al.*, 1984). It is clear that X would also react with *o*-phenylenediamine to give, *inter alia*, the quinoxaline derivative XI.

HUMIFICATION AND THE SUBSEQUENT STEPS IN TANNING

The glycosylamine V and any Schiff-base derivatives formed by X would subsequently undergo the complex series of rearrangements and eliminations of water, carbon dioxide and ammonia referred to collectively as 'non-enzymic browning' or 'the Maillard reaction' (Maillard, 1916, 1917; Olsson *et al.*, 1977, 1978). The liberation of carbon dioxide and ammonia by peat has been demonstrated, but interpreted as evidence for a microbiological mechanism of diagenesis (Waksman, 1930).

The following preliminary observations indicate some of the main characteristics of this complex transformation:

(1) When the free-acid form of sphagnan is heated alone in aqueous solution, it slowly develops the characteristic, monotonal absorption spectrum of natural aquatic humus, in which there is a nearly exponential dependence of absorbance upon wavelength (Smidsrød & Painter, 1984). The rate of these changes is highly concentration-dependent; hence the reaction is not unimolecular. The solubility of the polymer at low pH, as indicated by the proportion remaining in solution when the pH is adjusted to 1·5, simultaneously decreases from 100% to < 50%.

(2) When the free-acid form of sphagnan is heated in solution in the presence of arginine or another primary amine, the spectral changes occur more rapidly, and the resultant, artificial humic acid, isolated by precipitation at pH 1·5 and washing, contains

nitrogen. A part of the nitrogen is retained by the acid-insoluble, dark brown chromophore after its isolation by prolonged boiling with mineral acid.

(3) After reduction of the reactive carbonyl groups with sodium borohydride, the spectral changes occur much more slowly.

(4) Carbon dioxide is liberated, the percentage of carbon in the isolated polymer increases, and the density of the precipitate obtained upon mixing with aqueous gelatin decreases.

It is therefore clear that humification occurs spontaneously, even in the absence of amines, but that nitrogen becomes covalently incorporated into the humic acid molecule whenever amines are introduced at a sufficiently early stage in the transformation. In the natural situation this condition would be met, because protein and sphagnan co-exist in the living moss, and other proteins are introduced at the surface of the bog. These proteins must be the origin of the polypeptide moiety that is normally present in peat humic acids (Bremner, 1967). Even though they usually make up no more than 20% of the humic acid molecule, humic acids could be validly regarded as 'heavily tanned proteins', especially as the proportion of protein in some leathers is as low as 50% (Gustavson, 1956). This unusual description of a humic acid helps to relate the discovery of preserved bodies to a more fundamental aspect of peatland ecology.

CONCLUSIONS

(1) There is no evidence for the presence in *Sphagnum* moss or peat of substances that are significantly toxic to micro-organisms.

(2) The low intensity of microbial activity in the sub-surface layers of *Sphagnum* peat is unlikely to be due to the low pH and anoxic conditions, because acidophilic anaerobic bacteria have been found there. A simple lack of nutrients is a more likely explanation.

(3) *Sphagnum* holocellulose, its soluble autohydrolysis product, sphagnan, and the aquatic humus derived from sphagnan by a Maillard reaction can suppress the growth of micro-organisms by sequestering essential metal cations.

(4) Under the mildly acidic conditions in a *Sphagnum* peat-bog, sphagnan undergoes a Maillard reaction with ammonia, amino-acids and the free amino-groups in polypeptides. It can do this without first being hydrolysed to monosaccharides because of the

presence of reactive carbonyl groups in residues of D-*lyxo*-5-hexosulopyranuronic acid. The resulting humic acids are extremely resistant to microbial attack, especially under anoxic conditions. Microbial growth is therefore suppressed because of a lack of accessible nitrogen.

(5) The collagen in animal remains is tanned by the Maillard reaction with sphagnan. The cross-linking that is essential for leather formation is made possible by the multiplicity of reactive carbonyl groups in sphagnan.

(6) Reaction with sphagnan probably inactivates proteolytic and other enzymes secreted by putrefactive bacteria. Most of the nitrogen in animal remains would then be inaccessible to the bacteria.

EXPERIMENTAL

Materials

The moss was *Sphagnum acutifolium* Lindb., collected in North Trøndelag, Norway. It was either frozen at −20°C until required ('fresh moss'), or dried in a current of air at 60°C. The dry moss was milled to pass a 20-mesh (1·25 mm) sieve, and extracted exhaustively with boiling acetone, followed by boiling methanol, to give 'extractive-free moss'. Moss holocellulose was prepared by several modifications of the chlorite procedure, as described elsewhere (Andresen *et al.*, 1987). The preparation of pure sphagnan (previously named 'fragment D') from the holocellulose, and the isolation of aquatic humus from peat-bog water, have been described (Smidsrød & Painter, 1984).

General methods

Carbon and nitrogen were determined with a Carlo Erba elemental analyser (Model 1104). Thin-layer chromatography of phenolic acids and polyphenols was carried out on cellulose in aqueous 2% (v/v) acetic acid or butan-1-ol acetic acid and water (4:1:5 v/v; upper layer). Spots were located by their fluorescence under ultraviolet light, and with the diazotised *p*-nitraniline, silver nitrate, or ferric chloride and ferricyanide reagents (Smith, 1960; Hathway, 1960). Column chromatography was carried out on cellulose eluted with aqueous 25% (v/v) acetic acid, polyamide ('Perlon') eluted with water, silica gel eluted with butan-1-ol

ethanol and water (40:11:19 v/v), or Chelex-100 (Cu^{2+} form) resin eluted with water followed by glycine-HCl buffers (pH 5·0→3·0). Column effluents were examined by measuring their absorbance at 280 nm, and by t.l.c.

Extraction of free phenols

Fresh moss (50 g, dry weight) was disintegrated in a homogeniser with ethanol (500 ml), and the resultant slurry was filtered. The filtrate was concentrated to dryness. The residual syrup containing pigments and waxes was washed by decantation with petroleum ether, and then extracted by shaking with water (4 × 50 ml). The aqueous extracts were pooled, filtered through cotton wool, and then extracted by shaking with ethyl acetate (3 × 100 ml). The organic layers were pooled, dried over anhydrous sodium sulphate, filtered, and concentrated to dryness, to give 450 mg of a crude mixture of polyphenols.

Extraction of combined phenols

A portion (10 g) of moss residue remaining after extraction with ethanol was suspended in butan-1-ol (500 ml). Concentrated hydrochloric acid (100 ml) was added, and the mixture was stirred at 98°C for 2 h. It was then cooled and filtered, and the filtrate was concentrated to dryness. Hydrogen chloride was removed from the residue by the repeated distillation of added methanol. The residue was extracted and worked up as before, to yield 90 mg of additional polyphenols as a yellow syrup.

Measurement of antimicrobial activity

Bactericidal activity was measured by the Rideal-Walker test (Croshaw, 1981), with *Pseudomonas aeruginosa* as the test organism. For the measurement of bacteriostatic (growth-inhibitory) activity, *Azotobacter vinelandii*, strain E (Larsen & Haug, 1971), was grown in 20 ml shake-cultures with the medium used by Norris and Jensen (1958), containing 0.1 mM calcium chloride. Growth was monitored turbidimetrically at 500 nm for 20 h at 30°C. The apparent phenol coefficient was calculated from the concentration of test material allowing the same rate of growth as phenol (0·1% w/v). The latter rate was one-seventh of that of the controls. Samples of sphagnan, humic acid prepared in the laboratory from sphagnan (Painter, 1983*b*), and natural aquatic humus gave apparent phenol coefficients of 10–20.

Simulation of *Sphagnum* diagenesis

Extractive-free moss (70 g) was washed with 0·05 N hydrochloric acid, and then with water until the washings were neutral. It was then made into a thick slurry with degassed distilled water (2 litres) and heated under nitrogen at 98°C. After 24 h, the mixture was cooled and filtered, and the insoluble part was heated again with distilled water (2 litres) at 98°C. This was repeated daily for 10 days. The 10 filtrates were concentrated separately *in vacuo* in 100 ml each, and dialysed against water (3 × 1 litre). The non-dialysable parts were freeze-dried; the combined yield of light-brown solid was 16 g. The dialysates were concentrated separately *in vacuo* to 100 ml each, and extracted by shaking with ethyl acetate (3 × 100 ml). The organic layers were dried (sodium sulphate), filtered, and concentrated to dryness. The residues (combined yield, 650 mg) were found by t.l.c. to contain a complex mixture of polyphenols. The aqueous layers (combined yield, 4·5 g) were found by t.l.c. to contain monosaccharides.

Preparation of quinoxaline derivatives

The free-acid form of the extractive-free moss (2 g) or its holocellulose (1 g) was stirred at 20°C in water (100 ml) with *o*-phenylenediamine (100 mg) for 24 h. It was then collected by filtration and washed with 0·5 M sodium chloride followed by water. The free-acid form of sphagnan (500 mg), in water (50 ml), was stirred at 20°C with *o*-phenylenediamine (100 mg) for 24 h. The solution was then dialysed against 0·5 M sodium chloride, followed by water, and freeze-dried.

Humification of sphagnan

Aqueous solutions (2–20% w/v) of sphagnan (H^+ form) were heated at 98°C, either alone or with amino-acids (0·2–2·0% w/v) in Teflon-capped tubes under nitrogen for 1–30 days. Portions were then diluted appropriately for measurements of absorption spectra and qualitative tests with gelatin. The pH of 2% (w/v) solutions was adjusted to 1·5 with hydrochloric acid, and the precipitated humic acid was collected, washed and dried for measurement of its yield and C and N contents. The chromophores of the humic acids were isolated as almost black, insoluble precipitates after hydrolysis of the humic acid in 0·5 M sulphuric acid at 98°C for 24 h.

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