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Preliminary communication

Carbohydrate origin of aquatic humus from peat

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Humic acids are widely considered to be products of the microbial oxidation or autoxidation of lignin and polyphenols[1–3]. Although most preparations contain carbohydrates[1–6], which can only be removed by boiling with water[5,6] or mineral acids[1,3], these are usually regarded as impurities[3].

The alternative view, that humic acids are partly or wholly carbohydrate in origin, is supported by Maillard's classical studies on the "browning reaction" between sugars and amino acids[7], and by Marcusson's demonstration that uronic acids can be converted, *via* furan derivatives, into aromatic material[8]. Recent work by Theander's group[9a] has confirmed and extended the findings of both workers. It has also been suggested[9b] from studies of *Sphagnum* moss and peat of various ages, and of phenol formation from carbohydrates, that the latter are the main sources of humic compounds in this type of peat.

The slow process of humification that takes place in peat bogs is unique in several respects that simplify interpretation. Beneath the surface layer of living *Sphagnum* moss, the bogs are anaerobic, and the level of microbial activity is generally low[10]. Moreover, the ratio of lignin* to holocellulose in the insoluble, peat fraction increases with age (depth below the surface)[11]. It is therefore arguable that the soluble brown-polymer, "aquatic humus" that is liberated into the ambient water[12] originates more from carbohydrate than from lignin, and is probably formed by a reaction that is neither oxidative nor microbial.

In considering the chemical reactions that could occur, it should be noted that *Sphagnum* mosses have no roots, and normally derive their cations from precipitation. These are usually insufficient to neutralise the high carboxyl-content of the moss[13]; in new fronds of *Sphagnum quinquefarium*, we found that only 10–20% of the carboxyl groups were neutralised. The well-known acidity of peat-bog water derives from inorganic acids, liberated by cation exchange, and from the soluble humic acids themselves[13].

We now report strong evidence that aquatic humus from peat originates primarily from the acid-catalysed dehydration and partial decarboxylation of residues of D-*lyxo*-5-hexosulopyranuronic acid (5-keto-D-mannuronic acid, 5KMA) present in the holocellulose of *Sphagnum* moss[14]. The first step is the autohydrolysis of the holocellulose, catalysed by

**Sphagnum* lignin is low in methoxyl[4], and may therefore not be considered as "true" lignin.

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its own acidity, and the liberation of a soluble glycuronoglycan containing residues of 5KMA.

Origin of the acidity – After removal of cations by washing with acid followed by water, extractive-free *S. quinquefarium* had a cation-exchange capacity (determined by direct titration with base) of 0.66 mequiv./g, and yielded ~50% of chlorite-holocellulose with a capacity of 1.36 mequiv./g. The corresponding figures, obtained by addition of an excess of alkali (to hydrolyse esters), followed by back-titration, were 0.80 and 1.60 mequiv./g, respectively. The lignin therefore contributed little or nothing to the acidity of the living moss.

Initial products of autohydrolysis. – When the free-acid form of the extractive-free moss was heated in distilled water (initial pH, ~6) at 98° under nitrogen, it liberated a soluble glycuronoglycan (**A**) and a mixture of phenolic acids into solution. The latter (~1%) were recovered by extraction into diethyl ether, and found to include 3,4-dihydroxybenzoic acid, detected chromatographically. Initially, **A** contained the same acid, covalently bound, but it was rapidly split off upon further autohydrolysis.

When the chlorite-holocellulose was autohydrolysed under the same conditions, it yielded **A**, free from phenolic acids, directly. The ultimate yield of **A** after 10 days was ~30% from the moss, and ~60% from the holocellulose. The fibrous residue from the moss contained all of the original lignin, as indicated by the loss in weight upon chlorite treatment.

A sample of **A** isolated from the holocellulose after 12 h of autohydrolysis contained residues of 5KMA (27%), D-galacturonic acid (25%), L-rhamnose (19%), and smaller amounts of D-glucose, D-galactose, D-mannose, D-xylose, and L-arabinose[13,14]. It was free from nitrogen and aromatic material. The 5KMA was estimated as phenylhydrazine taken up by **A** from aqueous solution[14].

Artificial humic acid from glycuronoglycan **A**. – After heating for 14 days at 98° under nitrogen, a 20% solution of **A** (H^+ form, 5 g) contained ~1.5 g of a dark-brown polymer (**B**) that precipitated below pH 2, and had an equivalent weight of 450. Its sodium salt contained 47% of carbon. Upon heating in 0.5M sulphuric acid at 98° for 12 h, **B** liberated galacturonic acid, rhamnose, and traces of other sugars, together with ~60% of an almost black, acid-insoluble chromophore (**C**) that had an equivalent weight of 425 and contained 61% of carbon (Na^+ salt). The p.m.r. spectrum of **C** in $NaOD/D_2O$ at pD 14 is shown in Fig. 1. Aromatic protons, which resonate downfield from the HOD signal[15], represented 25% of the total.

When a solution of **C** in 0.1M sodium hydroxide was kept at 20° under nitrogen for 6 days, the equivalent weight decreased to a constant value of 140. A similar change was brought about by 10% sodium borohydride in 0.1M sodium hydroxide at 20° for 48 h; after this treatment, **C** again became susceptible to acid hydrolysis (0.5M sulphuric acid, 98°, 12 h), liberating a mixture of six or seven aliphatic acids. These were resolved by two-dimensional t.l.c. on cellulose [ethyl acetate–pyridine–acetic acid–water (5:4:1:3) and then 1-butanol–acetic acid–water (50:25:25)] and detected with Bromocresol Green. The acids were not identified, but the pattern of spots provided a convenient "finger print" for the humic acid.

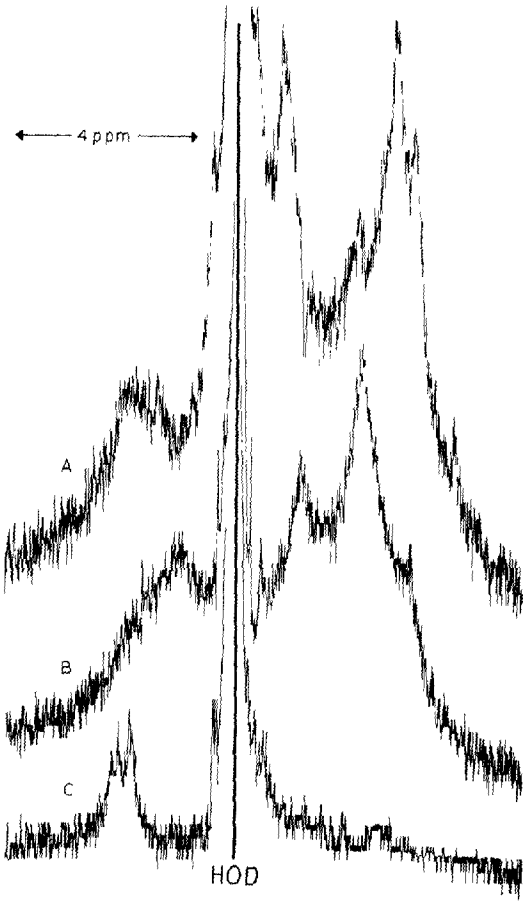

Fig. 1. 100-MHz P.m.r. spectra in NaOD–D_2O at pD 14 of (A) chromophore isolated from natural peat-bog humus, (B) artificial humus prepared from glycuronoglycan containing 5KMA, and (C) artificial humus prepared by phenolase oxidation of 3,4-dihydroxybenzoic acid.

That the humification process was due primarily to the 5KMA residues in **A** was shown by experiments with the monomeric sugars. The rate of decomposition of 5KMA (H^+ form), monitored spectrophotometrically, was highly concentration-dependent, and was accompanied by the evolution of carbon dioxide. The rates of decay of the other sugar components of **A** were much greater in the presence of 5KMA than in its absence. These other monosaccharides became incorporated into the humic acid, at least in part, without substantial modification, because they were partly released on subsequent hydrolysis with sulphuric acid. Whole, polymeric segments of **A** must have been incorporated, unmodified, into **B**, because they could be recovered as a non-dialysable, white glycan after removal of the brown chromophore by bleaching with chlorite.

Artificial humic acid from 3,4-dihydroxybenzoic acid. – This was prepared by aeration of a solution in McIlvain buffer, pH 6.5, in the presence of mushroom phenolase[16] ("tyrosinase", Sigma). The product (Na^+ salt) contained 50% of carbon and had an equivalent weight of 185, which changed very little upon keeping in alkaline solution, provided that oxygen was rigorously excluded. Its p.m.r. spectrum (Fig. 1) showed aromatic protons only. Upon reduction with alkaline borohydride, acid hydrolysis, and two-dimensional chromatography as described above, a "finger print" of twelve spots, which included the starting material, was obtained. Two of the spots might have been identical with two of those from **C**, but the others were different, and probably aromatic, as judged by their u.v. fluorescence.

Aquatic humus from peat. – Water was collected from a hole dug in a *Sphagnum* peat bog, shortly after a heavy shower that had been preceded by a long, dry period. This provided a conveniently high concentration of humic acid (~75 mg/L), and some assurance that it had not been exposed to oxygen for long. The humic acid was isolated by precipitation as its cupric salt, regeneration with ethylenediamine tetra-acetic acid, re-precipitation with acid (pH 2), and washing with water. It had an equivalent weight of 682, and contained 48% of carbon and 2.4% of nitrogen (Na^+ salt). Upon chlorite-bleaching[17], it yielded 46% of its weight as a non-dialysable, white glycan, qualitatively similar in composition to **A**.

The chromophore was isolated (59%) by hydrolysis in 0.5M sulphuric acid at 98° for 12 h. Its sodium salt contained 57% of carbon and 1.5% of nitrogen, and the equivalent weights, determined before and after treatment with alkaline borohydride, were 435 and 160, respectively. The "finger print", obtained by hydrolysis of the borohydride-reduced material and two-dimensional chromatography, was essentially identical with that given by the chromophore of carbohydrate origin (**C**), except that the spots were of different intensity, and one was apparently absent. The p.m.r. spectrum of the unreduced material (Fig. 1) indicated the presence of 12% of aromatic protons.

Discussion. – Apart from the higher temperature, and the wholly arbitrary choice of concentrations, the conditions of autohydrolysis in the laboratory were reasonably similar to those obtaining in the peat bog. These conditions liberate free phenolic acids from the moss; upon subsequent exposure to oxygen, these would obviously give brown polymers similar to that prepared in the laboratory. The amounts would be relatively small, however, and the natural humus, at least when it is freshly leached from the peat, is clearly very different in structure.

In comparing the p.m.r. spectrum of the natural humus with those of the two artificial products (Fig. 1), the nitrogen content of the former must be considered. It is probably present mainly as amino acids and polypeptide[5], which would then comprise ~10% of the isolated chromophore. This would contribute mostly aliphatic protons to the spectrum, and may explain some of the differences. The arbitrary conditions used to prepare the artificial chromophore from glycuronoglycan **A** probably explain most of the differences, however, and a systematic study of conditions is in progress.

In conclusion, the inference is very strong that aquatic humus from peat is primarily carbohydrate in origin.

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