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HUMIC ACID—I

ESR SPECTRA OF HUMIC ACIDS

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Abstract—Alkaline solutions of "humic acid" (HA) isolated from some eighty different soils and peats give ESR spectra indicative of the presence of remarkably stable semiquinone ion radicals. The type of spectrum is largely determined by the pH of the soil but not by the age of the soil or the nature of the plant cover, and the examination of the HA's isolated from lake muds provides useful evidence concerning the history of the sediment.

The HA's isolated from or derived by oxidation of coals gave ESR spectra which were frequently similar to those obtained from more basic soils, but artificial HA's produced by the action of acids or micro-organisms on carbohydrates, or by the atmospheric oxidation of alkaline solutions of catechol and pyrogallol derivatives, gave either no ESR signal or the ESR spectra were readily distinguishable from those shown by the HA's from soils.

The absorption of oxygen by alkaline solutions of HA's has also been examined.

THE organic matter of soils and peats consists of a mixture of plant and animal products in various stages of decomposition together with substances synthesized from the breakdown products. A large number of relatively simple compounds of known structures and belonging to well-known groups such as carbohydrates, acids, esters, amino acids, glycosides and bases have been isolated, usually by solvent extraction of the soil. The remainder of the soil organic matter consists of a mixture of amorphous brown or black products referred to as "humus", and partially decomposed plant and animal residues which can be removed with acetyl bromide. "Humus" has been divided into fractions differing in solubility in water, alkalis, acids and organic solvents, but as different workers have used different terminologies, considerable confusion has arisen.

In view of the heterogeneity of the fractions, the sub-divisions are arbitrary and in many ways unsatisfactory.[1] In our work, the term "humic acid" (HA) is used for that portion of "humus" which is soluble in sodium hydroxide solution and precipitated by acidification of the alkaline extract. This definition is widely used, although Oden[2] would remove the alcohol-soluble portion of the precipitate as "hymatomelanic acid"; it is, however, doubtful whether such a sub-division is desirable. The term "fulvic acid", denoting the portion of "humus" which remains in the aqueous liquors after the acidification of alkaline extracts, is still retained by many workers although this fraction includes polysaccharides, peptides, amino acids and relatively simple phenols as well as compounds of unknown structure.

Attempts to purify HA by chromatographic, electrophoretic, or exchange-resin techniques have not been very profitable and in our hands Deacidite K and Sephadex G25 resins were unsuccessful. It is now generally accepted that the HA is a mixture

[1] S. A. Waksman, *Humus* (2nd Edition) p. 63. Williams and Wilkins, Baltimore (1938).
[2] S. Oden, *Kolloidchem Beihefte* **11**, 75 (1919).

of closely related macromolecules and that the composition is determined by the source and isolation techniques. Elementary analyses of HA's from various soils have given values ranging from C, 49–62; H, 3–6; N, 0·4–5·0%, and mol wts from 1,000–300,000 are reported. Early researches showed that carboxyl, alcoholic and phenolic OH, MeO and quinone groups were present in HA, but it is still uncertain whether the nitrogen is an integral part of the HA structure or whether it is associated with physically attached impurities. Degradations involving fusion with alkali,[3] oxidation with nitric acid,[4] permanganate,[5] alkaline nitrobenzene,[6] or copper oxide[7] gave small yields of phenols such as catechol, resorcinol, and phloroglucinol, aldehydes including *p*-hydroxybenzaldehyde, vanillin, syringaldehyde and acids such as *p*-hydroxybenzoic, vanillic, protocatechuic, veratric and isohemipinic acids. Most of these products were also obtained by similar methods from lignin, which Shmuk,[8] Waksman,[9] and others regarded as the precursor of HA.

Until a few years ago no satisfactory methods were available for the characterization of HA. Not only was it impossible to distinguish between HA from different sources, but according to Erdtman,[10] any "brown or brownish-black substance of unknown constitution" could be regarded as HA. Burges and Hurst,[11] have recently shown that sodium amalgam reduction of HA released a number of phenols, phenolic acids and phenolic alcohols, and on TLC examination they provided a pattern characteristic of the overlying vegetation which frequently distinguished between the HA's from different sources. Some of the phenols, based upon resorcinol or phloroglucinol structures were attributed to flavonoid precursors, whilst others based upon catechol types were regarded as lignin-derived units and it was concluded that besides lignin, flavonoids and possibly other naturally occurring polyphenols may be concerned in the formation of HA as suggested by Trussov.[12]

HA is altered considerably in composition and in properties by boiling with water, acids, or alkalis, when carbohydrates, metals and phenolic and amino acids are eliminated. This will be discussed later, but is important that any such purification treatment should be indicated by the use of terms such as "acid-boiled humic acid" (ABHA). Failure to appreciate this point may be responsible for some of the variations in the reported elementary analyses and mol wts, and some of the aromatic degradation products mentioned above may arise from phenolic acid residues which would have been removed by hydrolysis of the HA's.

The HA used in our preliminary experiments was kindly supplied by Professor A. Burges, and had been isolated from the B_1 horizon of a podzol formed under *Pinus sylvestris* from Delamere Forest, Cheshire. The acid was extracted from the dried

[3] F. Hoppe-Seyler, *Z. physiol. Chem.* **13**, 66 (1889).
[4] A. A. Shmuk, *Trudy kuban s.-Kh. Inst.* **1**, 2 (1924).
[5] R. I. Morrison, *Chem. & Ind.* 231 (1955).
[6] R. I. Morrison, *J. Soil Sci.* **9**, No. 1, 130 (1958); 14, No. 2, 210 (1963).
[7] G. Green and C. Steelink, *J. Org. Chem.* **27**, 170 (1962).
[8] A. A. Shmuk, *Byull. Pochvoveda* **5**, 7 (1930); *Pedology* **25** (3), 5 (1930).
[9] S. A. Waksman and K. R. N. Iyer, *Soil Sci.* **34**, 43 (1932).
[10] H. G. H. Erdtman, *Proc. Roy. Soc. A.* **143**, 177 (1933).
[11] [9] H. M. Hurst, N. A. Burges and P. Latter, *Phytochemistry* **1**, 227 (1962); [9] N. A. Burges, H. M. Hurst and B. Walkden, *Geochim. et Cosmochim. Acta* **28**, 1547 (1964).
[12] A. G. Trussov, *Zh. opyt. Agron.* **17** (1916); *Contrib. to the Study of Russian Soils* **26–27**, 1 (1917) Petrograd.

soil by 50% lactic acid and freed from carbohydrates and proteins by boiling with 6N HCl. The visible and UV spectra of the ABHA in sodium hydroxide solution were monotonic, with no observable bands, and the IR spectrum in nujol or KBr disc was largely featureless, but showed OH and carboxyl (1715 cm^{-1}) bands and a band at 1615 cm^{-1} attributable either to a carboxylate anion or to aromatic structure. In order to remove the ambiguity of the 1615 cm^{-1} band, the NMR spectrum in

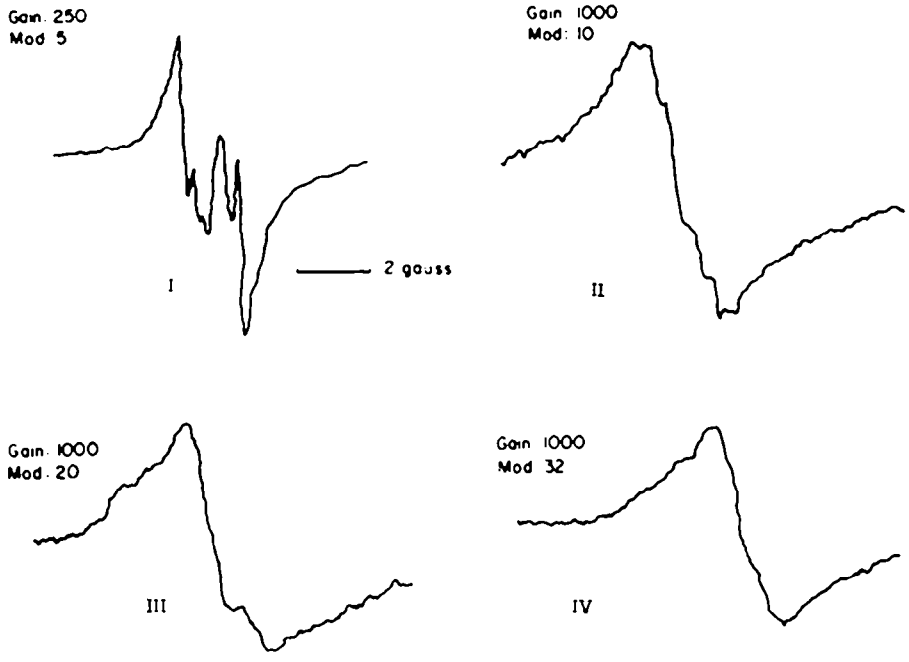

FIG. 1

alkaline solution was studied, but no low field signal around τ 2·7, indicative of aromatic structure, was observed. The absence of this low field signal could have been due to (a) a low concentration of aromatic protons, either because of the sparing solubility of the ABHA, or because of heavily substituted benzene rings, or (b) the strong relaxation effect of the spin of unpaired electrons. Consequently the ESR spectrum was examined. The solid ABHA showed strong absorption at g = 2·00, which was some 4·1 gauss in breadth between points of maximum slope, and a solution in 0·1N NaOH gave the derivative curve (I), Fig. 1. which is a plot of the gradients of the absorption curve against the magnetic field strength. It was concluded that ABHA was a free radical, although the unsymmetrical nature of the curve suggested some heterogeneity. The hyperfine structure (four-lines) indicated that the unpaired electron was interacting with two non-equivalent protons with coupling constants 1·2 and 0·35 gauss; this may be of greater significance when more is known about the chemical structure. The ESR signal was observable with solutions of ABHA in 0·1–0·5N NaOH or KOH, but not in 0·2N $NaHCO_3$, and as a weak signal only was observed with a solution in 0·2N Na_2CO_3, it was concluded that a phenoxide ion was

[18] N. A. Burges and P. Latter, *Nature, Lond.* **186**, 404 (1960) and Ref. 11*b*.

responsible for the signal. The ESR signal was rapidly destroyed by the addition of sodium dithionite, or by reducing alkaline solutions of the acid with hydrogen in the presence of a Pd–C catalyst, and as it was recovered by exposure of the reduced solutions to the air, it was concluded that the paramagnetic species was a radical of the semiquinone ion type. These observations, the position of the signal at $g = 2{\cdot}00$, and the breadth of approximately 1·75 gauss between extreme peaks* on the derivative curve cannot be attributed to paramagnetic metal impurities or chelated derivatives.

After completion of these preliminary experiments, it was discovered that Rex[14] and Steelink[15] had shown that HA exhibited ESR absorption. Working with solid HA both had obtained structureless signals with properties consistent with semiquinone ion radicals, but whilst Rex believed the radicals were simple C_6–C_3 fragments trapped in the HA micelle, Steelink concluded, especially from further studies[15] in organic solvents, that the radical was a paramagnetic semiquinone ion, co-existent with a diamagnetic quinhydrone. The trapped radical idea of Rex, which was criticised by Steelink on the basis of fractionation studies and the effects of temperature, was also inconsistent with the following observations:

1. The ESR signal of ABHA in sodium hydroxide solution had two clear hyperfine splittings of 0·35 and 1·2 gauss, probably too small for a C_6–C_3 fragment.
2. The signal was observed in solution where trapped radicals should be freed.
3. The ready reduction and recovery of the signal suggested that occlusion of small free radicals by micelles was unlikely to produce the stability required for their persistence through the variety of conditions which obtain during the formation of HA and during the isolation of ABHA.

We believe, with Steelink, that HA itself is the free radical, or a mixture of free radicals of the semiquinone ion type, and although unproven it was attractive to imagine that the peculiar stability of the radical, indicated by the retention of the signal after boiling the acid with 6N HCl or 4N NaOH, may be due to association with an extended conjugated system; this system may also account for the intense colour of HA†.

The production of the ESR spectrum with hyperfine structure is of considerable interest, both in connection with the chemical constitution of HA and also as a possible physical method for the characterisation of HA's. However, before this ESR technique could be applied adequately it was necessary to determine whether the signal depended upon the method of isolation, or the source of the acid.

Methods of isolation of "Humic acid"

The 50% lactic acid isolation technique[13] was not generally applicable to all soils, and, in any case, it had low extracting power which was not improved by the use

* It is more usual to quote the total hyperfine splitting, which in this case is 1·55 gauss. However, we prefer to use the breadth between peaks on the derivative curve, as this enables us to deal with the structureless ESR signals discussed later.

† A brief account of these early experiments was given at the symposium on "Natural Quinones" held at New Delhi on 4th–5th October, 1963 and reported in the Bulletin of the National Institute of Sciences of India, No. 28, 57–60 (1965).

[14] R. W. Rex, *Nature, Lond.* **188**, 1185 (1960).

[15] C. Steelink and G. Tollin, *Biochem. Biophys. Acta* **59**, 25 (1962); C. Steelink, T. Reid and G. Tollin, *Ibid.* **66**, 444 (1963); C. Steelink, *Geochim. et Cosmochim. Acta* **28**, 1615 (1964). See also H. Kleist and D. Mücke, *Experientia* **22**, 136 (1966).

of 100% lactic acid. Sodium fluoride was a poor extractant, but 0·2M sodium oxalate, tetrasodium pyrophosphate,[16] or formic acid, particularly in the presence of acetylacetone,[17] were more effective; the HA's recovered from the solvents after boiling with 6N HCl gave good ESR spectra. Sodium hydroxide has been widely used for the isolation of HA; it has high extracting power, but has been suspected of producing chemical changes. However, the HA isolated from a 0·2N NaOH extract of the B_1 layer of Delamere Forest podzol, after boiling with 6N HCl, gave an ESR spectrum indistinguishable from that of a specimen isolated by lactic acid, and consequently in all subsequent work, alkaline extraction was used under the standardized conditions described in the Experimental. The treatment with 6N HCl, which was essential for the display of strong ESR signals, produced in the case of the ABHA from Delamere Forest podzol, a loss of about 40% in weight.

Measurement of ESR spectra of humic acids from different sources

A Varian Associates V-4500 spectrometer and recorder were used, and 1% solutions of HA's or ABHA's in 0·1N NaOH were examined in flat quartz cells (0·25 mm thick), in order to minimize absorption of the microwave power by the aqueous solutions. All acid samples displayed an interesting behaviour towards oxygen; acids from some source were more sensitive than others but, in general, excess oxygen resulted in an ESR signal of reduced strength, and oxygen deficiency produced a signal of diminished resolution. A satisfactory explanation of these observations cannot be suggested at the moment, but reliable and reproducible spectra can only be obtained by careful adherence to a standard technique. After observation of the initial ESR signal, the solution was transferred to a small conical flask and shaken with air for several minutes before re-admission to the ESR cell. Usually, shaking for 3 min was effective, but unless the signal strength had been halved, it was desirable to repeat the shaking with air until this diminution in strength was realised. The solution was then allowed to remain in the cell, where it was effectively out of contact with air, and where, presumably, the excess oxygen was gradually and irreversibly exhausted, and the recovery of the ESR signal was followed during 30–60 min. The destruction and recovery of the signal could be repeated several times.

As a result of collaboration with the Bangor, Merlewood, and Monks Wood stations of the Nature Conservancy, and with the Windermere Laboratory of the Freshwater Biological Association, it has been possible to examine a wide range of soils formed under varying types of plant cover. The soils were extracted with sodium hydroxide, the HA's were boiled with 6N HCl, and the ESR spectra of the resulting ABHA's were observed in alkaline solution. Standard conditions were used in the extraction, hydrolysis, and examination of spectra, and in particular, the oxygenation cycle outlined above was always used to ensure that four-lined spectra were not missed because of oxygen effects. Seventy-nine soils are arranged as far as possible in descending order of spectral resolution in Tables 1 and 2, which also include, where available, information concerning the overlying vegetation, the depth and pH of the soil, the yield of HA, the oxygen absorption, and the percentage loss in weight on boiling the acid with 6N HCl. The ESR spectra are divided into two classes. Class I, containing ABHA's from 51 sources, showed four-lined spectra with a breadth of 1·75–1·9

[16] J. M. Bremner, *J. Agric. Sci.* **46**, 247 (1955).

[17] J. W. Parsons and J. Tinsley, private communication.

Table 1. Sources and properties of "acid boiled humic acids" with class I ESR spectra

Source	Nature of Soil	Depth (cm) or Horizon	Plant Cover	pH of Soil	*Yield of HA (%)	Loss on 6N-HCl Boil (%)	Oxygen Uptake μl/mg. HA	Oxygen Uptake μl/mg. ABHA	"X" Value
Class I spectra									
†[1]Moor House A.9	Blanket bog peat	45–50	*Calluna, Eriophorum, Sphagnum*	3·45	9·0	26	16·0	33·5	2·1
[6]Bagshot	Podzol	B_1 Horizon	*Betula, Calluna, Peteris*	3·8	2·4	46	3·8	14·1	3·7
[3]Edale A.3	Podzol	B_1 Horizon 40–45	*Vaccinium Agrostis, Deschampsia*	3·9	0·8	37	—	—	—
[6]Delamere Forest	Podzol	B_1 Horizon	*Pinus*	3·05	4·6	43	5·4	18·4	3·4
[6]Whitelow	Podzol	B_1 Horizon	*Calluna*	3·9	6·0	39	4·8	17·8	3·7
[9]Red Tarn Moss A.1	Blanket bog peat	40–50	*Calluna, Eriophorum, Sphagnum*	3·5	4·2	31	—	—	—
[1]Moor House A.8	Blanket bog peat	40–45	as above (Moor House)	3·4	8·4	32	15·1	38·1	2·5
[1]Moor House A.7	Blanket bog peat	35–40	as above (Moor House)	3·5	9·5	35	11·1	32·5	2·9
[1]Moor House A.6	Blanket bog peat	30–35	as above (Moor House)	3·4	8·3	34	15·3	38·4	2·5
[1]Moor House A.5	Blanket bog peat	25–30	as above (Moor House)	3·35	5·8	29	15·7	36·9	2·4
[1]Beinn Eighe A.4	Podzol	Upper A. 0—15	*Pinus, Calluna, Vaccinium, Sphagnum*		0·2	43	—	—	—
[1]Beinn Eighe A.5	Podzol	Middle A. 45–60	as above (Beinn Eighe)		0·1	50	—	—	—
[1]Beinn Eighe A.6	Podzol	Upper B_1 105–124	as above (Beinn Eighe)		1·6	38	—	—	—
[1]Beinn Eighe A.7	Podzol	Middle B_1 150	as above (Beinn Eighe)		0·3	48	—	—	—
[1]Beinn Eighe A.8	Podzol	250	as above (Beinn Eighe)		6·1	45	—	—	—
[10]Draved, S. Jutland	*mor*	65	*Quercus*	3·5	8·4	25	—	—	—
[1]Stribers Moss	Raised bog peat	25–40	*Calluna, Sphagnum*	3·2	7·2	37	10·3	31·2	3·0
[6]Ringinglow A.	Blanket bog peat	30–45	*Calluna, Sphagnum*	3·3	19·6	43	4·8	20·4	4·2
[1]Simon Fell	Blanket bog peat	25–40	*Calluna, Sphagnum*	2·8	20·0	36	10·3	31·1	3·0
[6]Edale A.2	Podzol	A_1 Horizon 20–25	as above (Edale)	3·4	2·8	27	—	—	—

[8]Edale A.1	Podzol	H layer 10–15	as above (Edale)	3·2	16·5	20	—	—	—
[1]Moor House A.4	Blanket bog peat	20–25	as above (Moor House)	3·4	8·6	44	9·0	34·7	3·9
[1]Moor House A.3	Blanket bog peat	15–20	as above (Moor House)	3·4	3·4	48	9·1	36·4	4·0
[9]Tregaron Bog B.2	Bog peat	60–65	*Calluna, Erica, Eriophorum, Sphagnum*	3·6	5·2	37	—	—	—
[9]Tregaron Bog B.4	Bog peat	165–170	as above (Tregaron B.)	3·8	12·2	20	—	—	—
[4]Fox House Soil G.	Acid grassland	7–15	*Graminae*	4·6	2·4	48	4·4	23·7	5·3
[4]Fox House Soil P.	Acid grassland	7–15	*Graminae*	3·8	3·2	48	4·5	22·9	5·1
[1]Antarctic moss A.3	Soil beneath peat layer	20–25	*Polytrichum, Dicranum*	4·3	15·0	48	—	—	—
[1]Beinn Eighe A.3	Podzol	H Layer	as above (Beinn Eighe)	3·4	3·7	32	—	—	—
[8]Ringinglow B.	Blanket bog peat	approx. 200	as Ringinglow A.	2·9	27·8	26	18·4	32·4	1·8
[1]Moor House A.2	Blanket bog peat	10–15	as above (Moor House)	3·1	1·0	46	18·5	50·1	2·7
[4]Macaulay Institute soil	Garden soil	—	none; cultivated soil	5·3	2·3	53	5·5	32·3	6·1
[8]Red Tarn Moss A.2	Forest humus	—	*Betula*	4·0	16·1	44	—	—	—
[1]Ridley Wood A.2	*mor* humus	Upper A.	*Fagus*	3·5	3·4	37	5·4	22·4	3·5
[8]Heathwaite Moss A.1	Raised bog peat	100	*Sphagnum*	4·0	3·7	27	—	—	—
[8]Heathwaite Moss A.2	Fen peat	250	*Sphagnum*	4·3	3·1	21	—	—	—
[4]Totley Wood A.	*mor*	0–8	*Quercus, Acer*	3·6	11·2	52	7·9	33·2	4·2
[4]Totley Wood B.	*mor*	0–6	*Quercus, Acer*	3·3	7·6	50	6·3	28·8	4·5
[9]Tregaron Bog A.1	Bog peat	20	*Molinia, Calluna, Scirpus, Erica*	3·4	8·6	35	—	—	—
[9]Shoulthwaite A.1	Raised bog peat	50	*Calluna, Sphagnum, Eriophorum*	3·8	3·8	64	—	—	—
[1]Roudsea Wood	*mor*	H Layer	*Quercus*	3·3	4·8	50	6·3	28·8	4·5
[1]Ridley Wood A.1	*mor*	H Layer	*Fagus*	3·2	13·2	40	9·7	36·8	3·8
[9]Tregaron Bog A.2	Bog peat	40	as above (Tregaron A.)	3·3	1·9	33	—	—	—
[1]Bogle Crag	*mor*	H Layer	*Quercus, Pteridium, Deschampsia*	3·65	10·0	53	6·3	30·0	4·8
[9]Tregaron Bog B.5	Bog peat	200	as above (Tregaron B.)	4·0	2·9	33	—	—	—
[9]Tregaron Bog B.1	Bog peat	20	as above (Tregaron B.)	3·3	3·4	40	—	—	—
[9]Tregaron Bog A.4	Bog peat	130	as above (Tregaron A.)	3·8	2·6	31	—	—	—
[9]Tregaron Bog A.5	Bog peat	280	as above (Tregaron A.)	4·0	3·9	40	—	—	—
[9]Tregaron Bog B.3	Bog peat	135	as above (Tregaron B.)	3·8	3·5	46	—	—	—
[1]Antarctic Moss B.2	Peaty soil	6–10	*Bryum*	4·0	6·5	69	—	—	—
[1]Antarctic Moss B.3	Peaty soil	10–15	*Bryum*	4·2	1·7	60	—	—	—

Table 2. Sources and properties of "acid boiled humic acids" with class II ESR spectra

Source	Nature of Soil	Depth (cm) or Horizon	Plant Cover	pH of Soil	*Yield of HA (%)	Loss on 6N-HCl Boil (%)	Oxygen Uptake μl./mg. HA	Oxygen Uptake μl./mg. ABHA	"X" Value
Class II Spectra									
†[1]Roudsea Tarn fen A.	Fen soil	0–15	*Calamagrostis*	4·6	6·5	52	4·7	24·4	5·2
[5]Esthwaite N. fen A.	Fen peat	30–40	*Salix*	5·1	7·6	42	—	—	—
[5]Holme Fen A.1	Fen peat	approx. 20–40	*Sphagnum*	5·0	1·5	20	—	—	—
[6]Millport			*Acer*	3·9	6·6	54	7·8	37·2	4·8
[1]Roudsea Tarn fen B.	Fen peat	0–15	*Alnus, Phragmites, Calamagostis*	6·05	7·4	46	6·5	26·5	4·1
[1]Moor House A.1	Surface vegetation	0–10	as above (Moor House)	3·1	1·6	53	9·0	44·6	4·9
[1]Simon Fell	Blanket bog peat *mull*		*Agrostis, Festuca*	6·1	2·1	57	5·5	32·4	5·9
[5]Holme Fen A.2	Fen peat	150	as above (Holme Fen)	5·0	2·6	33	—	—	—
[1]Antarctic moss A.1	Surface vegetation	0–8	*Polytrichum, Dicranum*	4·1	2·3	60	—	—	—
[1]Antarctic moss A.2	Peat layer	8–15	*Polytrichum, Dicranum*	4·5	4·5	55	—	—	—
[1]Antarctic moss C.	Peat	8–15	*Bryum*	4·2	6·2	45	—	—	—
[1]Antarctic moss B.1	Peat	3–6	*Bryum*	4·1	8·2	50	—	—	—
[1]Esthwaite N. fen B.	Alluvial soil	0–15	*Calamagrostis, Phalaris, Ulmaria*	4·6	5·2	64	3·6	24·8	7·0
[6]Macaulay Institute	Peat		*Phragmites*	3·5	8·8	33	13·7	40·2	2·9
[5]Woodwalton Fen	Fen peat	10	*Phragmites*	4·8	10·6	44	7·9	25·9	3·3
[5]Shoulthwaite A.2	Fen peat			4·0	3·0	69	—	—	—
[1]Elterwater Wood	Alluvial soil		*Alnus*	6·0	1·5	53	4·7	31·4	6·6
[1]Roudsea Tarn Fen C.	Fen peat		*Phragmites, Crataegus*	5·2	10·2	44	7·5	28·2	3·8

[1]Humphrey Head A.	*mull*	0–15	*Quercus, Fraxinus, Taxus*	6·5	1·2	63	6·6	38·9	5·9
[1]Humphrey Head B.	*mull*	0–10	*Fraxinus, Mercurialis*	7·0	1·4	55	9·2	44·5	4·9
[1]Humphrey Head C.	*mull*	0–10	*Taxus*	6·8	1·2	61	7·5	41·1	5·5
[5]Anston Stones Wood A.	*mull*	0–10	*Tilea, Mercurialis*	6·9	1·1	65	5·8	34·0	5·9
[5]Anston Stones Wood B.	*mull*	0–10	*Ulnus, Taxus*	6·7	1·0	63	5·4	33·0	6·1
[5]Anston Stones Wood C.	*mull*	0–10	*Ulnus, Taxus*	6·8	1·8	54	8·4	38·0	4·5
[1]Beinn Eighe A.2	Podzol *mor* humus	F-layer	as above (Beinn Eighe)		3·8	42	—	--	—
[1]Beinn Eighe A.1	Podzol *mor* humus	Surface vegetation	as above (Beinn Eighe)		2·2	33	--	—	—
[6]Sonning meadow soil	Meadow soil		*Graminae*	7·2	0·8	72	2·8	28·5	10·1
[7]Norfolk Broad Farm	Fen peat		—	5·8	—	50	8·9	34·8	3·9

* as percentage of the dried EtOH–benzene extracted soil.

† The superscript numeral refers to the station supplying the soil:

1 = Merlewood
2 = Freshwater Biological Association
3 = Monks Wood
4 = Marine Station, Millport
5 = Local
6 = Department of Soil Science, Aberdeen University
7 = Dr. G. Lawson, Birmingham University
8 = Professor A. Burges, Liverpool University
9 = Bangor
10 = Dr. J. Iversen, Geological Survey of Denmark

gauss, and with the lines in the same relative positions, although some spectra were better resolved than others; the range of spectra of Class I soils is illustrated by curves (I) and (II) in Fig. 1. Class II, containing 28 samples, gave ill-defined, structureless spectra, without clear peaks but with the same overall breadth of 1·75–1·9 gauss, as shown by curves (III) and (IV) in Fig. 1.

Some of the main conclusions drawn from these experiments are:

1. ABHA's from all soils gave ESR spectra of breadth 1·75–1·9 gauss, which were susceptible to oxygen effects.

2. In all cases, the ESR signals were removed by reduction with sodium dithionite, and recovered by exposure to air; all signals were therefore attributable to semiquinone ion radicals.

3. Four-lined Class I spectra were obtained from acid soils, pH 2·8–4·3, including acid or bog peats, humus containing horizons of podzols and *mor* humus; the low acidities of Fox House G and Macaulay Institute soils are possibly due to cultivation. The structureless, Class II spectra were usually derived from more basic soils, pH 4·0–7·2, including fen peats and soils, and *mull* humus.

4. The nature of the ESR signal was largely independent of the depth or age of the soil. However, the virtually non-humified, uppermost layer may give results differing from deeper layers, and several examples were found where the surface layer of an acid soil yielded an ABHA, giving a Class II spectrum. Thus, a Moor House peat profile was examined at nine depths; the uppermost layer (0–10 cm) gave a Class II signal, the second layer (10–15 cm), consisting of slightly humified material, gave a fair Class I signal, and the deeper samples, taken every 5 cm until reaching a depth of 50 cm, showed increasingly stronger and better resolved Class I spectra. Irregular results would be anticipated in soil profiles showing discontinuities and grenz-horizons resulting from climatic or vegetational changes. Thus, Red Tarn Moss A.1 (40–50 cm) was a blanket peat[18] giving rise to an ABHA showing a good Class I ESR spectrum whilst sample A.2, from a lower, humified layer, was a forest soil giving a less well-resolved spectrum. The vegetation at Tregaron Bog[19] however, shows little alteration with the grenz-horizons, and samples taken from different depths at two sites in the bog all gave rather poor Class I spectra.

The samples from Tregaron Bog were dated from 1500B.C. to recent times, and consequently, the nature of the ESR signal was largely independent of the age of the soil, and a further striking example of this was provided by the ABHA from Draved Forest, South Jutland, where a well-resolved Class I spectrum was obtained from *mor* humus, the pollen of which was about five thousand years old.[20]

5. The ESR spectra depended upon the overlying vegetation in so far as this corresponded with the pH of the soil. It is particularly interesting to observe that Class I spectra were given by the deeper soils below the Antarctic mosses, which contain little, if any, lignin and, consequently, other plant phenolic substances must contribute to the HA responsible for the four-lined spectra.

6. Soils giving rise to Class II ESR spectra usually showed a greater loss in weight on boiling with 6N HCl than the more acidic soils. The loss in weight showed considerable variation, and the generalization is not without exceptions, e.g. Macaulay

[18] W. Pennington, *Proc. Roy. Soc. B.* **248**, 205 (1964); **161**, 310 (1965).
[19] J. Turner, *Proc. Roy. Soc. B.* **161**, 343 (1965).
[20] J. Iversen, *J. Ecol.* **52** (Suppl.) 59 (1964).

TABLE 3. SOURCES AND PROPERTIES OF "ACID BOILED HUMIC ACIDS" FROM LAKE MUDS

Source	Depth below Mud surface (cm)	pH of sample	*Yield of HA (%)	Loss on 6N-HCl boil (%)	Oxygen uptake μl./mg. HA	Oxygen uptake μl./mg. ABHA	"X" Value
Class I ESR Spectra							
Seathwaite Tarn A.2	100–115	4·8	5·1	40	—	—	—
Seathwaite Tarn A.3	150–165	—	7·7	47	—	—	—
Seathwaite Tarn A.5	250–268	5·25	6·1	60	—	—	—
Floutern Tarn	440–455	4·3	5·1	69	—	—	—
Floutern Tarn	300–305	4·5	7·6	49	—	—	—
Angle Tarn	175–180	5·2	2·2	60	—	—	—
Seathwaite Tarn A.1	50–65	5·1	6·1	70	—	—	—
Seathwaite Tarn A.4	200–215	5·1	2·5	55	—	—	—
Seathwaite Tarn A.6	300–315	—	4·6	40	—	—	—
Seathwaite Tarn A.7	350–365	—	6·6	56	—	—	—
Thirlmere	18–34	5·95	1·6	48	7·4	27·5	3·7
Thirlmere	0–18	5·8	4·6	59	5·8	29·4	5·05
Class II ESR Spectra							
Blea Tarn A.1	50–70	—	2·0	43	—	—	—
Blea Tarn A.2	100–120	5·25	2·8	41	—	—	—
Blea Tarn A.3	140–160	5·5	2·1	43	—	—	—
Blea Tarn A.4	200–220	5·65	1·5	45	—	—	—
Blea Tarn A.6	300–320	5·8	1·7	50	—	—	—
Seathwaite Tarn A.8	400–415	—	4·7	51	—	—	—
Seathwaite Tarn A.10	473–488	—	2·2	60	—	—	—
Seathwaite Tarn A.9	450–465	—	1·6	48	—	—	—
Rostherne	15–50	4·2	3·8	47	7·1	30·2	4·3
Esthwaite deep	0–13	4·4	6·5	55	9·8	31·6	3·2
Blea Tarn A.5	250–270	5·6	2·6	46	—	—	—
Blea Tarn A.7	320–332	5·7	1·8	50	—	—	—
Seathwaite Tarn A.11	493–508	—	1·4	54	—	—	—
Seathwaite Tarn A.12	510–520	5·5	1·2	57	—	—	—
Seathwaite Tarn A.13	520–535	5·1	0·9	68	—	—	—
Esthwaite shallow	0–13	4·3	6·0	60	8·2	34·9	4·3
Rostherne A.	3000	5·9	2·0	50	8·5	29·1	3·4
Rostherne B.	1500	5·0	2·0	42	11·4	34·0	3·0
Esthwaite	450	5·9	1·5	57	4·3	18·6	4·4

* As percentage of the dried EtOH–benzene extracted soil.

Institute phragmites peat, Holme Fen A.1 and A.2, Antarctic mosses B.2 and B.3, Shoulthwaite A.1.

Further assistance from the Windermere Laboratory of the Freshwater Biological Association has led to an examination of thirty-one samples of ABHA's prepared from lake muds, which were isolated from varying depths in a number of lakes, by means of a corer. The results, included in Table 3, showed no relationship between the ESR spectrum and the pH of the mud; this of course, is not surprising, because the pH of the mud was largely determined by the pH of the lake water, and may not represent the pH at which the HA was produced. It is now widely recognised, on the basis of chemical[21] and pollen analyses,[18] that lake sediments are derived largely from inwashed soils from the drainage basin, and the ESR spectra of the muds provide some striking confirmations of this hypothesis. The two deep cores from Floutern Tarn gave Class I

[21] F. J. H. Mackereth, *Proc. Roy. Soc. B.* **161**, 245 (1965).

spectra, which are consistent with the pollen contents, indicating that these sediments arose largely from an inwash of acidic sphagnum peat. The vegetation histories of Blea Tarn and Seathwaite are well-known.[18] There has been little deforestation at Blea Tarn until very recently, and consequently, little change has taken place in the types of organic matter entering the lake. In agreement with this the seven mud samples from the lake all gave Class II spectra, which is also entirely consistent with the absence of the micro-fossils characteristic of *mor* humus, and with the presence of much juniper on the surrounding fell sides. At Seathwaite, on the other hand, there was considerable deforestation in prehistoric times, followed by the development of acid grassland and heather; pollen analysis indicated that about 350 cm down the mud profile, the *mull* began to change to *mor* humus, and the latter became increasingly predominant as the surface was approached. In agreement with these observations, the ABHA's from the six lower layers gave structureless, Class II ESR signals, but those from the seven higher layers, commencing at a depth of 350 cm, gave Class I spectra, indicative of *mor* soils.

Oxygen absorption of "Humic" and "acid boiled humic acids"

In an attempt to discover reasons for the peculiar influence of oxygen upon the ESR spectra, the oxygen absorptions of many HA's and ABHA's were measured at 25° and during 6 hr in a Warburg manometer. Very small absorption occurred in sodium carbonate or bicarbonate solutions, but solutions in 1% NaOHaq rapidly absorbed oxygen. The oxygen uptakes are shown in Tables 1, 2, and 3, together with the ratio,

$$X = \frac{\text{Uptake in } \mu\text{l./mg of ABHA}}{\text{Uptake in } \mu\text{l./mg of HA}}$$

The absorption of oxygen could not be correlated with the ESR spectra, but a rough connection between the "X" and the ESR spectra of the soils was detected. HA's giving Class I ESR signals usually had lower "X" values (1·8–4·8) than those from soils giving Class II signals (4·0–7·0), but there are several exceptions, e.g. Fox House G and P, Macaulay Institute garden soil and phragmites peat, Woodwalton Fen, and the few lake muds examined do not conform.

The increased oxygen uptake of ABHA's was only partly accounted for by the removal of inert carbohydrates and proteins during the hydrolysis, but in almost all cases it was necessary to assume in addition that some of the compounds removed during the hydrolysis were chemically combined in the HA, and that their removal liberated new groups, such as phenolic groups, capable of reacting with oxygen. Thus, in the case of the HA from Ringinglow A, the ABHA absorbed more than twice the amount of oxygen than that calculated from the elimination of inert groups: 3 mg of HA absorbed $3 \times 5{\cdot}75 = 17$ μl. of oxygen but gave 1·86 mg of ABHA, which absorbed $1{\cdot}86 \times 21{\cdot}15 = 38$ μl. of oxygen.

Solubility of "acid boiled humic acids" in alkali

It was observed that two types (A and B) of material were present in varying proportions in many ABHA's from different sources. Type A dissolved rapidly in 3 min in 0·1N NaOH, and was unaffected either in ESR spectrum or in solubility, after boiling with 6N HCl. Type B material, on the other hand, dissolved slowly in

alkali during a period of 8 hr, although in a few cases, e.g. Humphrey Head C, complete solution required either 3 days at 15° or ½ hr at 100°. Acidification of the alkaline solutions of these Type B materials yielded acids which re-dissolved readily in alkali, but which reverted to the slowly soluble forms after boiling with 6N HCl. These observations were made with ABHA's from the following sources: Simon Fell, Ringinglow A and B, Bogle Crag, Totley Wood A and Humphrey Head C, and it was noted that the ESR signals from materials of Type B were never better in structure and always of lower intensity than the corresponding Type A fractions. A possible explanation of the observations would be that Type A material is an acid incapable of lactonisation, whilst Type B material is lactonic.

"Humic acids" from coals

A number of black, amorphous, alkali-soluble substances, kindly supplied by Dr. G. J. Lawson of the Department of Mining and Mineral Engineering, The University of Birmingham, and obtained directly or indirectly from coals, have been examined (Table 4). These samples, prepared by direct extraction of the coals with sodium hydroxide solution, yielded acid-boiled products which gave structureless ESR spectra with breadths of 2–3 gauss. On the other hand, alkali extraction of a technical product,* obtained from a West German brown coal, gave an acid-boiled product, characterized by a typical Class I HA ESR spectrum, with a breadth of 1·8 gauss. Seven acid-boiled products, prepared by oxidation of coals in air at 150°, were examined; five gave structureless ESR spectra, indistinguishable from Class II HA spectra, with breadths of 1·8–2·0 gauss, but two samples from Mitchell Main and Dalton Main dull coals were distinguishable only by their greater breadth (ca. 4 gauss). An acid sample obtained by permanganate oxidation of a high rank Ryder bright coal, also gave a structureless ESR spectrum with a breadth of ca. 4 gauss. All these samples, however, showed small percentage losses on boiling with 6N HCl and low "X" values (Table 4), which were more consistent with the HA's from peats, podzols and *mor* soils, which usually gave Class I ESR spectra.

Artificial "humic acids"

Some early workers[22] suggested that carbohydrates were precursors of HA, but the black, amorphous products obtained by boiling cellulose or sucrose with hydrochloric acid were incompletely soluble in sodium hydroxide, and the alkaline solutions gave little, if any, evidence of free radical components in the ESR spectrometer: extremely weak, very broad and structureless spectra were observed. Similalry, the black amorphous "aspergillin" prepared by the action of micro-organisms on glucose[23] and kindly supplied by Professor W. B. Whalley, did not give an ESR signal either before or after boiling with 6N HCl.

Eller *et al.*[24] drew attention to the similarity between HA's and the complex products formed by the oxidation of some phenols. A number of these "artificial phenolic humic acids" have been prepared, but this work is still in progress, and a brief outline only is included in the present paper. Alkaline solutions of a variety of phenols and

* Sold by Gee Lawson Chemicals Limited, London W.1.

[22] H. Braconnot, *Ann. Chim.* **61**, 187 (1807); J. Marcusson, *Ber. Dtsch. Chem. Soc.* **58**, 869 (1925).
[23] N. A. Lund, A. Robertson and W. B. Whalley, *J. Chem. Soc.* 2434 (1953).
[24] W. Eller, *Ber. Dtsch. Chem. Soc.* **53**, 1469 (1920); *Liebig's Ann.* **431**, 133 (1923); **442**, 160 (1925).

Table 4. Sources and properties of "acid boiled humic acids" from coals

Source	Isolation Procedure	Loss on 6N-HCl Boil (%)	Oxygen Uptake μl./mg. HA	Oxygen Uptake μl./mg. ABHA	"X" Value	Spectra	Breadth Gauss
Gee Lawson Co. soft brown coal	0·2N-NaOH extraction	15	6·6	14·1	2·1	structure	1·8
Dopplerite	0·2N-NaOH extraction	20	—	—	—	no structure	2–3
Outcrop	0·2N-NaOH extraction	18	—	—	—	no structure	2–3
Outcrop	0·2N-NaOH extraction (HF treated)	24	—	—	—	no structure	2–3
Mitchell Main bright	Oxidation at 150° with air NaOH extraction	18	17·8	23·3	1·3	no structure	1·8–2
Dalton Main bright	NaOH extraction	17	—	—	—	no structure	1·8–2
Thailand lignite	NaOH extraction	21	—	—	—	no structure	1·8–2
Wyllie	NaOH extraction	16	—	—	—	no structure	1·8–2
Pakistan	NaOH extraction	18	—	—	—	no structure	1·8–2
Dalton Main dull	NaOH extraction	13	—	—	—	no structure	4
Mitchell Main dull	NaOH extraction	17	11·8	17·8	1·5	no structure	4
Ryder bright	Oxidation with $KMnO_4$	18	8·3	13·3	1·6	no structure	4

quinones, including catechol and some 4-alkylcatechols, quinol, pyrogallol, 1,2,4-trihydroxybenzene, *o*- and *p*-benzoquinone, hydroxy-*p*-benzoquinone, protocatechuic, caffeic, gallic and 2,3,4-trihydroxybenzoic acids, purpurogallin, 2,3,4,2',3',4'-hexahydroxydiphenyl, 2,3,6,7-tetrahydroxydibenzofuran and catechin have been oxidized either by air or by potassium iodate. Some of the products were soluble and others insoluble in water, but in all cases the ESR spectra differed either in structure, breadth, strength, or in behaviour towards oxygen from the spectra of the HA's isolated from soils, peats, coals, etc. The "X" value and the loss on boiling with hydrochloric acid were measured in a few cases only, and fell within the limits of 1·1–2·0 and 30–40% respectively. In all cases, the "artificial humic acids" differed decisively from the naturally occurring acids.

EXPERIMENTAL

Standard extraction of soils with sodium hydroxide. The air-dried soil was defatted with a mixture (10 vols) of equal vols EtOH and benzene, and dried at 90°. The defatted soil (20 g) and 0·2N NaOH (240 ml), after standing for 17–18 hr with occasional shaking, were centrifuged; in a few cases it was desirable to pass the extract through a muslin filter before centrifuging. The supernatant liquors were filtered through a sintered glass funnel (No. 4) and the filtrate was acidified with dil HCl to pH 1, again centrifuged and the liquors decanted from the ppt. The collection of the HA was greatly facilitated by a modification of Forsyth and Fraser's technique,[35] in which the moist ppt was frozen to −50°, allowed to thaw, and the solid collected on a sintered glass funnel (No. 4) and dried at 90°. The yield at this stage varied from 0·2–5·5 g according to the soil source.* The HA was then boiled for 20 hr with 6N HCl (100 volumes) and the ABHA collected on a sintered glass funnel, washed until free from chloride, and dried at 90°. The yield varied from 30–75% according to the source of the HA.

Experiments on Ringinglow A showed that extraction with 12 vols 0·2N NaOH gave the same yield of better quality ABHA than two consecutive extractions, each with 6 vols of alkali. Thus, one extraction with 6 vols gave a 9% yield of ABHA with an ash of 0·75%; a second extraction with 6 vols gave a further 4% yield with ash of 4·4%. On the other hand, one extraction with 12 vols gave a 13% yield of acid with ash of 0·5%, and a second extraction with 12 vols gave a further 1·5% yield with ash of 4·1%.

* In view of observations made in Part 2, it may be desirable to modify the standard conditions for extraction by introducing a 24-hr boil with water (30 vols) before subjecting the HA to hydrolysis with 6N HCl. It is unlikely that this modification will alter the classification of the soils according to their ESR spectra, although so far, it has only been possible to confirm this impression for the cases of the soils from Ringinglow A, Roudsea Tarn fen A, and Woodwalton Fen.

[35] W. G. C. Forsyth and G. K. Fraser, *Nature, Lond.* **160**, 607 (1947).