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HUMIC ACID—II

STRUCTURE OF HUMIC ACIDS

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Abstract—The action of water and acids on "humic acid" (HA) is described, and it is concluded that HA is a complex of a polycyclic aromatic "core" responsible for the ESR spectrum which is attached to polysaccharides, proteins, relatively simple phenols and metals.

Oxidation of the "core" with permanganate and decarboxylation of the resulting acids gives a neutral oil containing β-methylnaphthalene, anthraquinone, fluorenone, xanthone and homologues. Zinc dust distillation of the "core" gives a yellow distillate containing naphthalene, anthracene, benzofluorene, pyrene and benzopyrenes, perylene and benzoperylene, triphenylene, chrysene, coronene, carbazole, acridine and benzoacridines, and possibly complex benzofurans together with homologues and hydrogenation products.

The products from the permanganate oxidation and the zinc dust distillation, obtained in very small yields, were separated by chromatography into fractions which were analysed by UV and mass spectrometry.

The nature of attachment of the polysaccharides, proteins, phenols and metals to the polycyclic aromatic core is discussed.

It was concluded in Part I that the ESR spectra of HA and ABHA were best accounted for by postulating that the acids were stable semiquinone ion radicals and that the stability was probably associated with extensive conjugation. In addition, it was shown that ABHA gave a much stronger ESR signal and absorbed oxygen more rapidly than HA. The objects of the work now reported were to discover reasons for the acid activation of HA and to obtain, if possible, evidence of the nature of the extensively conjugated system in the HA structure.

The hydrolysis of HA by water or acids was studied by earlier workers. Forsyth[1] showed that boiling water removed from HA's derived from a variety of sources, about 20% as a polysaccharide mixture yielding on acid hydrolysis glucuronic acid, galactose, glucose, mannose, arabinose, xylose and ribose. Duff,[2] using water at 85°, found that hydrolysis of the polysaccharides yielded rhamnose and two O-methylated sugars in addition to those detected by Forsyth.[1] Deuel *et al.*[3] showed that small yields (ca. 1%) of ether-soluble products containing vanillin, *p*-hydroxybenzoic, protocatechuic and vanillic acids were also produced when HA was boiled with water.

Acid hydrolysis of HA's has yielded amino sugars[4] together with the pentoses, hexoses, uronic acids and phenols mentioned above. Hydrolysis, usually with 6N HCl

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[1] W. G. C. Forsyth, *Biochem. J.* **41**, 176 (1947); **46**, 141 (1950).

[2] R. B. Duff, *J. Sci. Food and Agric.* **3**, 140 (1952); *Chem & Ind.* 1513 (1954).

[3] T. Jakab, P. Dubach, N. C. Mehta and H. Deuel, *Z. Planzenernähr., Düng Bodenk.* **96**, 213 (1962).

[4] J. M. Bremner, *Biochem. J.* **47**, 538 (1950); *J. Sci. Food and Agric.* **9**, 528 (1958).

[5] H. M. Hurst, private communication.

also removed a large amount of nitrogen from HA in the form of α-amino acids, and paper chromatographic techniques[6–8] have shown the presence in the hydrolysate of the 22 amino acids usually found in protein hydrolysates, and in approximately the same ratio; the isolation of diaminopimelic acid[9] is however abnormal.

Similar results were obtained during our experiments on the action of water and acids on the HA isolated by the standard method described in Part I, from Ringinglow I peat. The carbohydrates were detected by the Molisch test, and phenols and α-amino acids were examined by TLC using tetrazotized benzidine and ninhydrin sprays respectively; protocatechuic, vanillic, syringic and 3,5-dihydroxybenzoic acids, probably predominated in the phenolic pattern, but these and other products were not investigated in any great detail. When HA was treated with water for 40 hr at room temperature or for 48 hr at 100°, losses in weight of 10 or 40% respectively were observed, and further extractions with hot water removed small and decreasing quantities of organic matter. The solution contained polysaccharides, protein-like substances giving a ninhydrin test only after subsequently boiling with 6N HCl, phenols and other unidentified organic matter. A loss in weight of 15% was observed when HA was stirred for 24 hr with cold 6N HCl, and the extract contained a significant amount of iron in addition to the products mentioned above. When HA was boiled for 24 hr with 0·5N HCl a loss in weight of 30% was observed, and the extract contained carbohydrates, phenols, α-amino acids and iron. After boiling with 6N HCl for 24 hr, HA lost 40% in weight, but carbohydrates were absent from the extract, which however contained phenols, α-amino acids and iron. Additional small losses in weight resulted from further extractions with 6N HCl or 4N NaOH at 100°, or by reduction with sodium amalgam;[10] in all cases the extracts contained phenols, but not carbohydrates, α-amino acids or iron.

The absence of carbohydrates in the filtrates after hydrolysis with hot 6N acid, was probably due to their decomposition during extraction, and this was supported by examination of the water extracts When the early hot water extracts were boiled with hydrochloric acid, black insoluble products resembling HA were precipitated; these gave structureless ESR spectra and they probably arose from the coupling of the carbohydrates or their breakdown products such as furfural, either with themselves, with phenols or with other constituents of the extracts. Later hot water extracts, on the other hand, contain little or no carbohydrate, and on boiling with acids they gave small yields of black precipitates showing the four-lined ESR spectra characteristic of the ABHA's isolated from acid soils; it is suggested that these precipitates are formed by coagulation of small amounts of HA dispersed in the aqueous extracts.

The HA's recovered after boiling with water showed increases in C value with repeated extractions, but no significant alterations in H, N or ash values, and they gave ESR signals with typical four-lined structures. Treatment of HA with warm 0·5N HCl led to an increase in C and a reduction in ash value, whilst boiling with 6N acid resulted in an increase in C and very significant reductions in N and ash

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[6] J. M. Bremner, *Biochem. J.* **47**, 538 (1950); *J. Agric. Sci.* **46**, 247 (1955); J. M. Bremner and K. Shaw, *Ibid.* **44**, 152 (1954).

[7] F. Sowden and D. Parker, *Soil Sci.* **76**, 201 (1953).

[8] M. N. Kononova and I. V. Alexandrova, *Pochvovedenie* **5**, (1956).

[9] J. Carles and G. Decau, *Sci. Proc. Roy. Dublin Soc. Ser. A.* **1**, 177 (1960).

[10] N. A. Burges, H. M. Hurst and B. Walkden, *Geochim. et Cosmochim. Acta* **28**, 1547 (1964).

values. The ABHA's produced either by 0·5N or 6N acid gave strong four-lined ESR signals which persisted after boiling with 6N HCl for 144 hr, and after a further 24 hr boil with 4N NaOH. After sodium amalgam reduction however, the recovered acid gave a structureless and very weak ESR signal.

Extensive conjugation, such as that offered by a complex polynuclear aromatic structure, seemed to provide a reasonable hypothesis for the stability of the free radical responsible for the ESR spectrum of ABHA, and evidence for such aromatic structures has been obtained (a) by oxidation with potassium permanganate and subsequent decarboxylation of the acid mixture with quinoline and copper sulphate, and (b) by zinc dust distillation of ABHA. Both techniques (a) and (b) gave extremely complex mixtures from which pure substances were rarely obtained, but partial separation by chromatography and analysis of the fractions by UV and mass spectrometry has given valuable information concerning the constitutions of the components which are frequently present in minute quantities.

Kumada *et al.*[11] obtained anthraquinone and β-methylnaphthalene (isolated as its picrate) by permanganate oxidation of HA and subsequent decarboxylation of the acid mixture. This work has been repeated with ABHA; the neutral products of the decarboxylation, obtained in about 0·5% yield, were separated by a combination of column and TLC as described in the Experimental and anthraquinone and β-methylnaphthalene were isolated and identified by m.p., mixed m.p., and by their UV and mass spectra. In addition fluorenone (I), giving a 2,4-dinitrophenylhydrazone, m.p. and mixed m.p. 296–300°, and xanthone (II), m.p. and mixed m.p. 168–170° were isolated and their identities confirmed by UV and mass spectra. Mass spectrometry also showed the presence of small amounts of substances with mol wts 128, 210 and 222 consistent with naphthalene, methylxanthones and methylanthraquinones respectively, whilst molecular ions at *m/e* 168, 178 and 194 indicated the presence of the hydrocarbons $C_{13}H_{12}$, $C_{14}H_{10}$ and $C_{15}H_{14}$ respectively.

Attempts to identify the carboxylic acids before decarboxylation were unsuccessful. The acid mixture was methylated with diazomethane and the methyl ester yielded on distillation a considerable amount of dimethyl oxalate and smaller quantities of two yellow viscous oils, (A) and (B), b.p. 130°/1 mm. and 210°/0·6 mm., respectively. Oil (A) gave an NMR spectrum with 3 bands in the MeO region 6–6·4 τ, of which the band at 6·08 τ was stronger than the other two bands; the ratio of aromatic ring protons shown at 1·1–2·7 τ to methoxy protons was 1:8. The NMR spectrum of oil (B) showed 3 peaks in the MeO region, but in this case the band at 6·08 τ was much stronger than the other two bands and the ratio of aromatic ring protons to methoxy protons was 1:6·5.

Distillation of ABHA with zinc dust at 500° in a stream of hydrogen gave a 3% yield of a pale yellow oil, showing in ether solution an intense green–blue fluorescence in visible or UV light. Small amounts of acidic and basic substances were removed in dilute sodium hydroxide and hydrochloric acid respectively, and the residual neutral oil was separated by chromatography on a silica gel column into nine fractions. Each fraction was then separated into sub-fractions by TLC as described in the Experimental. Anthracene and 2,3-benzofluorene (III), isolated in crystalline and fairly pure forms, were recognized by their UV and mass spectra, and a combination

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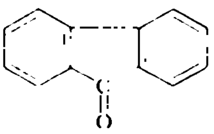
[11] K. Kumada, A. Suzuki and K. Aizawa, *Nature, Lond.* **191**, 415 (1961).

of VPC, UV and mass spectrometry provided convincing evidence of the presence of naphthalene, α- and β-methylnaphthalenes and higher homologues of naphthalene. Examination of the sub-fractions by UV and mass spectrometry indicated the presence of a wide range of other aromatic hydrocarbons including pyrene (IV), perylene (V), 1,2-benzopyrene (VI), 3,4-benzopyrene (VII), triphenylene (VIII), chrysene (IX), 1,12-benzoperylene (X), and coronene (XI), together with homologues, unidentified hydrogenation products, products of higher mol wt and non-aromatic substances. Other fractions contained carbazole (XII) and homologues, and complex benzofuran structures were probably present in the fractions of high mol wt. The small basic fraction from the zinc dust distillation contained a mixture of homologous acridines



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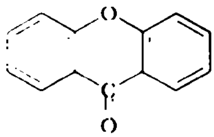

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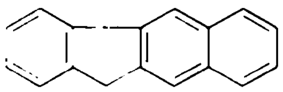

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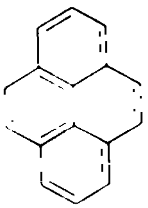

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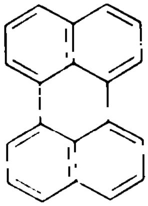

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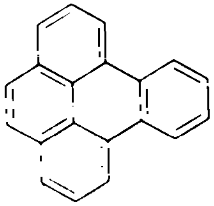

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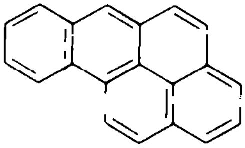

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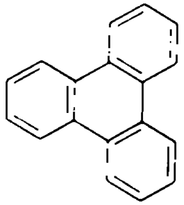

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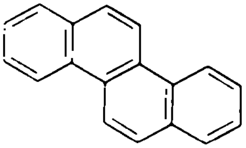

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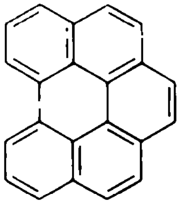

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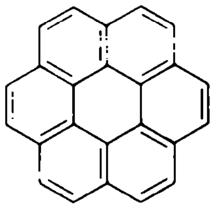

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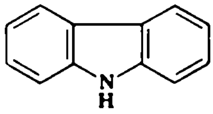

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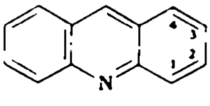
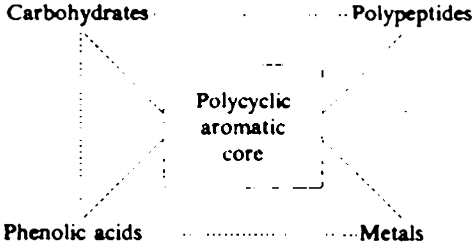


XIII

(XIII) and 1,2- or 3,4-benzoacridines. The small yield of yellow distillate was not improved by Clar's technique,[12] using zinc dust in a zinc chloride–sodium chloride melt, and a diminished yield was obtained by distillation with zinc dust in a stream of nitrogen. In all cases most of the material was recoverable from the zinc dust as an alkali-insoluble, infusible black product (Found: C, 77·1; H, 3·2; N, 1·0; O, 8·9; ash, 6·2%) which is being examined further.

The differences in ESR spectra of ABHA's derived from acid and basic soils reported in Part I cannot be attributed to observed differences in behaviour towards hydrolysis or distillation with zinc dust. The HA from the more basic peat from Woodwalton fen reacted similarly to the HA from the acid Ringinglow I peat towards water and acids, and the ABHA from Woodwalton fen gave on distillation with zinc dust a yellow distillate which yielded the same products as those derived from Ringinglow ABHA; the evidence suggested that the distillate from the Woodwalton fen acid had a smaller perylene content, but quantitative data is not available.

In view of the small yields, the products from the zinc dust distillation may arise from secondary reactions but this is improbable in the case of products from the permanganate oxidation, and consequently it is believed that the existence of a polynuclear aromatic structure in ABHA is established. This polycyclic aromatic core probably contains the stable semiquinone ion radicals responsible for the ESR spectra, and the general structure XIV may be advanced for HA, although the mode of attachment of the hydrolysable groups is uncertain.



XIV

Deul *et al.*[13] found that the carbohydrate content of "fulvic acids" was reduced from 25 to 5% by gel filtration on Sephadex G. 75, and the nitrogen content was reduced from 2·1 to 1·3% by ion exchange chromatography, and concluded that the carbohydrates were contaminants of the "fulvic acids". The evidence concerning the protein-like constituents was regarded as inconclusive, but many workers including Waksman[14] and Swaby[15] considered that the stability towards chemical and biochemical attack of the peptides was an indication that they were an integral part of the HA molecule. The removal of carbohydrates and phenolic acids by means of cold or hot water is not necessarily evidence in favour of physical absorption; readily hydrolysable depsidic, ester and glycosidic linkages are frequently encountered in

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[12] E. Clar, *Ber Dtsch. Chem. Soc.* 72, (1939).

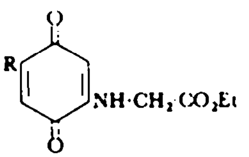
[13] N. Roulet, N. C. Mehta, P. Dubach and H. Deuel, *Z. Planzenernähr. Düng. Bodenk.* 103, 1 (1963).

[14] S. A. Waksman, *Humus* (2nd Edition) p. 189. Williams and Wilkins, Baltimore (1938).

[15] R. J. Swaby and N. J. Ladd, *Trans. Int. Soil Conf., New Zealand, Commissions IV and V* (1962); N. J. Ladd, *Aust. J. Biol. Sci.* 17, 153 (1964).

other fields, e.g. the gallo- and ellagitannins,[16] and the hydrolysis of HA would probably be facilitated by the presence of carboxyl or other acid groups in the molecule. It has also been shown that the acid hydrolysate from HA is not readsorbed upon the ABHA formed, but again this is inconclusive as the physical state of the residual acid has possibly undergone some change during the hydrolysis. However, the increased oxygen absorption of ABHA reported in Part I is greater than can be accounted for by the removal of inert groups only, and suggests that some of the hydrolysable groups are chemically attached to the HA structure.

Various workers including Swaby[15] and Handley[17] have suggested that the stability of the nitrogenous components of HA towards micro-organisms may be caused by their chemical combination with quinone groups by reactions similar to those frequently postulated in earlier theories of tanning. Fischer and Schrader[18] showed that p-benzoquinone condensed with glycine ethyl ester to give XV (R = $NH{\cdot}CH_2CO_2Et$) and Dr. P. Shanmugan of A. C. College, The University of Madras, has repeated the preparation of this compound, prepared the analogous compounds XV (R = Me and OMe) from p-toluquinone and methoxy-p-benzoquinone respectively, and showed that all three substances lost glycine on hydrolysis with 6N HCl just as the nitrogen was removed from HA by acid hydrolysis; in all three cases black amorphous substances were produced, presumably by further condensation of the hydroxy-p-benzoquinones resulting from the removal of the glycine. Substances of type XV however, are unlikely to lose their nitrogen by treatment with water, and the removal of peptide-like substances by treatment with cold or hot water suggests either physical attachment or alternatively, attachment by hydrogen bonding between the amide groups of the peptide and phenolic groups, as shown in structure XVI or XVII.



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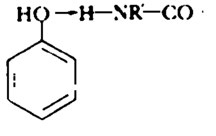
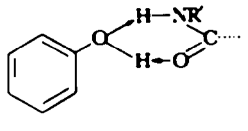


XVI



XVII

EXPERIMENTAL

UV spectra were determined in a Perkin–Elmer Model 137; ϵ_{max} values were not estimated either because of shortage of materials or because the values were meaningless in cases of mixtures.

All mass spectra were determined in an A.E.I., MS9 mass spectrometer, using 70 eV-ionizing electrons. The source temp was maintained at 200° and samples were introduced into the spectrometer via a direct insertion probe. Where mixtures were encountered, the molecular ion peaks (M^+) were identified by repeatedly scanning the appropriate part of the spectrum while decreasing the ionising voltage within the range 10–20 eV. High resolution mass measurements were determined with an accuracy of ± 7 ppm. The strengths of the low voltage spectra are referred to those of the base peak (b) by the following conversion: strong (s), medium (m) and weak (w) peaks imply 70–100%, 40–70% and 20–40% respectively of the base peak (b) intensity.

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[16] R. D. Haworth, *Proc. Chem. Soc.* 406 (1961); E. Haslam, *Chemistry of Vegetable Tannins* pp. 92, 93, 126, 129, Academic Press, London (1966).

[17] W. R. C. Handley, *For. Comm. Bull. No.* 23 *Mull and Mor Formation in Relation to Forest Soils* (1954).

[18] E. Fischer and H. Schrader, *Ber. Dtsch. Chem. Soc.* 43, 525 (1910).

The VPC was done on an F and M Model 720 instrument, using a silicone rubber SE 30 column of 2 ft length.

Unless otherwise stated, the HA used was isolated from Ringinglow I peat with 0·2N NaOH as described in Part I; it had C, 49·9; H, 4·9; N, 2·1; ash, 5·4%. A few experiments were made with HA isolated from Woodwalton fen; it had C, 51·0; H, 5·0; N, 3·0; ash, 3·2%.

Analysis of hydrolysates

Water hydrolysates were adjusted to pH 1 and the small black ppt removed on the centrifuge. The liquors from the acid hydrolysates (for which this precipitation was unnecessary) were continuously extracted with ether to remove phenols. The ether extract was dried ($MgSO_4$), the phenols were recovered and detected by two-dimensional, TLC on silica gel plates, using AcOEt–benzene (45:55) and chf–AcOH (9:1), as solvent systems, and spraying with tetrazotized benzidine soln.

After removal of the phenols, the liquors were evaporated to dryness in a rotary evaporator at 15 mm and 25°, and the residue treated as follows: (a) Fe was detected by the addition of a soln of $K_4Fe(CN)_6$ and the formation of Prussian blue, (b) carbohydrates were detected by Molisch test with α-naphthol and (c) α-amino acids were detected by TLC on silica gel, developing the plates with a soln of MeOH–chf–17% NH_4OH (2:2:1 by vol) before spraying with ninhydrin soln.

In the case of alkaline hydrolysates or filtrates from reduction with NaHg, the alkaline liquors were acidified, evaporated to dryness under reduced press and the phenols isolated from the salt by continuous extraction with ether; the extract was dried, evaporated and the residue tested for phenols as described above.

Action of water on HA

(a) HA (10 g) was stirred at room temp for 40 hr with water (300 ml). The residue (9 g) was collected and the filtrate contained carbohydrates, peptides (giving a positive ninhydrin test after boiling with 6N–HCl), phenols and traces of Fe.

(b) HA (10 g) was boiled for 24 hr with water (1000 ml). The residue (X) (7·5 g) had C, 54·1; H, 4·9; N, 1·4; ash, 4·7%. The filtrate contained carbohydrates, phenols, peptides and traces of Fe and after boiling with an equal vol HCl, it yielded a black ppt (0·4 g) which, when dissolved in dil NaOHaq, gave a structureless ESR signal. The residue (X) (7·5 g), when boiled with water (750 ml) for a further 24 hr, was reduced in wt to 7·0 g; the filtrate contained no carbohydrate, showed very weak tests for Fe, phenols and peptides, and after boiling with 6N HCl acid, gave a black ppt (0·05 g) which when dissolved in NaOHaq gave a 4-lined ESR signal. Further treatments with boiling water resulted in small losses (0·1 g) in wt; after concentration the filtrate gave negative tests for Fe and carbohydrates, very weak tests for phenols and peptides, and on boiling with HCl, yielded a black ppt (0·01 g) giving a 4-lined ESR signal.

Action of hydrochloric acid on HA

(a) HA (10 g) was stirred at room temp for 24 hr with 6N HCl (1000 ml). The residue (8·5 g) C, 54·0; H, 5·0; N, 1·5%, and the filtrate contained Fe, carbohydrates, phenols, peptides and small quantities of α-amino acids.

(b) HA (10 g) was boiled for 24 hr with 0·5N HCl (1000 ml). The residue (7·0 g) had C, 57·2; H, 4·6; N, 1·4; ash, 1·5%, and the filtrate contained Fe, carbohydrates, phenols and α-amino acids.

(c) HA (10 g) was boiled for 24 hr with 6N HCl (1000 ml). The residue (6·0 g) had C, 59·5; H, 4·4; O, 31·0; N, 0·3; ash, 0·5%, and the ash was reduced to 0·25% after treatment for 1 hr with cold 20% HF. The HCl filtrate contained Fe, phenols and α-amino acids, but no carbohydrates.

Subsequent treatments with boiling 6N HCl resulted, each time, in small losses (0·1 g) in wt; the filtrates, after concentration gave negative tests for Fe, carbohydrates and α-amino acids and weak tests for phenols. When the residual ABHA was dissolved in NaOHaq, well-developed, 4-lined ESR spectra were obtained, and the signal was retained after boiling ABHA (5 g) with 4N NaOH (250 ml) for 24 hr; the filtrate gave negative tests for Fe, carbohydrates and α-amino acids, but yielded an ether-soluble oil (0·05 g) containing phenols. A further liberation of phenols (0·24 g) was observed when a soln of ABHA (5 g) in 0·25N NaOH (500 ml) was reduced with 3% NaHg.[16]

The HA from Woodwalton fen gave similar results and the resultant ABHA had C, 59·4; H, 4·3; N, 1·6; ash, 1·4%.

Oxidation of ABHA *with potassium permanganate*

ABHA (30 g) was dissolved in N NaOH (300 ml) and maintained at 55–60° for 1½ hr, during the gradual addition with stirring of a soln of $KMnO_4$ (120 g) in water (1000 ml). The MnO_2 was separated in a centrifuge, washed well with water and the combined liquors and washings acidified with conc HCl (60 ml). The ppt (1·2 g) was removed in a centrifuge and the clear liquid concentrated under reduced press to 500 ml, filtered and continuously extracted with ether for 3 days. The extract was dried ($MgSO_4$), the solvent removed and the residue (7·1 g) heated for 6 hr at 240° with quinoline (35 ml) and anhyd $CuSO_4$ (3·5 g). The mixture was diluted with ether, washed with dil HClaq and NaOHaq dried and the solvent removed, leaving a neutral oil (0·12 g).

The neutral product (0·3 g) in CCl_4 (5 ml) was absorbed on a column of neutral alumina (15 g) and separated by successive elutions with CCl_4, benzene and benzene–chf (4:1) into 5 fractions.

Fraction 1 (0·08 g), which showed a blue fluorescence and a naphthalene chromophore with λ_{max} (n-hexane), 225, 275, 281, 287, 302, 311, 319 mμ was separated by TLC on silica gel plates, using pet. ether (b.p. 40–60°) for elution into 2 fractions, 1A and 1B. *Fraction* 1A (0·03 g) was a liquid with a mass spectrum showing M^+ at *m/e* 142, 168, 178, 194, associated with the presence of methylnaphthalene, $C_{13}H_{12}$, $C_{14}H_{10}$ and $C_{15}H_{14}$ respectively. *Fraction* 1B (0·03 g) had m.p. 31°, undepressed by admixture with β-methylnaphthalene, gave λ_{max} (n-hexane) 225, 270*, 276, 286*, 305, 312, 319 mμ showed a strong M^+ at *m/e* 142, and a weak M^+ at *m/e* 128 indicated the presence of a trace of naphthalene.

Fraction 2 (0·025 g), which was pale yellow and partly crystalline, was crystallized several times from MeOH, and gave anthraquinone (0·01 g), showing λ_{max} (EtOH), 253, 272, 326 mμ; the mass spectrum had M^+ at *m/e* 208 and fragment ions at *m/e* 180(M-CO) and 152(M-2CO) identical with that of an authentic specimen of anthraquinone. The mother liquors were separated by TLC on silica gel plates, using benzene–chf (9:1) for development into (a) a small impure fraction showing M^+ at *m/e* 222, indicative of a methylanthraquinone and (b) a major fraction giving a pale yellow band with R_f value identical with that of fluorenone (I) and showing λ_{max} (EtOH), 249, 257 mμ characteristic of fluorenone, but some background absorption was probably responsible for the absence of the weak bands at 287, 295 mμ present in the UV spectra of authentic fluorenone. The 2,4-dinitrophenylhydrazone had m.p. 296–298° (dec), undepressed by admixture with fluorenone 2,4-dinitrophenylhydrazone.[19]

Fraction 3 (0·03 g), which solidified almost completely, gave after fractional crystallization from MeOH, (a) anthraquinone (0·001 g), characterized as described above for fraction 2, and (b) xanthone (II; 0·01 g), m.p. and mixed m.p. 168–170°, with λ_{max} (EtOH) 233*, 240, 261, 288, 337 mμ, and with M^+ at *m/e* 196 and fragment ions at *m/e* 168(M—CO) and 139(M—CO—CHO) characteristic of xanthone.

Fraction 4 (0·04 g), obtained by elution with benzene, was divided into 4 parts by TLC on silica gel. One part had λ_{max} (EtOH)240, 253*, 285*; ν_{max} (chf) 1660, 1710, 1750 cm^{-1} and showed M^+ at *m/e* 196, 210, consistent with the presence of xanthone and methylxanthones respectively.[6] The other components were not identified.

Fraction 5 (0·05 g), eluted with benzene–chf gave weak bands at ν_{max} (chf) 1660, 1710, 1754 cm^{-1}, but the components of the mixture were neither separated nor identified.

Zinc dust distillation of ABHA

A mixture of ABHA (0·5 g) and Zn dust (30 g, purified by washing successively with 0·1N HCl and EtOH, drying in a desiccator, and heating for 1 hr at 300–350° in a stream of H) was introduced into a Pyrex Tube (diam 10 mm) and a further quantity (10 g) of Zn dust added on the exit side. H was passed through the tube for 15 min and the contents were then heated in a H stream at 500–550° for 15 min. The combined yellow distillates (1·65 g) from 60 g ABHA were dissolved in ether (200 ml), filtered from insoluble material (0·2 g), washed successively with dil NaOHaq (2 × 25 ml) from which a small acid fraction (0·02 g) was recovered, 1N HCl (2 × 25 ml) from which a small basic fraction (0·055 g) was recovered, and water (2 × 25 ml). Removal of the solvent from the dried ($MgSO_4$) ether extract gave a neutral oil (1·04 g), which was shown by preliminary TLC on silica gel plates, and development with pet. ether (b.p. 40–60°) to be a very complex mixture showing UV fluorescence between R_f values 0·0 and 0·62, and a ceric sulphate spray revealed the presence of non-fluorescent substances at R_f 0·92.

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[19] N. Campbell and M. Wang, *J. Chem. Soc.* 2186 (1949).

Fractionation of the neutral oil

The neutral oil (1·04 g), dissolved in pet. ether (b.p. 40–60°; 25 ml), was filtered from traces of insoluble black tar and absorbed on a column of silica gel (40 g). Successive elution with pet. ether (b.p. 40–60°; 250 ml) gave the colourless semi-solid fraction 1 (0·32 g) and the yellow, partly crystalline fractions 2–7 weighed 0·26, 0·125, 0·055, 0·035, 0·03 and 0·02 g respectively. Fraction 8 (0·125 g), an orange coloured gum was eluted with pet. ether (b.p. 40–60°)–benzene (1:1; 250 ml) and fraction 9 (0·12 g) was removed by MeOH (50 ml) as a brown oil.

Fractions 2 and 5 were fractionally crystallized from MeOH. Fraction 2A (0·26 g) gave colourless plates (0·0015 g), m.p. 188–190° of impure anthracene, λ_{max} (n-hexane) 247*, 252, 274, 288*, 292*, 298*, 319, 324, 335, 339, 352*, 356, 370*, 375 mμ and the mass spectrum showed M^+ at *m/e* 178(s), 192(w), the latter corresponding with a trace of methylanthracene. The mother liquors were treated as described below. Fraction 5A (0·035 g) gave colourless plates (0·0015 g), m.p. 194–205° of impure III which, when mixed with an authentic specimen (m.p. 203–205°),[10] had mixed m.p. 200–203°; it had λ_{max} (n-hexane) 216, 239*, 255*, 258, 263, 266*, 287, 293*, 303, 316, 324*, 332*, 340 mμ and the mass spectrum showed M^+ at *m/e* 216(s), 230(w) and a strong fragment ion at *m/e* 215. The weak peak at *m/e* 230 indicated the presence of a trace of methylbenzofluorene impurity, and the absence of a fragment ion at *m/e* 229 suggested that the Me group was situated at position 9 and was eliminated during the fragmentation. That the strong fragment ion at *m/e* 215 arises partly by elimination of the Me group from 9-methylbenzofluorene was confirmed (a) by the presence of a metastable peak at *m/e* 201; Calc. for $230^+ \rightarrow 215^+$, *m/e* 201, and (b) by experiments with 9-methyl-1,2-benzofluorene[11] which gave a mass spectrum with M^+ at *m/e* 230, and a strong fragment ion at *m/e* 215 but not at *m/e* 229.

The non-crystalline parts 2B and 5B from the mother liquors of fractions 2 and 5, together with the remaining fractions 1, 3, 4, 6, 7, 8 and 9, were repeatedly separated by chromatography on thick silica gel plates, using pet. ether (b.p. 40–60°) and pet. ether–benzene mixtures as solvents. Fluorescent bands, detected by exposing the plates to UV light, were removed and eluted. Other organic material was detected by spraying the edges of the plates with 2% ceric sulphate in 2N H_2SO_4, and the bands corresponding with the blackened areas were extracted. Each fraction was divided into about 10 sub-fractions, marked by letters A, B, C, D etc. in order of decreasing R_f value; letters W, X, Y, Z were used by sub-fractions detected by ceric sulphate. Some of the sub-fractions were separated further on silica gel plates, and sub-script numerals were used to indicate the separations in order of decreasing R_f value. The properties of the more significant fractions only are shown in Table 1.

Assignments and comments on Table 1

Fraction 1. The UV spectrum of fraction 1A indicated the presence of a naphthalene chromophore, and VPC at 125°/5°/min showed the presence of naphthalene, a mixture of α- and β-methylnaphthalenes and 5 more highly alkylated derivs with retention times 3·4, 5·5, 7·5, 7·9, 9·9, 11·8 and 14·3 min respectively. α- and β-Methylnaphthalenes were separated by using the columns at 110° when naphthalene, β- and α-methylnaphthalenes had retention times 5·8, 12·2 and 12·9 min respectively, which were unchanged by the additions of authentic samples of these 3 components. The order of intensities was confirmed by the mass spectrum; the M^+ at *m/e* 142 and 156, together with fragment ions at *m/e* 141 and 155, indicated that the main components were methyl- and dimethyl- (or ethyl-) naphthalenes with less naphthalene (*m/e* 128) and probably homologues $C_{13}H_{14}$, $C_{14}H_{16}$ and $C_{15}H_{18}$ with *m/e* 170, 184 and 198 respectively present in smaller amounts.

The non-fluorescent fraction 1Y, detected by ceric sulphate spray, showed little absorption in the UV and consequently was largely non-aromatic. VPC at 125°/5°/min showed the presence of 9 components with retention times 5·0, 7·2, 8·8, 9·7, 12·2, 14·7, 17·2, 19·4 and 21·8 min respectively. The mass spectrum showed no doubly charged ions and confirmed the presence of at least nine non-aromatic constituents, forming a homologous series from *m/e* 196 to 308, and high resolution mass spectrometry on the peak at *m/e* 252 established the hydrocarbon, $C_{18}H_{36}$ structure. (Found: 252·28161. Calc. for $C_{18}H_{36}$, 252·28168.)

Fraction 2. Fractions other than fraction 2A, yielding crystalline anthracene (above), were complex mixtures. The UV spectra of fractions 2B and 2D indicated the presence of anthracene and

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[10] J. Thiele and A. Wandscheidt, *Liebig's Ann.* **376**, 269 (1910).

[11] G. M. Badger, *J. Chem. Soc.* 535 (1941).

TABLE 1

Fraction	Weight (mg)	Fluorescence in UV	λ_{max} (n-hexane)	Mass Spectrum
1A	50	violet	221•, 224, 275, 319, 323•	s ⊙141^b, 142 m 156, ⊕155, 168 w 128, 170, 184, 198
1Y	135	—	228, 270•	s 252^b m 238, 266 w 196, 210, 224, 280, 294, 308
2B	99	violet	214, 245•, 251, 319, 335, 356, 375	—
2D	15	pale-blue	215, 245•, 253, 272, 275, 287, 320, 336, 358	s 202^b, 206 m 192, 204 w 216, 218, 220
2D$_1$	—	pale-blue	213, 231, 240, 253, 260, 272, 295•, 300, 305, 320, 335, 364, 385	s 202^b m 200, 206, 218 w 204, 216
2D$_2$	--	pale-blue	221, 244•, 252, 272, 281•, 293, 319, 335, 346, 349	—
2E	15	pale-yellow	215, 245•, 255, 262	s 192^b m 178, 206 w 220
2Z	65	—	226, 250, 280, 324	· ·
3D	80	green-blue	218, 247, 254, 272, 287, 320, 335, 360	s 204^b, 192 m 202, 206 w 218
4C	35	green-yellow	218, 230•, 240, 253, 262, 278, 288, 303, 316, 336, 340, 397	s 216^b, ⊕215 m 230 w 192, 218, 232
5B	10	green-blue	217, 255, 263, 277•, 288, 304, 316, 336, 340	—
5D	5	orange	220, 253•, 258, 266, 276, 288, 296, 316, 331, 346, 364, 378•, 381•, 384, 402•, 407, 429, 434	s 228^b m 252 w ⊕215, 216, 242
5D$_2$	—	blue	222, 249, 257, 267, 286, 306•, 320, 340•, 350•, 361	s 228^b m 280, 294, 308, 322, 336 w 242, 266, 278, 292, 306, 320, 334
5D$_4$	---	yellow	227, 250•, 258, 269, 284, 296, 345, 364, 378•, 381•, 384, 402	s 242^b, 228, 252 m 254

Table 1 (cont'd).

Fraction	Weight (mg)	Fluorescence in UV	λ_{max} (n-hexane)	Mass Spectrum
5D$_{11}$	—	yellow	223, 237, 255, 267, 277, 289, 305•, 316, 331	s 252'
6A	15	blue	218, 256, 264, 288, 303, 316, 340•, 343	s 230' m 216, ⊖215
6B	15	green	220, 257, 265, 289, 316•, 342, 362, 378, 381, 384, 402, 435	s 242', 244 m ⊖229, 230, 252, 254, 256 w ⊕215
6D	5	violet	218, 245, 252, 289, 316, 331, 347, 366, 385, 408, 429, 434	s 252' m — w 216, ⊖215
7D	—	purple	222, 245, 252, 276, 288, 300, 316, 331, 345, 362, 383, 407, 429, 434	s 252' w 266, 276
7F	5	yellow	222, 257, 301•, 313, 345, 363, 390–440	s 252' w 266
8C	35	orange	215, 260, 320•, 334•, 366•, 386, 406, 431•, 437	s 242', 256 m 252, 254, 268 w 266, 270
8F	20	yellow	280–290, 378, 410•, 432•, 439	s 290' m 292, 302, 304, 306 w 276, 278, 300, 316, 318, 320, 328
8F$_2$		yellow	253, 284, 287•, 297, 301, 322, 338	s 300' w 314, 316, 318, 332, 344, 352
8J	20	pale-yellow	258, 285•, 293, 325•, 335•	s 167', 181 m 202 w 195, 216
8J$_1$		yellow	220, 258	s 336', 252, 280, 294, 308, 322, 350, 362, 364, 372 m 266, 278, 306, 320, 334 w 304, 318, 332, 346
8J$_2$		yellow	232, 244•, 254•, 285, 290, 302•, 318, 331, 375•	s 167', 181 w 195
9H	15	orange	λ_{max} (methanol) 230•, 255•, 395	s 382', 368 m 396, 410 w 354, 424

• shoulder; ⊖ fragment ion; ' base peak; s, m, w — strong, medium, weak (see p. 1674).

pyrene (IV) chromophores respectively, and the mass spectrum of fraction 2D suggested the presence of methylanthracene and/or methylphenanthrene (*m/e* 192), pyrene (*m/e* 202) and hydrogenated derivatives (*m/e* 204 and 206), and small amounts of methylpyrene (*m/e* 216[*]) with its di- and tetrahydro derivatives (*m/e* 218, 220). Fraction 2D was separated by further chromatography; fraction $2D_1$ gave a UV spectrum almost indistinguishable from that of pyrene and showed a mass spectrum with M^+ at *m/e* 202; the peak at 293 mμ in the UV spectrum of fraction $2D_2$ may be attributed to a phenanthrene chromophore.

Fraction 2E gave M^+ at *m/e* 178, 192, 206 and 220. The absence of doubly charged ions and the UV spectrum showed that the material was largely non-aromatic, and a series $C_{13}H_{22}$, $C_{14}H_{24}$, $C_{15}H_{26}$, $C_{16}H_{28}$ would be consistent with the data. Fraction 2Z gave a UV spectrum which was predominantly naphthalenic.

Fraction 3. The UV and mass spectra of all the sub-fractions were similar to those from fraction 2.

Fraction 4. The benzofluorene[†] chromophore indicated by the UV spectrum was strongly supported by the mass spectrum of fraction 4C, m.p. 169–187°, with a strong M^+ at *m/e* 216 and a strong fragment ion at *m/e* 215 corresponding with benzofluorene, and a moderately strong M^+ at *m/e* 230 was attributed to a 9-methylbenzofluorene (see p. 1667). The weak M^+ at *m/e* 192 may be due to the presence of traces of methylanthracenes or phenanthrenes, and the two weak ions at *m/e* 218 and 232 may be produced by small amounts of dihydro derivatives of methyl- and dimethylpyrenes respectively.

Fraction 5. The benzofluorene chromophore characterizing the crystalline fraction 5A was retained in fraction 5B and 5D, but the latter also contained 3,4-benzopyrene (VII) responsible for the bands at 364, 378[*], 384, and 402[*] mμ, small amounts of V giving bands at 407, 429 and 434 mμ, and traces of VI responsible for the band at 331 mμ. The mass spectrum of fraction 5D was consistent with the interpretation of the UV data, but further fractionation gave additional information. The mass spectrum of sub-fraction $5D_3$ showed a strong M^+ at *m/e* 228, corresponding with $C_{18}H_{12}$, and the UV spectrum with strong peaks at 257 and 267 mμ suggested the presence of VIII and IX respectively; smaller amounts of higher mol wt hydrocarbons were also present. Sub-fraction $5D_4$ gave a typical VII UV spectrum and the mass spectrum with M^+ at *m/e* 252 was in agreement, although other unidentified compounds were also present. Sub-fraction $5D_{11}$ showed a characteristic VI UV spectrum and a strong M^+ at *m/e* 252.

Fraction 6. The UV spectrum of fraction 6A, m.p. 160–170° resembled that of fraction 5B and the mass spectrum suggested the presence of a 9-methylbenzofluorene (see p. 1677). The UV spectrum of fraction 6B resembled that of fraction 5D in showing the presence of benzofluorene, perylene and 3,4-benzopyrene. The mass spectrum of fraction 6B confirmed the presence of a 9-methylbenzofluorene (*m/e* 230; fragment ion at *m/e* 215) and also suggested the presence of a dimethylbenzofluorene with one Me group only attached to position 9 (*m/e* 244; fragment ion at *m/e* 229; metastable ion at *m/e* 215 corresponding with $244^+ \rightarrow 229^+$); reduction products were also present in this fraction. The UV spectrum of fraction 6D indicated considerable perylene content, which was confirmed by the mass spectrum (*m/e* 252); traces of benzofluorene (*m/e* 216; fragment ion at *m/e* 215) were also indicated.

Fraction 7. This fraction did not give very satisfactory UV spectra, and in general, the sub-fractions resembled the corresponding ones in fraction 6. The mass spectrum of fraction 7D showed M^+ at 252, 266, 276 corresponding with perylene, a methyl homologue and X; the UV spectrum was consistent with the assignment. The mass spectrum of fraction 7F showed a strong M^+ at *m/e* 252, for which the hydrocarbon structure $C_{20}H_{12}$ (Found: 252·0945. Calc. for $C_{20}H_{12}$, 252·0939) was established by high resolution measurements; the UV spectrum of 7F, however, lacked some of the characteristic perylene peaks.

Fraction 8. The UV spectra of all sub-fractions were diffuse, but that of fraction 8C showed the presence of perylene and the benzopyrenes. This was confirmed by the mass spectrum which showed M^+ at *m/e* 252, but homologues and reduction products were also present. High resolution measurements on the strong M^+ at *m/e* 242 indicated the structure $C_{19}H_{14}$ (Found: 242·1105. Calc. for $C_{19}H_{14}$, 242·1095) which however was not identified. Fraction 8F gave a mass spectrum showing a strong M^+ at *m/e* 290 and a large number of weaker ions corresponding with homologues and hydrogenation products. Further fractionation gave fraction $8F_9$ showing a strong M^+ at *m/e* 300

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[*] The absence of a fragment ion at *m/e* 215 excludes benzofluorene.

[†] Both 1,2- and 2,3-benzofluorenes are included.

assigned to XI, and the UV spectrum with sharp peaks at 297, 301, 322 and 338 mμ supported this conclusion. Fraction 8J gave a mass spectrum with M^+ at *m/e* 167, 181 and 195 suggesting the presence of nitrogen-containing components. High resolution studies on *m/e* 181 indicated the formula $C_{13}H_{11}N$ (Found: 181·0873. Calc. for $C_{13}H_{11}N$, 181·0915) of a methylcarbazole. This would explain the non-basic properties of the fraction, and it was inferred that the *m/e* 167 and 195 represent carbazole and a dimethylcarbazole respectively. Further separation yielded fraction $8J_1$ with a characteristic carbazole UV spectrum, and a mass spectrum with M^+ at *m/e* 167, 181 and 195 corresponding with carbazole and homologues. Fraction 8J probably also contained non-aromatic constituents; this was confirmed by the UV spectrum and the absence of doubly charged ions in the mass spectrum of sub-fraction $8J_1$.

Fraction 9. Fraction 9H gave a very diffuse UV spectrum, and the mass spectrum showed M^+ at *m/e* 354, 368, 382, 396, 410 and 424, of which the first was weaker than the rest. High resolution techniques on *m/e* 354, 382 and 396 established the formula $C_{28}H_{18}$ (Found: 354·1395. Calc. for $C_{28}H_{18}$, 354·1408) for the former, but the two others were oxygen-containing substances $C_{29}H_{18}O$ (Found: 382·1340. Calc. for $C_{29}H_{18}O$, 382·1358) and $C_{30}H_{20}O$ (Found: 396·1484. Calc. for $C_{30}H_{20}O$, 396·1514) respectively. The oxygen-containing compounds are probably complex polybenzofurans, and the hydrocarbon $C_{28}H_{18}$ may be produced by loss of carbon monoxide in fragmentation of $C_{29}H_{18}O$.

Fractionation of the basic fraction from zinc dust distillation of ABHA

The basic fraction (see p. 1676; 0·055 g) was dissolved in ether (30 ml), filtered and the filtrate separated by TLC on thick silica gel plates, and developed by chf into 15 fractions (A, B, C, D etc.), distinguishable by their fluorescences in visible and UV light. The UV and mass spectra of fractions D, I and L are given in Table 2.

TABLE 2

Fraction	Fluorescence in UV	λ_{max} (MeOH)	Mass spectrum
D	yellow	236, 254, 267, 275, 285*, 332*, 345*, 365, 385	s 229(b), 243 m 257
I	pale-blue	236, 250*, 270–280, 328*, 335*, 343	s 169(b) m 155, 183, 197
L	pale-yellow	236, 243–259, 317*, 345*, 372	s 179, 193(b) m 203 w 207

Assignments and comments on Table 2

Fraction D. The UV spectrum indicated the presence of 1,2- or 3,4-benzoacridines, and the mass spectrum with M^+ at *m/e* 229, 243, 257 suggested that the main constituents of the fraction were benzoacridine with the methyl and dimethyl (or ethyl) homologues.

Fraction I. The mass spectrum indicated the presence of a base, $C_{11}H_9N$, with 3 of its homologues, but these have not been identified.

Fraction L. The UV spectrum was not very characteristic, but the mass spectrum suggested the presence of acridine or an isomer with methyl and dimethyl (or ethyl) homologues and a component, $C_{15}H_9N$.

Condensation of glycine ethyl ester with quinones (with Dr. P. Shanmugan)

2-(N-*Carboethoxymethylamino*) 5-*methyl*-1,4-*benzoquinone* (XV; R = Me). *p*-Toluquinone (4·3 g) and glycine ethyl ester (1·8 g) were mixed in benzene (50 ml), and after 12 hr water (200 ml) was added. The dried ($MgSO_4$) benzene layer was diluted with pet. ether (b.p. 40–60°) and the dark-red ppt was crystallized from pet. ether (b.p. 40–60°) containing a little benzene; dark-red plates, m.p.

104–105° (Found: C, 59·0; H, 5·8; N, 6·1. $C_{11}H_{13}O_4N$ requires: C, 59·2; H, 5·8; N, 6·3%) were obtained.

2-(N-*Carboethoxymethylamino*) 5-*methoxy*-1,4-*benzoquinone* (XV; R = OMe), prepared similarly from methoxy-*p*-benzoquinone (5 g) and glycine ethyl ester (1·8 g) separated from pet. ether (b.p. 40–60°) in orange-red needles, m.p. 152–154°. (Found: C, 55·0; H, 5·6; N, 6·1. $C_{11}H_{13}O_5N$ requires: C, 55·2; H, 5·5; N, 5·9%.)

Both XV (R = OMe) and XV (R = Me) were refluxed with 2% HCl for 1 hr; the solns, which were filtered from the black amorphous ppts, both contained glycine, which was identified by paper chromatography.

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