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Studies on the more Soluble Complexes of Soil Organic Matter

1. A METHOD OF FRACTIONATION

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This paper is intended as the first of a series dealing with the so-called 'fulvic acid' fraction of soil organic matter. Fulvic acid (after Oden, 1919) is that part of soil organic matter which is peptized by alkali but remains in solution on precipitation of the 'humic acids' with acid. It is proposed in this paper to set forth the method of fractionation adopted and in subsequent papers to deal in more detail with the character of the isolated complexes.

The fulvic acid fraction has not up to now received, in comparison with the humic precipitate, the attention it warrants. As it contains the more soluble soil organic constituents, it should be the fraction most intimately associated with plant growth, and thus be of the utmost importance. Moreover, from recent work by Tiurin (1944) it appears to be the fraction of soil organic matter which effects the mobilization of the sesquioxides, during podzolization.

Little work has been done on the actual isolation of the components of this fraction since Schreiner & Shorey (1909, 1910, 1911) isolated several simple degradation products (amino-acids, purine bases, sugars) in small yield. Since that early period, however, the methods of isolation of organic constituents from natural sources have been greatly improved and augmented.

Since the fulvic extract consists of water soluble components it was thought that it might be fractionated by a method using a chromatographic or general adsorptive technique. After tests under varying conditions with activated alumina, charcoal, various grades of fuller's earth and other clay adsorbents, charcoal was selected as the most suit-

able. The charcoal used was of the quality described as 'animal, purified' (Hopkin & Williams, Ltd).

It was found that if the fulvic extract was adjusted to pH 2.5-3.0 and then run through a layer of charcoal of suitable dimensions, over 90% of the organic matter was adsorbed by the charcoal. This organic matter could be subsequently extracted with various solvents and thus separated into distinct fractions. The details of a method were determined and applied to the fulvic extracts of four soils.

EXPERIMENTAL

Materials

The soils used were as diverse as possible and consisted of samples of

- (1) Heather raw humus,
- (2) Gardener's 'turf' soil,
- (3) *Sphagnum*-cottongrass peat,
- (4) Pine raw humus.

Preparation of the fulvic extracts

A weight of the original oven-dried (at 65°) soil containing 100 g. of organic matter was stirred with 10 l. of 0.5N-sodium hydroxide for 48 hr. It was then allowed to settle and the supernatant dark liquor siphoned off. This suspension was extremely difficult to filter by the usual methods. It was found, however, that when the solution was run through a wide cylinder packed with cotton wool to remove the finely suspended material it could be readily filtered under pressure through a sintered glass funnel (pore size 5-10 μ). Filtration of 10 l. by this method took on the average only 24 hr. To the residue a further 10 l. 0.5N-sodium hydroxide was added and the stirring, settling, and siphoning repeated. The residue was then washed by

adding and siphoning off 10 l. of distilled water. The combined filtrates and washings were acidified with hydrochloric acid and, after the 'humic' precipitate had settled, the acid solution was siphoned off. A sufficient excess of acid was added to retain the sesquioxides in solution. The humic precipitate was redissolved and reprecipitated as before, and the supernatant liquid added to the acid solution. In general, the total volume of the acid solution with washings from the humic precipitate was about 30 l. The pH of this solution was adjusted to 2.5-3.0.

With the exception of the 'turf' soil, the pigments present in the extracts could themselves be used as indicators for the adjustment of the pH, and this was confirmed by quinhydrone electrode determinations. The solutions were straw-yellow at a lower pH and wine-red at a higher pH, passing through an orange point in the pH range 2.5-3.0. With the mineral soil, however, the aluminium and ferric hydroxides present apparently affected the colour changes. At pH 2.5-3.0 the hydrogen ion concentration was sufficient to prevent the precipitation of these hydroxides. The solution at this and lower pH values was a deep red. At pH values near neutral, however, the hydroxides were precipitated along with coloured organic matter, leaving a straw-yellow solution.

The solution at pH 2.5-3.0 was filtered through a very fine paper to remove any sediment present.

Fractionation of the fulvic extract

The fulvic extracts from the four soils (30 l. at pH 2.5-3.0) were filtered in a Buchner funnel with suction applied through pads of animal charcoal, which had previously been thoroughly washed with 0.1 N-hydrochloric acid. The pads were about 2 in. in depth and 6 in. in diameter. All the colour and most of the organic matter was adsorbed on the charcoal. After the 30 l. of extract had passed through, the pad was washed with 500 ml. of 0.1 N-hydrochloric acid and the washings added to the filtrate.

Fraction A was composed of the colourless filtrate and washings.

The charcoal was then eluted successively with the following solutions:

Fraction B. Acetone containing 10% water was first run through the pad. The first 500 ml. of filtrate generally contained most of the colour extracted by this solvent. The extraction was continued until the solvent came through colourless. Generally about 4.5 l. of the solvent were required.

Fraction C. Distilled water was then sucked through. The extract was a clear colourless liquid, which gave a flocculent white precipitate on addition of acetone. The extraction was continued till the extract no longer gave a precipitate on addition of excess acetone. Generally about 1 l. of distilled water was sufficient.

Fraction D. The organic matter remaining adsorbed on the charcoal was then completely eluted with 0.5 N-sodium hydroxide. A deep wine-red eluate was obtained. In general 4 l. were sufficient to elute all the colour.

The fulvic extract was thus separated into four distinct fractions two of which (A and C) were colourless and two (B and D) coloured. One deduction that can immediately be drawn from these results is that there are at least two pigments present in the fulvic extract of markedly different properties.

Individual fractions

Fraction A. This fraction was not fractionated further for the following reasons. With all the materials examined only a very small part of the organic matter was not adsorbed on the charcoal. From a consideration of properties it was obvious that nearly all of the many simple organic substances (amino-acids, purine bases, sugars, etc.) isolated by Schreiner, Shorey and co-workers (1909-13) would occur in this fraction. The filtrate, unlike the original extract, no longer gave a precipitate with ethanol, phosphotungstic acid, or lead acetate, showing that all the organic complexes had been adsorbed. Nitrogen was determined on samples of the filtrate, and by comparison with the nitrogen present before adsorption it was found that 70-80 % of the fulvic nitrogen had been adsorbed.

One litre portions of the colourless filtrates were carefully neutralized with sodium hydroxide. With the mineral soil there was a considerable precipitation of aluminium hydroxide on neutralization showing that the soluble aluminium salts had not been adsorbed. This alumina precipitate, unlike the precipitate obtained on neutralizing the original extract, contained no organic matter. The neutralized and, for the soil, filtered, solutions were taken to dryness under reduced pressure and extracted with ethanol. The residues left after the ethanol extraction contained no organic matter. The ethanol was then removed under reduced pressure and the residues extracted again with ethanol which after evaporation left in every case only a very small amount of a light brown syrup. This syrup was rich in nitrogen and gave a strong α -naphthol test for sugars. It reduced Fehling's solution and gave the phloroglucinol and orcinol tests for pentoses.

The study of this fraction was not continued further. It should, however, be pointed out that by this method the simple degradation products are easily obtained separately from the complex and colouring matters, and the fraction could, therefore, be worked up without the tedious use of metallic precipitants and clarifiers required for Schreiner & Shorey's (1910) method.

Fraction B. The first 500 ml. portion of the eluate from each of the four soils, was a deep wine-red colour. Generally, a small amount of a white flocculent precipitate, which proved identical with the material eluted with distilled water, formed in this first portion. The reason for this is presumably that at the start of the elution the acetone was first rapidly adsorbed by the charcoal, leaving the water it contained to elute this fraction but, since the charcoal soon became saturated with acetone, the acetone ran through and precipitated in the filtrate jar the water-eluted contaminant.

This material was filtered off to be added to the water-eluted fraction C. The total acetone eluate was transferred to a distillation apparatus and the acetone distilled off on a water-bath at 50–60°. The water was then removed under reduced pressure at the same temperature and the residue extracted with absolute ethanol.

All the colour and almost all the organic matter was soluble in ethanol; only a very slight colourless residue of a carbohydrate nature, with a very high ash (NaCl) content was left. This gave positive α -naphthol, phloroglucinol, and orcinol tests. The peat was the only material which gave a sufficient amount of this residue for a carbon estimation and the result, 38% C, agreed with the carbohydrate properties of the material which was nitrogen free.

The ethanolic solution of the bulk of the fraction was evaporated down under reduced pressure and excess ether added. The dark red amorphous precipitate was filtered, washed with ether, then redissolved in ethanol and reprecipitated with ether several times for purification. It was finally dried *in vacuo* and ground to a red powder.

	Yield (g./100 g. original organic matter)
Heather raw humus	3.39
'Turf' soil	2.13
Peat	2.51
Pine humus	4.35

The yields quoted are minimal, i.e. the amounts actually isolated after purification.

These powders were readily soluble in water, ethanol and acetone. On standing in the presence of air, the dry powders and their aqueous solutions slowly darkened in colour till finally, with the powders, black tars were obtained. The fresh powders all gave positive Molisch, phloroglucinol and orcinol tests showing the presence of sugars, and in particular pentoses. Their aqueous solutions also gave dark red to purple colours with ferric chloride solution indicating the presence of phenolic compounds; on hydrolysis with dilute acid a dark-coloured precipitate formed and the remaining solution strongly reduced Fehling's solution.

It is obvious from these properties that the substances are phenolic glycosides. They had the following carbon contents:

	Heather raw humus	'Turf' soil	Peat	Pine humus
Carbon (%)	43.71	45.24	47.31	46.35
Ash (%)	2.51	3.06	2.62	2.34

The isolation of these compounds in the yields shown from four widely diverse types of soil organic matter demonstrates that a quantitatively important glycoside fraction is widely distributed in

soil organic matter. Shorey (1913) isolated a glycoside in unstated yield from a New York soil.

The instability of these glycosides in air is interesting since it suggests that they may be intermediate products in the formation of humic acids. Both the carbohydrate and phenolic constituents are being investigated.

Fraction C. To the distilled water eluate which had been run through the charcoal, were added 3 vol. of acetone. A voluminous flocculent white precipitate was formed which was allowed to settle. The precipitate was washed with pure acetone by decanting, and then filtered in a Buchner funnel. To it was added the small precipitate obtained in fraction B.

The total precipitate was purified by redissolving in water, filtering, and reprecipitating with acetone. It was again washed with acetone by decanting, filtered, and finally washed with ether. It was then transferred to a Petri dish and dried *in vacuo* to a clear transparent gel membrane having somewhat the appearance of gelatin. It was brittle in thick sheets but flexible in thin sheets.

	Yield (g./100 g. original organic matter)
Heather raw humus	1.64
'Turf' soil	1.32
Peat	1.60
Pine humus	1.94

The physical appearance and properties of this substance tended to suggest that it was pure, and, moreover, identical from all four sources. This identity was confirmed since the material from all four sources on heating started to redden at 194° and decomposed to a black friable mass at 200°.

Depending on the method of drying the substance could be obtained in clear pliable sheets, as an easily powdered white mass, or as a hard brittle translucent gum. After drying it was but slowly dissolved by water, softening to a sticky gel before going into solution. It was readily soluble in alkali and was acid to litmus. It gave a titration equivalent of about 1500 when titrated with 0.02N-sodium hydroxide. Its high molecular colloidal structure was confirmed by its inability to dialyze through parchment. After hydrolysis with acid the hydrolysate strongly reduced Fehling's solution. The usual sugar tests were applied. It gave a strong positive α -naphthol test confirming its carbohydrate nature. Both the Seliwanoff and mucic acid tests were negative showing that neither ketoses, nor galactose nor galacturonic acid, were present. It gave, however, a positive saccharic acid test showing that glucose and/or glucuronic acid units were present in its molecule. It also gave positive phloroglucinol and orcinol tests indicating the presence of uronic acids and/or pentoses. The solution obtained by

hydrolysis for 4 hr. with 2 % (w/v) hydrochloric acid gave a positive naphthoresorcinol test showing that glucuronic acid was a component. The carbohydrate gave a reddish purple colour with iodine solution. Obviously the material was a polysaccharide, containing glucose and glucuronic groups and perhaps also pentose groups.

This first isolation of a polyuronic acid from soils confirms the many indications of the presence of uronic units obtained by the study of the evolution of carbon dioxide from soils on heating with acids (Shorey & Martin, 1930; Norman & Bartholomew, 1943). This carbohydrate, which can be obtained pure and in relatively large yield from a wide variety of soils, should be of considerable importance in the study of soil organic matter. A vigorous chemical study of its structure by the methods of the carbohydrate chemist is obviously warranted.

Fraction D. This fraction contains all the substances present in the original fulvic extract that have not been eluted by the previous solvents.

The anode, cathode and central liquids and the precipitate gave a negative biuret test, indicating that no proteins or other polypeptides were present. Hobson & Page (1932) also failed to obtain a positive biuret test with the total fulvic extract and assumed that this was due to interference by the colour of the fulvic acid. Most of the solutions in the present study were only lightly coloured or colourless, but despite this, no proteins were observed.

In dealing with the bulk of fraction D the 5 l. of alkali solution were first subjected to ordinary dialysis in a bell jar, with parchment clamped over the wide end, suspended in a tank of running distilled water. The dialysis was allowed to continue till colour just started to come through the membrane after about 1 week. The pH values of the solutions were then about 8-9. These solutions could then be electrodialed to completion quickly, each 750 ml. lot requiring only 24 hr.

The precipitate formed at the anode membrane was filtered off in each case and its properties further

Table 1. *Electrodialysis of soil fulvic fraction D*

Time (hr.)	Anode cell			Centre compartment	
	Volume (ml.)	pH	Colour of solution	Colour of solution.	pH
0	1st 700	—	Colourless	Wine red	—
1	2nd 700	12.5	"	"	—
2	3rd 700	—	"	"	—
3	4th 700	12.0	"	"	—
4	5th 700	—	"	"	—
20	6th 700	11.2	Faint yellow	"	—
27	7th 700	—	Deep yellow	Lighter red	—
45	8th 700	8.4	Red	Colourless solution	—
65	9th 700	—	Faint yellow	with ppt. on anode	—
90	10th 700	6.0	Colourless	membrane	6.27

The problem in isolating this fraction is to remove the alkali. Accordingly, electrodialedysis of the fraction was studied.

A 750 ml. portion of the deep wine-red sodium hydroxide solution from the 'turf' soil fraction D was electrodialed in a three-cell dialyzer against parchment with running distilled water in the cathode cell. The anode liquor was changed frequently, the temperature being allowed to rise to a maximum of 60° and the current to 2 amp. The changes observed are shown in Table 1.

After 90 hr. the central compartment was washed out with distilled water and the suspension filtered. The precipitate in the centre cell was obviously a mixture. It deposited at the anode membrane as a colourless film streaked with a red resin-like material. The filtrate from the centre chamber contained little organic matter.

The coloured anode solutions (6-9, Table 1) were collected, but although quite richly coloured they were found on evaporation to contain little organic matter. Indeed, most of the organic matter was included in the precipitate at the anode membrane.

studied. These precipitates would appear to be the main constituents in fraction D. They were dried *in vacuo* and ground to a brown powder.

	Yield (g./100 g. original organic matter)
Heather raw humus	1.68
'Turf' soil	2.60
Peat	0.38
Pine humus	1.56

	Analysis			
	C (%)	H (%)	N (%)	Ash (%)
Heather raw humus	48.44	5.49	4.17	2.16
'Turf' soil	47.25	5.61	5.87	4.10
Pine humus	—	—	3.50	1.47

Although relatively rich in nitrogen, solutions of the substances did not give any positive protein tests. They gave, however, positive phloroglucinol and orcinol tests for pentoses and contained, moreover, considerable amounts of phosphorus. They were acidic, being readily soluble in alkali, and were precipitated from solution by ethanol, lead acetate,

and by neutralization in the presence of aluminium salts.

This fraction appears to have properties similar to fractions isolated by Shorey (1913), Bottomley (1919), and Wrenshall & Dyer (1941).

The fractionation of fulvic nitrogen

The separation previously discussed is represented diagrammatically in Table 2. Nitrogen was determined in each fraction and the distribution of the fulvic nitrogen between these fractions was calculated. It is readily seen that the greater part of the nitrogen complexes is concentrated in fraction D.

Table 2. *The distribution of the total fulvic nitrogen between the individual fractions*

Fulvic extract 100			
Not adsorbed		Charcoal adsorbed	
		Eluted	
Filtrate fraction A	Acetone fraction B	Water fraction C	Alkali fraction D
Colourless	Coloured	Colourless	Coloured
<i>S</i> 21.3	<i>S</i> 7.9	<i>S</i> 3.4	<i>S</i> 52.4
<i>Pi</i> 31.7	<i>Pi</i> 12.2	<i>Pi</i> 7.3	<i>Pi</i> 40.9
<i>H</i> 24.8			<i>H</i> 45.3
<i>P</i> 30.4			<i>P</i> 37.8

S = percentage soil fulvic nitrogen in fraction.

Pi = percentage pine humus fulvic nitrogen in fraction.

H = percentage heather humus fulvic nitrogen in fraction.

P = percentage peat fulvic nitrogen in fraction.

The application of the methods of fulvic fractionation to the hydrolyzable fraction of the humic precipitate

It was observed during the purification and fractionation of the humic precipitate, not reported here, that as much as 50 % of it could be hydrolyzed with dilute acid. It is generally considered that this fraction consists mainly of hemicelluloses, possibly from bacterial gums (Winogradsky, 1929) but it was thought that a proportion of the hydrolyzable material might consist of the fulvic constituents already studied. All these constituents are acidic polymers and all are strongly adsorbed from acidic solutions. It was thought extremely likely that the humic acid, with its very large surface area, might adsorb on precipitation large amounts of the fulvic constituents which would only be very slowly removed by reprecipitation. Accordingly the following experiment was performed.

Humic precipitate (200 g.), which had been obtained from the soil, and purified by redissolving and reprecipitating twice, was refluxed for 1 hr. with 8 l. of distilled water at 100°. The suspension was filtered and the residue washed, dried and weighed. The weight was 162 g., leaving 38 g., or 19 % of the original, for the soluble fraction. The first filtrate was subjected to the fulvic fractionation.

The residue was suspended in 8 l. of distilled water and neutralized with dilute ammonium hydroxide. The solution was then refluxed for 2 hr., acidified and filtered. The residue was again washed, dried, and weighed. It weighed 128 g., and 34 g., or 17 % of the original, had gone into solution; it still contained 24.6 % of material hydrolyzable with 2 % (w/v) HCl.

The second filtrate was also subjected to the fulvic fractionation. The fractions obtained from the humic precipitate had all the properties, already described, of the fulvic fractions. The yields are shown in Table 3.

Table 3. *Yield of fulvic fractions from soil humic precipitate*

Fraction	First extract		Second extract	
	Yield (g.)	Humic precipitate (%)	Yield (g.)	Humic precipitate (%)
B	15.64	7.82	8.08	4.04
C	1.92	0.96	Trace	—
D	5.20	2.60	12.57	6.28

From these results it is seen that a considerable part (in this case 21.7 %) of the humic acid precipitate consists of constituents normally found in the fulvic extract. These contaminants of the humic acid appear to be removed only to a small extent by reprecipitation, more so by boiling water and completely by hydrolysis with dilute acid.

The fulvic fractionation of the water-soluble fraction of soil organic matter

The chief criticism which has been levelled against workers isolating organic constituents from soils is that the products obtained may not be actual *in situ* constituents of the soil but artefacts produced by the extractants used. It may be argued that the fractions isolated from the fulvic extracts are formed by the alkali used as extractant. To refute this possibility it is necessary to find out whether the same constituents can be obtained by the use of an extractant unlikely to produce any changes in the products.

It was thought that they might be obtained simply by extracting soils with water, although naturally in much smaller yields. Accordingly, a weight of the 'turf' soil equivalent to 100 g. of organic matter was refluxed for 1 hr. with 4 l. of distilled water, and then filtered and washed with a further 1 l. of distilled water. The extract had a pH of 4.38. Portions of the extract were taken for determination of nitrogen and organic matter and the remainder subjected to the usual adsorption on charcoal after adjusting the pH to 3.0. The eluate was colourless. Its nitrogen and organic content were measured. The charcoal was then eluted with acetone, water and alkali as before and the yields obtained are shown in Table 4.

These extracts were much lighter coloured than those from the fulvic extracts but the products obtained had properties identical with those of the fulvic constituents previously discussed.

Table 4. *Fractionation of water-soluble fraction of soil organic matter*

Water-soluble organic matter (g./100 g. organic matter)		Water-soluble nitrogen (mg./100 g. organic matter)	
Total water extract	5.98	Total nitrogen	210
Fraction A	3.15		
Fraction B	0.38		
Fraction C	1.06		
Fraction D	1.14		
Nitrogen adsorbed (complex nitrogen)		37.6 %.	
Organic matter adsorbed (complex substances)		47.3 %.	

As might be expected, a much higher proportion of the simpler substances (fraction A) was observed here. These results show that the fulvic constituents are present in the soil as such. Fraction C especially is easily extracted by water.

SUMMARY

1. A considerable advance has been made in the study of the fulvic extract of soil organic matter. A new method has been introduced whereby the fulvic extract is separated by a selective adsorption technique into four distinct fractions.

2. These fractions have been obtained from the fulvic extracts from four widely diverse types of soil.

3. A preliminary examination of the chemical properties of the fractions has been made.

4. A pure polysaccharide containing uronic acid groups has been isolated from the four soils. The presence of a glycosidic fraction in the four soils has also been shown.

5. The fulvic nitrogen has been shown to be in part contained in nitrogenous acidic complexes and in part as simple degradation products.

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A Polarographic Study of Acid-insoluble Fractions from Iodo-proteins

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In the study of iodinated proteins, attempts to correlate the thyroxine iodine and the biological activity of these thyro-active substances have met with varied success. Deanesly & Parkes (1945) express the view that most of the acid-insoluble iodine as obtained by the accepted method (British Pharmacopoeia, 1932) is not true thyroxine iodine and that more evidence is required before the actual relationship between biological activity and thyroxine content can be measured.

More recently, Reineke & Turner (1945) claim to

have obtained close correlation between biological activity and the thyroxine content of iodinated casein. The method adopted was to hydrolyze the iodinated protein in a baryta solution and to extract the hydrolysate with *n*-butanol. The thyroxine content was then calculated from iodine estimations done on the purified *n*-butanol extracts.

The present paper describes some preliminary work on the application of the polarographic wave of thyroxine to certain acid-insoluble fractions obtained from the baryta hydrolysis of iodinated proteins.