CHAPTER VII

Chromatographic Analysis
INTRODUCTION

The fractions into which Oden (01) divided soil humus are definitely not single substances; but it is not yet known with certainty how complex these fractions may be. In order to throw light on this aspect of the problem of soil organic matter attempts have been made by a number of workers in resolving humic acids by the application of chromatographic techniques.

Adsorption column chromatography was tried by Hock (H1) who attempted to resolve natural humic, hymatomelanic and fulvic acids through columns of alumina, examining for fractionation with both visible and ultraviolet light. Recently Kononova et al (K2), using alumina as well as starch columns, claimed an improvement as judged by the same criteria. Forsyth (F1) separated natural humic acid into two fractions by selective adsorption on a bed of active charcoal followed by elution with suitable solvents. More recently Bromfield, Coulson and Davies (B2) attempted fractionation of humic acid on a column of celite '535' using acetone-water and acetonitrile-neutral sodium pyrophosphate as gradient eluants. Using a large number of synthetic ion-exchangers like 'Zeo-Karb' 225 and 226, Amberlite G.14, De-Acidite G, the cellulose ion-exchangers DEAE - cellulose and ECTEOLIA - cellulose, Coulson et al (C1) found no good fractionation of humic acids. By developing a column of cellulose by a gradient of iso-propanol into ethyl acetate (1:1), then water
into the iso-propanol, Sowden and Deuel (8) achieved some separation of fulvic acids into several distinct components, some of which were found to fluoresce.

PRESENT WORK

In the present investigations on soil organic matter some preliminary attempts have been made to resolve the crude humic and the alcohol soluble humato-melanic acids on columns of cellulose and humic acid on an alumina column.

EXPERIMENTAL

(1) About 151 to 160 mgs. of electrodialysed humato-melanic acid was mixed with a little of the chromatographic cellulose powder (pretreated) (No.123. Made in Germany) and this was layered on a column of cellulose (79 cm X 1.2 cm) which had been prepared by using a slurry in 100% dioxane. Elution was started with a mixture of dioxane and water in the ratio of 9:1. The proportion of water, the diluting liquid, was increased in discontinuous increments. The composition of the final eluting liquid is a mixture of dioxane and water in the ratio of 1:9. Fractions (10 ml.) were collected at the rate of 12 drops per minute.

(11) 134 mgs. of crude humic acid was mixed with a little of the cellulose powder and made into a paste by adding a buffer of pH 4.92. The paste was placed suitably on the cellulose column (85 cm X 1 cm) which had been set first in pure water and then washed several times with an acetate buffer of pH 4.92 which was also the eluting liquid. The pH was increased in discontinuous increment as shown in the elution
The pH of the final eluting liquid was 6. Fractions (10 ml) were collected at the rate of 10 drops per minute. An attempt has been made to resolve humic acid on alumina column using pyridine-water as an eluant. The proportion of water, the diluting liquid, was gradually increased at the rate of 10%.

The progress of fractionation was followed by measuring the percentage transmission at a fixed wavelength (535 mµ) in Leitz colorimeter using a cell of 1 cm. thickness. The percentage transmission readings were plotted against tube nos. as shown in Fig. VII. 1.1 which is the elution diagram.

RESULTS AND DISCUSSION

Hymatomelanic acid as evident from the elution diagram (Fig. VII. 1.1) consists of two main fractions. One fraction, distinctly yellow in colour, travelled down the column rapidly when the eluant composition was 9:1 dioxane water mixture. Collection was started when this yellow band came near the end of the column. The fractions in tubes 4 to 11 were collected together, the eluant was removed by evaporation on water bath, the residue was taken in spectral alcohol. The major portion of hymatomelanic acid came out between the tubes 56 to 75 in a finely dispersed state. The eluant composition was 1:9 dioxane-water mixture. This fraction was brown in colour. The materials in tubes 62 to 75 were collected together. Dioxane was removed by evaporation on water bath. The suspension was electrodialysed and kept for spectral fluorescence study.
The elution diagram shows definitely that resolution of crude humic acid on cellulose column is not a good one. Only two main peaks are discernible. The first peak preceded by a slight hump appears between the tubes 11 to 20. The eluant buffer had a pH of 4.92 in this zone. The major portion of crude humic acid came out between the tubes 56 to 72 when the eluant buffer had a pH of 5.8. The flat peak in this zone indicates that further resolution of the material in this region is possible. Further study with the materials appearing under the peaks has not been carried out.

The two fractions from hymatomelanic acid exhibit greenish fluorescence. The highly mobile fraction fluoresces with maximum appearing in the region 508 - 514 m\(\mu\) in its fluorescence emission spectrum (Fig. V. 1.4). The less mobile fraction exhibits a maximum in the region 530 - 534 m\(\mu\) (Fig. V. 1.4).

Resolution of alcohol and acid insoluble humic acid on an alumina column was attempted using pyridine-water as an eluant. The major portion of humic acid remained strongly adsorbed on alumina column, which was found too difficult to be eluted. Only a small fraction was isolated when the pyridine-water composition was 70:30. The material in this fraction was precipitated with acid and the precipitate was purified by electrodialysis. Infra-red spectra (Fig. V.3.4) of this fraction revealed no difference from that of the parent humic acid.

During the fractionation study on cellulose and alumina columns it was observed that humic acids at first separated into several distinct zones; but as the zones travelled downward, spreading of the zones and consequent overlapping between them...
occurred. This made the resolution ill-defined.

The results obtained in the present investigation give grounds for believing that the apparent but usually unsatisfactory evidence of the separation of humic acids into fractions by adsorption column chromatography does not represent a separation of chemical entities but is essentially a separation of different size particles.
FRACTIONATION OF ORIGINAL HUMIC ACID ON CELLULOSE COLUMN

- BUFFER pH = 9.2
- BUFFER pH = 5.2
- BUFFER pH = 5.8
- BUFFER pH = 6.0

COLUMN: CELLULOSE (85 cm X 1 cm BED)
FRACTION SIZE: 10 ml
FLOW RATE: 10 drops per minute
LOAD: 15-20 mg of HYMATOMELANIC ACID

COLUMN: CELLULOSE (79 cm X 1.2 cm BED)
FRACTION SIZE: 10 ml
FLOW RATE: 12 drops per minute
LOAD: 15-160 mg of HYMATOMELANIC ACID

FIG. XIII. 1
FRACTIONATION OF HYMATOMELANIC ACID ON CELLULOSE COLUMN

TUBE NO.

% TRANSMISSION AT 529 m.μ.
CHAPTER VII
Section 2
PAPER ELECTROPHORESIS

Introduction

The transport properties of humic acid in free solution are known since the days of Sprengel in 1826. Fractionation of humic acids by paper electrophoresis was initiated by Robinson, Martin and Page (R1), Swaby (S5), and Bremmer and Arnold (B1). Swaby (S5) and Bremner and Arnold (B1) observed only one component by this means whilst others claimed more than one component. Pavel (P1), Welte (W4), Scheffer et al (S6), e.g. Pavel et al (P1) observed several fluorescent areas on paper electrophoresis. It was observed that synthetic humic acids prepared by the oxidation of polyphenols showed similar patterns on paper (S6). Continuous paper electrophoresis failed to produce any significantly different fractionation of the humic acids as far as could be judged from the amino acids of the fractions (S5). Resolution of humic acids by paper ionophoresis was attempted by Pospishil (P7), Noda et al (N2). Resolution of humic acids by continuous paper electrophoresis was attempted by Burges (B15) and Kaurichev et al (K9). Burges noted that the electrophoretic separation of humic acid isolated from the B1 horizon of a podzol into bands is due to separation into particle size groups rather than chemical entities.

Object of the present investigation

To examine the heterogeneous character of soil organic matter preparations, paper electrophoretic study of crude humic-
humic- and hymatomelanic acids was carried out.

Experimental

Paper electrophoresis was carried out on whatman chromatographic paper (No.1) cut into strips 56 cm. X 6 cm. and at 13 V/cm. in two buffers of pH 6.77 and 9.11 respectively. About 0.1 mg material dissolved in alkali was spotted at a distance of 12.5 cm. from one end of the paper. The experiment was run continuously for 2 hours.

Results and Discussion

The electrophoretograms (shown in Figs. VII.2.1 - 2.3) reveal that some fractionation, though unsatisfactory, of humic materials have occurred. Usually three main fractions are recognized.

(i) A small immobile component.
(ii) A principal mobile component.
(iii) A streaky fraction with migration values between those of (i) and (ii).

Hymatomelanio acid is more mobile than crude humic- and humic acids. The electrophoretograms exhibit greenish fluorescence under U.V. light.
FIG. VII.2.1
HYMATOMELANIC ACID
(pH 9.1)

FIG. VII.2.2
CRUDE HUMIC ACID
(pH 9.1)

FIG. VII.2.3
HUMIC ACID
(pH 9.1)