Macromolecular changes of humic substances induced by interaction with organic acids

A. PICCOLO, S. NARDI* & G. CONCHERI*

Dipartimento di Scienze Chimico-Agrarie, Università di Napoli 'Federico II', Via Università 100, 80055 Portici, Italy, and
*Dipartimento di Biotecnologie Agrarie, Università di Padova, Via Gradenigo 6, 35131 Padova, Italy

Summary

Two different humic materials, one from a forest soil and the other from wormcasts, were used to study the influence of mineral and organic acids on the conformational properties of humic substances. The macromolecular changes were followed by low pressure gel permeation chromatography after titrating humic material to low pHs with acids. All organic acids (mono-, di-, tri-carboxylic, and oxy-acids), added to humic solution prior to a gel permeation in an alkaline buffer, were able to shift the totality of absorbance of the humic chromatographic peak from high to low molecular sizes. Mineral acids, phenol, alcohols, dipolar aprotic solvents, could not produce the same shift and gave total absorbance at the column void volume as in the case of humic substances alone. The chromatographic peak shifted back to elution volumes proper of higher size molecules when the humic-organic acid mixture was back-titrated to high pHs before gel permeation. Elution in a much stronger alkaline buffer did not change the overall macromolecular behaviour.

These results suggest that humic substances behave as micelles in solution and that hydrophobic bondings play an important role in holding humic molecules together. The organic acids enter the interior of the humic micelle-like aggregates and alter the stereochemical hydrophobic arrangement of the humic material. In alkaline conditions the negative charges developed disrupt the apparent high molecular size configuration and disperse the humic aggregates into small micelles. Such conformational properties of humic substances appear to be a function of pH and of the concentration of organic acid.

Introduction

Humic substances are natural, organic substances ubiquitous in water, soil and sediments. Due to their chemical heterogeneity and polydisperse nature, the secondary chemical structure of humic substances is still ill-defined and so is their conformational arrangement (Stevenson, 1994). Visser (1964) first introduced the concept of rigid globular particles to account for the macromolecular structure of humic substances, i.e. molecular size, shape, and weight. Cameron et al. (1972) and Orlov et al. (1975) later attributed to humic material a more flexible nature and an ellipsoidal shape and proposed a randomly coiled polymeric conformation. Further experiments were interpreted with a macromolecular structure varying concentration, and the pH and ionic strength of the medium (Chen & Schnitzer, 1976; Ghosh & Schnitzer, 1980). Concurrently, Schnitzer (1978) suggested that humic substances are not single molecules but rather associations of molecules of different natural origin. Recently, he has shown by Transmission Electron Microscopy (TEM) that with increasing pH or humic substances concentration, a finely woven network of elongated fibres is formed which then coalesces into a sheet-like structure, perforated by voids of varying dimensions, which can trap or fix organic and inorganic components (Schnitzer, 1994).

Wershaw (1986) postulated an alternative description for the macromolecular structure, namely that humic substances in solution form mixed aggregates or micelles and that humic aggregates are held together by weak bonding mechanisms such as H-, and π-bonding, and hydrophobic interactions. Barak & Chen (1992) have recently endorsed the micelle-like model in which the hydrophilic ionized groups are located at the interface of the humic substances with solution, whereas the hydrophobic portions of the molecule are likely to arrange themselves in the interior of the macromolecule.

Nardi et al. (1988) showed earlier that acetic acid added to humic macromolecules could separate small-size humic fractions when the mixture was subjected to dialysis. The low molecular size fractions thus obtained stimulated specific biological properties in plants (Nardi et al., 1991). Piccolo
et al. (1992) related the structural characteristics of humic substances to plant biological activities and found that the small-size fractions were more active than the humic material from which they were separated. These properties were attributed to the increased concentration of acidic functional groups in low molecular size fractions separated by the acetic acid treatment. However, the molecular process of the separation of low molecular-size fractions from the main humic material remained unexplained.

We have investigated the mechanism of such disaggregating phenomenon activated by acetic acid. We followed the effect of different organic acids on the molecular size of humic substances by gel permeation chromatography. Moreover, since the macromolecular structure of humic substances depends on pH and ionic strength of the medium, we assessed the variation of these factors on the gel permeation behaviour of the humic materials.

**Materials and methods**

**Humic substances extraction and characterization**

Two different humic substances were obtained from air-dried samples of: (1) the A horizon of a Calcic-Luvisol under beech (*Fagus sylvatica*) from a site near Belluno, Venetia, Italy (HS₁), and (2) faeces of a mixture of *Allolobophora rosea* and *A. caliginosa* (HS₂) that are common earthworm species responsible for producing humus in soil. The casts were isolated from earthworms living in a soil substrate different from HS₁. Extraction was done with a 0.1 N NaOH solution at room temperature for 16 h under N₂ atmosphere as outlined by Stevenson (1994). Extracts were separated from suspended material by centrifuging at 7000 g at 10°C for 30 min. The supernatant solution was dialysed, using Visking tubes with 18 KD cut-off, against distilled water until the liquid outside the dialysis tube remained colourless. The humic solution was then purified by eluting through cation exchange resin Amberlite IR 120 in a protonated form and freeze-dried.

Humic extracts (HS₁ and HS₂) were characterized by elemental analysis using a Carlo Erba Elemental Analysers 1108. Quantitative 13C-NMR spectra of the extracts in solution were recorded using a Varian XL 300 NMR spectrometer at 75.4 MHz. Sample preparation and conditions applied to obtain suitable quantitative intensity distribution are reported elsewhere (Piccolo et al., 1992). The number of scans ranged from 80000–100000. The free induction decays (FID) were processed by applying a 50 Hz line broadening and baseline correction was done by the Varian software. The chemical shift was expressed in ppm on a scale relative to external sodium 3-methylsilyl-propionate (TSP) at 0 ppm. The spectra were divided into the following areas: 0 to 48 ppm, aliphatic C; 49 to 105 ppm, substituted C (amino acids, carbohydrates, etc.); 106 to 145 ppm, aromatic C; 146 to 165 ppm, phenolic C; 166 to 190 ppm, carboxyl C. Areas were measured by an automatic integrator.

**Preparative gel permeation chromatography**

HS₁ was redissolved in 0.02 M Na₂B₄O₇ buffered at pH 9.2 and applied on a K 100/100 Pharmacia column packed with Sephadex G-100. By elution with 0.02 M Na₂B₄O₇ (pH 9.2), the high molecular weight fraction (HFI) eluted at the void volume (<100 000 D) was separated from the low molecular weight fraction (<100 000 D) that diffused instead through the gel. The elution was repeated enough times to collect the required material to proceed with the experiment. The HFI fraction was then dialysed extensively against water and freeze-dried. An aliquot (114 mg) of this high molecular weight fraction was resuspended in 150 ml of doubly distilled water, dissolved by addition of KOH 0.5 m up to a solution pH of 11.8, and stored under N₂. This stock solution of HFI contained approximately 750 μg ml⁻¹ of humic material.

**Analytical gel permeation chromatography**

Samples from HFI, HS₁, and HS₂, treated with chemicals as described below, were applied on a LKB, K 16–70 column, packed with a BioRad P100 Biogel (molecular range: 5–100 KD). The gel packing solution and the eluent were a Na₂B₄O₇ 0.02 M solution buffered at pH 9.2. The elution was maintained at 22.2 ml h⁻¹ by a peristaltic pump (Gilson Miniplus 4), and the absorbance of eluates was recorded at 280 nm by a continuous flow spectrophotometer (Gilson Spectrochrom M). The column was previously calibrated using globular proteins from the Serva Fein Biochemica Kit MS II. In the experiment to evaluate the effect of ionic strength the gel permeation chromatograms were obtained by using the more concentrated 0.1 M Na₂B₄O₇ as gel packing solution and eluent.

**Addition of organic acids**

In a first experiment, inorganic and organic acidic compounds (see Table 1) were added to aliquots of the HFI stock solution to lower the pH of the humic solution from 11.8–2. The added chemicals, their final concentrations in the mixture, and the pH reached in these mixtures prior to gel permeation chromatography are reported in Table 1. A 1 ml aliquot of each mixture was used in an analytical gel permeation chromatography to evaluate the changes in molecular size distribution in comparison to the untreated humic material.

In a second experiment the effect of varying the pH of the humic solution with addition of organic acids prior to gel permeation was studied. A series of 25 mg samples of the original HS₁ and HS₂ were suspended in 4 ml of doubly distilled water, redissolved by the dropwise addition of 0.5 N KOH and titrated with acetic acid to lower the pH of the humic solution to 6, 4.5, 3.5, and 2. The molecular size distribution of these humic solutions was evaluated by the
Table 1 Percentage of absorbance of humic material eluted through gel permeation

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<th>Added Compounds</th>
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<th>Concentrationb (μmol l⁻¹)</th>
<th>% Absorbance in MW intervalsc</th>
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* pH of the final mixture of humic substances and added compound, prior to the application on the column.  
  a Concentration of the added compound in the final mixture with humic substances, prior to the application on the permeation column.  
  c % of absorbance, at 280 nm, measured as % of area under chromatographic peaks at elution volumes corresponding to molecular weight ranges assessed by a previous column calibration based on standard proteins (Serva, Kit MS-II).  
  d Monocarboxylic acids were added as pure solutions from the reagent bottle. Dicarboxylic acids were added at the same concentration used for acetic acid except for those whose water solubility was limited.  
  e Alcohols were added in mixture with water (v/v) or in mixture with a HCl solution of reported molar concentration. The dipolar aprotic solvents, acetone and dimethylsulphoxide (DMSO), were added only as HCl mixtures.
analytical gel permeation chromatography (Fig. 2, A to E).
Another set of humic solutions that had been previously
titrated to pH 2 by acetic acid, were then brought back to pH
3.5, 4.0, 6.0, and 8.5 with 0.5 N KOH and the resulting
molecular size distribution was checked again (Fig. 2, F to I).
The humic solution brought back to pH 8.5 was then roto-
evaporated under vacuum at 30°C to reduce the added organic
acids (Fig. 2, J).

A third experiment was done to verify if the variation in
chromatogram shape might have been due to changes in ionic
strength rather than to addition of the acetic acid. A second
series of HS2 alkaline solutions were titrated to the same lower
pHs with acetic acid, back-titrated to higher pHs with KOH,
and roto-evaporated as described above, but eluted through the
permeation gel with a more concentrated solution of the
Na2B4O7 alkaline buffer (Fig. 2). A series of HS1 solutions
(at pH 11.8) were again treated as above but using formic acid
to vary the pH of the humic solution. Both low and high ionic
strength alkaline buffers were used in these gel permeation
chromatograms (Fig. 3). All additions of organic acids as well
as the titration with formic and acetic acid were done with an
Orion Research 960 automatic titration apparatus.

**Results and discussion**

**Characteristics of the humic substances**

The solution-state NMR spectra of the two humic substances
used in this study are shown in Fig. 1. The relative distribution
of 13C intensities within the different ppm ranges obtained by
integrating the NMR spectra are reported in Table 2 together
with elemental analysis and ash content of the humic extracts.
The humic material extracted from the forest soil (HS1),
appears richer in aromatic, phenolic, and carboxylic C than
the extract from the earthworm faeces (HS2) that, in turn,
contains more aliphatic and O-alkyl carbon. Kögel-Knabner
et al. (1988) presented solid-state NMR spectra and relative
quantitative data for humus extracted from soils under beech
similar to the one of this study (HS1). However, these authors
found more aliphatic and aromatic C than we did and a
smaller content of O-alkyl and carboxylic C. Ziegler & Zech
(1992) reported solid-state quantitative data on the 13C distri-
bution in the casts produced by E. fetida, but their carbon
distribution was richer in aromatic and O-alkyl carbons and
poorer in alkyl carbon than our humus from wormcasts. These
differences may be due to variation in humic material but also
Macromolecular changes of humic substances

The content of elemental carbon in both HS₁ and HS₂ is more characteristic of fulvic than humic acids (Schnitzer, 1978). This is probably because our isolation method did not separate fulvic from humic acids and because of the nature of the original substrates which were not highly humified. Elution over a cation exchange resin, effectively reduced the ash content of HS₁ and HS₂ to the usual levels reported in humic substances studies (Table 1). Further purification with the traditional HCl-HF was avoided because of the alteration produced on the humic substances (Piccolo, 1988).

Effect of organic acids

The variations in molecular size distribution of the humic fraction HFI observed with the addition of different acids are summarized in Table 1. This table shows that HFI, when simply dissolved in the alkaline eluting solution produced a gel permeation chromatogram with only one peak eluted at the void volume (V₀), indicating that all of the recorded absorbance was related to humic material with nominal molecular size higher than the molecular weight cut-off of the permeation gel (100 KD). Similar chromatograms were obtained when the alkaline HFI solution was brought to pH 2 with mineral acids such as HCl and H₂SO₄. Conversely, a dramatic change in the chromatogram was observed when the HFI solution was lowered to pH 2.1 with organic monocarboxylic acids containing a different number of aliphatic carbon. In fact, the use of formic, acetic, propionic, and butyric acids, produced a single chromatographic peak (100% of total absorbance) but at the elution volume relative to material of molecular size less than 25 KD. Despite the different concentrations used (the pure acids were employed), the shift of the absorbance to larger elution volumes was similar for all monocarboxylic acids, except for benzoic acid, the solubility of which in water is so low that the most concentrated water solution (0.03 M) did not lower the pH of the humic solution below 4.1.

The addition of dicarboxylic acids to humic samples generally produced a similar shift of the chromatographic peak to larger elution volumes (Table 1), though the concentrations used varied according to the different solubilities of the acids in water. Fumaric acid represented an exception since it did not give the shift of the humic absorbance from void volume to larger elution volumes. Again, this difference can be attributed to the acid's poor solubility in water and to the consequent small amount of the acid in contact with humic substances. The tricarboxylic citric acid also produced the absorbance shift to lower molecular size regions, as did the oxyacids, glycolic and glyoxylic acids. Totally ineffective were the additions of alcohols (ethanol, butanol and glycerine), phenol, and of dipolar aprotic solvents (acetone and DMSO).

Fig. 2 Gel permeation chromatograms of HS₁ (I) and HS₂ (II) humic material that, before elution in 0.02 M Na₂B₄O₇ at pH 9.2, were treated as follows: (A) dissolved at pH 11.8 (B) titrated with acetic acid to pH 6 (C) to pH 4.5 (D) to pH 3.5 (E) to pH 2; (F) the material brought to pH 2 was further back-titrated with KOH to pH 3.5 (G) back-titrated to pH 4.5 (H) back-titrated to pH 6 (I) and back-titrated to pH 8.5; (J) the latter material at pH 8.5 was further roto-evaporated to attempt the elimination of the residual acetic acid.
Fig. 3 Gel permeation chromatograms of HS2 treated with acetic acid and eluted with a 0.02 M Na4B2O7 solution at pH 9.2 (I), and with a 0.1 M Na4B2O7 solution at pH 9.2 (II). The significance of the chromatograms are the same as in Fig. 2.

Fig. 4 Gel permeation chromatograms of HS1 treated with formic acid and eluted with a 0.02 M Na4B2O7 solution at pH 9.2 (I), and with a 0.1 M Na4B2O7 solution at pH 9.2 (II). The significance of the chromatograms are the same as in Fig. 2.
This model of conformational rearrangement due to the elute at volumes characteristics of low molecular size material. Observations for humic substances alone or for those treated with organic acids, whereas no size changes are observed after the treatment with mineral acids.

Disrupt by electrostatic repulsion the original configuration, forming smaller aggregates. The small micelles, thus formed, are already made unstable in their inner hydrophobic arrangement, small enough to diffuse separately through the gel pores and allow the material to resume the previous expanded configuration. In fact, there is no difference between the molecular size distribution of the humic substances alone and that obtained after the treatment with the mineral acids, and so we saw no change in the macromolecular arrangement of the humic material observed.

When organic acids are added, the aliphatic hydrophobic component of the molecules penetrates into the inner hydrophobic sites of the humic micelle and may disrupt its steremochemical arrangement, thereby altering the stability of the hydrophobic bondings that hold together the large hydrophobic part of the humic micelle-like aggregate (Wershaw, 1986). At the same time, the hydrophobic carboxyl groups of the organic acid are positioned at the interfaces between the micelles and water. During gel permeation the alkaline pH of the eluting solution rapidly dissociates these carboxyl groups as well as the humic acidic functions. The resulting negative charges disrupt by electrostatic repulsion the original configuration, already made unstable in its inner hydrophobic arrangement, into smaller aggregates. The small micelles, thus formed, are small enough to diffuse separately through the gel pores and elute at volumes characteristics of low molecular size material. This model of conformational rearrangement due to the disruption of inner hydrophobic bondings explains why the molecular size of the humic material is dramatically reduced by treatment with organic acids, whereas no size changes are observed for humic substances alone or for those treated with mineral acids.

Table 1 shows that the configurational change from high to low molecular size occurred only when humic material was added with organic chemicals containing relatively strong acidic carboxyl groups. When less acidic alcoholic and phenolic hydroxyl groups were present in the organic molecule no molecular size reduction was observed because no small micelles could be formed. In fact, the hydroxyl groups, are not acidic enough to supply the negative charges that, at the high elution pH, can disperse the small micelles by overcoming the inner hydrophobic arrangement of the humic material. The addition of the same organic chemicals mixed with HCl (Table 1) to lower the pH to those obtained with carboxylic acid, did not change their effect on the molecular size of humic substances. This confirms that organic hydroxyl groups and mineral acids, added either alone or together, cannot form small humic micelles.

The conformational change of humic material by interaction with organic acid is a function of the concentration of the acid in contact with the humic aggregate. This is shown by the results obtained with citric acid (Table 1). By reducing from 1.5 to 0.3 and 0.15 M the concentration of citric acid in the humic-organic acid mixture that was charged onto the gel permeation column, the shift of the absorbance towards larger elution volume was also concomitantly reduced. This behaviour indicates that the more concentrated the organic acid is, the more extensive is the dispersion of humic material into small submicelles.

To find out if hydrophobic bondings play a more important role in the aggregation of humic substances than hydrogen bondings, contrary to what is currently assumed (Schnitzer, 1978; Swift, 1989), humic material was treated with dipolar aprotic solvents such as acetone and dimethylsulphoxide (DMSO). Dipolar aprotic solvents are thought to be able to disrupt inter- and intra-molecular hydrogen bondings in humic substances (Piccolo, 1988; Hayes et al., 1989; Stevenson, 1994). No change of macromolecular structure was revealed by the molecular size distribution of the humic material after addition of dipolar aprotic solvents, thereby confirming that hydrophobic bondings are mostly responsible for the stability of humic conformational structures.

Effect of pH variation

To assess further the role of pH changes on the molecular size distributions, we performed another experiment on the two

entire HS₁ and HS₂ humic extracts. The gel permeation chromatograms of HS₁ and HS₂, which were both previously titrated with acetic acid to progressively lower pHs and then back-titrated with KOH to high pHs again, are shown in Fig. 2. I and II, respectively. Although the molecular size distribution of the untreated (pH 8.5) HS₁ and HS₂ materials appeared slightly different (IA and IIA), the presence of acetic acid at progressively lower pH induced the progressive modification of the first high molecular size peak at the void volume (V₀) and the concomitant increase of the diffused low-molecular-size peak at larger elution volumes (I and II, B-D). When the humic solutions reached pH 2 with additions of acetic acid, the chromatographic peak at V₀ had completely disappeared while a sharp peak at large elution volumes was revealed (I and II, E). As in the HF₁ experiment, this behaviour may be explained with the disruption of the hydrophobic arrangement of humic material by acetic acid. The organic acid penetrates into the inner hydrophobic sites of humic micelles and by the dissociation of its carboxyl group in the alkaline eluting solution favours the dispersion of small humic micelles by electrostatic repulsion.

By increasing the pH of the acetic acid-humic solution again, the chromatograms of both HS₁ and HS₂ revealed a reverse trend. The peak at large elution volumes was progressively reduced, whereas at the void volume increased concomitantly (I and II, F-I). Such reversible effect on the humic configuration further confirms the proposed hydrophobic mechanism. It indicates that the disruption of the humic macromolecule and the dispersion into small micelles by organic acids occurs in the dynamic conditions of gel permeation when the sub-micelles are separated from each other, and that it is not permanent. In fact, when the complete dissociation of the carboxyl groups of the acetic acid-humic mixture was achieved (pH 8.5) before application to the gel column, the conformational change caused by the alteration of the humic hydrophobic arrangement by the methyl end of the acetic acid, did not appear (I and II, I). The humic micellar aggregates, being still in close contact, can form new hydrophobic bondings and find a stereochemically stable configuration that allows the reformation of large micelles. The subsequent gel permeation chromatography then shows an apparent high molecular weight configuration. Conversely, when the dissociation of the acetic acid carboxyl groups occurs directly in the gel column the small humic micelles are dispersed away by the eluting solution and cannot reaggregate into larger size configurations (I and II, E). In the other hand, if the pH of the acetic acid-humic mixture is not raised enough to ensure the complete dissociation of the acetic acid carboxyl groups (I and II, F-H), the hydrophobic rearrangement of the humic micellar aggregates is unstable. When this mixture is placed on to the elution column the residual carboxyl groups dissociate and can still induce a partial dispersion of submicelles and a macromolecular configuration of intermediate size.

Despite the capacity of humic material to re-establish stable hydrophobic bondings and apparent high molecular sizes, the chromatograms never resumed the same appearance as that observed with direct titration to the corresponding pH value. The chromatograms of the back-titrated samples revealed sharper low-molecular size peaks (e.g. I and II, G) than the chromatograms of the directly titrated samples (I and II, C). The original shape of the chromatograms obtained for the untreated HS₁ and HS₂ was not resumed even for the samples roto-evaporated at 30°C under vacuum to eliminate the added acetic acid (I and II, J). These results indicate that the insertion of organic acids produce a degree of permanent alteration into the hydrophobic arrangement of humic micelles. This may be due to the impossibility to re-establish exactly the original configuration of the micellar structure and/or to the persistence of acetic acid molecules. We found by ¹³C-NMR spectroscopy that acetic acid residues were still present in the roto-evaporated samples. These residues may have prevented the original hydrophobic aggregation of humic molecules reforming into the previous large micelle-like structure and the same peak shape at the void volume.

**Effect of ionic strength**

Ghosh & Schnitzer (1980) pointed out that the conformational structure of humic substances was also a function of the ionic strength (I) of the medium and that, for I ≥ 0.05 M, macromolecules can be considered in fully coiled shapes with characteristics similar to those of uncharged polymers and spherocolloids. Berdén & Berggren (1991) have shown that by increasing the eluent's ionic strength elution volumes for the humic material diffusing through the column are enhanced. However, the elution volumes did not increase significantly after I ≥ 0.15 M. Chin & Gischwend (1991) also emphasized that the separation of humic substances by size exclusion chromatography depends strongly on ionic strength of both the mobile phase and sample matrix. They explained their results by dynamic coiling and uncoiling of the humic molecules.

We evaluated the effect of ionic strength to see whether it influences the micellar behaviour of humic substances. The previous titration and back-titration were repeated on the HS₂ material but with a more concentrated solution, namely 0.1 M Na₂B₄O₇, as gel column eluent (Fig. 3, II). This solution ensures an ionic strength greater than 0.3 M that exceeds the values previously indicated for the complete coiling down of humic macromolecules (Ghosh & Schnitzer, 1980; Berdén & Berggren, 1991). The chromatograms obtained with the stronger eluent did not differ substantially from those obtained with the weaker solutions (Fig. 3, I). Evidently, the smaller sizes of humic substances and the consequent increase of elution volume cannot be due to an extreme coiling of the macromolecules but another mechanism must explain this behaviour. Nevertheless, the increased ionic
strength did shift somewhat the diffused peak towards larger eluting volumes (Fig. 3, II, A-D) as compared to those with the less concentrated Na₂B₄O₇ solution (Fig. 3, I, A-D). The full peak at low molecular sizes, when the pH was finally brought to pH 2 with acetic acid (Fig. 3, II, E) was slightly sharper, with three somewhat resolved peaks, than that obtained for 0.02 M Na₂B₄O₇ (Fig. 3, I, E), which revealed only two peaks. Such peak shape suggests that a more efficient separation into small micelles of well different sizes is obtained with the stronger solution. Moreover, this solution seemed to reduce the reaggregation into larger configurations and to maintain more polydispersion when the humic-organic acid solution was back-titrated to high pHs. In fact, the shift of the peak back to the void volume (Fig. 3, II, F-J) appeared less definite than for the elutions with less concentrated Na₂B₄O₇ (Fig. 3, I, F-J). These effects may be attributed to the compression of the random coil in the stronger solution. This restricts the stereochemical freedom of the heterogeneous components of the humic micelle and, consequently, the capacity to form aggregating hydrophobic bonds with nearby micelles.

The HS₂ humic material was also used to verify if formic acid produced the same changes in humin configuration as for acetic acid and if different ionic strengths of the gel eluent were as uninformative as in the case of acetic acid (Fig. 4). In weak solution the pattern of peak shifts from small to large elution volumes when the pH was lowered with formic acid and back from large to small elution volumes during alkaline back-titrations to high pH (Fig. 4, I) was similar to that for acetic acid. Furthermore, a stronger solution (Fig. 4, II) did not change the chromatographic behaviour, although it produced the same slight effects as for the acetic acid experiment, namely, an easier transformation into small aggregates at low pH (Fig. 4, I, A-E, and II, A-E) and an incomplete resumption of the high-molecular-size peak in the back-titrations (Fig. 4, I, F-I, and II, F-I). In formic acid, the stronger 0.1 M Na₂B₄O₇ solution favoured a larger reduction of the low-molecular-size peak and a concomitant more pronounced backshift towards intermediate elution volumes. This suggests the formation of medium-size humic aggregates eluting at intermediate volumes (Fig. 4, I, H-J, and II, H-J).

These series of gel chromatograms for the entire HS₁ and HS₂ humic materials confirmed the same pattern of conformational changes observed for the HF₁ humic fraction. Moreover, the substantial similarities of the chromatograms obtained by elution at different ionic strengths suggest that the observed shift was due to true changes in the steric conformation of the humic aggregates more than adsorption phenomena on the gel matrix caused by the presence of organic acids. The formation of small micelle-like humic fractions rather than a retardation in elution because of adsorption on the gel, was further confirmed by performing the same experiments on other gel matrices (unpublished results).

Conclusions

We showed that humic substances from two different sources behave in solution as large micellar aggregates composed of smaller molecules. This micelle-like structure of humic material may be thought of as having the hydrophilic components mainly at the solution interphase, whereas the hydrophobic constituents form a hydrophobic phase in the micelle's interior (Tanford, 1980). Moreover, our results indicate that weak inter- and intra-molecular interactions, such as hydrophobic bondings, regulate the molecular size of humic aggregates in solution. The conformational properties of humic substances in solution being governed mainly by hydrophobic interactions is a new finding.

The mechanism by which humic substances aggregate indicated by our results helps to explain the findings of Ogner & Schnitzer (1970) and Wershaw & Pinckney (1980) who showed how terminal aliphatic ends of amphiphilic molecules were either trapped in, or difficult to extract from, humic substances. Such conformational properties of humic substances may lead to a reconsideration of the definition of the classical humic and fulvic fractions of the humified organic matter. Fulvic acids may be regarded as stable small micelle-like materials in which there are enough acidic functional groups to keep the fulvic micelles dispersed in solution. Conversely, humic acids are formed because micelle-like aggregates, having few dispersive negatively charged groups, can get closer to each other. Then, favourable stereochemical configurations lead to the formation of hydrophobic interactions that are strong enough to bind humic molecules together and to cause their flocculation into aggregates of apparent high molecular weight.

References


