CHAPTER III

INVESTIGATIONS ON THE HUMIC SYSTEMS
(A) EXTRACTION, FRACTIONATION AND PURIFICATION

Materials and methods

A typical brown forest (Mollic Haplaquept) surface soil sample (0-15 cm) from Darjeeling, West Bengal, India, was used. The method of extraction, fractionation and purification was broadly as suggested by Kononova (1966), Schnitzer and Khan (1972) and Stevenson (1982), with some modifications of the latter owing to the high ash content of humus. Problems in purifying humic samples of high ash content were also reported by Griffith and Schnitzer (1975).

To air-dried and powdered soil sample, 0.5 N Na$_2$CO$_3$ solution was added in a solution:soil ratio of 10:1. The bottle was flushed with N$_2$, shaken for 1 hour, and allowed to settle.

Prior decalcification of the soil was avoided because it was observed that on acidification of the soil with dilute HCl, quite a large amount of fulvic acid was solubilised which would be lost on subsequent washing. Apparently the fulvic acid is present as a fulvate in combination with Ca$^{2+}$ or Mg$^{2+}$ or similar cations. This is quite likely with brown forest soils of Himalayan foothills which remain relatively cool and moist throughout the year.

The supernatant was siphoned off and the solution centrifuged to remove residual clay. Following acidification with dilute
HCl to pH 2.0, the precipitated humic acid (HA) was separated from the fulvic acid (FA) by centrifugation. The HA was redissolved in Na$_2$CO$_3$ solution and centrifuged. It was then precipitated with dilute HCl, separated from the solution by centrifugation, and shaken for 36 hours with a large excess of 0.5% (v/v) HF-HCl mixture to reduce ash. The solids were subsequently washed by centrifugation, dialysed and freeze-dried.

The popular method of recovering FA from the aforesaid acid solution after removal of HA includes neutralisation and subsequent evaporation under reduced pressure at moderate temperatures (40°-50°C), using a flash evaporator, followed by freeze-drying. A disadvantage of this procedure is that chemical changes can occur through condensation of FA with other reactive organic components, if any, or polymerisation of FA itself; moreover, the problem of removing inorganic constituents has not been lessened (Stevenson 1982). Alternatively, FA can be separated as Ba-salt (Tyurin 1940; Sowden & Deuel 1961) at pH 7.0 under N$_2$ (Chatterjee & Ghosh 1981). However, with the present sample, when the pH of the FA solution was raised to about 7.0, the entire FA precipitated as fulvate due, possibly, to the presence of divalent ions in the system. Naturally, further addition of Ba$^{2+}$ was not necessary. The fulvate was dialysed and then passed through H-resin (Amberlite IR-120). However, it was observed that the resin was incapable of exchanging the metal-fulvate completely as a consequence of which
a greater part of FA did not pass through the resin column and the small portion that could, was contaminated with insoluble particles of fulvate. Other grades of H-resin (like Dowex-50) too, behaved similarly in spite of the fact that the total exchange (capacity) of the resin was at least 2000 times that of FA. Apparently the metal ion was held too tenaciously by the FA to permit exchange. Mention may be made, in this connection, that in spite of repeated passages through H-resin, Schnitzer and Ghosh (1982) were able, only to reduce and not to remove completely, the metal ions from the laboratory-prepared FA-Cu(II) and FA-Fe(III) complexes. Therefore, in order to remove as much of the metal ions as possible, prior to resin-treatment, the following method was tried: Excess of acid (0.1 N HCl) was added to the fulvate to release the metal ions and the solution was electrodialysed. It was observed that a precipitate forms as excess acid was removed. The suspension was again acidified and the process repeated. The dialysed material, which could never be completely freed of precipitate, was then added to a bottle containing H-resin and was shaken for 24 hours. The FA was filtered free of resin and any insoluble fulvates that may remain, and then freeze-dried. This revised methodology naturally eliminates the usual ash-removal of FA-systems as has been done by repeatedly passing them through H-resin columns (Schnitzer 1978).

For a few studies, HA and FA samples were also obtained from an alluvial (Aeric Fluvaquent) surface soil sample (0-15 cm) of Baruipur, West Bengal, India, following the aforesaid procedure.
The only difference in methodology was that, decalcification was not avoided in this case because the soil sample contained some CaCO₃-concretions.

Further, for some comparative studies, a HA sample from Beaverhills, Alberta, Canada (Haploboroll) and one FA sample from Armadale, Prince Edward Island, Canada (Cryaquod) were used. They were kindly supplied by Dr. Morris Schnitzer of Chemistry & Biology Research Institute, Agriculture Canada, Ottawa, Canada. These two samples have been exhaustively studied by Schnitzer and his co-workers in many of their investigations and a number of their analytical characteristics, both degradative and non-degradative, may be obtained from the literature (Schnitzer 1978).

Analysis of the humic samples for carbon and hydrogen were done by dry combustion, for sulphur by the oxygen-flask combustion, and for nitrogen by the automated Duma method; oxygen was determined by difference. Ash content of the samples was determined by igniting them at 750°C for 4 hours.

The ratio of optical densities of dilute aqueous HA or FA solutions at 465 and 665 nm, or the $E_4/E_6$ ratio, was determined (Chen et al. 1977; Ghosh & Schnitzer 1979) by dissolving the samples in 0.05 N NaHCO₃; the sample concentration (w/v) was varied between 0.001 and 0.01% to check
concentration-independence of the $E_4/E_6$ ratio; pH of the resulting solutions were around 8.5. A Hitachi Double Beam Spectrophotometer (Model 100-60) was used.

Results

Elemental composition and $E_4/E_6$ ratios of humic samples are presented in Table 4.
(B) INFRARED, X-RAY AND THERMAL ANALYSES

Materials and methods

IR spectra of the samples were noted on a Perkin Elmer 577 Instrument within a scanning range of 4000 to 400 cm\(^{-1}\), using pellets containing KBr as matrix.

XRD of the samples was recorded on a Philips PW 1140 X-ray Diffractometer using Ni-filtered CuK\(_\alpha\) radiation at a scanning speed of 2°2\(^\circ\)/min using sample holders.

Thermogravimetry was carried out on a Gebrüder-Netzsch Instrument (No, 404) using 20 mg sample and a heating rate of 10°C/min. Weight loss and temperature were automatically noted on a Siemens Recorder.

Both HA and FA samples (Haplaquept from Darjeeling) were subjected to study; however, FA could not be used for XRD and TG analyses because of scarcity of sample. In tropical soils, the proportion of FA is often extremely low.

Results and discussion

IR absorption of HA and FA samples are shown in Fig. 6. Since quantitative spectroscopy was not done, absorption intensities of the HA and FA cannot be compared. The spectra show extensive
overlapping of absorptions, which is characteristic of humic substances. Considerable suggestions for assignments of the spectral bands are available in the literature (Farmer & Morrison 1960; Kononova 1966; Theng et al. 1966; Schnitzer & Khan 1972; Flaig et al. 1975; Stevenson 1982). The major absorption bands of the humic substances (Schnitzer 1978; Stevenson 1982) lie in the following regions: 3400 to 3300 cm\(^{-1}\) (H-bonded OH or O-H stretch), 2940 to 2900 cm\(^{-1}\) (aliphatic C-H stretch), a trough in the region of 2700 to 2400 cm\(^{-1}\) (H-bonded COOH), 1725 to 1720 cm\(^{-1}\) (C = O of COOH, C = O stretch of carbonyl), 1660 to 1630 cm\(^{-1}\) (C = O stretch of amide, quinone C = O, C = O of H-bonded conjugated ketone, aromatic C = C, COO\(^-\)), 1620 to 1600 cm\(^{-1}\) (aromatic C = C, H-bonded C = O of conjugated ketone), 1590 to 1517 cm\(^{-1}\) (COO\(^-\) symmetric stretch, N-H deformation and C = N stretch of amide), 1460 to 1450 cm\(^{-1}\) (aliphatic C-H), 1400 to 1390 cm\(^{-1}\) (COO\(^-\), aliphatic C-H, O-H deformation and C-O stretch of phenolic OH), 1280 to 1200 cm\(^{-1}\) (C-O stretch and O-H deformation of COOH), and 1170 to 950 cm\(^{-1}\) (C-O stretch of polysaccharides, Si-O of silicate impurities).

The assignments are, however, not free of ambiguity. For example, the 3400-3300 cm\(^{-1}\) band is considered to be primarily due to O-H stretching vibrations (Flaig et al. 1975). The spectra of methylated derivatives, however, show strong 3400 cm\(^{-1}\) absorptions (Stevenson & Goh 1974). Though it has been assumed that this may be due to residual OH, one cannot rule out the possibility that N-H stretching vibrations contribute significantly to the 3400 cm\(^{-1}\)
absorption. The broad trough shape absorption is probably due to extensive overlapping of OH and NH vibrations with \( = \text{C}-\text{H} \) stretching modes of aromatic and aliphatic structures. Assignments for absorptions in the 1650-1600 cm\(^{-1}\) are also riddled with controversy. This is generally attributed to \( \text{C}=\text{C} \) skeletal vibrations of aromatic structures together with hydrogen bonded quinones (Flaig & Salfeld 1959; Wagner & Stevenson 1965; Theng & Posner 1967). It has been pointed out (Stevenson 1982) that aromatic compounds that show 1600 cm\(^{-1}\) absorption should show a more intense 1500 cm\(^{-1}\) band; therefore, the reversal of this situation for HA suggests that other structures contribute to absorption in this region. Moreover, many workers (Schnitzer et al. 1959; Wagner & Stevenson 1965) failed to detect an increase in 1660 cm\(^{-1}\) absorption when the quinone groups were supposedly freed of hydrogen bonding by acetylation; other workers, however, detected the 1660 cm\(^{-1}\) absorption of the free quinone group (Moschopedis 1962; Mathur 1972). Therefore, there appears to be some doubts regarding the contribution of aromatic \( \text{C}=\text{C} \) and \( \text{C}=\text{O} \) of quinones, to the 1600 cm\(^{-1}\) absorption. In this regard, it may be mentioned that though the 1500 cm\(^{-1}\) band for aromatics is generally stronger than the 1600 cm\(^{-1}\) band, this is not so, when the ring is conjugated with any double bond such as \( \text{C}=\text{O}, \text{C}=\text{C}, \text{NO}_2 \), etc. Such conjugation produces, in most cases, a very marked enhancement of the intensity of all bands including the 1600 cm\(^{-1}\) band relative to 1500 cm\(^{-1}\) band (Bellamy 1975). This
explains the lack of a stronger 1500 cm\(^{-1}\) band relative to the 1600 cm\(^{-1}\) one. In addition, aliphatic C = C conjugated with the benzene ring, which also absorb a little above 1600 cm\(^{-1}\) (Bellamy 1975) but do not absorb at 1500 cm\(^{-1}\), might also contribute to the 1600 cm\(^{-1}\) band of the humic substances. The presence of such olefinic bonds in humic structures has been proved by Orlov et al. (1962). This is not unusual since humic substances as well as lignin are known to contain structures of the cinnamic type (Flaig et al. 1975). Primary N-H deformation vibrations may also be responsible for absorptions in the 1600 cm\(^{-1}\) region (Bellamy 1975). It thus appears, that the absorption in the 1600 cm\(^{-1}\) region is possibly owing to the aromatic rings in conjugation with C = O or C = C, aliphatic C = C as well as NH\(_2\) groups, either individually or in combination. In conclusion, this may be stated that though the IR spectra of humic samples may produce worthwhile information but in general, it is useful only for gross characterisation and is not that informative and reliable to reveal finer structural details.

Results of XRD on the HA sample are presented in Fig. 7 and Table 5. The nature of the diffraction pattern indicates a predominantly amorphous structure. However, a broad band, centred by a peak at 3.66 Å, suggests a certain degree of order, possibly in the three-dimensional structure of the molecule. Weak broad diffractions are also observed at around 9 Å and
7.5 Å which might be due to silicate impurities and also at about 4 Å and 2.4 Å which are probably due to molecular arrangement in the humic structure itself. Other workers have also obtained bands at 3.5 Å together with minor peaks or humps between 4 and 5 Å as well as between 1.5 and 3 Å (Kasatochkin et al. 1964; Kodama & Schnitzer 1967; Pollack et al. 1971).

The band near 3.5 Å, known as the 002 band, has been attributed to the ordering of condensed aromatic layers normal to their planes (Kasatochkin et al. 1964); this spacing is known to increase either because of the decrease in the number of aromatic rings or because of imperfections in the planar carbon network. The band between 4 and 5 Å, the so-called γ-band, possibly arises from the irregular packing of aromatic layers due to the presence of aliphatic edge groups around the aromatic clusters, which may prevent the layers from packing at a close distance (Kodama & Schnitzer 1967). The structure of HA was visualised as containing flat condensed aromatic networks to which side-chains are attached while FA, on the other hand, was assumed to consist of a broken network of poorly condensed aromatic rings with appreciable numbers of disordered aliphatic chains round the edges (Kasatochkin et al. 1964; Kodama & Schnitzer 1967).

The main reactions governing the thermal behaviour of humic substances with increasing temperature are as follows:
(a) dehydration, (b) further dehydration and loss of functional
groups, mainly decarboxylation, and (c) decomposition of the 'nuclei' (Schnitzer & Hoffman 1964; Schnitzer & Kodama 1972a; Schnitzer 1972). Thermogravimetry of the HA sample, as presented in Fig. 8, shows two regions of weight loss; the first, from 300° to 520°C and the second, from 520° to 630°C. Weight loss below 400°C is believed to be due to the elimination of functional groups and aliphatic components while that above 400°C, is the decomposition of the aromatic nucleus. In the region 150°-250°C, decarboxylation occurs which overlaps with (i) the elimination of phenolic groups at 150°-200°C, and (ii) cleavage of methoxyl groups around 250°-300°C (Schnitzer & Khan 1972; Flaig et al. 1975; Stevenson 1982).
(C) DETERMINATION OF TOTAL ACIDIC GROUPS

The total number of acidic groups in humic substances are usually taken to be equal to the sum of its carboxyl and phenolic hydroxyl groups. Strictly speaking, such a definition may seem to be somewhat erroneous. In fact, some phenolic hydroxyl groups may be so weakly dissociated that they are never neutralised in aqueous solution by inorganic bases. Such groups cannot, therefore, in general be considered acidic. However, even those phenolic hydroxyl groups which do not take part in acid-base reactions in aqueous solutions, may be capable of forming complexes with transition metal cations, with the consequent liberation of H\(^+\) ions. Thus, the inclusion of all phenolic hydroxyl and carboxyl groups in the definition of 'total acidity' is justified, as a means of assessing reactivity towards all cations, instead of a mere 'neutralisation or exchange capacity'. More precisely, (cation) exchange capacity is dependent on the environment (mainly the exchanging cation and the medium) and hence may not include contributions from all the carboxyl and phenolic hydroxyl groups, whereas inclusion of all such groups is imperative in the total acidity concept.

Total acidic groups in humic substances are usually determined by different methods that can be classified into two categories (van Dijk 1960): (1) Organo-chemical methods, that include exhaustive methylation with diazomethane and determination of
the methoxyl group by the well-known Zeisel method, reaction with diborane and determination of $H_2$ produced, etc. (Stevenson 1982). The disadvantages of these methods include tediousness, incomplete methylation, or side reactions (van Dijk 1960).

(ii) Inorganic methods in which the total acidic groups are determined by titration with alkali (Posner 1964; Kononova 1966; Schnitzer & Khan 1972).

Of these, the most popular is the 'baryta titration' method in which the humic sample is allowed to react with excess of $Ba(OH)_2$ and the unconsumed alkali back-titrated with acid (Schnitzer & Gupta 1965; Schnitzer 1972). In spite of its widespread acceptance, this method suffers from certain disadvantages. A fairly large amount of sample is required in comparison to, say, direct titrimetric methods. Moreover, reproducibility of the method is somewhat doubtful since all the steps in the whole process must be carried out under strictly $CO_2$- and $O_2$-free conditions. Lastly, whether $Ba(OH)_2$, would be capable of reacting with all weak phenolic hydroxyl groups appears to be also doubtful; weakly acidic phenolic hydroxyl groups cannot usually be neutralised except in some non-aqueous media (Siggia 1963).

It was, therefore, attempted to formulate a method that would combine the advantages of the direct titrimetric procedures, viz., speed, accuracy and small sample requirement, without its usual limitation of incomplete reaction and low CEC values. With
this aim in view, the non-aqueous titration technique appears to be the most suitable for the determination of acidic groups.

Non-aqueous titrations for the determination of total acidity in humic substances, have been carried out in dimethyl formamide (DMF), pyridine, ethylene diamine, etc., using titrants such as tetrabutyl ammonium hydroxide, sodium isopropylate, or KOH in isopropyl alcohol (van Dijk 1959, 1960; Stevenson 1982). In general, the results of these investigations indicate that the end-points can be better obtained than with titrations in aqueous solutions. Total acidity obtained, is always much higher relative to the Ba(OH)$_2$ method (van Dijk 1960; Stevenson 1982).

In this connection, it may be pointed out that the total number of proton donating groups of the humic substances depends on the basicity of solvents used. Thus, in solvents more basic than water, like acetone, DMF, or pyridine, more groups would become acidic which would not normally have been acidic in water. An example is van Dijk's (1960) titration of salicylic acid in water and DMF using NaOH and NaOC$_3$H$_7$ respectively. The former shows the presence of only a single acidic group whereas with the latter, two acidic groups are evident.

Although non-aqueous titrimetric methods give better and more 'complete' values of total carboxyl and phenolic hydroxyl groups, these have not been popularly adopted for the determination of total acidity. One of the reasons might be the time consuming
steps in the preparation and purification of the reagents for titration. Titrants like sodium isopropylate, sodium methyleate or tetrabutyl ammonium hydroxide that are commonly used (van Dijk 1960; Stevenson 1982) have to be prepared just prior to titration. It was, therefore, attempted to formulate a method for determining total acidity that would retain the advantages of the non-aqueous method and at the same time be rapid and simple.

**Materials and methods**

The samples used in this study were Haplaquept HA and FA (Darjeeling), Haploboroll HA (Beaverhills), Cryaquod FA (Armadale), and a salicylic acid (GR SM).

All samples were first titrated pH-metrically with standardised 0.1 N NaOH in aqueous medium. Subsequently, trial experiments were performed using salicylic acid and Cryaquod FA in a series of media of decreasing dielectric constant. These two samples were first titrated pH-metrically in 50:50 (v/v) ethanol–water, ethanol (Bengal Chemical, Calcutta; purified as stated in section E of this Chapter) and acetone (GR E. Merck) using 0.1 N aqueous KOH, methanolic KOH or ethanolic KOH (standardised with oxalic acid using phenolphthalein as indicator). Conductometric titrations were also done in the same set of media and using the same titrants; an additional titration was done in acetone medium using 0.1 N KOH in isopropyl alcohol (GR E. Merck) as titrant.
All titrations were performed as follows: To 20 mg of sample, 40 ml of solvent was added. Both pH and conductometric titrations were done under N₂ in a vessel over baryta as a double-check to maintain CO₂-free atmosphere. An Elico (Model LI-10) pH Meter with glass-calomel electrodes and an Elico (Type CM-82) Conductivity Bridge with a dip-type cell operating at 50 cycles/sec were employed. It must be mentioned that HA and FA being sparingly soluble in acetone and ethanol, the neutralisation reaction is a little slow and, therefore, about half-a-minute must be allowed, before a reading is noted.

Results and discussion

Results of titrations in aqueous media using 0.1 N NaOH are reproduced in Fig. 9 and Table 6. As expected, values of total acidity are much lower, for the Haploboroll HA and Cryaquod FA, than that obtained by the abovementioned 'baryta titration' method, viz., 6.6 and 12.4 me/g respectively (Ghosh & Schnitzer 1980a). It may be noted that the end point on titration of salicylic acid corresponds to exactly half the total acidic groups, i.e., only COOH groups were neutralised.

It is well-known that exchange capacities determined by Ba(OH)₂, even on direct titration, are much higher than with NaOH (Gillam 1940; Chatterjee & Bose 1952; van Dijk 1959; Schnitzer & Skinner 1963). Flaig et al. (1975) compiled such information
and it can be seen that the exchange capacity determined with Ba(OH)$_2$ or Ca(OH)$_2$ is sometimes double or more, than that determined with NaOH. Gamble (1970) titrated the same Cryaquod FA as is used in the present investigation, both conductometrically and potentiometrically using 0.1 N NaOH and 0.1 N KOH as titrants respectively; exchange capacity as calculated from the curves and data presented is between 6.5 and 7.0 me/g, i.e., much lower compared to, 12.4 me/g as determined by the 'baryta titration' method (Ghosh & Schnitzer 1980a). However, the purpose of carrying out the NaOH titration was to observe whether the end point obtained, thus, might correspond to the total COOH as was shown by the salicylic acid system stated above. The data in Table 6 reveal that the amount of alkali (NaOH) consumed is even much less than the content of COOH group for both the Haploboroll HA and Cryaquod FA samples, reported as 4.5 and 9.1 me/g respectively, by Ghosh & Schnitzer (1980a). It may be concluded that some COOH groups in humic substances are too weak to react even with NaOH and, therefore, phenolic OH groups, which are in general weaker in acidity than the COOH, are even weaker and probably do not react with NaOH at all.

Preliminary non-aqueous pH-metric titrations with salicylic acid and Cryaquod FA revealed that the neutralisation occurs above pH 12 that corresponds (in the case of salicylic acid) only to the COOH group. Therefore, it is not possible to observe the complete course of the titration using a pH-meter. Potentiometric titrations using glass-calomel electrodes, though allows larger addition of
alkali, suffers from the drawback that prolonged contact at high pH with strongly dehydrating solvents causes serious damage to the electrodes.

To observe neutralisation behaviour at high alkali concentrations, conductometric titrations were, therefore, thought to be preferable. Titrations in the abovementioned media using aqueous methanolic or ethanolic KOH did not show any sharp end point. In the course of neutralisation, conductance of the solution increases and continues to increase after neutralisation. Therefore, unless there is a significant difference in the change of conductance before and after neutralisation, end points will be difficult to detect. With this in view, titrations were done with a titrant of even lower dielectric constant, viz., KOH in isopropyl alcohol. Fig. 10 shows the titration behaviour of salicylic acid in acetone with the abovementioned titrant. The end point corresponds to the neutralisation of both COOH and phenolic OH groups. Whereas the initial increase in specific conductance is rapid, after complete neutralisation, the specific conductance increases more slowly (Fig. 10). Total acidity of Cryaquod FA (Fig. 11 & Table 7) is much higher than that obtained, as mentioned earlier, by the 'baryta titration' method. The tropical Haplaquept FA sample has lower value of total acidity than that of the Cryaquod FA. Total acidity value of Haploboroll HA is almost the same as that obtained by baryta titration.
It is concluded that conductometric titrations of humic substances in acetone using KOH in isopropyl alcohol as titrant may be used for the determination of total acidity. The method is simple; no prior purification or synthesis of reagents is required; small amounts of sample are sufficient (10-20 mg) as compared to about 100 mg for 'baryta titration' method; it is rapid, accurate and highly reproducible. It may be mentioned here that carboxylic and phenolic acids are known to be amenable to titration by alcoholic KOH in acetone or alcohol media (Siggia 1963). Some compounds which have been successfully titrated using NaOH/KOH in methanol/isopropyl alcohol and in acetone/ethanol media include carboxy acids, phenols, salicylic acid, shellac acid, phenolic and sulphonic acid groups in lignin sulphonnic acids, etc. (Ashworth 1964). The use of more basic solvents like pyridine or DMF, therefore, does not appear to be necessary for the determination of total acidity of humic substances.
Humic substances, being macromolecules, the study of their molecular shape, size and weight is of primary importance to the soil chemist. More specifically in regard to such shape, the latest concept is that the macromolecular configurations of HA and FA molecules are not unique; they vary with the changes in the environment; they behave like rigid spherocolloids at high sample concentrations, low pH, or in the presence of sufficient amounts of neutral electrolytes, but they are flexible linear colloids at low sample concentrations, provided that the pH is not too low or that the ionic strength is relatively low, conditions that normally prevail in soils (Ghosh & Schnitzer 1980a). However, the determinations of molecular size and weight are complicated by the fact that during the extraction and fractionation, a range of molecular sizes (and weights) is invariably obtained; subsequent repeated fractionation even by the most sophisticated methodology may result in a narrower range but the range exists. Therefore, in the determination of molecular weights by conventional methods such as osmometry (Ghosh & Mukherjee 1971), vapour pressure osmometry (Hansen & Schnitzer 1969), surface pressure measurements (Ghosh & Schnitzer 1980a), sedimentation at the ultracentrifuge (Flaig & Beutelspacher 1968), diffusion measurements (Cameron et al. 1972), etc., only an average value of the molecular weights is obtained, viz., $M_n$ (number-average), $M_w$ (weight-average),
However, the above methods fail to give any idea about the distribution of molecular weights, i.e., polydispersity of the sample, which is so vital when one deals with heterogeneous systems. Some idea of the polydispersity of a sample can be obtained by comparing \( \bar{M}_n \), \( \bar{M}_w \), and \( \bar{M}_z \) values. The sample is considered monodisperse when \( \bar{M}_n = \bar{M}_w = \bar{M}_z \). Evaluation of polydispersity by comparison of \( \bar{M}_n \) and \( \bar{M}_w \) values obtained from different methods, as for example, from osmometry and ultracentrifugation respectively, may be criticised since errors result from differences in the limitations (inclusive of theoretical treatment) and accuracies of the respective measurements. In this respect, light scattering is a unique technique, enabling both \( \bar{M}_n \) and \( \bar{M}_w \) to be calculated from the same experimental data. This is, therefore, the only reliable method for obtaining an idea of the polydispersity of humic systems.

The only reported study on light scattering by humic substances was by Orlov and Gorskova (1965). However, their data are questionable because they did not make requisite corrections for a system which is known to fluoresce (Ghosh & Schnitzer 1980b) and causes absorption in the visible region (Ghosh & Schnitzer 1979). Moreover, they determined \( \bar{M}_w \) only, and hence were unable to evaluate the polydispersity of the systems.

Viscosity measurements of these humic substances were carried out in order to have an approximate comparison of molecular weights obtained from the light scattering measurements with the viscosity-average molecular weights.
Theory

A direct proportionality exists between the intensity of scattered light at a particular concentration and the molecular weight (Tanford 1967):

\[
\frac{Kc}{R_\theta} = \frac{1}{\bar{M}_w} + 2Bc + 3Cc^2 + \ldots \quad (1)
\]

where \( c \) is the sample concentration; \( R_\theta \) the intensity of scattered rays, which is dependent on angle of observation, \( \Theta \), and wavelength of primary radiation, \( \lambda \); \( \bar{M}_w \) the weight-average molecular weight; \( B \) and \( C \) are virial coefficients; and \( K \) an optical constant given by:

\[
K = 2\pi n_0^2 (dn/dc)^2 N \lambda^4 \quad (2)
\]

where \( n_0 \) is the refractive index of the solvent; \((dn/dc)\) the refractive index increment of the solution; and \( N \) the Avogadro number.

According to equation (1), intercept on the \( Y \)-axis of a plot of \((Kc/R_\theta)\) against \( c \) is a measure of \( \bar{M}_w^* \). This is essentially extrapolation of \( c \) to zero at constant \( \Theta \). Further, by combining this experiment at different scattering angles and extrapolating both (i) \( c \) to zero at constant \( \Theta \) and (ii) \( \Theta \) to zero at constant \( c \), according to Zimm (1948), one can determine the value of \( \bar{M}_n \), the number-average molecular weight, by drawing asymptote to the curve.
and measuring the intercept on the Y-axis as described by Stacey (1956).

In coloured solutions, the intensity of the incident light is diminished by absorption before reaching the scattering volume and the scattered light is similarly attenuated, before reaching the walls of the cell. Brice et al. (1953) have shown that accurate scattering ratios may be determined even when considerable colour is present. The equation reads:

\[ I_s = A I_o f_1 f_2 f_3 \]

where \( I_s \) is the measured scattered intensity, \( I_o \) the incident intensity and \( A \) the light scattering constant; \( f_1 \) represents the attenuation of the primary scattered beam by absorption, \( f_2 \) the effect of the finite size of the scattering volume and \( f_3 \) the effect of the component of the incident light reflected at the exit face.

\[ f_1 = e^{-2aI}; \quad f_2 = 1 + \frac{W^2}{24} \alpha^2 + \left( \frac{W^2}{576} + \frac{W^4 + h^4}{1920} \right) \alpha^4 + \ldots; \]

\[ f_3 = (1 + 0.043 T_a^2)/1.043; \]

where \( W \) is the effective width of scattering volume viewed by the receiver, \( h \) the width of primary beam in the direction of transverse viewing, \( 2l \) the cell thickness, \( \alpha \) the absorption coefficient, and \( T_a \) the transmittance of the solution relative to the solvent.
Correction for fluorescence is done by utilising the fact that whereas scattered radiation is polarised, fluorescent radiation is not (Brice et al. 1953). Three separate quantities are measured: (i) A scattering ratio with analyser vertical, $R^V$; (ii) a scattering ratio with analyser horizontal, $R^H$; (iii) the depolarisation of the fluorescent light using an auxiliary filter. The equation for the true scattering ratio, corrected for fluorescence, is:

$$\frac{I_s^V}{I_t^V} = \frac{(R^V - R^H/P_f)/(1-P_s/P_f)}{P_f}$$

where $P_f$ and $P_s$ are the depolarisation of the fluorescent and scattered lights respectively, and the notations $V$ and $H$ correspond to analyser vertical and horizontal respectively.

The value of $P_s$ is usually 0.02 or smaller and is, hence, small compared to $P_f$. The denomination of the above equation is, therefore, taken to be equal to unity. This correction due to fluorescence as suggested in equation (4) is incorporated in the evaluation of $R_o$.

For a neutral macromolecule in solution, the reduced viscosity, $(\eta_{sp}/c)$, usually increases with increasing concentrations, $c$, while for a salt-free polyelectrolyte like HA or FA, the curve rapidly rises with diminishing concentration (Tanford 1967; Ghosh & Mukherjee 1971; Ghosh & Schnitzer 1980a). In presence of a sufficient amount of neutral electrolytes, the polyelectro-
lytic behaviour is suppressed and the $(\eta_{sp}/c)$ versus c plot is linear having a positive slope with the c-axis; intrinsic viscosity $[\eta]$, can be evaluated from the intercept on the c-axis, and the viscosity-average molecular weight, $\bar{M}_v$, can be determined from the well-known modified Staudinger equation which is as follows:

$$[\eta] = K \times \bar{M}_v^\alpha$$

(5)

where the values of $K$ and $\alpha$, the two constants, are 0.0350 and 0.65 respectively (Ghosh & Schnitzer 1980a).

Root-mean-square average end-to-end separations, $(R^2)^{\frac{1}{2}}$, of a macromolecule can be determined from the following equation (Flory & Fox 1950):

$$\frac{3}{2} (R^2)^{\frac{3}{2}} = \left(\bar{M}_n [\eta]\right) / \phi$$

(6)

where $\phi$ is a constant having a general value of $2.1 \times 10^{21}$.

**Materials and methods**

All the chemicals used for light scattering measurements were of Analar grade. Dust-free water was prepared by slow distillation of conductivity water. Solutions of HA and FA (both Fluvaquent from Baruipur) were prepared by dissolving the samples in 0.1 N NaCl where their macromolecular structures do not change with sample
concentration (Ghosh & Schnitzer 1980a) and adjusting the pH strictly to 7.0, so that the pH-effect on colour (Ghosh & Schnitzer 1979), fluorescence (Ghosh & Schnitzer 1980b) and free-radical content (Ghosh & Schnitzer 1980c; Varadachari 1981) is nearly uniform in all the solutions. Both the solvents and solutions were filtered several times through 4G sintered crucibles and were collected directly into the cells.

Measurements were carried out with the aid of a Brice-Phoenix Light Scattering Photometer (Series 1999-77) equipped with a Brice-Phoenix Differential Refractometer at a wavelength of 546 nm (Ghosh et al. 1976).

The solutions for viscometric studies were prepared as described for the light scattering measurements. The measurements were done with the help of a Ubbelohde viscometer (capacity: 12 ml) having a flow time of 260.0 sec with water at 30°C ± 0.1.

Results and discussion

Zimm (1948) plots for HA and FA solutions are presented in Fig. 12; \((Kc/R_q)\) plots are curved, so that by drawing asymptotes to the curves and extrapolating to the ordinate, \(\bar{M}_n\) can be obtained from the relation \((Kc/R_q) \rightarrow 0 = \frac{1}{2} \bar{M}_n\) (Tanford 1967). The values of \(\bar{M}_w\) were obtained, according to equation (1), as described earlier. Fig. 13 represents the \((\eta_{sp}/c)\) against \(c\) plots.
The values of $\overline{M}_w$ and $\overline{M}_n$ obtained by light scattering measurements are close to the $\overline{M}_v$ (viscosity-average) data (Table 8). The values of $\langle R^2 \rangle^{1/2}$ (Table 8) show that the HA molecules, which are completely coiled at the salt concentration used (Ghosh & Schnitzer 1980a), have an end-to-end separation of 170 Å as compared to 138 Å for FA molecules. These data too are in reasonable agreement with those reported by Ghosh and Schnitzer (1980a) on similar systems. Polydispersity of the samples may be estimated from the ratios of $\overline{M}_w$ to $\overline{M}_n$. It can be seen (Table 8) that both HA and FA are highly and almost equally polydispersed. It may be concluded that light scattering is a useful tool for estimating polydispersity of humic substances. However, back scattering by these systems is a limitation to the accuracy of this technique, and can only be solved by an improvement in instrumentation.
The greatest stumbling block of the humic scientists is that they need one unique measurement by which they can identify humic substances under any condition. The degradative methods produce information only about the gross chemical composition and about the so-called 'building blocks'. Amongst the non-degradative methods, quite a large number deals mainly with the molecular weight determinations. Some others are mostly concerned with surface and electrochemical properties. IR spectra of the humic materials are very diffuse and overlapping thereby causing much ambiguity in interpreting the observations. UV and visible absorption spectra are featureless, absorption decreases monotonically with increasing wavelength and gives little structural information. ESR spectra is devoid of hyperfine splitting under normal conditions, and NMR (proton and $^{13}$C) data are not much encouraging either, and so is the X-ray diffraction. Summing up of the information from all these methods, a detailed list may be obtained from Schnitzer and Ghosh (1979), one may find valuable observations and interesting results but the aforesaid 'characterisation problem' still remains to be solved. Usually a spectroscopic tool is recommended for this purpose because of its relatively easy, rapid and reproducible natures. A thorough search of literature has been done (Kononova 1966; Schnitzer & Khan 1972; Flaig et al. 1975; Schnitzer 1978; Hayes & Swift 1978; Stevenson 1982) for a clue. The only redeeming feature is probably exhibited by the fluores-
cence excitation spectra of the humic materials.

Fluorescence excitation spectroscopy is a relatively recent tool in the study of humic substances (Datta et al. 1971), although it has already been well-established that in aqueous medium, HA, FA (Ghosh & Schnitzer 1980b), and hymatomelanic acids (Ghosh & Mukherjee 1972), exhibit characteristic spectral bands at 465, 465 and 360, and 470 nm respectively. Our up-to-date knowledge of the subject more or less accepts one of the theoretical predictions of Seal et al. (1964) that the humic fluorophore is an aromatic moiety with electron donating functional groups which was later experimentally supported by Ghosh and Mukherjee (1972) during their course of investigations with the hymatomelanic acids. The present study, primarily, aims at confirming the aforesaid concept by performing experiments with other humic systems. Once this theoretical basis is properly established, one may advocate with a fair degree of certainty that the fluorescence excitation spectral band which is noted to be characteristic of the humic molecules, around 465 nm in aqueous solutions, may be used as their distinguishing features. One cannot guarantee at present, but this approach appears to be closest to the solution of the 'characterisation problem' of humic substances under diverse experimental conditions.

Theory

The effects of solute-solvent hydrogen bonding on the electronic spectra of organic molecules are of two types (Nagakura & Baba
1952). In the first type, \( n-n^* \) bands of proton donors such as phenols, carbazoles, etc., move towards longer wavelengths as a result of hydrogen bonding with proton acceptors like ethers, alcohols, etc. In the second type, the absorption or fluorescence bands of ketones, aldehydes, pyridiazines, etc. (arising from singlet-singlet \( n-n^* \) transitions), undergo a blue shift as a result of hydrogen bonding with proton donors like alcohols. The influence of hydrogen bond formation on electronic transition has been discussed in terms of Franck-Condon principle (Pimentel 1957). The resultant frequency shift is equal to \( W_0 - W_1 + w \) where \( W_0 \) and \( W_1 \) are the energies of hydrogen bond formation in the ground and excited states respectively, and \( w \) is the excitation energy according to the Franck-Condon principle. Factually, by changing the solvent from a pure hydrocarbon where the solute molecules are not hydrogen bonded with the solvent molecules, to another solvent like an alcohol or an ether where there is solute-solvent hydrogen bonding, the most prominent variation (Mataga \textit{et al.} 1956a, 1956b) is a shift in the fluorescence maximum identical with that of the absorption one. Its magnitude may be nearly equal to that of the absorption spectrum or slightly longer than it, when there is extra-stabilisation of the hydrogen bond in the excited state due to electron migration to that from the ground state.
**Materials and Methods**

All the non-aqueous solvents used, except ethanol, were of E. Merck (Darmstadt) GR quality; ethanol was supplied by Bengal Chemical, Calcutta. These solvents were further purified, as described below, with meticulous care to keep them free from contamination by water. Cyclohexane was distilled over metallic sodium and was kept over sodium wires. Both diethyl ether and dioxane (1:4) were treated with ferrous sulphate, distilled and re-distilled over metallic sodium. Tetrahydrofuran was treated with lithium aluminium hydride, distilled and re-distilled over metallic sodium. All these three ether solvents were stored over metallic sodium. Alcohols were refluxed with solid caustic potash, distilled and re-distilled over metallic aluminium powder, stoppered and kept in a desiccator. Two HA samples used in the present investigation, viz., Haplaquept (Darjeeling) and Fluvaquent (Baruipur), were dissolved in the solvents immediately before recording the spectra. Their solubility in non-aqueous solvents is very low. It may be noted that the approach here is purely qualitative and depends exclusively on the nature of the spectra; as the experimental limitation does not permit us to record the fluorescence intensity on an absolute scale, the determination of humic concentration in different solvents was not done because that was not necessary.

Spectra were recorded with the help of a Farrand Spectrofluorometer using a silica cell of 1 cm thickness. The emission wavelength
was fixed at 520 nm, and the wavelengths of excitation radiation were varied automatically from 500 to 220 nm.

Results and discussion

Fluorescence excitation spectra of the HA samples in non-aqueous media are shown in Fig. 14 and the spectral maxima are presented in Table 9. Both the samples showed identical spectral behaviour in every respect.

To characterise the samples, spectra were also run in aqueous solution (Fig. 15) adjusted to pH 7.0; a characteristic excitation band at 465 nm as is well-known in the literature (Ghosh & Schnitzer 1980b) was noted in each case. Thus, the possible use of fluorescence excitation spectral band as the characteristic property of humic substances is reaffirmed. The subsequent task is to determine the nature of the fluorophore. It has been pointed out earlier that the solubility of HA samples in non-aqueous media is extremely low. In order to be sure that the solubilisation of HA in organic media do not cause any adverse effect on the fluorescing moiety, a portion of each of these non-aqueous solutions were dried in vacuum, residue extracted with water, and spectra recorded again. In each case, re-appearance of 465 nm band was observed.
In cyclohexane, a single fluorescence excitation band at 360 nm was noted with both the samples; it is most likely that the fluorophore is not hydrogen bonded in this solvent. On changing the solvent either to an ether (diethyl ether, tetrahydrofuran or 1,4 dioxane) or to an alcohol (methanol, ethanol or n-butanol), a red shift of the fluorescence excitation maximum to 370 nm was observed. Ethers usually act as proton acceptors to \( \pi \)-electron systems which undergo a \( \pi-\pi^* \) transition (Mataga et al. 1956a); while alcohols can act both as proton acceptors to \( \pi \)-electron systems giving a \( \pi-\pi^* \) transition (Mataga et al. 1956a), as well as proton donors to nitrogen heterocycles undergoing a \( n-\pi^* \) transition (Mataga et al. 1956b). Since a red shift is observed here, both the alcohols and ethers have unequivocally acted as proton acceptors to \( \pi \)-electron systems, which indicates that the nature of the fluorophore is an aromatic one with electron donating functional groups similar to phenols, carbazoles, etc., and not a nitrogen bearing group. This may also be noted that the hydrogen bond energy ranges from 1 to 7 K.Cals/bond (350 to 2500 cm\(^{-1}\)), hence a red shift cannot be as large as \( \Delta \nu \) (Franck-Condon principle), i.e., should not exceed 2500 cm\(^{-1}\) (Pimentel 1957). In this experiment, a shift of 750 cm\(^{-1}\) was observed, which is within the above limit.
TABLE 4

Elemental composition (dry, ash-free basis)

<table>
<thead>
<tr>
<th>Geographical origin</th>
<th>Soil series</th>
<th>Soil classification (USDA)</th>
<th>Depth of horizon (cm)</th>
<th>Soil pH (soil:water, 1:2.5)</th>
<th>Organic carbon (%)</th>
<th>Sample</th>
<th>Elemental analysis (%)</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Bengal (North), India</td>
<td>Darjeeling</td>
<td>Mollic Haplaquept</td>
<td>0-15</td>
<td>5.6</td>
<td>1.5</td>
<td>HA</td>
<td>55.6</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FA</td>
<td>45.6</td>
<td>5.3</td>
</tr>
<tr>
<td>West Bengal (South), India</td>
<td>Baruipur</td>
<td>Aeric Fluvaquent</td>
<td>0-15</td>
<td>6.2</td>
<td>0.9</td>
<td>HA</td>
<td>56.2</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FA</td>
<td>45.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Alberta, Canada</td>
<td>Beaverhills</td>
<td>Haploboroll</td>
<td>0-25</td>
<td>6.4</td>
<td>6.1</td>
<td>HA</td>
<td>56.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Prince Edward Island, Canada</td>
<td>Armadale</td>
<td>Cryaquod</td>
<td>15-22</td>
<td>4.0</td>
<td>4.2</td>
<td>FA</td>
<td>49.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>
TABLE 5

X-ray diffraction patterns

<table>
<thead>
<tr>
<th>Haplaquept HA</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>d (Å)</td>
<td>9.6 (b)</td>
<td>7.5</td>
<td>4.0 (b)</td>
<td>3.66</td>
<td>2.41</td>
</tr>
<tr>
<td>I</td>
<td>7</td>
<td>8</td>
<td>19</td>
<td>39</td>
<td>6</td>
</tr>
</tbody>
</table>
TABLE 6

NaOH-titratable acidity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acidity (me/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplaquept HA</td>
<td>3.97</td>
</tr>
<tr>
<td>Haplaquept FA</td>
<td>6.47</td>
</tr>
<tr>
<td>Haploboroll HA</td>
<td>3.62</td>
</tr>
<tr>
<td>Cryaquod FA</td>
<td>6.73</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>7.40</td>
</tr>
</tbody>
</table>

TABLE 7

Total acidic groups

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total acidic groups (me/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplaquept HA</td>
<td>7.20</td>
</tr>
<tr>
<td>Haplaquept FA</td>
<td>12.64</td>
</tr>
<tr>
<td>Haploboroll HA</td>
<td>6.30</td>
</tr>
<tr>
<td>Cryaquod FA</td>
<td>13.54</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>14.44</td>
</tr>
<tr>
<td>Sample</td>
<td>$\overline{M}_w$</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Fluvaquent HA</td>
<td>4650</td>
</tr>
<tr>
<td>Fluvaquent FA</td>
<td>3850</td>
</tr>
</tbody>
</table>
### TABLE 9

**Fluorescence excitation spectral bands in non-aqueous solvents**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Excitation spectral maxima in nm</th>
<th>Haplaquept</th>
<th>HA</th>
<th>Fluvaquent HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexane</td>
<td>360</td>
<td>360</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>370</td>
<td>370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>370</td>
<td>370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dioxane (1 : 4)</td>
<td>370</td>
<td>370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>370</td>
<td>370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>370</td>
<td>370</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-Butanol</td>
<td>370</td>
<td>370</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
% WEIGHT LOSS

TEMPERATURE (°C)

HA
Specific conductance \times 10^5 \text{ ohm}^{-1} \text{ cm}^{-1}

pH

me base/g Salicylic acid

- **NaOH (Medium: Water)**
  - pH-metric
- **KOH in Isopropyl alcohol (Medium: Acetone)**
  - Conductometric
Specific conductance $\times 10^5$ ohm$^{-1}$ cm$^{-1}$

- **Haploboroll HA** (Beaverhills)
- **Cryaquod FA** (Armadale)
- **Haploaquept HA** (Darjeeling)
- **Haploaquept FA** (Darjeeling)

![Graphs showing specific conductance vs. me KOH/g for different soils](image-url)
Medium:
(a) Cyclohexane
(b) n-Butanol
(c) Ethanol
(d) Methanol
(e) Diethyl ether
(f) Tetrahydrofuran
(g) Dioxane(1:4)
(a) Haquept HA
(b) Fluvouent HA
Medium: aqueous