Journal of Chromatography, 177 (1979) 130–134

CHROM. 11,956

Note

Characterization of soil humic acid by combined polyacrylamide disc electrophoresis and chromatic reactions

MASSIMO CASTAGNOLA
Istituto di Chimica dell'Università Cattolica del Sacro Cuore e/o Centro della Chimica dei Recettori e delle Molecole Biologicamente Attive, C.N.R., Rome (Italy)
CORRADO NIGRO
Istituto Sperimentale per La Nutrizione delle Piante, Rome (Italy)
GIOVANNI BATTISTA MARINI-BETTÒLO and ADRIANO MILANA
Istituto di Chimica dell'Università Cattolica del Sacro Cuore e/o Centro della Chimica dei Recettori e delle Molecole Biologicamente Attive, C.N.R., Rome (Italy)
and
RAMON GARCIA DE LAS HERAS
Istituto de Edafologia y Biologia Vegetal, Consejo Superior de Investigationes Cientificas, Madrid (Spain)
(Received May 8th, 1979)

Zone electrophoresis has been employed in the study of humic acids, with paper[1–7], cellulose[8] and agar and polyacrylamide gels[9–11] as supports. Moreover, paper electrophoresis has made possible the separation of humic acids in the most important pedological soils which have three fractions of different mobilities (mobile, immobile and semimobile), and the determination of their polymerization grade as well their average composition of brown and gray humic acids. Recent research[8] has also indicated the possibility of obtaining, from the electrophoretic pattern of humic acid fractions on a cellulose column, some information on the organic fertility of the soil, which is important for the evaluation of the potential fertility of the soil.

Among the different separation techniques the most promising is disc electrophoresis on polyacrylamide gels; this method make possible the separation of polymer fractions on the basis of molecular weight and thus constitutes an important approach to the study of the chemical structure and the properties of humic substances. Polyacrylamide gels have also been used in the characterization of humic acid by electrofocusing[12–14].

In previous experiments[15] we have shown that the use of urea solutions at different concentrations, facilitating the disaggregation of humic acid fractions, allows an increase in their resolution. The most difficult problem, however, is the separation, identification and characterization of the various humic fractions; disc electrophoresis, although not suitable for separations on a preparative scale, in this case allows the identification and characterization of some chemical properties of the various fractions by the use of chromatic reagents. The use of dyes in electrophoresis

for staining proteins gives limited results and does not reveal all the fractions separated. For this reason we have examined some specific staining methods which could give, in the electrophoretic migration pattern, some information on the presence of particular components of functional groups in the humic complex. These results could be useful in the evaluation of organic fertility of the soil.

We have thus studied the behaviour of two cyanines, blue alcian and blue astra, often used to detect mucopolysaccharides and in general all polyoses with acidic properties, in the electropherogram of humic acids. Comassie brilliant blue and amido black, respectively a triphenylmethane and an azo dye, employed in the staining of proteins, were also studied.

We have assayed the PAS staining method, based on the oxidation of two adjacent OH groups followed by the identification of the resulting aldehyde with fuchsine bisulphite. This method is largely employed in the histological characterization of polysaccharides[16] and thus may be used to establish the presence of sugar residues in the humic fractions.

Finally we have examined the possibility of using the reduction of Fe^{3+} to Fe^{2+}, which can be revealed by staining with Prussian blue, to identify reducing groups present in the humic fractions.

MATERIALS AND METHODS

Eight samples of humic acids, extracted with 0.1 *M* NaOH and 0.1 *M* $Na_4P_2O_7$ according the method of Kononova[18], were purified by repeated precipitations and lyophylization. The samples were obtained from typical soils of the Lazio region surrounding Rome, as reported in a previous paper[19].

Electrophoresis in polyacrylamide gel was performed according to a procedure previously described by us[15], both under standard conditions and with 4.0 *M* urea solutions. The staining procedures were modified as follows.

Blue alcian. The fixing of the gels was performed with a 10% solution of lead acetate in 10% acetic acid for at least 6 h. The staining was carried out with 0.2% Alcian Blau 8GS (C.I. 74240) in 7% acetic acid for at least 6 h; destaining in 10% acetic acid required at least 24 h (standard method).

Blue astra. The same procedure was employed as with blue alcian.

Amido black. The gels were stained for 2.5 h with a 1.0% solution of amido black (C.I. 20470) in 7% acetic acid without previous fixing treatment. The plates were treated after staining for 24 h in 10% acetic acid in order to eliminate the excess of the dye.

Comassie brilliant blue. Gels were fixed in a 20% sulphosalicylic acid for 12 h and then treated with a 0.25% solution of Comassie brilliant blue (C.I. 42660) for 6 h and for at least 24 h with 7% acetic acid.

Periodic acid –Schiff (PAS) reaction[17]. The gels* were treated with 12% trichloroacetic acid for 30 min, washed two or three times with water and dipped for 50 min in 1.0% periodic acid solution in 2.0% acetic acid. After thoroughly washing with water until the IO_4^- had completely disappeared, the plates were treated with

* To avoid possible contaminations, saccharose is eliminated from the spacer gel and the sample is applied suspended in Sephadex G-200 (0.4 mg/100 ml).

fuchsine sulphite* in the dark for 50 min. They were then washed three times (10 min each) with 0.5% metabisulphite solution and repeatedly washed with water. The plates were kept under 5% acetic acid.

Prussian blue[11]. The gels were dipped into a 0.5% solution of $FeCl_3$ in 0.1 *M* HCl for 10 min, treated with a 2.0% potassium ferricyanide solution for 20 min and repeatedly washed and kept under 0.1 *M* hydrochloric acid.

Reagents

Lead acetate, trichloroacetic acid, periodic acid (for analysis; E. Merck, Darmstadt, G.F.R.), blue astra (for histology, Merck), Alcian Blau 8GS (Standard für Mikroskopie; Fluka, Buchs, Switzerland), Comassie Brilliant Blue R-250 (Serva, Heidelberg, G.F.R.), Amido Schwarz B-10 (Erba RS; Carlo Erba, Milan, Italy), ferric chloride and potassium ferricyanide (Erba RP), basic fuchsine and sodium metasulphite (Erba RPE).

RESULTS AND DISCUSSION

First, the analogous electrophoretic behaviour of all the different humic

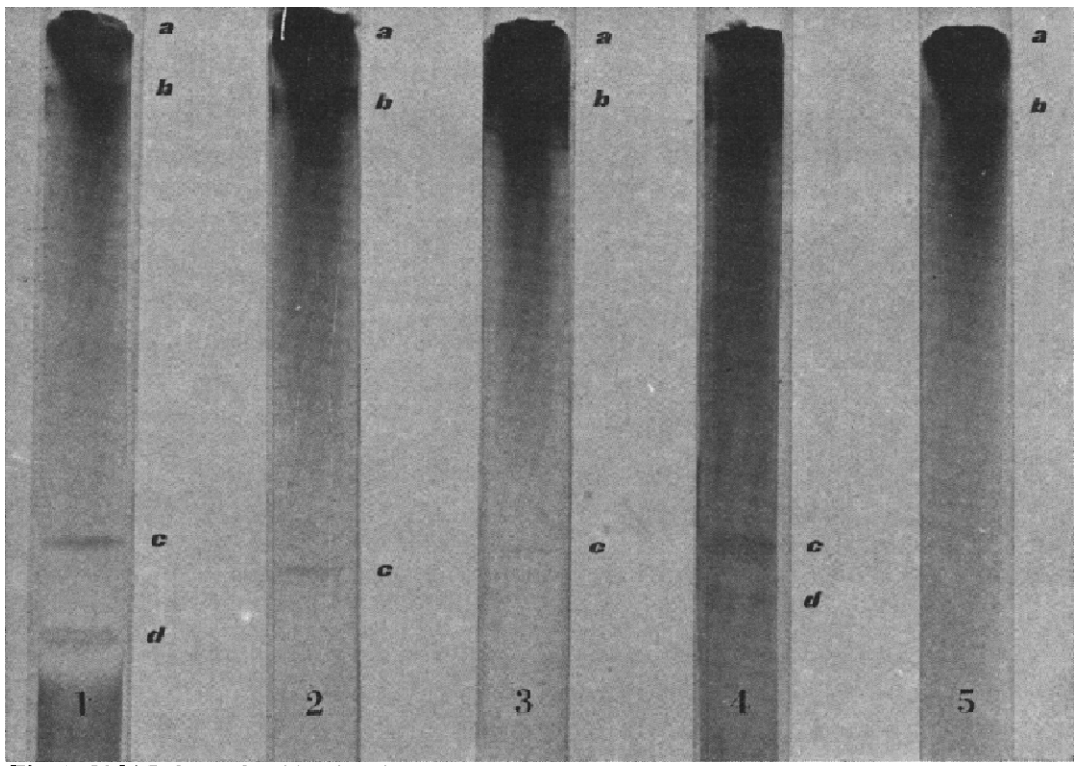

Fig. 1. 20% Polyacrylamide disc electrophoresis of humic acid under standard conditions: 1 = without stain; 2 = blue alcian; 3 = blue astra; 4 = Prussian blue reaction; 5 = PAS reaction.

* A 0.5-g amount of basic fuchsine (C.I. 42510) and 0.5 g of sodium metabisulphite were dissolved in 100 ml of 0.15 *M* HCl. The solution was shaken every 2–3 h until all the fuchsine was transformed into the sulphite (*i.e.*, the solution is practically colourless). The solution was then treated with carbon black and filtered. Kept at 4°, it can be stored for 15 days[20].

acids under examination (so far investigated) should be emphasized[19]. We report as an example the results obtained with the humic acid separated from the soil of Arcinazzo (soil No. 5 of ref. 19), derived from old limestones and containing 11.9% of organic matter. In the photographs we do not show the gels stained with amido black and Comassie brilliant blue, because they gave unsatisfactory results, confirming that no protein is present. Thus the nitrogen bonded to the humic complex should be attributed to the presence of free amino acids.

The electrophoretic patterns obtained with blue alcian and blue astra are similar and show a group of homogeneous fractions, One of these (a)[15] does not penetrate in the gel, owing to its high molecular weight and its shape. A second fraction (b) of intermediate electrophoretic mobility, has a lower molecular weight than fraction a, and two other fractions (c, d) have higher electrophoretic mobility and thus still lower molecular weights. By treatment with urea, which as previously reported disrupts the hydrogen bonds, a new fraction (e) originates. This indicates that a part of the high-molecular-weight fractions (a and b) is formed of aggregates not covalently bonded but stabilized by hydrogen and ionic bonds.

Both blue alcian and blue astra are therefore suitable for selective non-specific staining of all the fractions obtained from humic acids. This is evident from a comparison of gels 2 and 3 and those fixed, but not coloured, reported for gel 1 (Fig. 1). The staining with fuchsine bisulphite and Prussian blue establishes particular chemical properties of the various fractions of humic acids present. The results for the gels 3 and 4 (Fig. 1) show that the fractions obtained by electrophoretic separations can be

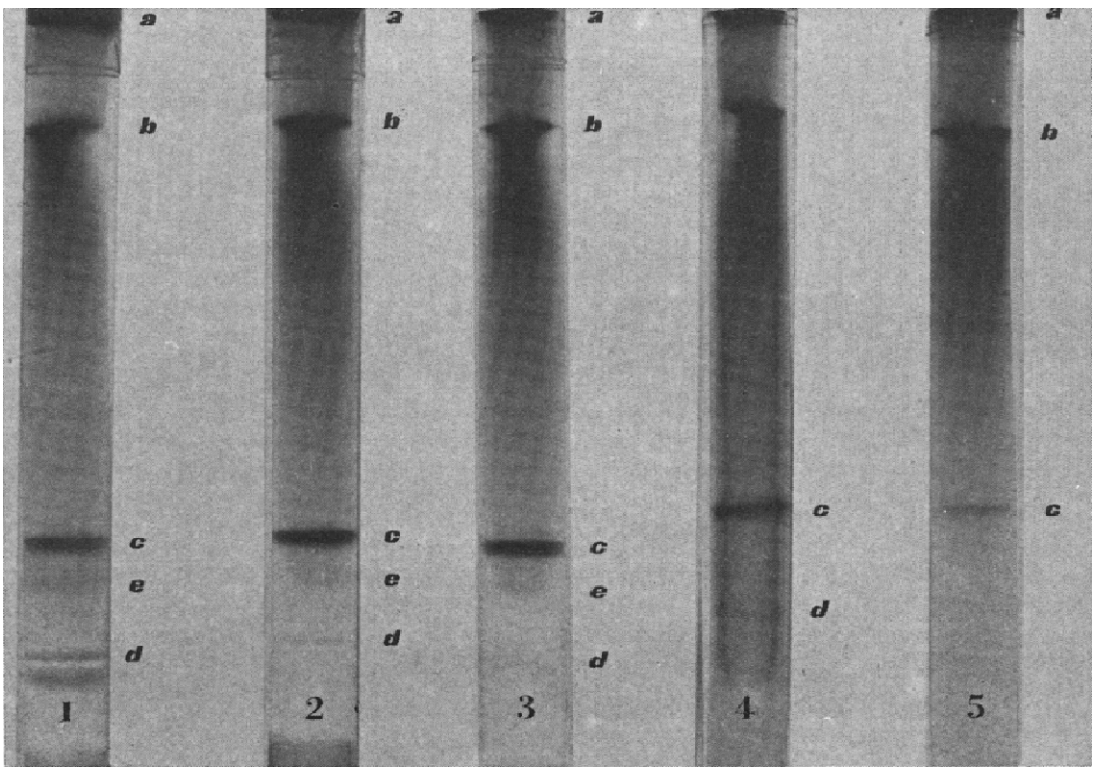

Fig. 2. 20% Polyacrylamide disc electrophoresis of humic acid in 4.0 *M* urea. Details as in Fig. 1.

divided into two groups. The first is formed by the fractions a and b of high and medium molecular weight. They are both intensely stained with the PAS reagent and do not give the Prussian blue reaction. This can be attributed to the fact that present in these fractions are substances with various adjacent OH groups, which by themselves do not have reducing properties (*e.g.*, polysaccharides). Thus, it can be inferred that polysaccharides are present in these fractions, which do not present free glycoside or aldehyde groups (negative Prussian blue reaction). This is confirmed by other results reported in the literature[21–23].

The second group formed by fractions c and d of high electrophoretic mobility are not coloured with PAS reagent, but give a positive Prussian blue reaction. Thus we may argue that these fractions do not contain polysaccharides (negative PAS reaction), and that their reducing properties, indicated by the Prussian blue reaction, may be attributed to other reducing functional groups, *e.g.*, *ortho*- and *para*-diphenols which are easily oxidized to quinones, not present in fractions a and b.

These data are confirmed by the results obtained with humic acids in the presence of urea solutions (3 and 4, Fig. 2). In this case a new band is formed of high mobility, most probably originating in the high-molecular-weight fractions which react only with PAS reagent.

Therefore it seems that the high molecular-weight fractions a and b are formed by a molecular aggregate resulting from binding through weak forces such as hydrogen or ionic bonds. On the other hand, we may assume that c and d are not derived from disaggregation of fractions a and b, but may be considered to differ both in their structure and chemical properties.

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