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LOW TEMPERATURE SCANNING ELECTRON MICROSCOPY OF CLAY AND ORGANIC CONSTITUENTS AND THEIR RELEVANCE TO SOIL MICROSTRUCTURES

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Abstract

Low temperature scanning electron microscopy (LTSEM) is essential for studying the microstructure of small-sized soil constituents such as clays and organic matter. These hydrated and swelling materials naturally undergo drastic changes in water potential and water content in soils. The cryofixation of clays and organic macromolecules by immersion in cryogens and their LTSEM observation are evaluated. The microstructures of clay minerals, polysaccharide macromolecules and their associations, as revealed by LTSEM, contribute to a better understanding of the water retention properties and physical stabilities of these major soil constituents.

Key Words: Structure, soil, clay, organic matter, polysaccharides, microorganisms, water retention, aggregation, LTSEM, TEM.

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Introduction

Low temperature methods in electron microscopy have been established and continuously improved for observing biological material. They have also been successfully applied to other fields of research, such as polymers, foods, and soils. Soils are at the interface of the biological and mineral world. In many aspects, they drastically differ from rocks: soils are loose, hydrated and partly organic materials and contain living organisms. Therefore, traditional scanning electron microscopy (SEM) or transmission electron microscopy (TEM) techniques may alter their structures.

A major need in soil science is to bridge the gap between the molecular and crystal structures on one hand, and the macroscopic properties of soils, such as water movement and retention, aeration, and mechanical stabilities, which play a major role in biological activities, on the other hand.

The purpose of the present paper is to present the application of low temperature scanning electron microscopy (LTSEM) to soil microstructures, its aims, achievements and limitations.

Soils are very heterogeneous materials mainly constituted of minerals, quartz and inherited silicates, carbonates, oxides and clays, which may occur in a range of nanometric to millimetric sizes. Soils also are comprised of organic constituents (usually 1-10% weight/weight, w/w). These include the vegetation and the remains of soil inhabitants at various stages of decomposition, from small debris, to humic macromolecules and simple organic molecules.

Soil macroscopic properties are dominated by the amount and nature of their smallest constituents: clay minerals and organic substances. These exhibit very high surface areas, as a result of their small particle size: a few square meters to several hundred square meters per gram. Clay minerals have a net negative charge (0.1-1.5 meq/g) and humic substances exhibit a high functionality (e.g., carboxyls, phenolic acids). Clays and organic constituents are thus responsible for retaining cations and organic molecules such as nutrients or pollutants.

Table 1. Water energy levels in soils: equivalence between water activity, matric potential (suction pressure), osmotic potential (ionic strength), and relative humidities (Kelvin's equation), and consequent maximum size of water-saturated pores (Jurin-Laplace's law) and decrease in solution freezing point.

Water activity	Suction pressure (MPa)	RH (% at 25°C)	Ionic strength (M)	Maximum size of water-saturated pores (μm)	decrease of water freezing temperature (°K)
.99999	.001		2.2×10^{-4}	150	
.99993	.01	99.99	2.2×10^{-3}	15	
.9993	.10	99.96	2.2×10^{-2}	1.5	
.9927	1.0	99.29	2.2×10^{-1}	.15	
.989	1.58	98.88	3.3×10^{-1}	.095	-1.1
.967	4.64	96.66	1.0	.032	-4
.927	10	92.69	2.2	.015	-10
.800	30	80.00	6.0	.005	
.695	50	69.50		.003	
.493	100	49.30		.0015	-90

Soils are triphasic materials: they comprise the above described solid constituents, with a liquid phase, i.e., the soil solution, and a gaseous phase, i.e., the soil atmosphere. The proportions of these three phases in soils are not constant but fluctuate widely with wetting and drying events such as rain, irrigation, freezing and thawing. Water is retained by soils by short range hydration forces, long range forces and capillary or osmotic forces. The relationships between the various dimensions expressing soil-moisture tension, the maximum size of the water filled pores, and the freezing corresponding points are given in Table 1.

It was essential to the study of the organization of clays and organic constituents to develop observation methods, at the electron microscope resolution, that could preserve the wet state fabrics.

Soil Sample Preparation for Scanning Electron Microscopy

Given the size of soil constituents and structural units, scanning electron microscopy is an appropriate tool to analyze soil microstructures (Smart and Tovey, 1981). A major point is that, prior to introduction in the high vacuum of the SEM, samples have to be dehydrated or frozen (Smart and Tovey, 1982).

When subjected to drying, soils lose water and may even shrink as a whole. The surface tension of water is high (73 dynes/cm^2), and the capillary forces created at air/water menisci may exceed 100 bars in small pores leading to considerable shrinkage and distortion of the original structure. This shrinkage is major in the case of clays, organic constituents or soil biota (Fig. 1). Whereas, the very surface of soils regularly experiences dry conditions (relative humidity, RH 50%; water potential: Ψ > 100 MPa), deeper soil horizons, or soils in water-logged situations, may never reach such dry states. Therefore, the microstructures resulting from air dried soil samples may represent very temporary structures or even artefactual ones.

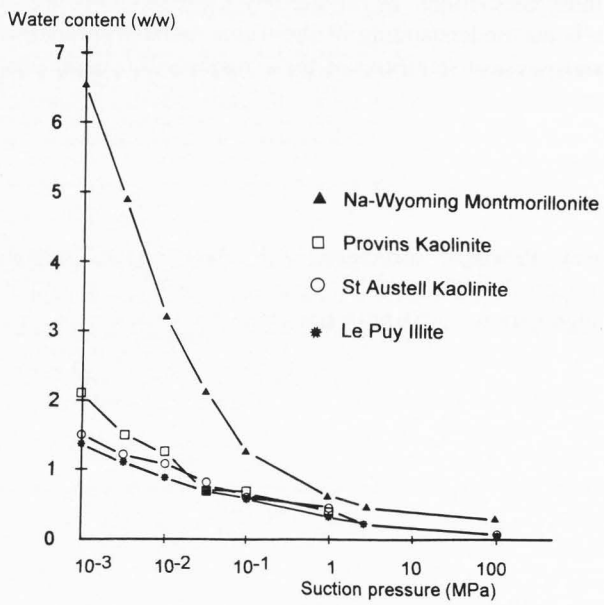

Figure 1. Water retention of clays (from Tessier, 1984).

The deleterious effects of surface tension can be overcome by critical point-drying of the samples. Specific chemical treatments are then needed to stabilize the organic constituents during the exchange of water by solvents, and these may be deleterious as well (Crang, 1988). The result may not be satisfactory: for example, samples with low stability, such as kaolinites, can be severely disrupted (Tessier, 1987).

Freezing is another way to immobilize the microstructures in their hydrated state and allow soils to be examined in their natural states when low temperature SEM is used. Freezing has additional advantages: the mechanical strength of water and hydrophilic materials is enhanced by freezing. It allows the specimens to be fractured or even dissected. Planar fractures can be obtained, revealing the inner part of samples, which is not affected by the sampling process (Delage *et al.*, 1982; Tessier and Berrier, 1979).

Cryofixation has its own set of artifacts and limitations, the ideal goal obviously being the vitrification of the liquid phase, or microcrystalline (nm) ice formation that does not perturb the structures (Robards, 1991). Several sophisticated methods have been set up that allow water or dilute solutions to be vitrified (Dubochet *et al.*, 1982). Unfortunately, such methods are not realistic options for soils. The quenching may be optimized by an appropriate choice of coolant (Ryan *et al.*, 1987) and by increasing the velocity of entry in the coolant (Robards, 1991), but the main limit is that fast freezing methods require very small samples. Even the best freezing methods at ambient pressure, cannot freeze below a depth of 10-20 μm. This is a result of the low thermal conductivity of water and ice, and the consequent slow rate of heat withdrawal from the specimens. Solid or "gelly" samples, such as soil samples, cannot be easily manufactured into spray droplets (Bachman and Mayer, 1987) or thin sandwiches (Müller *et al.*, 1980) without altering their microstructures. Thus, a compromise has to be made between the necessity to excise a piece of soil that is small enough to freeze well and the inevitable damage associated with the sampling process.

Two sets of methods, hyperbaric freezing (Moor, 1987) and the use of cryoprotectants (Franks *et al.*, 1977), ensure proper "deep" cryofixation. To our knowledge, the applicability of hyperbaric freezing to soil has not been evaluated yet. High pressure freezing allows good freezing conditions on reasonably large samples (0.5-1 mm), but the presence of air and air-water interfaces probably precludes the possibility of using it with soils. On the other hand, cryoprotectants are not used because: (i) the osmotic potential directly affects the arrangement and structure of clay particles, and (ii) polymeric cryoprotectants may obscure the morphology after deep etching (μm) is performed.

Freezing soils or clays is obviously not freezing pure water. Soil solutions contain salts and organic solutes. These increase the osmotic potential and thereby change the freezing conditions, in particular, they may decrease the ice nucleation temperature and limit crystal growth (Robards and Sleytr, 1985). A significant proportion of the water is "bound water," that is adsorbed to clay surfaces or to organic or mineral macromolecules. These water molecules have different chemical and physical properties from bulk water and respond differently to changes in temperature: bound water may be unfreezable, and has reduced evaporation rates (Bachman and Mayer, 1987; Homshaw and Chaussidon, 1978).

Given the large size of soil or clay samples that are cooled (0.1-2 mm), freezing should lead to ice crystallization with large crystals (> 10 nm). However, both the strong binding of some of its water and the composition of the solution may restrict crystallization and crystal growth. A detailed analysis of the microstructures after cryofixation is therefore required. This will be discussed in the following sections for clayey and organic constituents.

LTSEM Processing of Soils

The observation of samples in LTSEM involves several steps: (i) cryofixation, (ii) fracturing, partial freeze-drying, and coating, and (iii) examination and analysis at a low temperature in the SEM. Each step is critical to the quality of observations.

A major point, quite specific to clays and soils science, is that the hydration state needs to be known or maintained because the soil solution composition (i.e., osmotic potential), the matric potential and the previously applied stresses strongly affect the microstructures. Several methods are used to maintain or control these parameters: pressure plates ($0 < \Psi < 2$ MPa), polyethylene glycol solutions ($0.05 < \Psi < 7$ MPa), and desiccators with saturated solutions (RH 98-0 %; $1.5 < \Psi <$ 1000 MPa). How physical and geochemical parameters may affect the microstructures of soil constituents is presented below.

Soil or clay samples are usually cryofixed by plunging them into melting nitrogen (nitrogen slush 63K), liquid freon (113K), or other coolants. For this purpose, they are mounted on metallic stubs using a variety of sample holders (Robards and Sleytr, 1985). After freezing, the specimens are transferred in an evacuated transfer device to the low temperature preparation chamber of the microscope. For example, our laboratory is equipped with a Hexland CT1500 system with a cryochamber dedicated to a Philips 525 SEM. In this chamber, the specimens are fractured with a cooled blade.

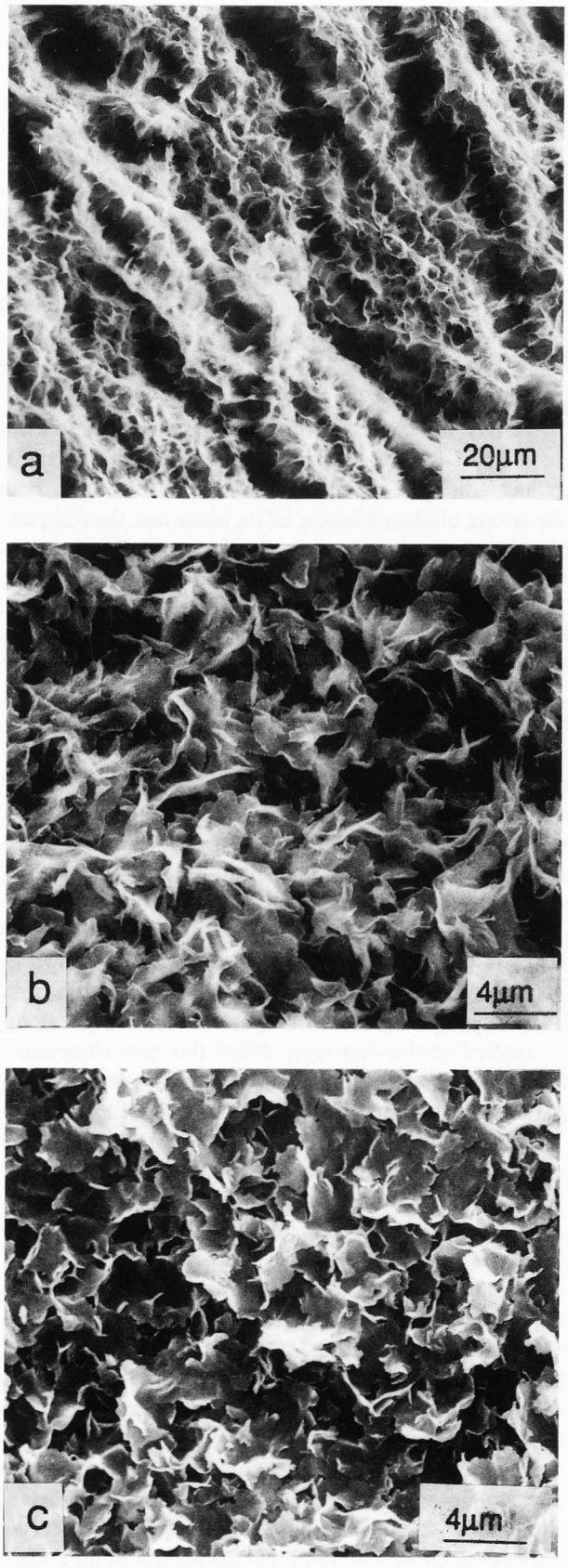

Figure 2 (at left). Calcium smectite microstructure. (**a**) Large sample (diameter, ϕ = 1 cm) frozen in liquid nitrogen and observed with LTSEM (partial freeze drying); (**b**) small sample (ϕ = 1 mm) frozen in freon at its melting point and observed with LTSEM (partial freeze drying); and (**c**) sample prepared after critical point drying.

Samples may then be observed fully hydrated, so that water films and menisci are observed (Campbell, 1983; Campbell and Porter, 1982). Most often a partial freeze-drying process (etching) is performed to remove superficial ice and reveal the microstructures. The ice sublimation process can be visualized in the SEM. Ice sublimation is performed at temperatures < 193K (-80°C) at a vacuum of 10^{-4} Pa, in order to avoid ice recrystallization and growth (Robards and Sleytr, 1985). Different sublimation times (2-30 minutes) are used, depending on the nature of the sample and on its water content. Samples are then sputter coated with gold under an argon atmosphere at 187K (-180°C). Metallization with gold limits the resolution to 5-10 nm, which is the size of deposited gold particles (Jeffree and Read, 1991). After introduction into the cryostage (at < 100K), the samples are observed and analyzed. During all steps of specimen preparation, special care is needed to maintain a clean high vacuum using cold traps to prevent the sample from being contaminated by condensing water vapor.

Clay Microstructures

Presentation

When strongly hydrated, clays are very soft, sticky and deformable materials. Most clay minerals are characterized by a plate-like shape and a small size (< 2 µm). The basic unit of the clay is a **layer** of 1.0 or 0.7 nm thickness. The space between successive stacked layers is called **interlayer**. Each clay crystal can be defined as a stacking of parallel layers. Among the clay minerals in nature two groups may be distinguished: (1) clays with hydrated and expandable interlayers, i.e., smectites and vermiculites, and (2) clays with anhydrous and non-expandable interlayers such as kaolinites and illites. The study of clay hydration requires both, characterizing interlayer water (when present), and water located within pores of the clay particle assemblages.

Since Mering (1946), Norish and Raussel-Colom (1962), and Posner and Quirk (1954), many reports have shown that "crystal swelling," i.e, interlayer swelling of clays is controlled by the nature of compensating cations and by the nature and concentration of electrolyte solutions.

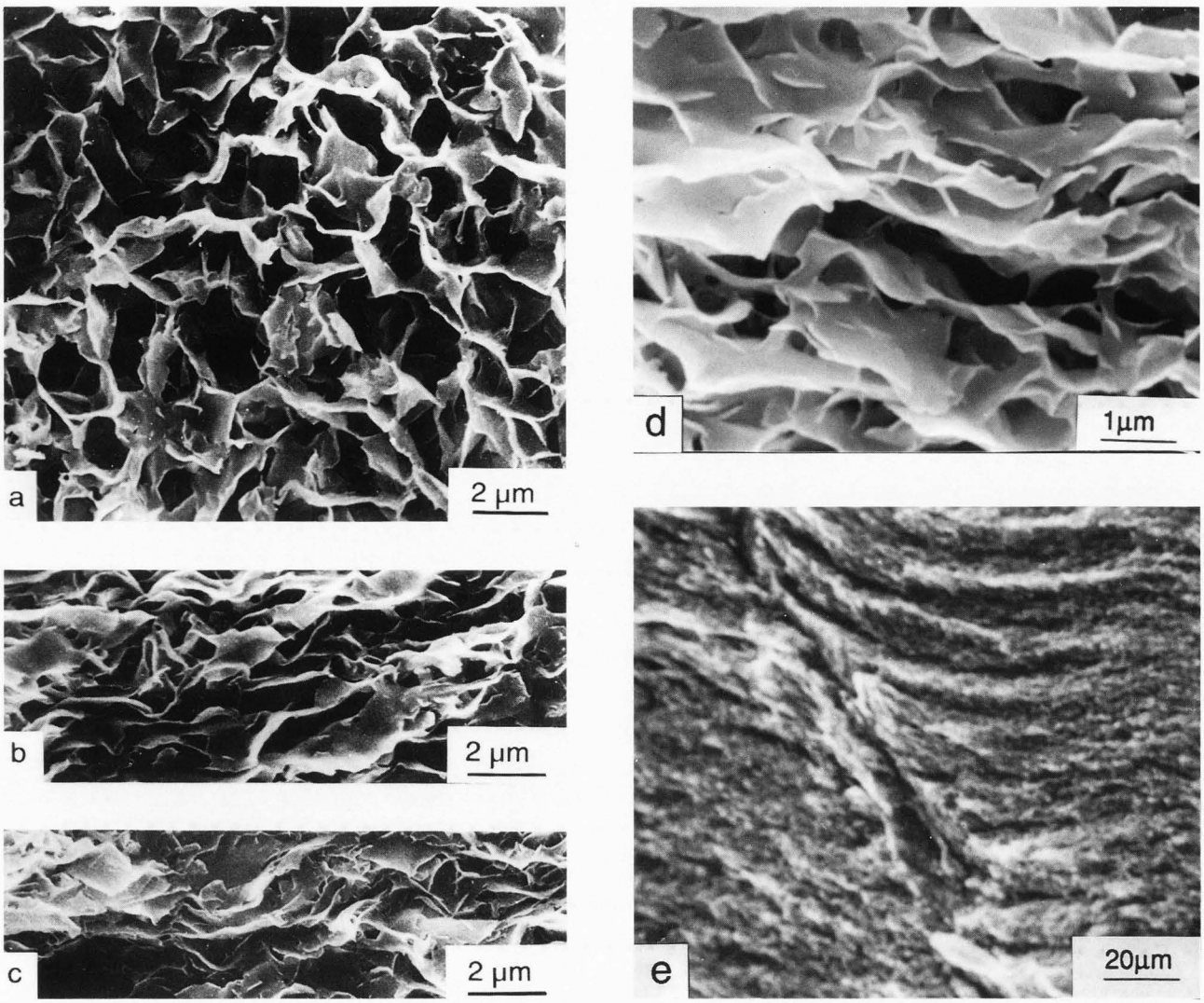

Figure 3. Microstructural changes of smectites with water content, LTSEM observations, (partial freeze drying). Dehydrating of a NaCl 1 M montmorillonite: (**a**) 0.0032 MPa, water content = 3.69 g/g; (**b**) 0.1 MPa, water content = 1.14 g/g; and (**c**) 1 MPa, water content 0.82 g/g. The surface area of the micrographs 3a to 3c is proportional to the apparent volume of the sample. The shrinkage of the sample from 0.0032 MPa to 1 MPa water potential can then be visualized. (**d**) Ca smectite rewetted from 100 MPa to 0.0032 MPa; and (**e**) same sample at lower magnification showing shear plane formation (from Tessier, 1984, 1991).

One of the first attempts to visualize the spatial arrangement of clay particles in a strongly hydrated state was reported by Weiss *et al.* (1952). Clay smectites were examined after freezing large samples (1 cm) in liquid nitrogen, and the observed structures were similar to those shown in Figure 2a. Considerable distortions of the clay microstructure were observed, i.e., 100 μm elongated pores due to ice crystallization. Since then electron microscopy techniques, including LTSEM, have allowed clay microstructures at the hydration states, prevailing in soils, to be resolved.

Smectite clay paste microstructure

The microstructures of smectites prepared with calcium and magnesium as the exchangeable cation, or with sodium at a high salt concentration, were well described using different techniques (X-ray diffraction, TEM, water adsorption isotherms; Emerson, 1962; Tessier, 1984). Because these smectites have interlayer hydration limited to 1-3 layers of water molecules, both the type and the distribution of water in the microstructure are rather well known (Emerson, 1962; Touret *et al.*,

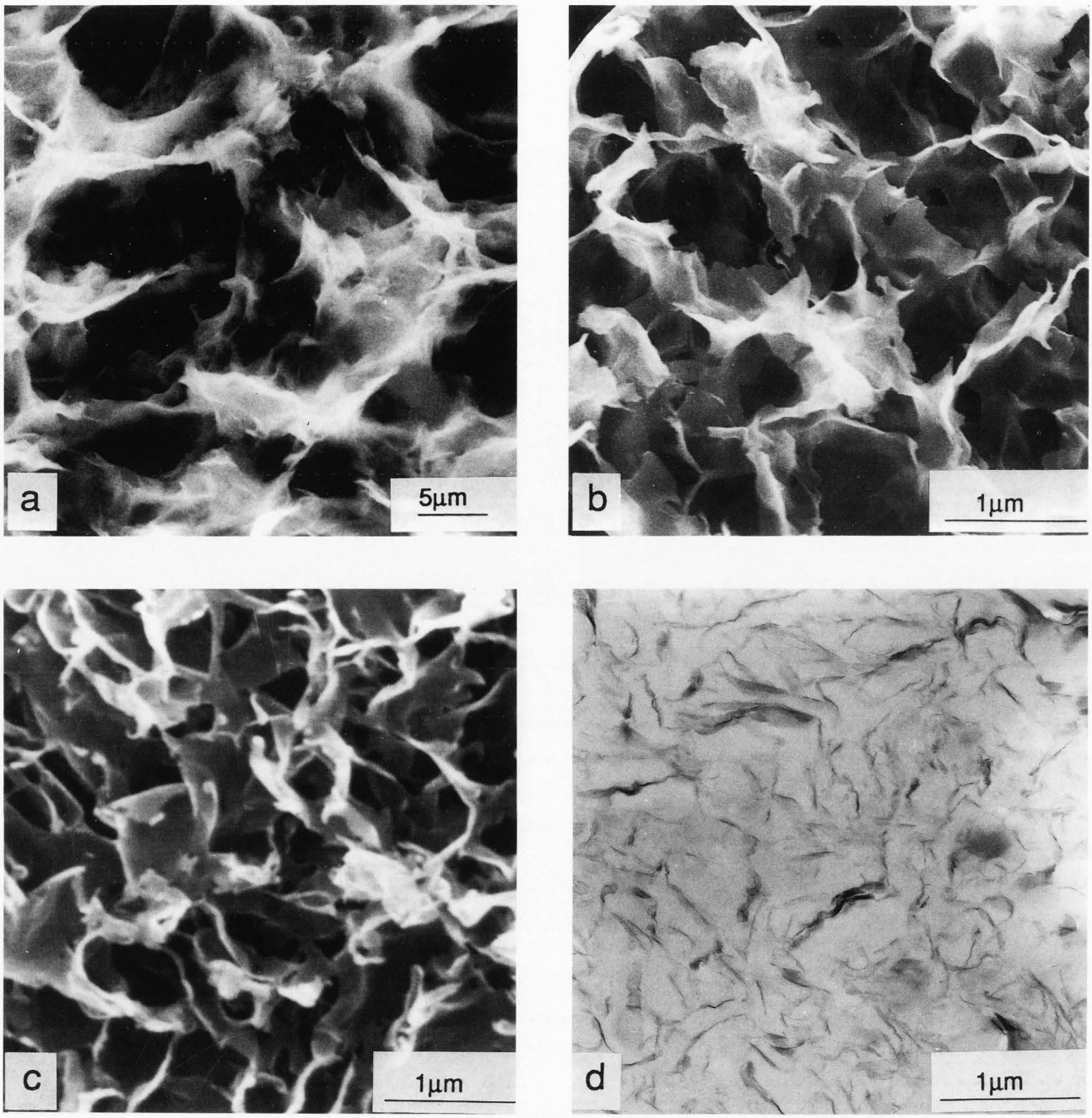

Figure 4. Sodium smectites prepared with dilute solution (10^{-3} M NaCl): (**a**) after freezing in liquid nitrogen, LTSEM; (**b**) after freezing in freon at its melting temperature, LTSEM, partial freeze drying; (**c**) after critical point drying; and (**d**) after embedding in an epoxy resin, TEM. Samples were partially freeze dried.

1990); e.g., in clay pastes equilibrated with 0.01 MPa suction pressure, the total water content is about 6 grams water per gram dry solid (w/w) while maximum interlayer water can be estimated at 0.36 g/g (w/w). Thus, most of the water is located between clay particles (Tessier, 1984). At this hydration state, the LTSEM shows the clay microstructure as an isotropic network of bent and twisted lamellae defining 1-2 μm pore spaces (Fig. 2b). An isotropic network microstructure is also observed after critical point drying (CPD) (Fig. 2c).

Table 2 gives basal spacings and particle thicknesses of a calcium smectite, and of a sodium smectite prepared with 1 M NaCl solution, at ambient temperature (293K), at 233K, and after solvent exchange for TEM inclusion

Table 2. Interlayers and particle thickness of smectite equilibrated with -0.0032 MPa water content (from Pons *et al.*, 1982; and Tessier, 1984). HRTEM = High resolution TEM.

Sample	Basal spacings	Number of layers per particle
Ca smectite, $CaCl_2$, 10^{-3} M, 293K (X-ray)	1.86	50
Ca smectite, $CaCl_2$, 10^{-3} M, 233K (X-ray)	1.56	~ 50 to > 100
Ca smectite, $CaCl_2$, 10^{-3} M, air-dry, (X-ray)	1.56	> 100
Ca smectite, $CaCl_2$, 10^{-3} M, (HRTEM)	1.26-1.56	~ 50
Na smectite, NaCl, 1 M, 293 K (X-ray)	1.86	~ 40

in a resin. It appears that few changes in basal spacings are caused by either freezing or solvent exchange, as also found by Annabi-Bergaya *et al.* (1980). This stability of interlayers can be explained by the presence of organized water molecules in the interlayers and by repulsion forces up to 5 MPa or above 100 MPa for 3 and 2 sheets of water molecules, respectively. Because of this high interlayer stability, water did not migrate when small specimens were frozen in nitrogen or freon at their melting temperatures and sublimated below 193K. It was concluded that the microstructure evidenced by LTSEM (or after CPD) was the true one, due to a face to face arrangement of clay particles.

The use of LTSEM made it possible to analyze how, and to what extent, the clay microstructure was affected by changes in water content. The impact of dehydrating a Na-smectite of high electrolyte content (1 M NaCl) on its microstructure is illustrated in Figure 3. At low suction pressure (i.e., high water content) the clay particles delineate pores 1-2 μm in diameter (Fig. 3a). Upon slight desiccation (0.1 MPa), the loss of water from interparticle pores results in increased capillary water tension. The interparticle pores become lenticular in shape as the particles get closer together (Fig. 3b). The network shrinkage and orientation is even more marked at 1 MPa suction pressure (Fig. 3c). These results were supported by X-ray diffractometry evidence of increased particle orientation (Tessier, 1978) and provided a microstructural explanation to the changes in clay permeability and rheological properties associated with drying. LTSEM also gave new insights on changes of smectites due to wetting and drying cycles. After drying and rewetting, Ca-smectite exhibits an oriented network microstructure (Fig. 3d) different from the original isotropic network. Strong drying causes particle aggregation which was confirmed by TEM and low angle X-ray scattering (LAXRS) (Ben Rhaiem *et al.*, 1986; Tessier, 1984). In addition, it was observed with LTSEM that the volume changes upon drying induced shear planes (Fig. 3e) (Tessier *et al.*, 1990).

It was concluded that the behavior and microstructure of calcium smectites, as well as of other smectites equilibrated with divalent cations, is strongly influenced by the stresses to which the material was previously subjected. In natural conditions, wetting-drying cycles, freezing, and mechanical compaction may induce such changes in microstructure and explain soil compaction and related properties due to climatic conditions or anthropic activity.

Sodium smectite (clay gel) microstructure

Sodium smectites are extensively used as additives in the oil industry, soil mechanics, foundries, as well as in cosmetics. When sodium smectites, or lithium or potassium smectites are prepared with dilute saline solutions (NaCl from ~ 10^{-4} to ~ 10^{-1} M for Na-clays), and at low suction pressures (< ~ 1 MPa), these clays exhibit thixotropic properties of gels.

Clay gels are very sensitive to the preparation methods that are used for SEM. An example is given with a sodium smectite sample, about 5 mm in size, plunged into nitrogen at its boiling temperature and observed after ice sublimation (~ 243K) (Fig. 4a), average pore size is 15 μm. This micrograph can be compared to the same sample that was frozen in freon at its melting point (113K) (Fig. 4b), average pore size is 0.8 μm. With boiling nitrogen, the formation of very large pores is due to large ice crystals, thus, revealing a considerable migration of water from the interlayers to the interparticle pore spaces (Anderson and Hoekstra, 1965).

Figures 4a and 4b can be compared to the same material which was observed after water substitution by methanol and CO_2 and critical point drying for SEM (Fig. 4c) or after water substitution by methanol and propylene oxide and embedding with a Spurr resin for TEM (Fig. 4d). With these preparative methods, the clay networks have pore sizes of 0.2-0.8 μm. Both pore sizes and particle sizes are small compared to those with LTSEM. Clearly, with low temperature techniques, it is very difficult to avoid water migration, and therefore, changes of clay gel microstructures.

In their study of clay hydration mechanisms, Pons *et al.* (1981) prepared gels at various temperatures. Changes in interlayer distances were observed upon

Table 3. Basal spacings and mean number of layers per particle in Wyoming sodium smectite as determined by HRTEM and LAXRS at 293K and 233K, at water content w/w = 9 (gel) and w/w = 0.14 (air dry). (From Pons *et al.*, 1981; Tessier, 1984).

Technique	Basal spacing (nm)	Number of layers per particle
HRTEM after embedding with resin	1.26-1.56	~ 5
LAXRS 233K	1.56	above 100
LAXRS 293K gel	3.5 to 10	~ 5
LAXRS 293K air-dry	1.26	above 100

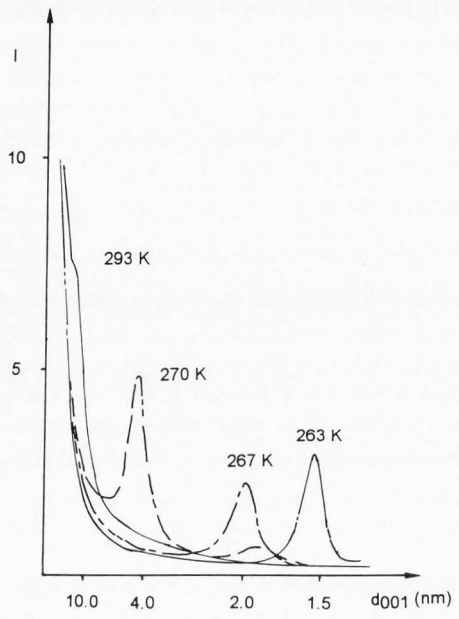

Figure 5. Low angle X-ray scattering diagrams of Li-smectite at different temperatures showing changes in interlayer distances (adapted from Pons *et al.*, 1982).

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freezing, showing a migration of water from interlayers to ice crystals as the temperature decreased (Fig. 5). A synthetic view of the results for Na-smectites is given in Table 3. Gels at ambient temperature (293 K) were characterized by large interlayer distances of 8 to 10 nm and few layers (5) per particle. Both the sample frozen at 233K and the air-dried sample had collapsed interlayers (1.56 nm) and thick clay particles (> 100 layers per particle). This microstructure differed from that of the original sample, i.e., the particles were clumped together to form larger entities.

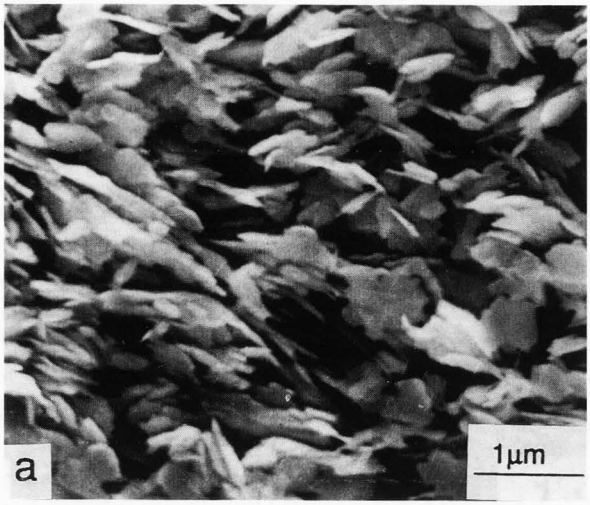
By contrast, with the critical point drying and the embedding technique, the number of layers making up the particles was equal to that of undisturbed gels. However, even with these techniques, the interlayer also collapsed and as a consequence interparticle pore size was presumed to be artefactually increased.

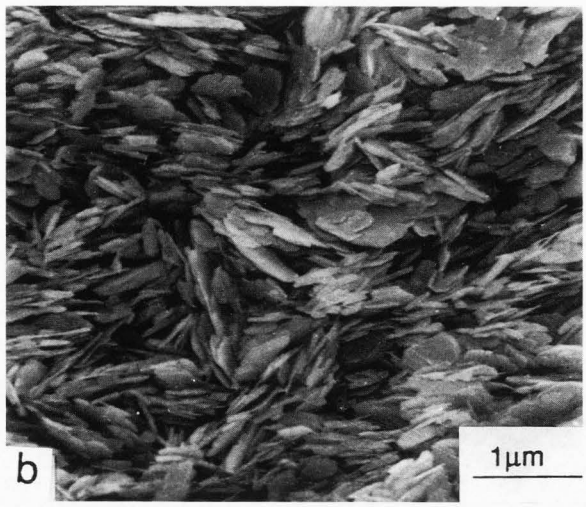
Figure 6 (on the facing page). Kaolinite and illite microstructures. LTSEM observation of St. Austell kaolinite at 0.001 MPa (**a**), and 0.1 MPa (**b**) (partial freeze drying), and corresponding orientation index obtained by X-ray diffraction (**c**). Le Puy illite: LTSEM (**d**), partial freeze drying; critical point drying (**e**), and TEM after embedding in epoxy resin (**f**). (From Tessier, 1984).

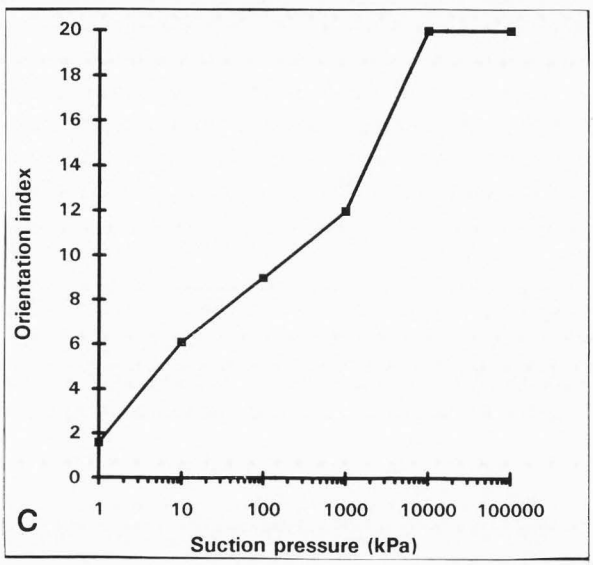
These results indicate that in sodium gels, water is mainly located in interlayer spaces and that interlayer hydration forces are very sensitive to various factors, such as, freezing. It also means that the magnitude of the interlayer repulsion forces does not exceed 1.5 MPa (Tessier, 1984). Thus, the preservation of the microstructure is largely dependent on the binding of water, which can be modified by lowering water contents and/or by increasing interstitial salt concentration. It is clear that for Na-smectites equilibrated with dilute solution at low stresses ($\leq$ 0.001 MPa), LTSEM observations were all characterized by an alteration of the microstructure. Tazaki *et al.* (1990) provided good microstructural observations of smectites at low water contents (i.e., low water potentials). To our knowledge, no preparative method has yet allowed the preservation of fully expanded interlayers in Na-smectites (~ 10 nm). Vali and Bachman (1988) were able to fix the true arrangement of particles in dilute suspensions of smectites and the gross size and shape of these particles, using fast freezing techniques, i.e., sandwich jet-freezing and spray-freezing. However, their high magnification micrographs reveal that ~ 10 nm interlayers are flattened to a few nanometers (Vali and Hesse, 1992). Nonetheless, the combination of various techniques has allowed gel microstructures of smectites to be revealed and properties such as water retention, swelling, hydraulic conductivity and rheological behavior could also be explained.

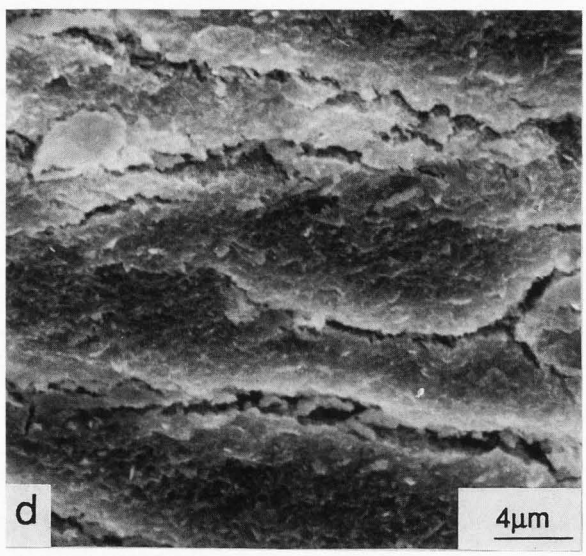
Kaolinites and illites

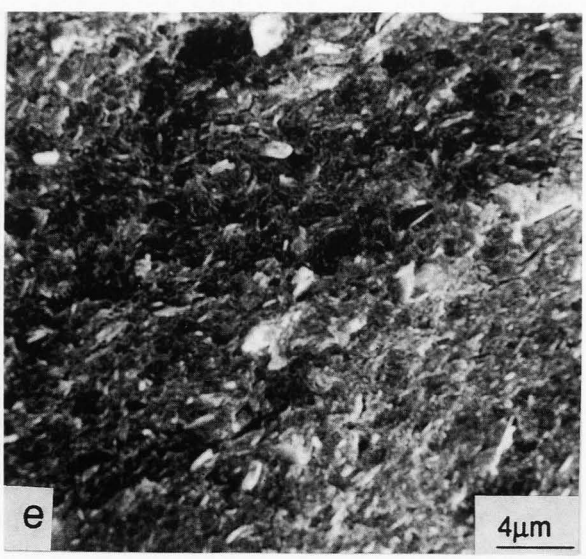
Kaolinite is the major constituent of tropical soils and common in temperate soils. It is frequently used in the ceramics and paper industry. Illites and related minerals are widespread in soils under temperate climate.


a
1μm


b
1μm


c
Orientation index
0
2
4
6
8
10
12
14
16
18
20
1
10
100
1000
10000
100000
Suction pressure (kPa)


d
4μm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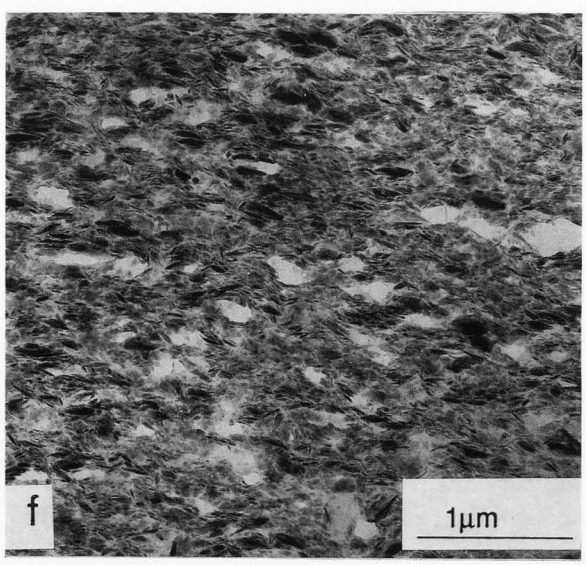

f
1μm

Two examples will be given in this section: coarse kaolinites and fine-sized illites.

The changes in clay microstructure of St. Austell kaolinite (with water content) are shown with LTSEM in Figures 6a and 6b. Discrete, coarse, and plate-like particles can be seen and their mutual parallel orientation increases with suction pressure as confirmed by X-ray particle orientation measurements and TEM (Fig. 6c) (Grunberger *et al.*, 1994; Tessier, 1978). The presence of very rigid particles in kaolinites is due to anhydrous and strong cohesive interlayer forces according to (Ben Ohoud and Van Damme, 1990), who concluded that clay particles in kaolinite have, because of particle rigidity, limited surfaces in contact as is observed with LTSEM (Fig. 6a) and consequently, poor interparticle physical stability.

Figures 6d, 6e and 6f are of illite, observed after low temperature preparation critical point drying, or observed with TEM. With LTSEM, large submacroscopic discontinuities were often observed in hydrated samples, in pure illites, as well as in illitic soils (Bruand and Tessier, 1987; Delage and Pellerin, 1984). The cracks, which were not observed with other preparative methods (Figs. 6e and 6f), were ascribed to water migration between pores upon freezing, as a result of the poor cohesion of the material and water being loosely retained by the clay matrix. The phenomenon was more marked when the clay particles were small, i.e., presumably when their size was close to that of ice crystals.

Organo-mineral microstructures

Organic compounds have a major role in the physical structure of soils. Various organic molecules are able to adsorb to soil minerals and to aggregate them and LTSEM has been used to demonstrate and analyze this aggregation (Metzger and Robert, 1985; Robert and Schmit, 1982; Vicente and Robert, 1981). Several studies have been focused on polysaccharides, because these are, among the organic constituents, particularly active in soil aggregation. In soils, polysaccharides are either inherited from plants or produced *in situ* by microorganisms and roots as extracellular polysaccharides (EPS).

The observation of polysaccharides in LTSEM

Polysaccharides are quite difficult to preserve for electron microscopy as a result of their strong hydration and the methods for preserving microbial EPS for SEM are the subject of much debate (Fraser and Gilmour, 1986; Graham and Beveridge, 1990; Jacques and Graham, 1989; Mutafschiev *et al.*, 1982; Ravenscroft *et al.*, 1991; Read *et al.*, 1983; Rhodes *et al.*, 1985; Van Doorn *et al.*, 1990).

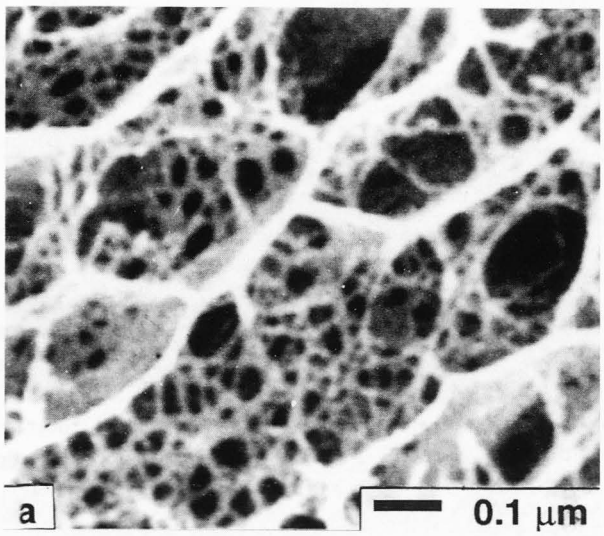
When observed by conventional LTSEM, EPS appear as glassy surfaces, and after etching, as networks of fibrils. For example, the fungal EPS scleroglucan

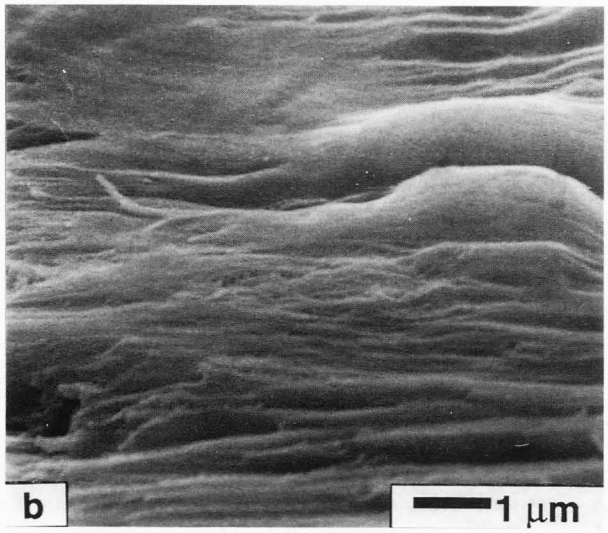
Figure 7 (on the facing page). LTSEM (partial freeze drying) and TEM observations of polysaccharides. **(a)** EPS scleroglucan, equilibrated with Ψ = 0.0032 MPa, water content 47 g/g dry sample; **(b)** EPS scleroglucan, Ψ = 1 MPa, water content 0.98 g/g.; **(c)** EPS scleroglucan adsorbed to kaolinite particles, Ψ = 0.0032 MPa, water content 1.5 g/g; **(d)** EPS scleroglucan adsorbed to kaolinite particles, Ψ = 0.0032 MPa, water content 1.5 g/g, TEM observation with silver staining of the polysaccharide; and **(e)** kaolinite-root mucigel association, equilibrated with Ψ = 0.1 MPa, EPS/clay ratio 1% w/w. (From Chenu and Jaunet, 1992a, b, c, d; Habib *et al.*, 1990e).

(Satia, France) is a weak gel at a 2% w/w concentration (i.e., 98 grams water per gram dry solid water content), and is observed as a network of fibrils with pores 0.05-1 μm in diameter (Fig. 7a). When scleroglucan is adsorbed onto clay, it appears as fibrils ~10 nm in thickness and 50-350 nm long, which are attached to the clay surfaces or to other fibrils (Fig. 7c). We also observed the kaolinite-scleroglucan complexes with TEM, after embedding in Spurr's resin and staining with silver proteinate (Chenu and Jaunet, 1990, 1992; Thierry, 1967). The polysaccharides were then visualized as a thin mesh of fibrils 1-5 nm thick and 20-100 nm long (Fig. 7d).

The size and shape of scleroglucan molecules are quite well known from physicochemical studies. Scleroglucan molecules have a high molecular weight, MW (~ 2 x 10^6), form rods of about 3 nm diameter with a persistence length of ~ 200 nn which may associate into aggregates (Biver *et al.*, 1986; Bluhm *et al.*, 1982; Yanaki and Norisuye, 1983). Random weak interchain association also occur (Stipanovic and Gianmateo, 1989). More recently, scleroglucan was shown to occur as worm-like structures of several hundreds of nm in length (Stokke *et al.*, 1993). Comparing our observations with this data leads to the conclusion that TEM preserved the microstructure very well and LTSEM fairly well. Some aggregation of chains did occur during the freezing: the scleroglucan fibrils are longer and thicker in LTSEM than predicted from literature data, or than observed with TEM. Such chain aggregation is probably due to an exclusion-concentration process (Kellenberger, 1987): as ice crystals nucleate and grow, the EPS fibers are concentrated in the remaining unfrozen solution. When the eutectic concentration is reached, this solution vitrifies (Bachman and Mayer, 1987).

The morphology of EPS as interconnected fibrils has often been questioned as being an artefact of specimen preparation. We conclude that it is a true microstructure of this polysaccharide and many others.


a
0.1 μm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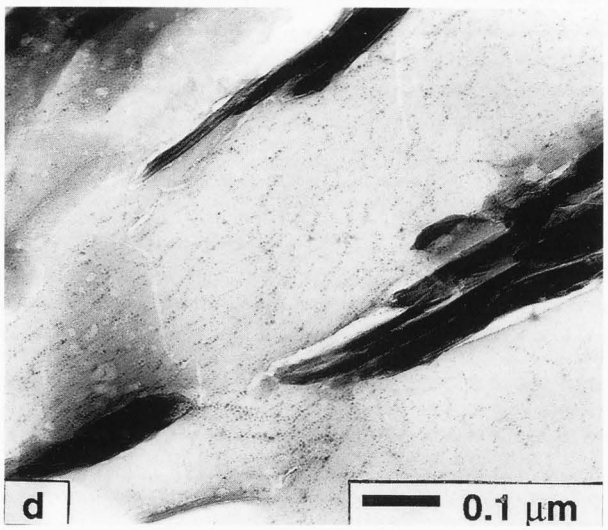
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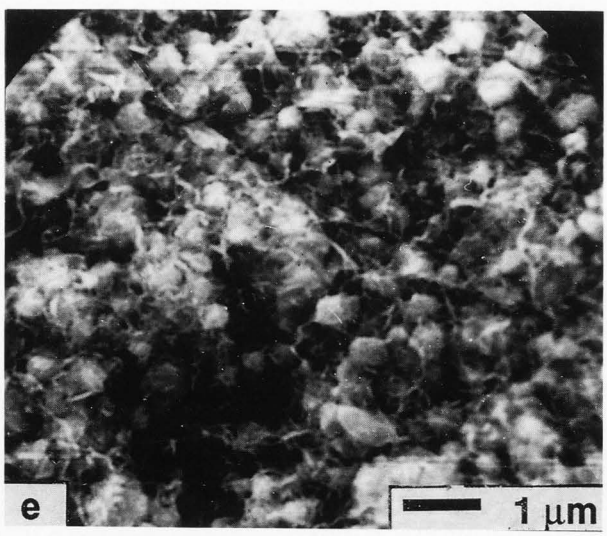

e
1 μm

The preservation of the microstructure of EPS in LTSEM is largely dependent on their water content at freezing, and on their conformational properties. As the water content in EPS decreased, the thickness of the observed fibers increased, whereas the pore size was not greatly affected (Chenu and Jaunet, 1992). Below water contents of about 1 gram water per gram dry solid (i.e., 50% w/w concentration), polysaccharides, such as, xanthan or scleroglucan appeared as non-porous solids, whose surface was glassy if unfractured, and rough after fracturing and etching (Fig. 7b) (Chenu and Jaunet, 1992). At such hydration states, the water is very strongly retained (Chenu, 1993) and the solution may freeze without phase separation or with very small crystals (Franks *et al.*, 1977). In addition, sublimation rates may be dramatically decreased (Bachman and Mayer, 1987). Polysaccharides having random coil structure with poor interchain association, such as, dextran or Na-polygalacturonate, invariably gave easily recognizable freezing artifacts (Chenu, 1985), similar to freezing artifacts recognizable in observations of other polysaccharides (Gu and Doner, 1992) or of humic substances (Chen and Schnitzer, 1976; Gu and Doner, 1992; Vali and Hesse, 1992). Hence, the cohesion of molecular assemblages is essential to their behavior upon freezing.

It is concluded that LTSEM of polysaccharides, and other soil macromolecules, can be performed with limited artifacts when water contents are low and when the polysaccharides have some inter-molecular cohesion. The preservation of organic macromolecules with LTSEM is better than that obtained with conventional methods such as air-drying, or critical point-drying (Chenu and Jaunet, 1992), which often led to not identifying or recognizing the presence of organic macromolecules.

Clay microstructure as affected by adsorbed polysaccharides

The formation of clay-polysaccharide associations in soils is one of the basic phenomena explaining the formation and stability of soil aggregates (Robert and Chenu, 1992).

Neutral or anionic polysaccharides, such as scleroglucan, xanthan or guars, did not greatly affect the general arrangement of clay particles to which they were adsorbed. The card-house structures of kaolinite, illites, as well as the networks of sheet-like particles of montmorillonite were generally preserved and the patterns of microstructure rearrangements with desiccation were essentially the same (Chenu, 1989; Loeber, 1992). However, the macroscopic properties of the clay were drastically modified. These organo-mineral assemblages are very cohesive and resistant to dispersion, even at millimetric scales (Chenu and Guérif, 1991; Chenu *et al.*, 1994). The clay particles were actually aggregated by polymer bridges that were visualized with LTSEM or TEM (Figs. 7c and 7d).

Figure 8 (on the facing page). The microstructure of a sandy and a clayey soil. C: clay; S: sand grain; V: vegetal remnant; the arrow points to crack in the clayey soil; white bars = 100 μm, Ψ = 0.01 MPa (courtesy of Hassink and Chenu). Samples partially freeze dried.

When ionic interactions prevailed, i.e., with anionic polysaccharides (e.g., alginate, polygalacturonic acid, carboxylic guar, or root mucigels) and when the clay was exchanged with di- or tri-valent cations (Ca, Al), the smectite microstructures were strongly modified. The clay particles were clustered into 1-10 μm microaggregates (Fig. 7e) (Habib *et al.*, 1990). Clay was also aggregated by the polysaccharides in this case, but the aggregates were smaller. This micro-aggregation presumably modified the macroscopic properties such as water retention and hydraulic conductivity.

In conclusion, observations carried out on clay-polysaccharide associations provide microstructural explanations for the fact that small amounts of organic compounds, in the order of 1% w/w, can drastically change the clays physical properties. LTSEM observations made it possible to identify the aggregation mechanism, i.e., polymer bridging of clay particles. Work on experimental clay-polysaccharide associations evidenced microstructural changes of clays due to adsorbed polysaccharides, and the same changes were recognized in the vicinity of microorganisms or roots (Dorioz *et al.*, 1993).

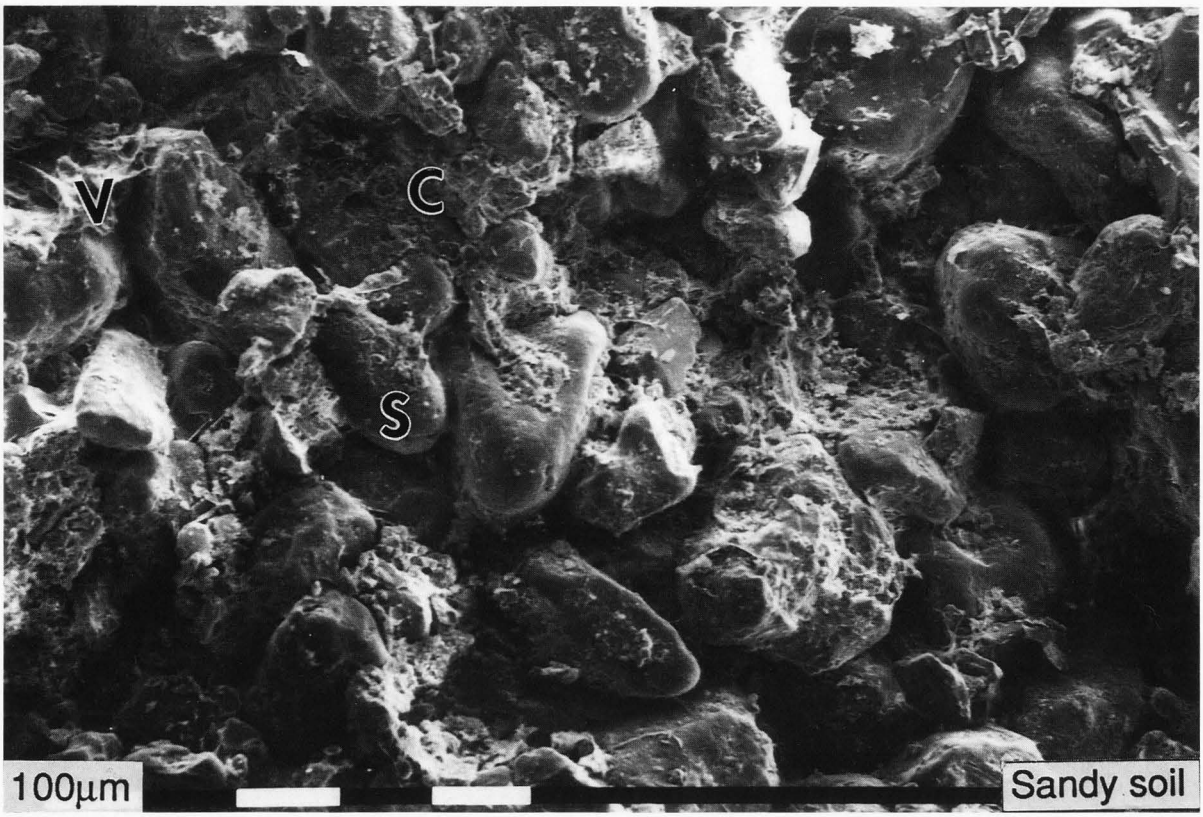
LTSEM as a Tool to Study Soil Microstructural Organization

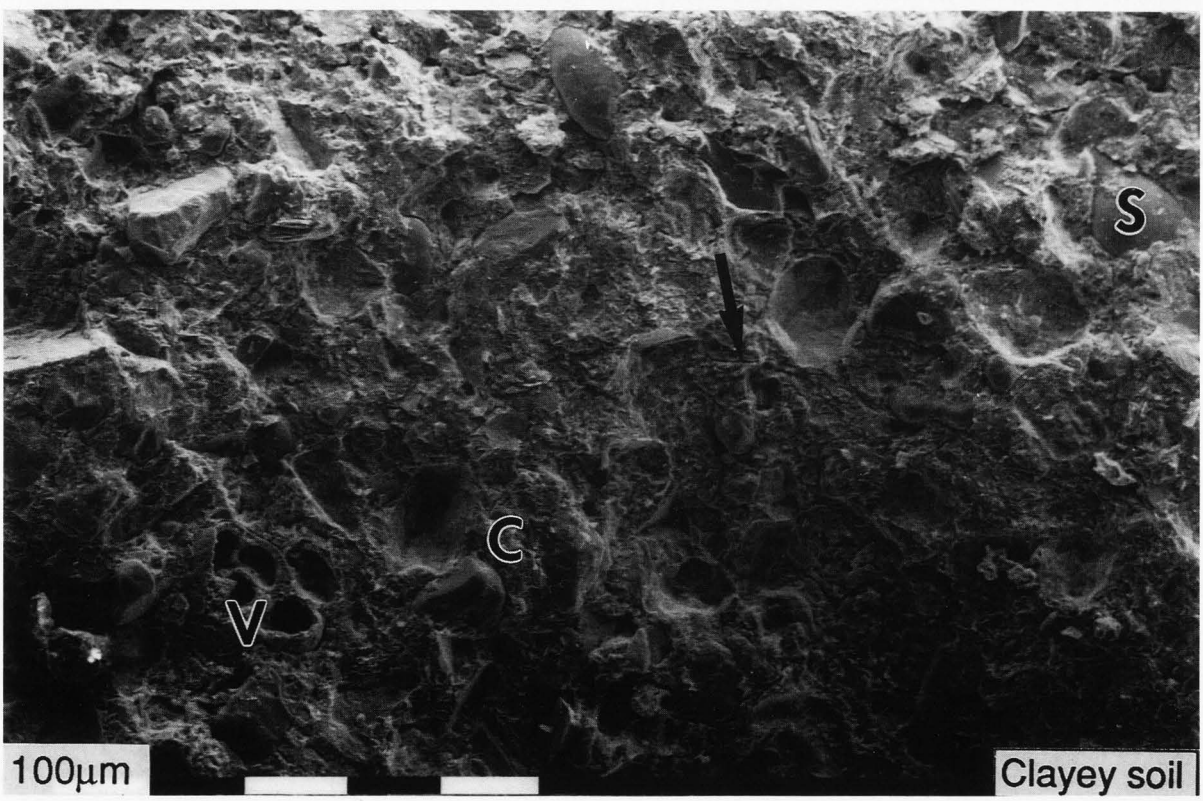
A major advantage of SEM is that it makes it possible to easily visualize the general soil fabrics in the 0.1 to 1000 μm range. Soils have complex hierarchical organizations ranging from particles and their elementary assemblages, to millimetric or centimetric crumbs. Microstructures and associated porosities may vary considerably among soils as exemplified in Figure 8 for a sandy and a clayey soil.

It was found that microstructures developed in clayey soils were highly comparable to those previously observed in fine illitic or smectitic clays (Wilding and Tessier, 1988). In other words, basic interpretations established for pure clays may be transposed for soils with high clay content.

Water-dependent microstructures in soils

The main interest in using LTSEM is to characterize hydrated natural soils at various water contents and to demonstrate how, and to what extent, the microstructures are water-dependent. The main contribution of


V
C
S
100μm
Sandy soil


S
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V
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Clayey soil

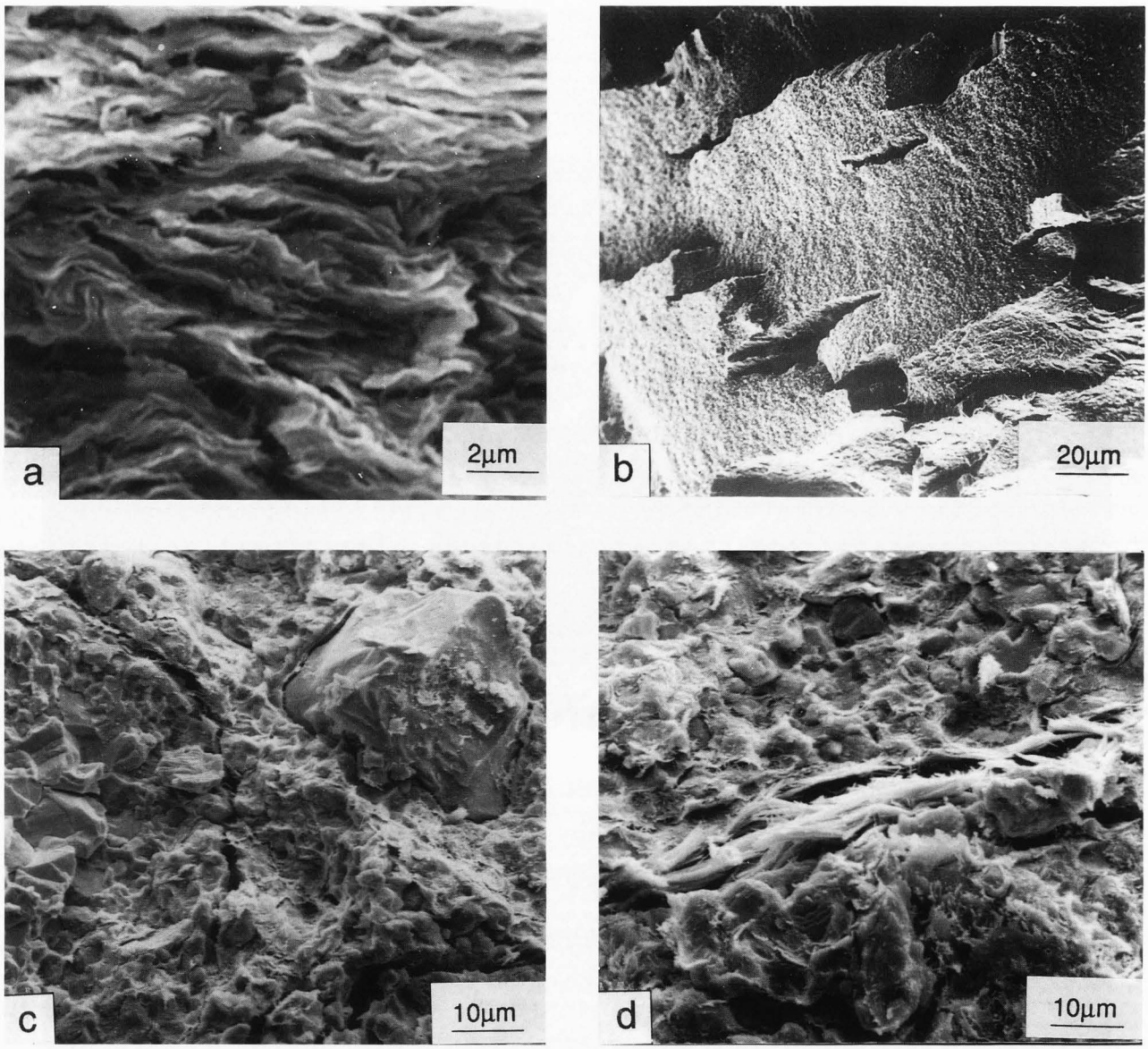

Figure 9. Soil microstructures. (**a**) Fabric of the clay mass in B horizon of Bethonvilliers soils, $\Psi = 0.001$ MPa; (**b**) cracks in the clay mass of B horizon in Versailles soil; (**c**) presence of cracks and (**d**) location of clay in desert soil fabric. (9c and 9d are from Marcoen *et al.*, 1994). Samples partially freeze-dried.

LTSEM was probably to demonstrate that water content variations in clayey soils do not correspond to changes in interlayers as commonly stated, but to clay particle rearrangements. Thus, the water holding capacity of soils is mainly related to the presence of clay matrix pores, ranging from 0.1 μm to about 2 μm in diameter (e.g., Figs. 2, 6 and 9a) (Bruand *et al.*, 1988; Tessier and Berrier, 1979).

Soil microstructures and aggregation

Different levels of organization are generally present in soils. LTSEM showed that clay particles and their associations with other constituents give rise to aggregates in natural conditions. Pore sizes that are observed with LTSEM are consistent with the pore size distributions that are deduced from water retention curves, or with mercury injection and permeability measurements. The observation of undisturbed soil samples at various states of hydration showed that cracking takes place upon soil dehydration, and that cracking affects all soil structure levels, even that of clay particle arrangement (Figs. 3f and 9b). Furthermore, the location of clay particles around, rather than between sand grains, explained specific physical properties in strongly irrigated

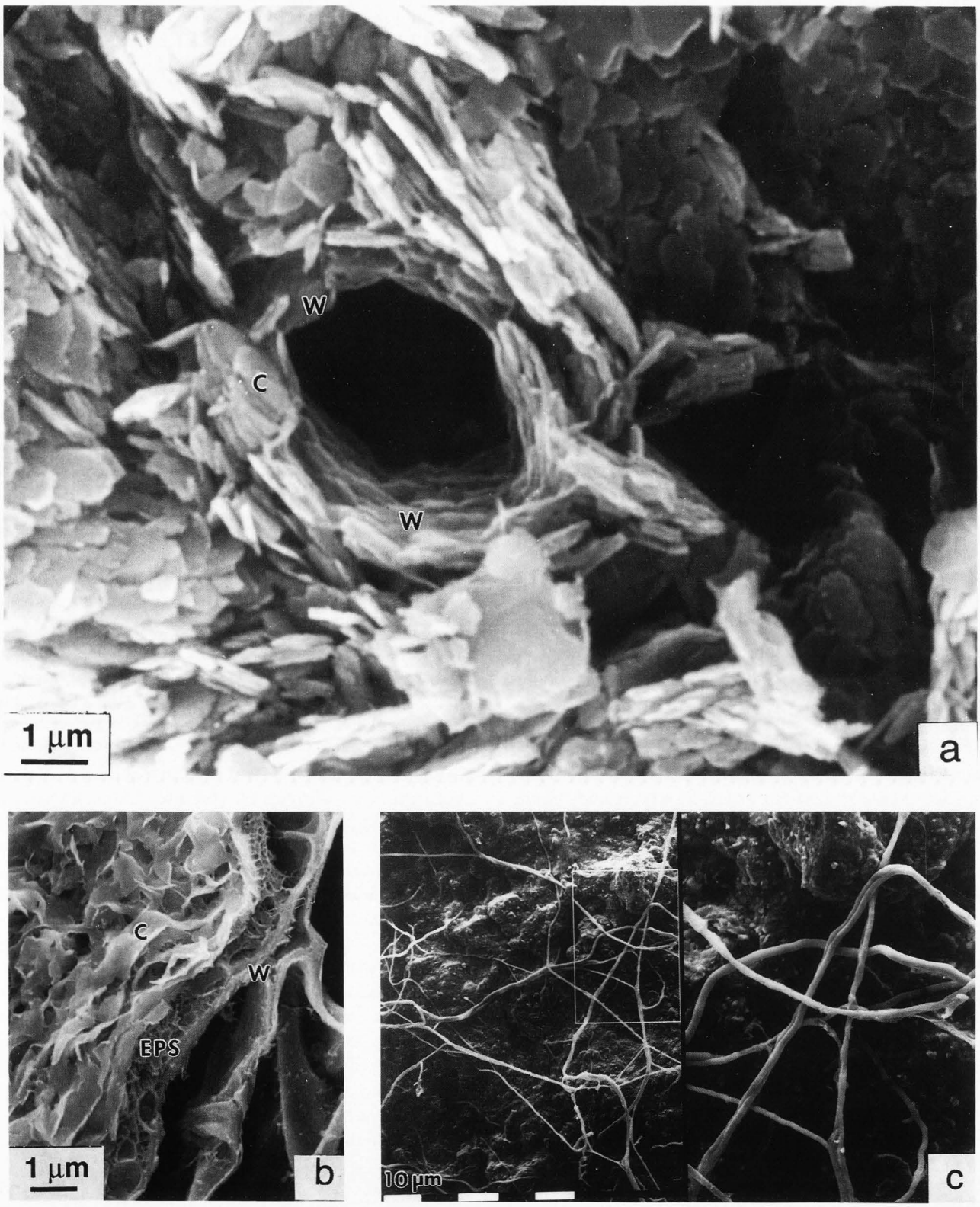

Figure 10. Biologically mediated aggregation. (**a**) Orientation of kaolinite clay particles by growing hyphae, $\Psi = 0.01$ MPa; (**b**) adhesion of smectite platelets to root absorbent hair, $\Psi = 0.01$ MPa (courtesy of J.M. Dorioz); (**c**) hyphae growing on soil aggregate and entangling soil particles (courtesy of J. Hassink and C. Chenu). Samples partially freeze-dried. W: cell wall; C: clay particles; EPS: extracellular polysaccharides.

desert soils (Figs. 9c and 9d) (Marcoen *et al.*, 1994).

The role of several soil constituents, such as, Al and Fe oxides or amorphous compounds, in soil organization and aggregation has been studied with LTSEM (Robert *et al.*, 1987). The contribution of soil biota to the formation and stability of soil aggregates has also been analyzed. Fungi and roots were shown to modify the fabric of their immediate soil environment. Clay particles adhere to the EPS-covered surface of roots and fungi (Campbell, 1983; Dorioz *et al.*, 1993), become oriented parallel to the cell walls of fungi or absorbent hair (Fig. 10a), and bound together by the extracellular polysaccharides or mucilages (Fig. 10b) (Dorioz and Robert, 1982; Dorioz *et al.*, 1993). Fungi also physically entangle the soil aggregates with their hyphae (Fig. 10c), the same phenomenon being observed with roots (Dorioz *et al.*, 1993). Soil biota was shown to modify the microstructure of its close microenvironment, at a scale which directly depends on the size of the organisms. Aggregates of various sizes are thereby initiated and stabilized by bacteria, fungi or roots in soils.

The habitats of soil micro-organisms

LTSEM has proved to be very useful in demonstrating qualitatively the distribution and spatial relationships of the microorganisms and soil components. A wide variety of aerobic and anaerobic microorganisms inhabit soils, with contrasting physiological requirements. Soils must therefore provide very different habitats, with different local physicochemical conditions. Some of these habitats were identified with LTSEM, thanks to an adequate preservation of both microorganisms and pore spaces. However, bacteria are often very difficult to locate in the soil matrix, because of their strong adhesion to clay surfaces, and because their cell dimensions are similar to those of clay particles (Campbell and Porter, 1982; Heijnen *et al.*, 1993). Bacteria in soils are located adhering to dry solid surfaces (Fig. 11a) where they may form biofilms (Fig. 11c), or located at the boundary of liquid menisci or within water-saturated pores (Fig. 11b) (Campbell, 1983; Roberson *et al.*, 1993). As regards to food sources, bacteria can be located in mineral environments, presumably oligotrophic, or close to food sources. LTSEM observations contributed to demonstrate that in soil, microhabitats with great differences in water conditions, nutrient availability or protection against predation could coexist over short distances (Heijnen *et al.*, 1993).

Furthermore, soil microhabitats are ever-changing micro-environments. For example, upon desiccation, the honeycomb structure of vertisol smectites shrink and the pores eventually close. Such drying events were demonstrated, by experimental studies and associated LTSEM observations, to be lethal for the bacteria inhabiting the smectite pore spaces (Schmit and Robert, 1984). It provided basic explanations for some cases of soil suppressiveness towards bacterial disease (Schmit *et al.*, 1990).

Microorganisms may themselves change the characteristics of their habitat, for example, by producing extracellular polysaccharides. In sand cultures of bacteria, the cells were shown to live surrounded by EPS shells (Roberson *et al.*, 1993). During desiccation, EPS formed a dense amorphous layer over bacteria (Fig. 11d), thereby creating a specific microenvironment for bacteria, in which water losses were probably reduced (Chenu, 1993). Such a microenvironment may explain the role of EPS in protecting bacteria against desiccation.

Conclusions

To summarize, proper understanding of the behavior of soils requires knowledge of their structure, i.e., the spatial arrangement of elementary particles and their interaction. The structure can be studied with visual observation techniques and scanning electron microscopy provides a range of magnifications that is appropriate to soil constituents. The fixation of hydrated microstructures with LTSEM has been assessed on pure constituents such as clay minerals and organic macromolecules. LTSEM does preserve most soil colloids well. An accurate use of LTSEM with soils should involve the control of several factors, in order to interpret the results and detect artifacts. Because soils are very divided systems, the energy state of water plays a major role in the behavior of soils upon low temperature treatments. Matric potential, i.e., the suction pressure of the interstitial solution, and the nature and concentrations of ions or of dissolved organics determine the type of water. Because the soil microstructures are affected by the history of previous stresses, studies of soil microstructure should also take this factor into account.

In soil science, freezing is often used to stop biological activity or to obtain, by freeze-drying, dry samples to be studied with other techniques. The use of LTSEM has helped to analyze the consequences of routine freezing treatments, which were often considered to be less drastic than chemical treatments, and to show that freezing may lead to severe alterations of the geometry and of the surface areas of the samples.

Among the fields open to LTSEM studies in soils, the main results are to be expected from: (i) water-related microstructural changes in soils, such as shrink and swell phenomena, soil fissuration and aggregation, and (ii) microbial ecology studies, i.e., studies of the spatial distributions of soil organisms and the consequences of water fluctuations and related microstructural changes on biological activities.

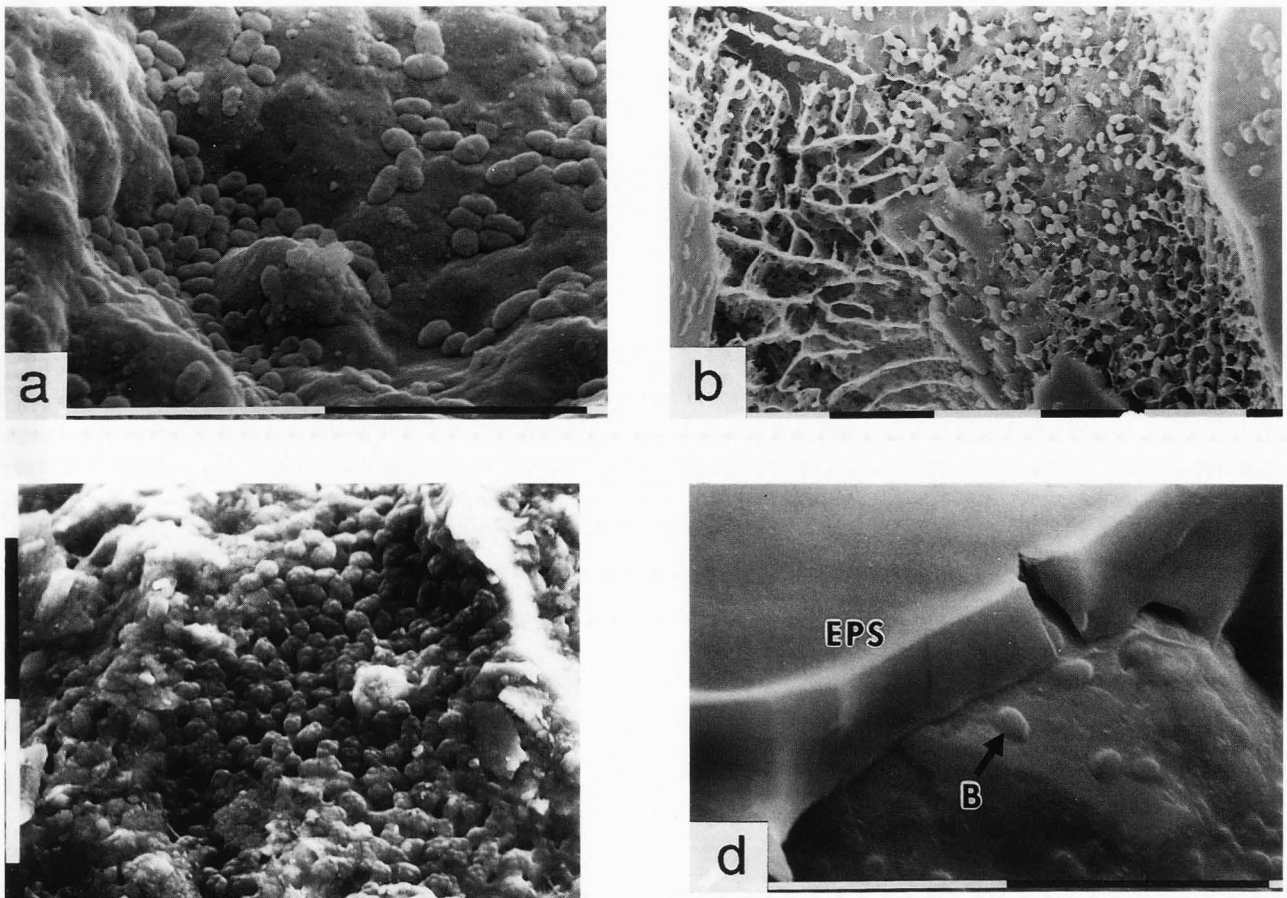

Figure 11. The habitats of soil microorganisms. (**a**) Bacteria (*Pseudomonas*) adhering to sand surfaces, white bar = 10 μm; (**b**) bacteria located within or at the boundary of a water meniscus between two sand grains, white bar = 50 μm, Ψ = 0.025 MPa, M: meniscus; (**c**) bacterial colony at the surface of soil aggregate, Ψ = 0.01 MPa, white bar = 10 μm; and (**d**) bacteria (*Pseudomonas*) growing on sand grains at -1 MPa, EPS: extracellular polysaccharide, white bar = 10 μm. Samples partially freeze-dried. Micrographs (a), (b) and (d) are reprinted from Roberson *et al.*, 1993; and (c) is courtesy of J. Hassink and C. Chenu.

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Discussion with Reviewers

K.P. Ryan: It is interesting to consider the problem of soil hydration. Soils can range from being totally waterlogged to being almost desiccated in hot conditions. Obviously LTSEM is apparently the ideal method for this type of specimen, but the preparation problems are similar to those for biological specimens, where perturbation and water loss during the handling process prior to freezing can induce changes. It may be of interest to apply some of the biologist's approaches such as using environmental chambers prior to freezing. Would it be of interest to pursue this type of approach, where different states of hydration could be experimentally controlled?
Authors: This is somewhat we already do by equilibrating the samples with given water potentials prior to freezing.

K.P. Ryan: Better freezing of the samples may be possible by putting small or thin samples on a metal foil support, rather than straight onto a conventional SEM support because this is quite a large heat reservoir and retards the cooling process. A suitable support can be made by fixing aluminium foil with epoxy glue to a wire frame shaped like a tennis racket or in the form of a "Y". after freezing, the specimen can be chipped off the support and glued to the normal stub using silver paint, preferably not too liquid, at -60°C on a low temperature "hot" plate. Would this be of use in soil research?
Authors: Yes, obviously this would improve the cooling rates of samples. We appreciate your suggestion.

N.K. Tovey: An early demonstration of LTSEM (albeit with no control of temperature) was reported by Tovey (1972). In that demonstration, I found that it was unnecessary to coat the samples as there remained sufficient conductivity within the frozen samples to minimize charge effects. Have the authors tried looking at uncoated samples? If so was charging present? Did they try low voltage observation to overcome the charging problem?
Authors: We often perform observations of uncoated samples prior to sublimation and coating. There are very often charging problems that reduce the quality of micrographs and we also found that metallization and high voltage ensure a better resolution. Observation at low accelerating voltages can also avoid the problems of charging, but high resolution in this mode of operation is only available with field emission microscopes which were not available to us.

N.K. Tovey: Figures 4a and 4b are dramatic illustrations of the problems of liquid nitrogen chilling, and mention freon and other liquids. Have the authors considered using liquid propane in a container chilled by liquid nitrogen as propane freezes at only just above the boiling temperature of nitrogen and thus provides a more rapid chilling than freon?
Authors: We agree that propane is a better coolant than nitrogen slush or freon. So far, safety considerations have impeded us from using propane.

N.K. Tovey: Have the authors attempted to measure the rise in temperature of their samples during transfer? Is it possible that these may warm up to temperatures above those used later and thus be a cause of damage?
Authors: During the transfer the samples are under vacuum. The temperature of the specimen holder is measured just after the transfer, in the preparation chamber. There is no significant warming up of the specimen holder during the transfer.

N.K. Tovey: What X-ray diffraction measurements did you use to obtain the orientation index (Fig. 7c). Is it a peak ratio (e.g., 002/020) or based on a X-ray texture goniometric parameter?
Authors: The orientation index is the ratio between maximum and minimum intensity of 001 X-ray diffraction peak. The results were obtained with a X-ray texture goniometer and a camera device.

N.K. Tovey: Would the authors please comment on their recommendation with regard to specimen preparation for normal SEM observations. Since samples in the field go through full drying cycles, presumably air drying would be sufficient for observing such samples already in a dry state. However, what about partially saturated soils (which would swell during fluid replacement, and shrink during drying), would you recommend freeze-drying here? Presumably critical point drying would be the best for fully saturated soils.
Authors: Even for samples which in the field go through full drying cycles, the dry state is not representative of wet states. Our investigations with LTSEM showed that changes of water content are associated with changes in the volume of the samples and of clay particle arrangement. Air drying is obviously enough for observing samples in the dry state but LTSEM (or CPD) are required to characterize the microstructure of wet samples, which is different from that of air-dried sam-

ples. Partially saturated soils are better preserved by LTSEM rather than by CPD as you point out. Even for saturated samples, CPD has two drawbacks: (i) the fracture planes follow the main structural discontinuities in the sample, so that only the aggregates external surfaces can be observed, and (ii) a collapse of the clay is frequently observed after CPD.

N.K. Tovey: The authors correctly emphasize the importance of size when using freeze-drying methods. For very soft sediments, is there not likely to be more damage in dissection than in actual freezing if samples as small as 0.5 mm are to be cut?

Authors: When soft samples are observed, the dissection causes some disturbance at the sample periphery. The thickness of this disturbed core is about 1-2 μm. This is also why we observe fresh fracture faces, obtained on the frozen samples.

Additional References

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