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Light and electron microscopy of stained microaggregates: the role of organic matter and microbes in soil aggregation

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Abstract. Micrographs from two semi-arid grassland soils – a cold temperate Mollisol (Chernozem) from western Canada and a hot Alfisol (Savannah Ochrosol) from northern Ghana – were examined by light and electron microscopy. Soil samples were stained with ruthenium red to show microbial polysaccharides. The stained soils were partially dispersed in water and subsamples of all particles and aggregates ($< 50\,\mu m$) were examined by light microscopy. Subsamples containing mineral particles $< 5\,\mu m$ as well as larger organic particles and aggregates of low density were observed by electron microscopy and analyzed with an electron microprobe (EDXRA). The ruthenium red stain adequately stained organic material for light and transmission electron microscopy and appeared to be relatively specific for polysaccharides of fungal and bacterial origin. This allowed the study of organo-mineral and microbial mineral associations in microaggregates, and pointed to the importance of organic matter and microbes in the mechanisms of aggregate formation and stabilization and structure in soils.

Introduction

Studies of soil organic matter and its transformations using density or particle size fractionations (Greenland & Ford 1964; Turchenek & Oades 1979; Tiessen & Stewart 1983) have shown that the biochemistry and turnover of organic materials are closely related to their size and to their associations with mineral soil components (Ladd & Amato 1980; Cameron & Posner 1979; Tiessen et al. 1984). Organic matter of colloidal size is characterized by a narrow C:N ratio, high aliphaticity and hydrolyzability indicative of recent microbial products (Anderson et al. 1981) while fractions $> 5\,\mu m$ contain numerous plant fragments in various stages of decomposition and in association with mineral particles (Tiessen & Stewart 1983).

These differences can be recognized in a concept that allows for two different sources of organic matter inputs:

- relatively course plant debris that is deposited within or upon the soil, and;

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- colloidal and soluble organic compounds from root exudates, microbial products or litter leachates.

Plant material enters the soil as particulates that can undergo biological/biochemical transformations or direct stabilization (Duchaufour 1976). Particulate organic matter is initially colonized by the microbial population and at the same time absorbs mineral particles and metal ions. Roots in particular show extensive mineral associations, and may act as binding agents for large soil aggregates while still functional (Foster et al. 1983). Labile portions of plant debris are consumed by the microbial population and will eventually enter the soil organic matter pool as microbial products. Microbial products, root exudates or soluble litter components can be distributed through the soil matrix where they may be adsorbed to mineral surfaces, precipitated with other organic compounds or inorganic ions, or immobilized in the biomass. Soluble compounds can interact with metals by chelation, coat aggregates and influence soil structure. Colloidal materials frequently act as nucleation points for aggregate formation and sesquioxide precipitation (Bruckert & Kilbertus 1980; Foster 1981a, 1981b; Tisdall & Oades 1982).

Tisdall & Oades (1982); Elliott (1986) and Emerson et al. (1986) examined in detail the associations between organic matter and soil mineral components as they form aggregates of different sizes and stabilities. These studies indicate that the physical dimensions and arrangements of organic matter in soil aggregates can greatly influence aggregate stability as well as the stability and turnover of the organic materials (Oades 1984). Aggregation and aggregate stability in turn influence soil physical characteristics such as erodibility, water holding and infiltration behaviour. This importance of the organo-mineral interactions has prompted an interest in the micromorphology of organic matter in the soils.

The physical arrangement of soil components has usually been studied on polished thin sections of impregnated soil cores by light or electron microscopy. Individual aggregates can be studied using scanning electron microscopy on suspension mounts (Riezebos & Lushenhouwer 1983). Smaller microaggregates ($< 5\,\mu m$) in addition, permit the use of transmission electron microscopy with its higher resolution and opportunity for specific staining. Specific staining techniques developed for cytology to facilitate the observation of different biological materials such as proteins, lipids or polysaccharides have to some extent been applied to environmental studies such as microbial ecology (Fletcher & Floodgate 1973; Balkwill & Casida 1979) or soil science (Foster 1981a). These methods facilitate a combination of chemical and morphological studies by showing selected organic materials in the context of the mineral soil.

Polysaccharides have been implicated in microbial adhesion (Fletcher & Floodgate 1973), in organo-mineral interactions of soils (Foster 1981b; Tiessen et al. 1984) and in the formation and stability of aggregates (Greenland et al. 1961; Cheshire et al. 1983). A substantial portion of the nonextractable soil organic matter (humin) may also be in the form of polysaccharides (Anderson et al. 1981).

The present study attempts to examine the organo-mineral interactions of polysaccharides and microbial adhesions as well as their role in soil aggregation. This was done using a modification of the ruthenium red (RR) stain developed by Luft (1971) in combination with transmission light and electron microscopy and energy dispersive X-ray analysis (EDXRA) of suspension mounted soil aggregates. Two soils were chosen; one a strongly structure Chernozem (Mollisol) with a high organic matter content that is known to be responsible for the aggregate stability of the soil; the other a weakly structured Savannah Ochrosol (Alfisol) with low organic matter content. The lability of the organic matter in the Savannah Ochrosol combined with a high iron activity would suggest that organic matter might play a secondary role in the stabilization of structure in this soil. Mechanisms of microaggregate formation in both soils were compared qualitatively by microscopy.

Materials and methods

Soil samples were obtained from a well aggregated lacustrian chernozemic silt loam soil (Haploboroll) under native prairie that contained 50 mg/g organic C and from a weakly aggregated Savannah Ochrosol loamy sand (Paleustalf) under savannah regrowth containing 8 mg/g C. Field samples from the Ah horizon were air-dried, crushed to 2 mm, and stored until further analysis.

Preliminary experiments had shown that organic matter could be stained using the RR/osmium tetroxide method of Cagle et al. (1972) but analysis of stained organic materials by EDXRA had shown the presence of OsO_4 without RR in several instances. In order to achieve a more specific staining of acidic polysaccharides the OsO_4 step was subsequently omitted. It was found that RR alone not only permitted light microscopy but also gave sufficient density for electron microscopy at 60 KV. The following procedure was adopted:

For the study of microaggregates and organo-mineral associations 1 g soil samples were suspended in 25 ml aqueous RR solution (0.15%), containing glutaraldehyde (3.6%), left for 30 min and then shaken for 1 h. The Savan-

nah Ochrosol was then centrifuged and washed with distilled water. The well aggregated Chernozem sample was sonified for 5 min at 50 W following the initial shaking. After a further 1 h shake to stain and fix possibly newly exposed surfaces the suspension was centrifuged and washed with distilled water. After several washes in deionized water flocculation was sufficiently low to allow the selective sedimentation of particles and aggregates of different sizes. The following samples were drawn from a depth of 10 cm in the suspension diluted to 50 ml:

- after 1 min settling time, containing all particle sizes other than coarse ($> 50\ \mu m$) mineral grains that would interfere with the light microscopy. The purpe-red RR stain allowed a clear distinction between microbial material and plant or humic materials. The light microscopy color plates are not reproduced in this communication.
- after 1 h, 12 min settling time, containing mineral particles $< 5\ \mu m$ diameter and larger organic (low density) particles and aggregates. This fraction was used for electron microscopy.

All suspensions were diluted and drop mounted on formvar and carbon coated microscopy grids, for observation in a Philips EM300 at 60 KV and a Philips EM400 with an EDAX X-ray microprobe analyser.

Ruthenium red was obtained from Sigma who indicate a 45% dye content in the preparation. The acutal RR content was found to be 60% by absorbance measurements following the procedure of Luft (1971). Only small amounts of ruthenium violet were shown by the spectral analysis. The dye content of 60% was used for calculation of the final RR concentration.

The EDXRA results are reported as relative peak heights giving the highest peak of each analysis at an arbitrary 100 units. This allows approximate comparisons of the relative abundances of the elements present in the analytical area. Due to differences in area, geometry and mass absorption, quantitative comparisons between different analyses are not possible.

Results

The partial dispersion in combination with the prefixation left most biological structures intact. The majority of large ($> 50\ \mu m$) aggregates were found (by light microscopy) to be based on plant debris, into which mineral particles have become entrapped. Older, more decomposed organic matter was seen as increasingly amorphous and intimately associated with mineral grains. Aggregates were frequently invaded by fungi or actinomycetes whose hyphae interconnect several organic and mineral primary aggregates to form secondary organo-mineral aggregates (Fig. 1). Many fungi showed specific

attachment sites to mineral particles, characterized by club-shaped thickening of the hyphae but branching points also served as nuclei for aggregate formation.

Final stages of decomposition were indicated by an unstructured matrix of organo-mineral associations which included particles of many different sizes or of largely bacterial and fine clay size. In addition to the inclusion of bacteria in mixed aggregates (Fig. 2), colonization of mineral grain surfaces was observed. Extensive polysaccharide deposits (ruthenium positive) were frequently observed on the mineral surfaces (Fig. 3).

Bacterial cell walls and glycocalices were well stained showing the external shapes of the organisms but few internal structures (Fig. 4) with the exception of some polysaccharide storage granules. Polar attachments of microbes to mineral components were frequent (Fig. 4) and in many cases the glycocalix extended to envelope minerals at the attachment site (Fig. 5). At the same time small mineral particles were adsorbed to microbial surfaces forming extensive coatings (Figs. 5, 6, 7).

The attachments of microorganisms frequently bridged 2 or more mineral particles or aggregates and thereby formed connective networks that stabilized microaggregates (Figs. 8, 9, 10). In many cases microbial cementations were still visible in aggregates after the cells themselves had disappeared (Fig. 11). In this way microbes acted as initiators for the formation of aggregates of mixed organo-mineral composition. In some such aggregates organic and inorganic cementations were difficult to distinguish (Fig. 11) unless analyzed by X-ray microprobe (Fig. 12). The gradual sorption of amorphous mineral components as well as crystalline particles to microbial cells resulted in the formation of organo-mineral aggregates of microbial or faunal size (Figs. 11, 13). Larger, elongated aggregates were formed by associations between actinomycetes or fungi and soil mineral components, forming strands of interconnected materials (Fig. 9). Aggregates of large particles contain pores that can fill with amorphous materials similar to the previously observed microaggregates (Fig. 14). Organic matter in such positions may be physically protected from decomposition.

Microprobe analysis of such microaggregates showed the presence of mineral components as well as ruthenium stain in microbial cells (Fig. 2, Table 1). Unless the appearance of the aggregate confirmed the presence of organic matter, this analysis is probably not conclusive since ruthenium may have been adsorbed to amorphous minerals present.

An example of the composition of specific identifiable structures such as microbial cells and their mineral associations is shown in Fig. 10. The central area of the cell contained P, Ca and Ru stain (area 10A, Table 1) while the more opaque portions contained silica in addition (Area 10B, Table 1). At

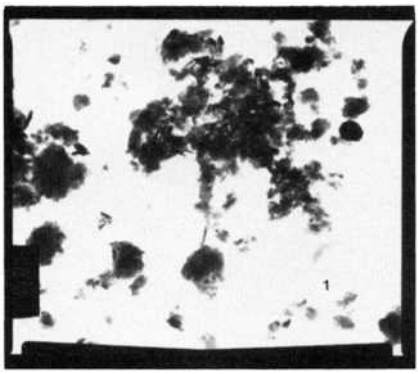

Fig. 1. Mixed organo-mineral aggregate. Note: grey, amorphous organic material; black clay platelets (Chernozem, field = 8 μm).

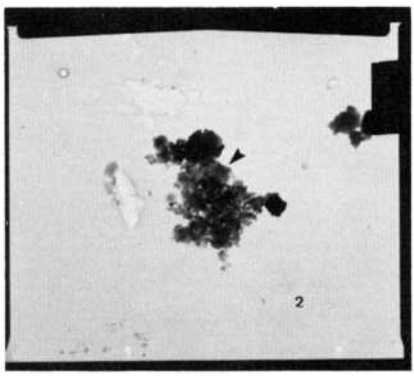

Fig. 2. Aggregate containing bacterium, EDXRA analysis includes entire aggregate (Chernozem, field = 8 μm).

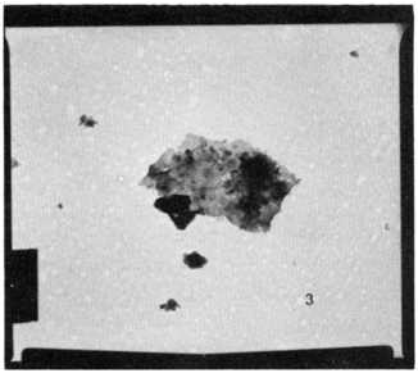

Fig. 3. Polysaccharide coat in mica (Chernozem, field = 8 μm).

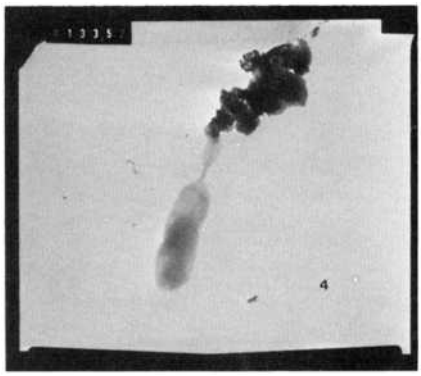

Fig. 4. Bacterium with polar attachment (Chernozem, field = 7 μm).

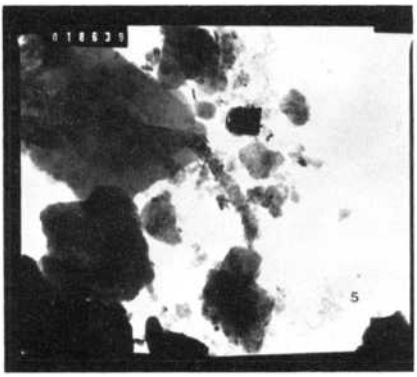

Fig. 5. Actinomycete, heavily coated with mineral particles and attached to mica or quartz sheet (Chernozem, field = 5.5 μm).

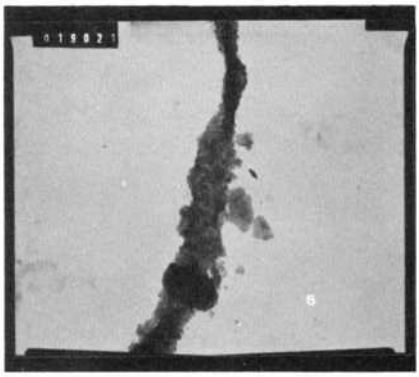

Fig. 6. Mineral coated actinomycete strand (S. Ochrosol, field = 4.5 μm).

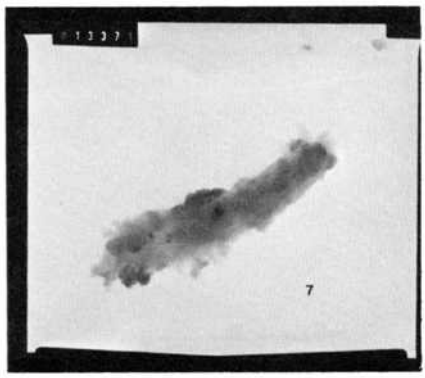

Fig. 7. Individual bacterium, note diffuse glycocalix with mineral associations (S. Ochrosol, field = 2 μm).

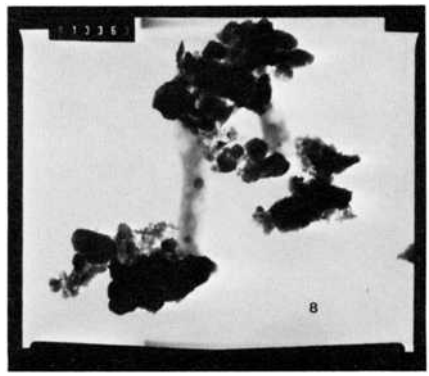

Fig. 8. Algal cells crosslinking aggregates (Chernozem, field = 9 μm).

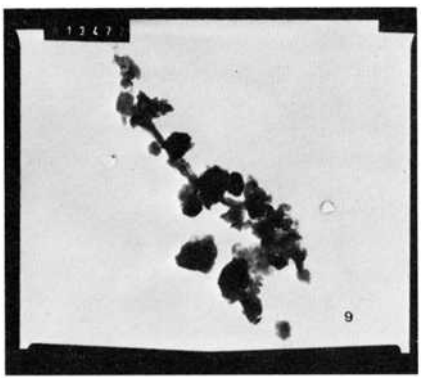

Fig. 9. Fungal or actinomycete strand (S. Ochrosol, field = 11 μm).

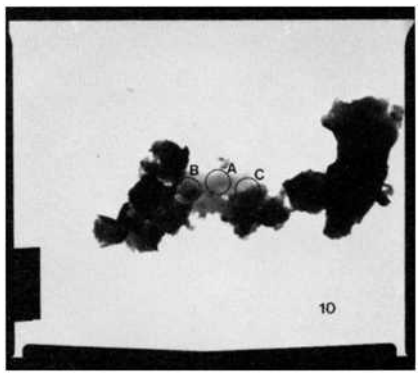

Fig. 10. Bacteria crosslinking aggregates. EDXRA areas are circled (Chernozem, field = 8 μm).

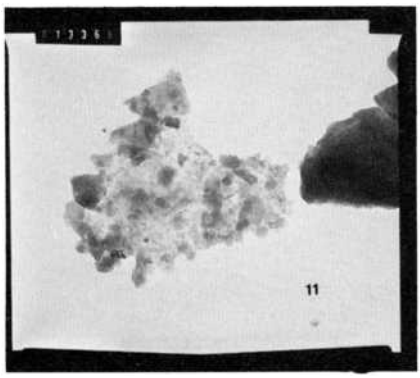

Fig. 11. Microaggregate of minerals cemented with polysaccharide (Chernozem, field = 3 μm).

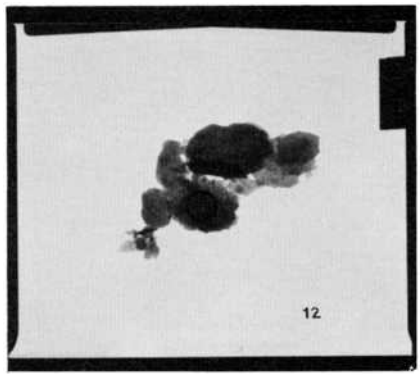

Fig. 12. The black non-crystalline particle consists entirely of iron (S. Ochrosol, field = 8 μm).

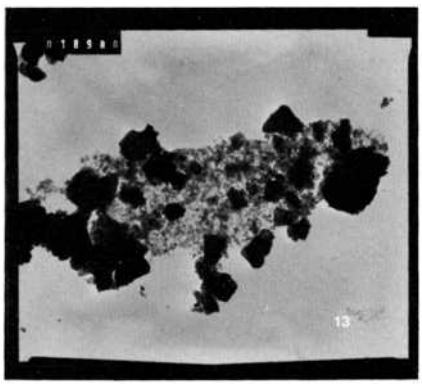

Fig. 13. Amoeba, heavily coated with minerals (S. Ochrosol, field = 13 μm).

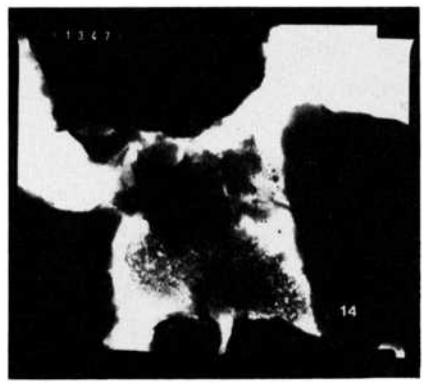

Fig. 14. Partially "occluded" organic material (Chernozem, field = 3 μm).

Table 1. Elemental compositions of selected areas by EDXRA (relative heights of the primary peaks of different elements, the highest peak of each analysis is arbitrarily set to 100).

Plate/area	Element					
	Si	Al	Fe	Ca	P	Ru
2	100	35	18	0	0	10
10 A	0	0	0	20	100	17
10 B	100	0	0	14	57	6
10 C	100	40	37	0	0	14
12	0	0	100	0	0	0

the point of attachment aluminum, iron and potassium were also found, indicating the presence of some layer silicates (area 10C). In this area the phosphorus signal was lost due to its proximity to the large silica peak.

Discussion

Ruthenium red stained organic material adequately for light and transmission electron microscopy. The stain appeared to be relatively specific for polysaccharides of fungal and bacterial origin providing some color differences between plant and microbial materials in the light microscopy. Small bacterial cells, actinomycetes and microbial products were visible in the transmitted electron beam. This allowed the study of organo-mineral and microbial-mineral associations of small ($< 5\,\mu m$) dimensions. The presence of stain in such structures could be confirmed in all cases by

elemental analysis (EDXRA). Since the charged ruthenium complex may have been adsorbed to amorphous mineral compounds, identification of organic materials had to rely on the evaluation of both stain and shape.

From these observations of organic materials and organo-mineral associations the following interpretations can be made. Organic matter entering the soil as particles (plant debris) can become the nucleus of aggregate formation by adsorbing small mineral particles. Fungal invasion and subsequent crosslinking of organic debris and mineral particles provides more extensive aggregation. At advanced stages of decomposition the organic material loses its structure as well as most of its volume. The aggregate thus formed is an intimate mixture of organic and mineral materials of varying dimensions. Such aggregates will usually measure one tenth to several millimeters across and may perform an important role in the overall aggregation and structure formation of soils (Tisdall & Oades 1982).

On a submicroscopic scale aggregation appears to be initiated by microbes living within the soil. Bacteria and actinomycetes and amoebae with extensive extracellular coats (glycocalices) and attachments to mineral surfaces were frequently observed. The adsorption of fine clay particles to bacteria which subsequently disintegrate leads to the formation of unstructured microaggregates of bacterial size. Active attachment of microbes to mineral surfaces and coating of such surfaces with glycocalix materials provides for the aggregation and cementation of microaggregates of varying sizes (1 to about 20 μm). Since such structures were normally observed separate from organic debris such organisms may feed on dissolved materials and form autogenous nuclei for aggregate formation. The high affinity of extracellular polysaccharides for polyvalent cations, particularly iron, can lead to the formation of organo-mineral cements, which, in the present study, may explain the variable elemental composition of microbial attachment sites.

This organic component of aggregate formation and soil structure initiation has long been neglected, partly due to the difficulties involved in its study. Our work presents preliminary information on the possible importance of such processes and supplements the increasing knowledge obtained from particle size and density fractions of organic matter and from an increasing number of "in situ" analytical and microscopic techniques. The role of organic components in the formation and stabilization of soil structure of a savannah soil with very low carbon content has been shown in several examples. It appears that these organo-mineral interactions are most important for the maintenance of a favourable structure despite the high sesquioxide activity in this soil.

Acknowledgements

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