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Examination of Thin Sections

Smeared or crushed paint on clothing can be examined as a thin section by smearing it onto a microscope slide and preparing a permanent mount with mountant and coverslip. This is examined on a transmitted-light microscope in plane-polarized light and under crossed polars. The refractive index of the pigments and fillers are estimated by observing their contrast in the mountant, or by the Becke line technique if the particles are particularly large. Fillers such as calcite or gypsum are provisionally identified by their birefringence seen under crossed polars. These techniques have been well described by McCrone. Flakes of paint can be characterized by cutting thin sections across the layers using a microtome. The flake is embedded in an epoxy or acrylic resin to form a block that is trimmed and cut. Sections are mounted on slides and examined on a transmitted-light microscope in plane polarized light and under crossed polars. Some layers that are very difficult to distinguish in a polished section viewed in incident light are clearly distinguished by this method. A technique has also been developed to mount the paint flake in ice or in gelatine on a Peltier stage. The thin sections are cut and mounted, but the majority of the flake is preserved for further analytical investigations.

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Environmental applications

During recent decades, a great deal of evidence has shown the importance of chemical processes at solid-solution interfaces in the global biogeophysicochemical cycles (surface complexation/adsorption of toxic heavy metals and anthropogenic organics, bioavailability of nutrients to plankton, weathering of minerals, precipitation of amorphous particles and growing of crystalline phases, phase transformations and defect structures in minerals, aggregation of living and nonliving species). For this reason, and in order to help understand the modulating role of particulate species in natural systems, microscopic methods, and particularly electron microscopy (EM), have become increasingly useful in environmental studies. [*See Mass spectrometry, environmental analysis*]

Contrary to classical physicochemical techniques employed for the identification of substances in environmental samples (atomic and molecular spectrophotometries, electrochemical analyses, chromatographic separations and their numerous detection devices) EM allows the direct visualization of virtually all kinds of nonsoluble species. Combined with one of the many detectors developed in the ever-growing area of surface analysis, EM becomes a very efficient characterization and analysis tool at the individual particle level; in this respect, energy-dispersive spectroscopy coupled to TEM is especially powerful. Although necessitating expensive and highly specialized procedures and instrumentation, EM is capable of providing information on objects with sizes down to the nanometer (10^{-9} m) range.

Scanning (SEM) and transmission (TEM) electron microscopy give complementary information on solid species. While the former yields images with a large depth of focus ($>10^3$ times better than light microscopy) and necessitates a relatively simple preparation of samples, it is limited to surface topography visualization; on the other hand, TEM usually possesses better spatial resolution than SEM and allows determination of fine structures and internal heterogeneities, in addition to sizes, forms, and porosities of particles. Therefore, the discussion below focuses on the use of TEM for aquatic and terrestrial samples; characteristic features of this technique are summarized in **Table 1**, and features of sampling procedures in **Table 2**. [*See Microscopy, transmission electron microscopy*]

SAMPLE TYPES AND SAMPLING PROCEDURES

Particulate species from aquatic, sedimentary, or terrestrial sources differ greatly in nature and surrounding environment, as well as the way they are studied by limnologists, pedologists, biologists or geologists. Globally speaking, particles originating from sediments, soils and rocks (hereafter called soil samples) comprise sizes between fractions of a micrometre (10^{-6} m) to millimetres, while aquatic particles represent a further challenge in their identification because their size ranges from nanometres to micrometres (larger particles being incorporated into sediment by fast sedimentation). Furthermore, soil samples are characterized by slower reactions/perturbations than water samples, because of the lower diffusivity of species in near-solid matrices.

Table 1 Summary of relevant features of TEM, with special attention to water and soil samples

Characteristic features		Remarks
Accelerating voltage	50–1000 kV	200–1000 kV for high resolution TEM (HRTEM) instruments
Spatial image resolution	0·2 nm	Atomic dimensions attainable under optimum conditions, but with no real meaning for environmental specimen (distinction between 1 nm and 2 nm particles is not feasible)
Practical magnification range	10^3 to 5×10^5	$>10^5$ accessible to HRTEM
Vacuum needed in the column	$<10^{-8}$ Pa	Sample must be dehydrated; ultrahigh vacuum necessary for ultrahigh resolution
Internal structure analysis	√√√	Necessitates ultrathin specimens; allows investigation down to nanometre (10^{-9} m) dimensions
Crystallographic information	√√	TEM in selected-area electron diffraction (SAED) mode; information on ≥ 20 nm^2 irradiated specimens allows identification of the crystalline phase (in SEM, ≥ 1 μm^2 surfaces are necessary); the use of hybrid instruments, such as field emission scanning/transmission EM (FES/TEM), can improve this
Surface structure analysis (other than by freeze-fracture)	√	Possible via preparation of surface replicas of the original specimen (difficult to obtain; time-consuming; produces artefacts; requires specialized instrumentation); freeze-fracture technology (vitrification, followed by fracturing of specimen for internal details, followed by exquisitely controlled replica formation) permits surface structure analysis on a per-particle basis with a minimum of artefacts, but also a maximum of cost and difficulty

Adapted from Kiss (1988) and Rossiter and Hamilton (1991).

Table 2 Selected sampling and fractionation procedures for the further characterization of nonsoluble species in natural aquatic and terrestrial samples

Sampling/fractionation procedures	Recommended use	Remarks
Dredge sampler	Bulk sampling in soil	Necessitates drying of sample prior to subsampling
Vertical corer	Bulk sampling in soil and sediment	Limited when used for anoxic soil/sediment sampling; necessitates subsampling
Sediment trap	Bulk sampling in water column	Time and depth integration of particles sedimenting in the trap
Bottle sampler	Bulk sampling in water column	Samples every particle present at sampling depth
Pump sampler	Bulk sampling in water column	Maximum particle size sampled limited by pumping conditions
Biological net sampler	Phyto- and zooplankton sampling in water column	Vertical or horizontal integration of biological population sampled Net apertures are generally limited to >10 μm
Sedimentation tank fractionation	Field fractionation of water samples	Particles collected at the issue of fractionation depend on sampling depth into the tank
Centrifugation and ultracentrifugation	Field and laboratory fractionation of water samples	Separation of particles according to their size and density
Filtration and ultrafiltration	Field and laboratory fractionation of water samples	Separation of sizes strongly depends on filtration conditions

Adapted from Bisdom and Ducloux (1982) and Buffle and Van Leeuwen (1992).

Environmental applications

Therefore, considering that soil samples contain much larger amounts of particles than aquatic samples, EM of the former in the micrometre to submicrometre range is a less complicated task, necessitating less sophisticated sampling precautions and specimen preparation than for aquatic particles. However, under certain circumstances (weakly mineralized topsoils rich in organic matter, sediments under anoxic conditions, soft clay-rich muds), it may be necessary to devote as much care to soil sampling as to water sampling. **Figure 1** lists particulate species that are encountered in aquatic and soil samples.

Nonperturbing Sampling of Aquatic Particulate Species

Be it living (viruses, bacteria, algae or cell fragments) or nonliving (organic – fulvic and humic acids, high-molecular-mass fibrillar biopolymers and proteins; or inorganic – clays, iron, manganese, silica, alumina or calcium particles being the most abundant ones), any nonsoluble species to be characterized by TEM must be sampled under nonperturbing conditions in order to keep its size, morphology and physicochemical properties in the original, native state. Therefore, aquatic samples for TEM must be isolated from the bulk solution with great care, and processed without delay. Special attention has thus to be paid to the bulk physical (temperature, light, agitation) and chemical (ionic strength, pH, O_2 concentration) conditions of the water in order to avoid modification of the sample by coagulation/disaggregation, precipitation/dissolution, or oxidation/reduction. This applies with special emphasis to fine amorphous submicrometre inorganic colloids generated *in situ*, generally possessing a fragile structure and subject to fast reactions of oxidation in O_2-rich waters or of reduction by light, and also to large superstructures involving long cross-linked flexible biopolymers onto the surface of which small inorganic particles can be attached.

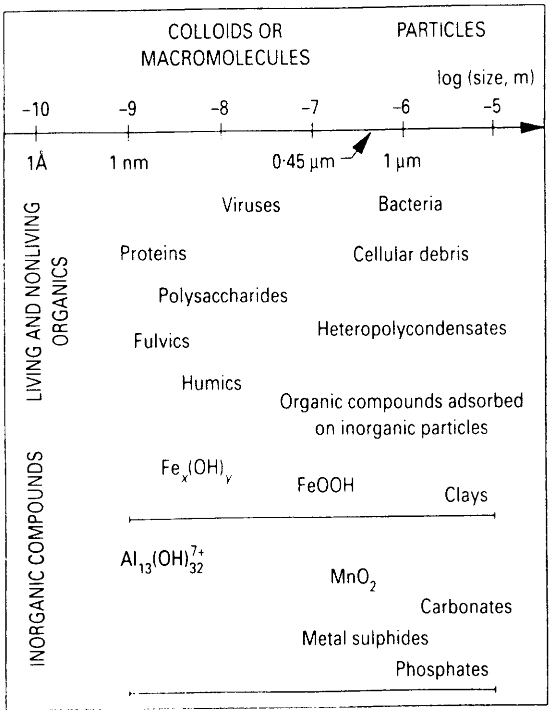

Figure 1 Overview of nonsoluble environmental species that can be analysed by TEM. (Adapted from Buffle and Van Leeuwen (1992), with permission.)

In any case, the kind of information searched for will dictate the complete sampling schedule, which will be different whether a global overview of all types/ morphologies of particles, or a quantitative determination of particle size/number distribution, or an ultrastructural characterization of a limited number of colloids is expected, and whether organic and/or inorganic colloids are to be studied. Highly specialized preparatory technology will be required, as will the use of split samples when the substructure of biota must be examined. However, because of the high heterogeneity characterizing natural aquatic samples, the origin of artefacts produced during the sampling procedure may usually only be assessed by a systematic preliminary investigation, consisting in sampling and fractionating the same water by different procedures, among which some of the most common are briefly described below, and summarized in Table 2.

Direct collection of natural waters is ideally the least perturbing sampling method: unfortunately, it is only applicable to certain specific environments, where particles to be studied are present in higher concentration than other nonsoluble species. This applies, for example, in surface waters, where photosynthesis takes place, when TEM of algae is to be performed, or at oxic–anoxic interfaces, where autochthonous inorganic particles can be formed, either chemically (iron oxyhydroxides) or bacteriologically (manganese oxides) under the influence of specific redox conditions not encountered elsewhere in the water column. These kinds of samples have to be processed (see Specimen Preparation below) as soon as possible in order to avoid even subtle changes in the physicochemical characteristics of the water. Direct sampling may also be chosen when a qualitative overview of the water content is expected. When applied to initially concentrated natural waters (total particulates $>10\ \mathrm{mg\ l^{-1}}$) prepared in whole mounts (particles directly deposited on grids, without further treatment), however, this procedure leads to artefacts in the visualization of particles with sizes smaller than *c*. 100 nm, because large ($>1\ \mu m$) particles will mask them. A dilution of the initial sample may then be necessary, but should only be done with an electrolyte representative of the raw water. Fractionation procedures are preferred for the study of aquatic particles, because of the very broad size range usually present in natural environments (1 nm–1 mm) and of the above-mentioned limitation. [*See Water analysis, particle characterization*]

When dense inorganic particles larger than *c.* >5 μm have to be studied, fractionation by natural sedimentation into sediment traps positioned in the water column or into temperature-controlled sedimentation tanks handled on the shore is suitable. Care should be taken not to leave the traps longer than several days in the water, because bioturbation inside the collected material may modify its composition and size distribution; likewise, raw water for sedimentation tanks must be collected by a high-flow-rate pump, in order to guarantee that even fast-sedimenting particles are sampled during their pumping from depth to surface. If smaller and less dense particles have to be studied, the supernatant of sedimentation tanks, instead of the bottom water layer, can be sampled; in this case, care should be taken to sample the very surface of the sedimenting solution when organic species (densities close to that of water) are of special interest, or to sample just below this surface when particulate organics have to be minimized. On the other hand, pumping flow rate can be substantially reduced if smaller and less dense particles must be studied, provided that their sedimentation rate is still smaller than the linear velocity of the pumped water.

Other ways of prefractionating samples include filtration/ultrafiltration and centrifugation/ultracentrifugation. Both techniques yield operational (i.e. method-dependent) fractions, and offer different advantages and disadvantages. Filtration is subject to coagulation of particles at the surface of membranes, when used under uncontrolled flow rate, stirring, and filtered volume conditions; on the other hand, centrifugation is less sensitive to fractionation conditions, but allows isolation of particles with similar composite size–density characteristics. In order to obtain distinct size fractions in the submicrometre range, it is usually necessary to fractionate samples in cascade, i.e. the filtrate/supernatant from the first filtration/centrifugation is used in the second filtration/centrifugation (with a smaller pore size membrane/higher centrifugal field), and so on, in order to gradually remove large particles that may hinder smaller ones. Nevertheless, owing to their complementarity, filtration and centrifugation procedures should be used in parallel for comparative purposes. [*See Centrifugation, low-speed separations; Centrifugation, ultracentrifugation; Membrane techniques, ultrafiltration*]

Sampling of Soil Particulate Species

Common procedures for soil sampling involve direct excavation with grabs, or extrusion with tubes that are pushed down into the soil. Small portions of core samples are then usually air-dried or oven-dried, or even freeze-dried. This dehydrated material can be either stored as it is or suspended in water for further specimen preparation.

It should, however, be pointed out that, while there exists a mass of literature on EM studies of soil samples, little attention is usually devoted to the possible artefacts caused by improper sampling conditions. For example, drying procedures are responsible for shrinkage of particulate species, especially with samples containing small amorphous material or those rich in organic matter; mucilaginous material is especially susceptible to shrinkage-induced artefacts. Dispersion in water, by means of disintegration/sonication of dehydrated samples, may also induce undesirable structural modifications for soft and even hard particles (dislocation of crystals, aggregation of small particles). Schemes mentioned in the previous section are also appropriate for fragile soils, and could be extrapolated without major difficulty for the nonperturbing preparation of most soil samples. Native associations between bacteria and mineral particles, as well as cell–cell associations within natural populations, cannot be analysed satisfactorily using highly perturbed samples.

SPECIMEN PREPARATION

Owing to the very poor penetrating power of electrons in air, and in order to avoid sample contamination from the sample environment, ultra-high-vacuum ($<10^{-8}$ Pa, depending on the data required) is needed in the column of an electron microscope. This means that samples, either from water or from soil origin, have to be dehydrated prior to analysis, or replicas must be used, as in freeze-fracture (not feasible for most environmental analyses). For TEM, furthermore, the image is formed by the differential elastic scattering of electrons through the solid objects to be visualized; so their thickness must be decreased to some 10–100 nm in order to obtain a high degree of optical resolution (thicker samples could be analysed by high-voltage TEM). Specimen preparation is therefore a key step in the complete EM process, as fine substructures in fragile particles may be altered, or even destroyed, because of inadequate preparation. [*See Microscopy, specimen preparation for electron microscopy*]

Ultrastructural Analysis

Classical specimen preparation for ultrastructural analysis by TEM requires that organic particulate components (carbohydrates, and especially proteins, lipoproteins and peptides synthesized by microorganisms) be fixed prior to subsequent steps. Unfortunately, while there exist a massive number of recipes for fixation of cells (containing large amounts of proteins), only scarce information is available for fixation of environmental organic matter. Fixation of samples containing algae, viruses or bacteria can be obtained by cross-linking proteins/carbohydrates with conventional mixtures of glutaraldehyde followed by osmium tetroxide or uranyl acetate in pH buffers; fixation is a long process (0·5–10 h, depending on specimen type), and should not be preceded by cleaning procedures (removal of undesired organic matter on the surface of diatoms and plankton skeletons, under acidic oxidizing conditions that may drastically perturb physicochemical conditions within the sample).

After fixation, the sample must be transformed into ultrathin slices; this is achieved on samples embedded into uniform polymeric resins possessing flexibility close to that of the particulate material and allowing extra rigidity to the sample. Embedding is usually done with hydrophobic resins (e.g. Epon™ or sometimes Araldite™), necessitating stepped predehydration of the initial sample in order to gradually replace water by an organic solvent (acetone, methanol, ethanol or propylene oxide) in which the resin is miscible. Dehydration steps are among the most prominent sources of artefacts, because of possible contamination, losses, and dramatic changes in the configuration of bioorganic macromolecules present in the sample. However, a new class of hydrophilic resin has appeared (Nanoplast™) that allows the direct embedding of aqueous samples, avoiding the tedious and perturbing predehydration steps; during polymerization of this resin, water is slowly eliminated by evaporation.

Embedded samples are then cut into ultrathin sections using an ultramicrotome; it is strongly recommended to use diamond knives for the sectioning of inorganic particles, because of particle hardness, which damages conventional glass knives. For high-resolution studies, the recommended section thickness should be close to 50 nm. During slicing, large electron-dense inorganic particles may be pulled out or smeared across the section to cause undesirable morphological artefacts. Trial and error, considerable effort, and development of skill by the operator are necessary for obtaining good-quality ultrathin sections; however, the procedure is undoubtedly time-consuming. Slices are finally placed on grids that are inserted into a specimen holder prior to TEM examination.

Even with a modern high-resolution TEM, obtaining the detailed ultrastructure of organic material may be complicated by a poor electron density of these objects; contrast must thus be enhanced by staining of the specimen prior to or after embedding. Stains are mixtures containing heavy metals (electron-dense, thus scattering the electron beam and producing a higher contrast) present in

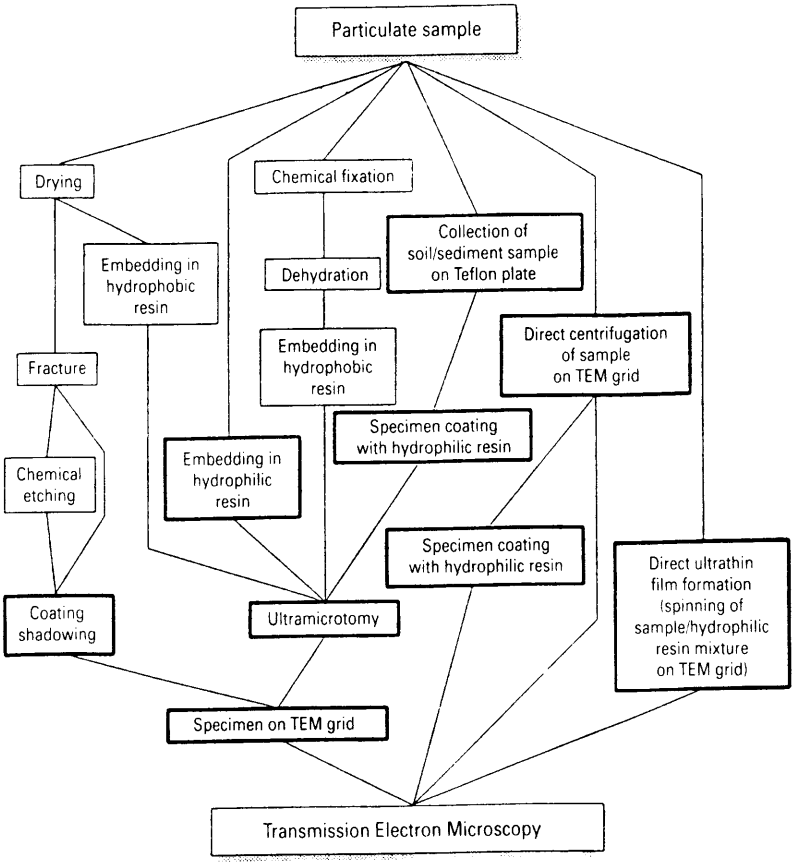

Figure 2 Schematic guidelines for specimen preparation modes; bold frames denote nonperturbing steps. (Adapted from Smart and Tovey (1982), Perret *et al.* (1991), Leppard (1992).)

different forms, depending on the type of organic material to be stained. Many kinds of specific stains are cited in the literature dealing with the EM of biological material; among these, many positive stains (i.e. stains that react with the material of interest, in contrast to negative stains, which enhance the contrast by darkening the background but not the relevant material) may be used with bioorganic macromolecules present in natural systems. However, great care should be taken to optimize the usual recipes given by the classical staining literature.

State-of-the-art Direct Ultrathin Films

From the preceding, we see that sampling and, especially, specimen preparation of particulate species, involve many lengthy steps, during which the integrity of the original sample may be seriously altered from the native state. For this reason, combination of the least-perturbing procedures is strongly recommended.

One of the most promising schemes includes the fast and direct preparation of ultrathin films (equivalent in thickness to ultrathin sections), eventually combined with one of the previously mentioned prefractionation steps; aqueous samples are mixed with a fresh hydrophilic resin, and then spun on grids placed on a disk rotating horizontally. The excess mixture is centrifuged away, and only an ultrathin film of resin (*c*. 100 nm thick) containing particulate species is retained on the grid, which can be studied directly after polymerization of the film. Fixation, predehydration, classical embedding and ultramicrotomy are thus avoided with this nonperturbing procedure. Further developments should improve existing pre- or poststaining methods for contrasting organic components within specimens.

Another state-of-the-art procedure consists of cascade centrifugation of freshly sampled water into centrifugation tubes on the bottom of which grids are held horizontally; during the centrifugation, particles of interest are collected on the grid, which may be further coated with a protective ultrathin film of fresh hydrophilic resin, as emphasized above. Biofibrils and macromolecular organic species possessing long, flexible chains may be subject to deformation/compression when they reach the grid. In the case of quantitative determinations, it may be necessary to dilute the sample or to centrifuge a smaller volume, in order not to cover the grid with too many particulates.

Both nonperturbing techniques mentioned above necessitate only a few operations, and should be preferred over other preparation schemes, for fragile and valuable samples. **Figure 2** summarizes the most prominent methods used for water and soil specimen preparation.

APPLICATIONS

For purely descriptive work on water, sediment and soil samples, one can use TEM to describe individual particles with regard to shape, size, size distribution, porosity, native electron opacity level (indicating heavy element distribution), heavy element composition, crystallinity, acquired electron opacity (from stains and molecular markers), internal occlusion of substances, and surface coatings.

One can also use TEM to describe aggregated particles for shape, size, size distribution, degree of packing, relative frequency of individual internalized particle types, distribution of colloid types with respect to the surface of the aggregate and frequent native associations within the aggregate.

For biologists, the ratio of viruses to microbial cells within an aggregate will be important, as will the nature of the association between microbes and other particles (e.g. the presence of organic fibrillar exopolymers). In some cases, TEM images may also be useful in classifying the microbes according to type.

Combination of energy-dispersive spectroscopy, selective electron diffraction, and morphological analysis will permit geochemists to speciate some inorganic particles. Astute use of molecular markers and literature information on biopolymers will allow the identification of many organic macromolecules according to family.

This great capacity for description should lead to valuable characterizations of particles and aggregates for use in ascertaining the mechanics of biogeochemical processes.

Spin-off applications include: (1) improved analysis of misfractionation by membrane filters accompanied by systematic improvements in the design of filters and filtration apparatus; (2) improvements in our understanding of occlusion phenomena whereby particles sort contaminants in such a way as to produce misleading chemical analyses (underestimates) for the contaminants; (3) better characterization of contaminant transport agents for improved modelling of dispersion.

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Minerals and crystals

Although microscopy can be used simply to determine the size of minerals and crystals, other methods are available to accomplish this task. In fact microscopy is the foremost technique for characterizing the morphology or shape of objects. Although crystal habit is determined by chemical nature, process conditions involving temperature, pH, additives such as growth restrainers or enhancers, shape modifiers, mixing, etc., are known to have a significant effect. Shape kinetic effects may occur during the crystalline growth. Breakdown, attrition and agglomeration can also modify the final shape of a crystal or a mineral, which is an important factor in powder handling and product quality control.

In contrast with other methods used in particle characterization, microscopy is the only method that allows the study of individual objects. Since minute quantities are used, the representativeness of the measurement sample is critical. One also has to be aware that on most occasions the image of the object produced by the microscope is a two-dimensional representation of the three-dimensional reality. Modern microscopy relies on image analysis to quantify the information contained in images, essentially to produce characteristic shape parameters. The purpose of shape characterization is to compute factors allowing discrimination between shapes of similar or dissimilar appearance or to compare them with a given pattern in order to reveal eventual flaws. The use of these shape-characterization techniques is not restricted to minerals and crystals, but many of them have been devised for that purpose. [***See*** *Particle size analysis, scattering techniques; Particle size analysis, mechanical techniques*]

TYPES OF MICROSCOPY

Optical microscopy is used for objects in the size range 0·8–150 μm. For objects larger than 150 μm, magnifying glasses are employed. Optical microscopy is impeded by its small depth of focus, which varies with magnification. Modern light microscopes are equipped with automated stage motion, autofocus systems and brightness control. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are also widely used. Particles in the size range 0·001–5 μm can be examined directly by TEM. High-resolution TEM (HR-TEM), scanning tunnelling microscopy (STM) and atomic force microscopy (AFM) are among the newest tools available to characterize solids at the atomic level. Environmental SEM (E-SEM) enables the study of samples without subjecting them to harsh vacuum conditions. With SEM coupled to energy-dispersive X-ray analysis (SEM/EDX), the chemical composition of particles can be determined at the same time as their size and morphology are assessed. [***See*** *Microscopy, transmission electron microscopy*]

SAMPLE PREPARATION

Minerals and crystals can be stored in a variety of containers – wagons, trucks, bags, heaps, etc. Nonflowing powders have no tendency to segregate and primary sampling can be done with a scoop before sample division. Free-flowing powders, in contrast, tend to segregate, and sampling should be done keeping this in mind. In any case, sampling from heaps should be avoided because they are highly segregated. For moving powders the best procedure is to collect the sample when the material falls at the end of the conveyor belt. The best sample division is obtained by putting the powder into motion in a stream. Several sample-dividing devices have been recommended for reducing the gross sample obtained from a process to the size of a laboratory sample. Table samplers, chute splitters and spinning rifflers are widely used. Further reduction is necessary to obtain an analysis sample (the spinning riffler is most suitable here) and then a measurement sample. In microscopy a few milligrams of material are necessary: the analysis sample can be dispersed into a viscous liquid and the measurement sample is extracted with a rod. Alternatively the analysis sample can be dispersed into a low-viscosity liquid and the measurement