face of soil particles is colonized (see *4*).

The development of the Stereoscan scanning electron microscope (*9*) provides a method for resolving most of these difficulties. Microorganisms can be examined in place on the surfaces of soil particles because an image is formed by electrons liberated from the surfaces examined. The degree of resolution (always better than 500 Å and sometimes better than 200 Å) and the depth of focus are superior to those obtained with the light microscope. As a result, an almost three-dimensional effect is produced. The method of specimen preparation is simple (*9*) and can be readily adapted for examining soil particles. A specimen holder, a small metal stub (diameter, 12.5 mm), is coated with an adhesive. While still sticky, the holder is pressed gently onto a freshly exposed soil surface. Upon removal, the soil particles remain fixed to the holder in approximately the same relation to one another that they had in the soil. Alternatively, soil particles may be smeared on the surface of the holder. The specimens are coated with a layer of gold-palladium alloy (about 800 Å thick) by means of a vacuum evaporation technique; this minimizes charging effects. The holder is then placed in the microscope. After the instrument is evacuated, the specimen is scanned by an electron beam. The electrons liberated from the surface of the specimen are detected by a scintillator-photomultiplier system and induce the formation of an image on a cathode-ray tube. This image may be photographed on either Polaroid or 35-mm film.

This technique was used to examine microorganisms on sand grains and humus particles taken from a sand-dune soil planted with *Pinus nigra* var. *laricio* (*10*) and bacteria on the root surface of *Trifolium repens*. Some sand grains were enmeshed entirely by fungal mycelium (Fig. 1a), although most particles were not so densely colonized. Different types of mycelium could be distinguished at higher magnifications. One commonly observed type was covered with small protuberances (Fig. 1, b and c) that presumably increased the surface area of the hypha, over which secretion of enzymes and absorption of nutrients could take place. Other mycelium was smooth and often much narrower in diameter; it frequently showed signs of collapse, probably due to desiccation during preparation (Fig. 1, d and f). It is impossible to say whether this finer mycelium was fungal or actinomycete in origin. Fungal spores were observed only rarely. Figure 1d shows such a spore which is about 3.0 μ in diameter. Other fungal structures observed included probable clamp connections, characteristic of basidiomycete mycelium (Fig. 1e). Sometimes, bacteria were seen to be associated with mycelium (Fig. 1f), usually in the form of coccoid rods. Bacteria were also observed as discrete colonies. Figure 1g shows part of a sand grain at a low magnification, with barely visible colonies at the points indicated. A few other cells were detectable, but the overall impression was one of sparse colonization. At higher magnifications (Fig. 1h), the colonies were seen to consist of up to 100 cells, although most colonies were smaller and single cells were frequent. The cells were almost spherical, although a few rods were visible. Definitely rod-shaped cells were seen on the root surface of *Trifolium repens* (Fig. 1i). One of the cells (bottom left) had projections on its surface, reminiscent of pictures of ectoparasitic bacteria (*11*).

The nature of the soil-particle surfaces was also visible. Mineral particles were often cratered (Fig. 1, b and g), but were comparatively smooth at high magnifications (Fig. 1h). Humus particles had irregular surfaces (Fig. 1, c–f). These irregularities make it clear that techniques which aim to remove dormant propagules from soil by washing (*12*) may not be successful because spores may become lodged in minute crevices that also provide niches for the growth of microorganisms.

Identification of microbial structures in the soil was based on a comparison of their size with organisms seen with the light microscope; comparisons with pure cultures were also made. The observation of surface mycelial structures should make possible a closer identification of mycelium than light microscopy provides. This will only be achieved when a collection of pure reference cultures is examined with the scanning electron microscope.

The technique has certain limitations. Dehydration and distortion of the organisms, especially of bacteria, occur. The degree of resolution is still insufficient for the examination of many of the surface structures of bacteria. However, this instrument has provided us with pictures of the hitherto invisible and ill-defined microhabitats in a relatively simple soil, indicating that it will help to solve a number of problems concerning the relation of cells to their environment in more complex soils. It should also enable us to determine whether the types of colony seen on contact slides are representative of those which occur in undisturbed soil.

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References and Notes

1. N. G. Cholodney, *Arch. Mikrobiol.* **1**, 620 (1930).
2. T. V. Aristovskaya and O. M. Parinkina, *Soviet Soil Sci.* 636 (1961).
3. H. J. Conn, *Tech. Bull. N.Y. State Agr. Exp. Sta.*, 64 (1918).
4. T. R. G. Gray, P. Baxby, I. R. Hill, M. Goodfellow, in *The Ecology of Soil Bacteria*, T. R. G. Gray and D. Parkinson, Eds. (Liverpool Univ. Press, Liverpool, 1967), p. 171.
5. N. A. Burges and D. P. Nicholas, *Soil Sci.* **92**, 25 (1961).
6. N. A. Krasilnikov and D. G. Zvyagintsev, *Dokl. Akad. Nauk SSSR* **123**, 366 (1958).
7. D. I. Nikitin, L. V. Vasil'eva, R. A. Lokhmacheva, *New and Rare Forms of Microorganisms* (Academy of Sciences of the U.S.S.R., Moscow, 1966, in Russian).
8. H. Jenny and K. Grossenbacher, *Proc. Soil Sci. Soc. Amer.* **27**, 273 (1963).
9. *Stereoscan Electron Microscope. B. Operation, Principles and Performance. List 178, sheet B* (Cambridge Instrument Co., London, 1965).
10. M. Goodfellow, I. R. Hill, T. R. G. Gray, in *The Ecology of Soil Bacteria*, T. R. G. Gray and D. Parkinson, Eds. (Liverpool Univ. Press, Liverpool, 1967), p. 500.
11. H. Stolp and M. P. Starr, *Antonie van Leeuwenhoek J. Microbiol. Serol.* **29**, 217 (1963).
12. S. T. Williams, D. Parkinson, N. A. Burges, *Plant Soil* **22**, 167 (1965).
13. I thank the Cambridge Instrument Co. for the use of their microscope.

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Disaccharidase: Localization in Hamster Intestine Brush Borders

Abstract. *Electron microscopy of isolated brush borders from epithelial cells of hamster intestine demonstrates 60-angstrom knobs attached to the lumen surface of the plasma membrane. Digestion with activated papain removes these knobs. Separation and recovery of the knobs and the plasma membrane are possible. The activities of the disaccharidases invertase and maltase reside in the knobs and are not found with the plasma membrane.*

The site of activity of invertase and maltase and their localization in the small intestine remain controversial. Therefore, the details of absorption of the associated sugars remain unclear. If the enzymes act predominantly extracellularly in the succus entericus, then hydrolysis of the disaccharides to monosaccharides probably occurs before transport into the cell. On the other hand, if the enzymes are pre-

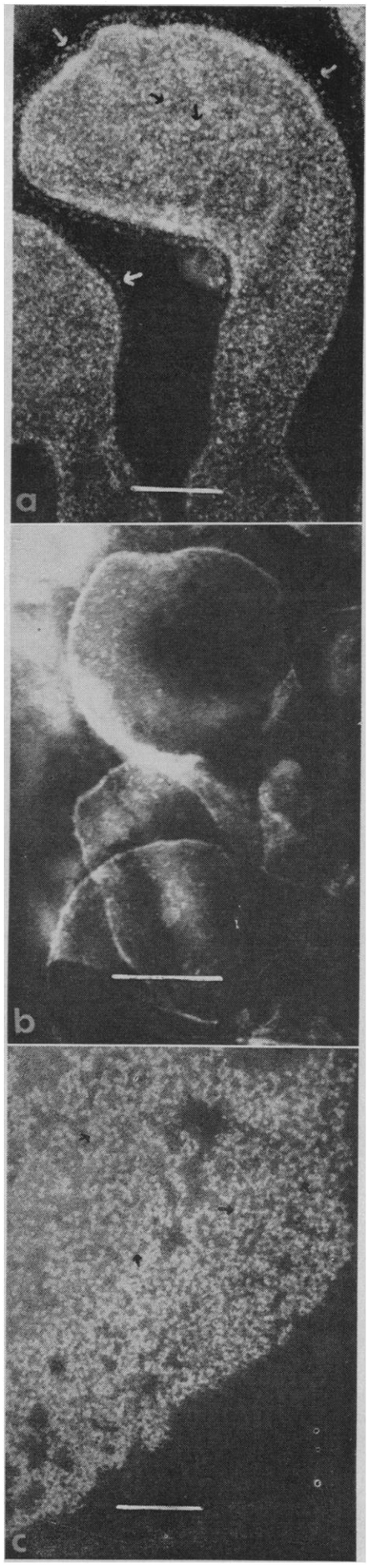

Fig. 1 (left). All specimens were prepared in 2 percent potassium phosphotungstate, *p*H 7.4. The marker corresponds to 0.1 μ. (a) The intact, undigested, isolated brush-border microvillus. The knobs are visible as projections along the edge or as globules when visible head-on (arrows). (b) The brush-border microvillus after digestion with papain. The knobs are no longer visible; instead the edge is clean, and no globules are seen. (c) The knobs as recovered in sediment 2. They measure about 60 Å, are globular, and tend to aggregate (arrows).

dominantly intracellular, then absorption of the disaccharides is likely, with subsequent hydrolysis to the monosaccharides occurring within the cell. Crane has briefly reviewed such data and has presented a theory of an arrangement of the enzymes "in an ordered proximity to one another, as in a mosaic" (*1*). He suggests that the brush-border plasma membrane of the intestinal epithelial cell is the site of this mosaic. My data support this view in part.

Isolation of the microvilli and terminal-web area of hamster epithelial cells —the brush-border preparation—has been described (*2*). This brush-border preparation has been separated into several fractions: one containing the plasma membrane, one containing the core structures, and others made up of unidentified globular elements (*3*). The membrane-containing fraction is associated with disaccharidase activities (*4*).

Electron microscopy of osmium-fixed tissue shows the brush border of the hamster intestinal epithelial cell to be covered by a fibrillar material—the fuzzy coat or glycocalyx which is external to the plasma membrane. Removal of this coating, which is predominantly mucopolysaccharide, from the microvillus is difficult (*5*). The coating varies in appearance according to the species and preparative technique (*5–7*). Electron microscopy of negatively stained brush-border preparations (Fig. 1a) reveals that the plasma membrane of the microvilli is covered on its luminal surface with particles, which I have called knobs, measuring 60 Å in diameter (*8*). In negatively stained preparations, the phosphotungstate forms an electron-opaque glass crystal upon drying. Therefore, those parts of the tissue which do not accumulate phosphotungstate are electron-lucent, and a negative image or the reverse of the familiar image of tissue fixed in osmium and stained with lead or uranyl acetate is obtained.

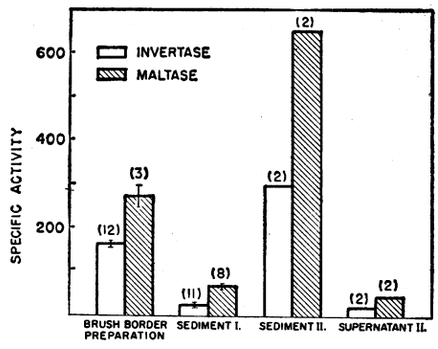

Fig. 2. The specific enzyme activities of the various sediments are shown. Numbers on the bars indicate the numbers of separate brush-border preparations used for the determinations. One standard deviation is indicated. Units for the specific activity are micromoles of disaccharide hydrolyzed per hour per milligram of protein.

The description of the elementary particle of the mitochondrial cristae and its association with specific enzyme activities (*9*) suggested to me that the knobs associated with the plasma membrane of the microvillus might contain disaccharidase activities. If true, this would localize these enzymes on the lumen side of the microvillus. Further, if the knobs could be removed and separated from the plasma membrane, then the enzyme localization would be identified as separate from an integral part of the membrane.

The brush borders were prepared from 15 cm of jejunum from two or three adult hamsters (*2*). Suspended in the ethylenediaminetetraacetate used for isolation, the brush borders were digested in a nitrogen atmosphere with papain activated by cysteine and 30 meq of K^+ per liter for 10 minutes at 37°C. The digestion mixture was cooled rapidly to 4°C and centrifuged at 90,000*g* for 90 minutes. The resultant pellet, sediment 1, was prepared for electron-microscopic examination and for protein (*10*) and enzyme (*11*) analysis. The supernatant was centrifuged further at 140,000*g* for 5 hours. A small clear pellet, sediment 2, was recovered. Sediment 2 and the supernatant were prepared separately for electron-microscopic examination and for protein and enzyme analyses.

Freshly prepared 2 percent phosphotungstic acid (PTA) brought to *p*H 7.4 with 1.0*M* KOH was used for negative staining. All specimens were prepared similarly. A drop of a 10 percent mixture of the fraction to be examined in 2 percent PTA was allowed to stand for 10 seconds on a grid thinly coated with carbon. The excess was removed,

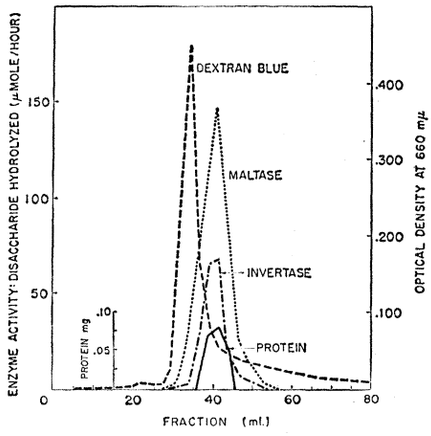

Fig. 3. The result of filtration through a Bio-Gel P-200 column (2 by 50 cm). The marker, dextran blue, was read at 600 mμ. Activities of invertase and maltase and the protein were similarly retarded in the column and were recovered in the same fractions.

and the grid was air dried. Examination in a Philips EM 200 at 80 kv was carried out within 3 hours of preparation.

The specific activities of invertase and maltase for the whole brush border, sediment 1, sediment 2, and the final supernatant are shown in Fig. 2. The highest specific activities are in sediment 2. The specific activity for invertase here is two times that of the brush-border preparation and 20 times that of sediment 1. Clearly the enzymes have been concentrated in this sediment.

Figure 1b reveals the appearance of the first sediment after its digestion with papain. Papain digestion has denuded the microvilli of the knobs, as indicated by the smooth appearance of the edge. Unit membranes are identified after osmium fixation of this sediment, confirming that this sediment contains the plasma membrane. Since relatively little specific enzyme activity was present in this sediment, the plasma membrane of the microvillus appears not to possess significant invertase or maltase activity.

Further, digestion with papain without cysteine, with cysteine, or with trypsin resulted in almost total recovery of the enzyme specific activities in sediment 1. For example, specific activity of invertase in sediment 1 was 100 times that in sediment 2 after digestion with papain not activated with cysteine. In these instances, as judged by electron-microscopic examination, sediment 1 contained the microvilli with the knobs still attached.

Sediment 2 contains numerous small particles, probably globular, 60 Å in diameter (Fig. 1c). The particles tend to aggregate. These particles are similar in size to those seen attached to the plasma membrane of the microvillus in the brush-border preparation. The specific activities of invertase and maltase were highest in this sediment.

Examination of negatively stained preparations of the final supernatant revealed no similar concentration of particles. Analysis also revealed very low specific activities of the enzymes.

Finally, sediment 2, which contains the knobs and enzyme activity, was suspended in a final volume of 1.0 ml of 0.1*M* phosphate buffer, *p*H 6.1, and applied to a Bio-Gel P-200 column (Fig. 3).

There is slight retardation of the enzyme activities and protein during passage through the gel column. The amounts of retardation are similar; the enzyme activities and protein appear in the same fractions collected. Electron microscopy of the sediment recovered from the tubes with these maximum activities reveals the presence of the previously described knobs. Negative staining of other fractions demonstrated no similar particles.

I conclude that digestion, with activated papain, of epithelial-cell brush borders isolated from hamster intestine removes knobs 60 Å in diameter from the plasma membrane. They are attached to the plasma membrane but are not an integral part of it. The activities of invertase and maltase in the brush border are associated with them. I believe these knobs and enzyme activities are contained in the glycocalyx or fuzzy coat, external to the plasma membrane of the microvillus.

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References and Notes

1. R. K. Crane, *Gastroenterology* **50**, 254 (1966).
2. D. Miller and R. K. Crane, *Anal. Biochem.* **2**, 284 (1961).
3. J. Overton, A. Eichholz, R. K. Crane, *J. Cell Biol.* **26**, 693 (1965).
4. A. Eichholz and R. K. Crane, *ibid.*, p. 687.
5. S. Ito, *ibid.* **27**, 475 (1965).
6. D. C. Pease, *J. Ultrastruct. Res.* **15**, 555 (1966).
7. J. H. Luft, *J. Cell Biol.* **23**, 54A (1964).
8. T. Oda and S. Seki, *Proc. Int. Cong. Electron Microscop. 6th* (1966), pp. 387–88; C. F. Johnson, *ibid.*, pp. 389–90.
9. H. Fernandez-Moran, *Circulation* **26**, 1039 (1962).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
11. A. Dahlquist, *Anal. Biochem.* **7**, 18 (1964).
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Glutaraldehyde Activation of Nuclear Acid Phosphatase in Cultured Plant Cells

Abstract. *Cultured tobacco cells exhibited a nuclear reaction in the cytochemical test for acid phosphatase after exposure to 2 percent glutaraldehyde. A slight preference for adenosine monophosphate over beta glycerophosphate as substrate was noted, but the enzymatic activity was not typically 5′-nucleotidase. Other aldehydes tested did not elicit the same enzyme response. Nucleolar localization was distinct from that in the rest of the nucleus. Assay for spectrophotometric measurements of enzymatic activity in vitro was developed as a modification of the Burstone procedure with naphthol AS-B1 phosphate and diazo blue B.*

The use of glutaraldehyde for routine fixation of biological materials for electron microscopy has stimulated study of the nature of its interaction with protoplasmic constituents, along empirical as well as theoretical lines (*1*). Chemical fixation generally causes a loss of histochemical staining ability (see *2*). It has been reported that glutaraldehyde does not alter acid phosphatase localization in some organisms (*3*).

We have found that treatment with 2 percent glutaraldehyde (*4*) specifically activates acid phosphatase in the nuclei of WR-132 culture cells. The suspension culture was derived from tobacco stem explant (*5*). Cytochemical localization of acid phosphatase in untreated cells is primarily at the cross-walls (Fig. 1A). This agrees with reports that a substantial portion of acid phosphatase activity in plant cells is associated with the walls, particularly those from tissue cultures (*6*). We observed that if the Gomori reaction for phosphatase (*7*) is performed subsequent to glutaraldehyde treatment, there is no longer a detectable wall reaction, but instead an intense positive reaction in the cell nucleus (Fig. 1B). The glutaraldehyde effect on acid phosphatase activity is not limited to experiments *in situ* since activation has been achieved with isolated nuclei. As observed, the phenomenon is unique for glutaraldehyde. Neither the monoaldehydes—formaldehyde, glyceraldehyde, propionaldehyde, crotonaldehyde, and isovaleraldehyde—nor the dialdehydes glyoxal and hydroxyadipaldehyde can serve as substitutes for glutaraldehyde. Substrates other than beta