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# Localization of the Enantiozymes of 6-Hydroxy-Nicotine Oxidase in *Arthrobacter oxidans* by Electron Immunochemistry†

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**During the course of growth of *Arthrobacter oxidans*, induction of the enantiozymes 6-hydroxy-D-nicotine oxidase and 6-hydroxy-L-nicotine oxidase occurred in the presence of DL-nicotine. Cryoultramicrotomed sections obtained from cells grown to stationary phase were gold immunolabeled. The results obtained demonstrate that both enzymes are localized in the cytoplasm.**

In *Arthrobacter oxidans*, the flavoprotein enantiozymes 6-hydroxy-D-nicotine oxidase (EC 1.5.3.6) and 6-hydroxy-L-nicotine oxidase (EC 1.5.3.5) are induced by DL-nicotine (1). The L-specific enzyme is composed of two identical subunits of $M_r$ 53,000, each containing one mole of noncovalently bound flavin adenine dinucleotide, and is synthesized during the logarithmic and stationary phases of growth (2, 5). The D-specific enzyme is a single polypeptide chain with an $M_r$ of 47,500, containing one mole of covalently bound flavin adenine dinucleotide, and is synthesized only during the late logarithmic or early stationary phases of growth (3, 6).

In continuing studies to ascertain the mechanism involved in the flavinylation of these two enzymes, it became desirable to determine their intracellular localization. The methods (9) recently employed to demonstrate the localization of NADP-isocitrate dehydrogenase in *Escherichia coli* were used for these studies and will be described.

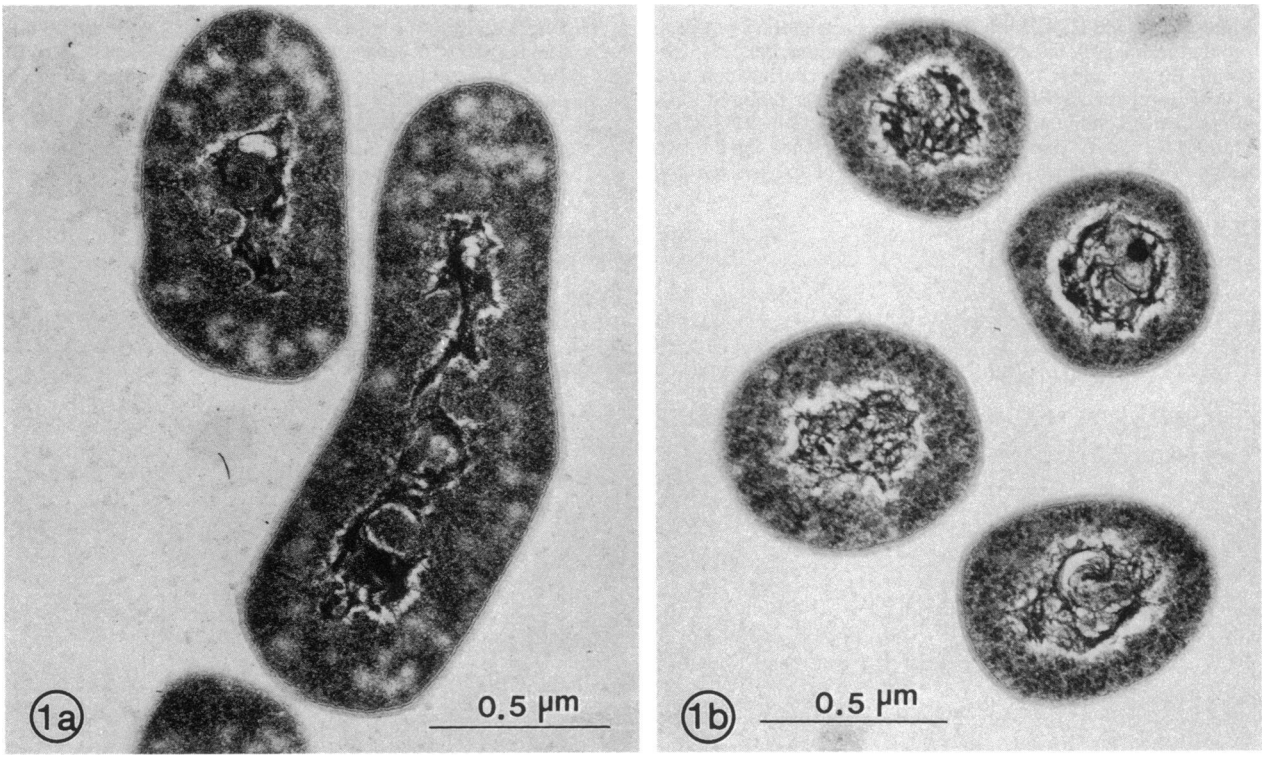


FIG. 1. Transmission electron micrographs of plastic-embedded, thin-sectioned *A. oxidans* cells demonstrating typical morphologies at different phases of growth. (a) Cells representative of logarithmic phase. (b) Cells representative of stationary phase.

The *A. oxidans* cells used in these studies have been described earlier (1). Cells were grown in 300-ml Erlenmeyer flasks containing 20 ml of mineral salts medium (4) containing 0.2% citrate as carbon source and supplemented with

† Dedicated to Karl Decker on his 60th birthday.
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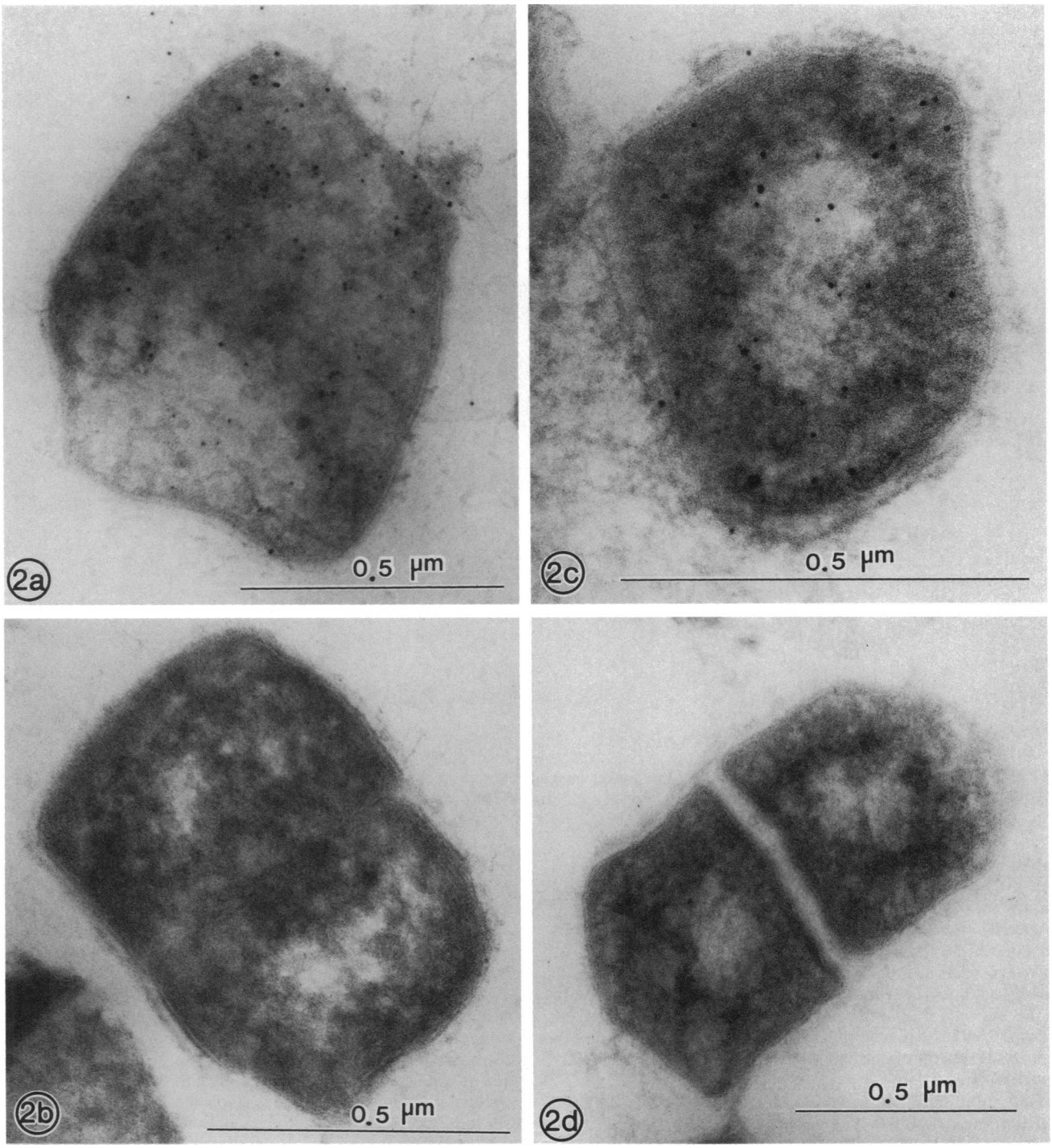


FIG. 2. Transmission electron micrographs of immunolabeled, cryosectioned cells from stationary phase of growth. (a) Gold immunolabeled section treated with 6-hydroxy-L-nicotine oxidase specific immunoglobulin G. (b) Corresponding control for panel a. (c) Gold immunolabeled section treated with 6-hydroxy-D-nicotine oxidase specific immunoglobulin G. (d) Corresponding control for panel c.

0.1% D,L-nicotine. The cultures were incubated at 31°C with vigorous aeration, and growth was monitored with a Klett colorimeter with a red filter (660 nm). At the appropriate time, 10 ml of the culture was harvested by centrifugation at 12,000 × *g* for 15 min at 4°C. The cell pellets were washed by suspension in 40 ml of 0.01 M sodium phosphate buffer (PB) (pH 7.5) and centrifuged as described above.

Ultrathin plastic sections of cells grown either to the logarithmic or to the stationary phase were obtained by fixing washed cells in 0.1 M sodium cacodylate-buffered glutaraldehyde (0.5%, pH 6.6) for 2 h. After three washes in 0.1 M cacodylate buffer, the cells were pelleted and suspended in 1% $OsO_4$–0.05 M sodium cacodylate fixative for 3 h. After a brief $H_2O$ wash, cells were enrobed in 2% agar (Difco Laboratories, Detroit, Mich.). Small pieces of the agar-immobilized cells were dehydrated with ethanol and

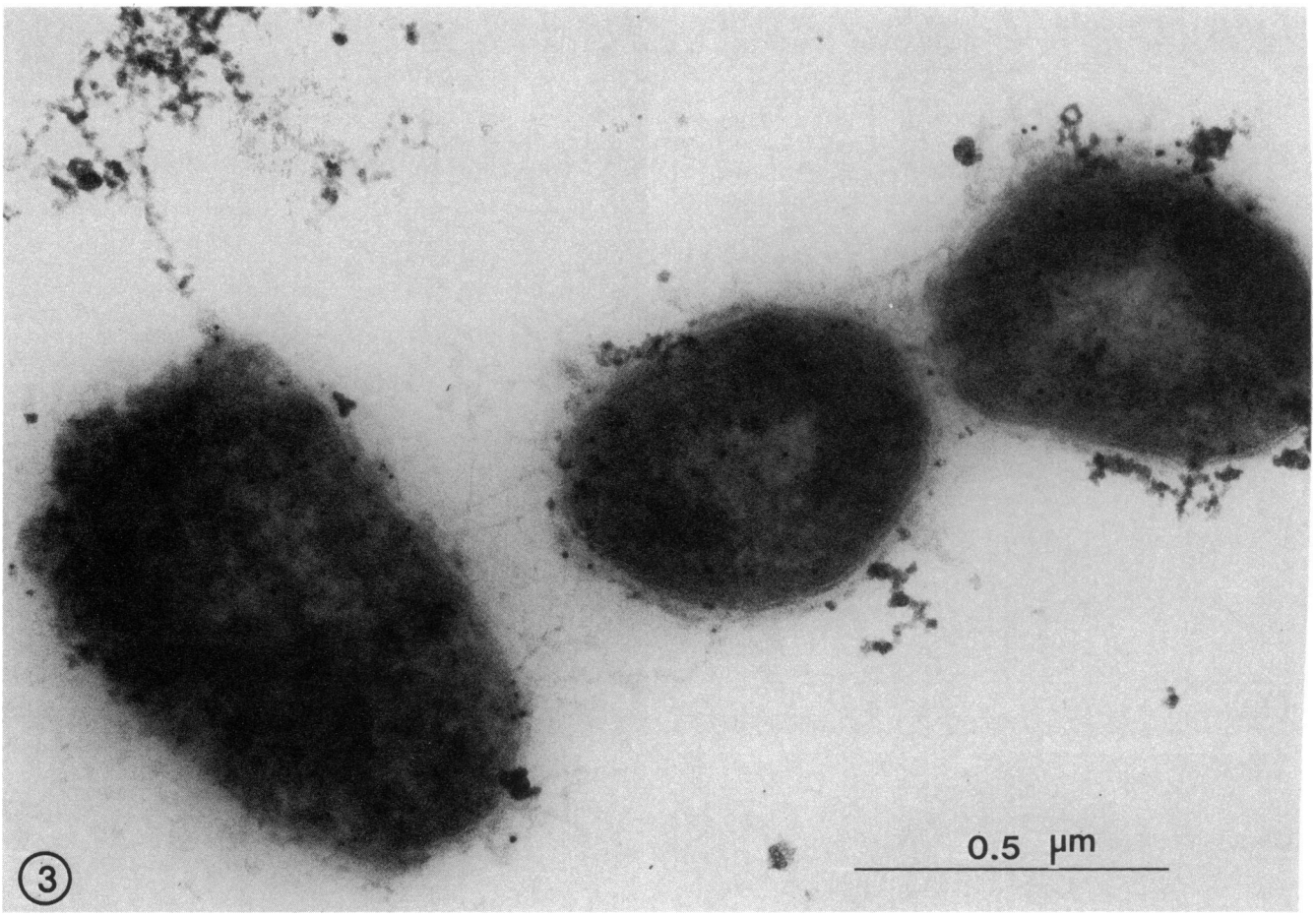


FIG. 3. Transmission electron micrograph at low-power magnification of cells gold immunolabeled with antibody raised against 6-hydroxy-D-nicotine oxidase.

embedded in Spurr resin (8). Thin sections were obtained with a diamond knife and stained with 2% aqueous uranyl acetate and Reynolds lead citrate (7). The sections were observed with a Philips EM 300 transmission electron microscope at 80 kV acceleration.

Frozen section immunolabeling was conducted with cells grown to stationary phase. Pelleted cells were washed with 0.1 M PB (pH 7.3) and suspended for 1 h at 4°C in 0.1% glutaraldehyde in PB. Preparations and conditions for obtaining frozen thin sections were carried out as previously reported (9).

Gold immunolabeling was performed by incubating the thin sections, retained on carbon-stabilized Formvar grids, for 1 h at 26°C with affinity-purified immunoglobulin G. These antibodies had been raised in rabbits against either the purified 6-hydroxy-L-nicotine oxidase or the 6-hydroxy-D-nicotine oxidase. After being thoroughly washed with PB, sections were treated for 10 min with protein-A gold colloid (10 to 15 nm, E. Y. Laboratories, San Mateo, Calif.) diluted 1:10 with 5% bovine serum albumin. The use of undiluted protein-A gold colloid did not result in increased labeling of the sections. Control samples were treated similarly except that purified immunoglobulin G (20 μg/ml) from preimmune serum was used. The sections were washed briefly with PB, fixed with 1% glutaraldehyde in PB, and stained with 2% aqueous uranyl acetate and finally with an adsorption stain by the method of Tokuyasu (10). Electron micrographs were recorded from these preparations with a Philips EM 300 transmission electron microscope at 100 kV acceleration.

Figure 1 demonstrates the morphological differences in *A. oxidans* cells harvested during the logarithmic (Fig. 1a) and stationary (Fig. 1b) phases of the growth curve. The 6-hydroxy-L-nicotine oxidase is synthesized first, while the culture is in logarithmic growth and the cells are rod form. Enzyme synthesis continues into the stationary phase. In contrast, 6-hydroxy-D-nicotine oxidase is synthesized only during the stationary phase of growth when the cells are coccoid. Aside from the shape of the cells, there are no obvious morphological differences which can be related to the time of induction or the synthesis of the enantiozymes.

The gold labeling shown in Fig. 2a and c demonstrates the intracellular distribution of 6-hydroxy-L-nicotine oxidase and 6-hydroxy-D-nicotine oxidase, respectively. Figures 2b and d show control sections which were treated with preimmune serum. The results obtained argue strongly against the possibilities that either of the enzymes is associated with the cytoplasmic membrane or occurs in the periplasmic space. Although these sections appear disfigured relative to those seen in Fig. 1b, which were obtained by conventional plastic embedment, the cell membrane, cell wall, nucleoplasm, and cytoplasm are clearly discernible. The light fixation employed in these studies (0.1% glutaraldehyde) to preserve

antigenicity may contribute to this deformation. It should be noted, particularly in Fig. 2a and c, that a leakage of the cytoplasm beyond the periphery of the cell occurred, presumably as a result of insufficient fixation. In Fig. 2a, some gold labeling can be observed on this material, suggesting that the cytoplasm has indeed leaked from the cell.

A field of cells at low magnification (Fig. 3) demonstrates the localization of the gold label in the cytoplasm of the cells, and the specificity of the reaction is indicated by the low background distribution of gold particles. The dispersement of the cells in the gelatin matrix employed in the ultramicrotomy under our cryogenic experimental conditions precluded the demonstration of a large number of cells in any one field of view. The distribution of gold label in cells treated with antibody against 6-hydroxy-L-nicotine oxidase was similar (data not shown).

These experiments demonstrate the usefulness of ultramicrotomy under cryogenic conditions and immunolabeling with protein-A gold in the cellular localization of bacterial enzymes. We recognize that the method may not provide the degree of high resolution currently available with other transmission electron microscopic techniques employing bacterial thin sections. Nevertheless, these studies provide information which will be extremely useful in continuing studies to determine the site and mechanisms involved in the flavinylation of 6-hydroxy-L-nicotine oxidase and 6-hydroxy-D-nicotine oxidase in *A. oxidans*.

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