THE DEMONSTRATION OF THE SUCCINIC DEHYDROGENASE SYSTEM IN *BACILLUS SUBTILIS* USING TETRANITRO–BLUE TETRAZOLIUM COMBINED WITH TECHNIQUES OF ELECTRON MICROSCOPY

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ABSTRACT

Activity of the succinic dehydrogenase system was studied in *Bacillus subtilis* utilizing combined techniques of cytochemistry and electron microscopy. Organisms were incubated in a medium containing tetranitro-blue tetrazolium (TNBT) which served as an electron acceptor. Enzymatic activity, as evidenced by deposition of TNBT-formazan, was found on membranous organelles associated with the cytoplasmic membrane and septal plasma membrane, the nuclear area, and the plasma membrane. Flagella, ~190 A in diameter, with thorn-like projections protruded through the cell wall. Tangential-oblique sections of the cell wall showed many pores ~ 220 A in diameter with a center-to-center spacing of ~450 A.

INTRODUCTION

Recent reports from our laboratories (1–3), on studies using combined techniques of cytochemistry and electron microscopy, showed that the succinic dehydrogenase system (SDH) was associated with the plasma membrane in the Gram-negative bacterium, *Escherichia coli*. It was decided to apply a similar technique, using tetranitro-blue tetrazolium (TNBT) as an electron acceptor, to study localization of SDH in the Gram-positive bacterium *Bacillus subtilis* because of the presence of membranous organelles in the cytoplasm that were presumed to possess respiratory activity (4, 6).

The fine structure of these membranous organelles has been well documented (4–11). This organelle, also named mesosome (6) or chondrioid (12), can assume a number of different configura-tions: (*a*) whorls of unit membranes (13) derived from the plasma membrane projecting into the cytoplasm; (*b*) combinations of tubules and vesicles originating from the plasma membrane; and (*c*) organelles consisting either of whorls of parallel membranes or tubulo-vesicular profiles contained within the nuclear area. It appears to have been established that the intranuclear organelle is connected to the plasma membrane by way of the cytoplasmic organelle (11). A number of functions have been suggested for these membranous organelles (7, 10, 11, 14): (*a*) they are mitochondrial equivalents and therefore are the locale of respiratory enzyme systems, and (*b*) they, especially the tubulo-vesicular organelles, are involved in cross-wall formation.

Recently workers have applied combined tech-

Composition of Incubating Media for Studying Activity of SDH in B. subtilis

Constituents	Experimental	Dye control	Competitive inhibition	Fixation control
0.2 M Na_2HPO_4/KH_2PO_4 at pH 7.2, ml	11.25	11.25	11.25	15.00
0.8 M Na_2 succinate, ml	3.75	3.75	3.75	None
TNBT*, ‡ (1 mg/ml), mg	15	None	15	None
Sodium malonate, mg	None	None	500	None

* See reference 3 for technique of dissolving TNBT.

‡ Obtained from Dajac Laboratories, Borden Chemical Co., Philadelphia.

niques of cytochemistry and electron microscopy in the study of respiratory activity of Bacillus subtilis. Vanderwinkel and Murray (10), using triphenyltetrazolium, associated redox activity with membranous organelles (mesosomes), but their technique involved acetone extraction of the formazan, leaving an empty space for the site of activity. van Iterson and Leene (14), using potassium tellurite, found enzymatic activity of the respiratory system localized in both membranous organelles and "rod-like elements" at the cell periphery; no enzymatic activity was found associated with the plasma membrane.

The desirable properties of a new dye, $2,2',5,5'$-tetra - p - nitrophenyl - $3,3'$ - $(3,3'$ - dimethoxy - $4,4'$-biphenylene)-ditetrazolium chloride (TNBT) offer a means to reinvestigate respiratory enzymatic activity in B. subtilis. These properties include: (a) insolubility in the common organic solvents used in electron microscopy (15); (b) lack of lipid affinity (16); and (c) small diameter of the formazan aggregates (30 to 40 A) (17, 18). The following report presents the results obtained with the combined techniques of cytochemistry and electron microscopy used to localize the SDH system in B. subtilis.

MATERIALS AND METHODS

A stock culture of Bacillus subtilis[1] was maintained at room temperature in a 3 per cent aqueous Trypticase Soy Broth with sodium succinate added to a final concentration of 1 per cent. For the experiments organisms were inoculated into 30 ml of broth which was oxygenated in a 500-ml Erlenmeyer flask. Organisms were harvested at a concentration of 5×10^9 bacteria per ml, using a Petroff-Hausser counting chamber, by centrifugation in 40-ml centrifuge tubes at 3300 RPM employing a clinical centrifuge. The supernatant was decanted and 11.25 ml of 0.2 M Na_2HPO_4/KH_2PO_4 buffer at pH 7.2 was added and the organisms resuspended. The bacteria were then subjected to the experimental conditions listed in Table I, and incubated for 7 to 60 minutes under either aerobic or semiaerobic conditions. Oxygen was bubbled through the broth to obtain the aerobic condition. The semiaerobic atmosphere was attained by burning an alcohol-saturated wick to suffocation in a tightly stoppered 40-ml test tube.

Following incubation the organisms were concentrated by centrifugation and treated in one of three ways: (a) fixed in 1 per cent osmium tetroxide buf-

[1] Obtained from stock cultures of Dr. R. J. Mandle, Department of Microbiology, Jefferson Medical College, Philadelphia.

Figs. 1 to 6 are of material not exposed to TNBT, whereas the cells of Figs. 7 to 13 were exposed to TNBT.

FIGURE 1 A portion of Bacillus subtilis is seen here delimited externally by a cell wall (cw) subjacent to which is found a tripartite plasma membrane (pm). The nuclear area consisting of fine filamentous material is indicated at n. The cytoplasm contains an abundance of ribonucleoprotein granules. At the top of the micrograph a whorl of unit membranes is observed originating from the plasma membrane; this configuration is identified as the membranous organelle (mesosome, chondrioid). $\times$ 120,000.

fered at pH 6.1 with the acetate-Veronal buffer of Michaelis (19) at room temperature for 12 hours; (b) fixed in 5 per cent glutaraldehyde at pH 6.1 with the Michaelis buffer at 4°C for 12 hours; and (c) fixed and stained according to the technique of Ryter and Kellenberger (19). During the first hour of fixation the bacteria were incorporated into agar at a final concentration of 1 per cent and sliced into 1 mm³ cubes. The organisms were then dehydrated in cold ethanol (starting with 50 per cent ethanol) and propylene oxide and embedded in Epon 812 according to Luft (20).

The blocks were sectioned with diamond knives using either a Porter-Blum microtome or an LKB Ultrotome. Thin sections were examined with either a Siemens Elmiskop I at 80 kv, with a double condenser and a 30 μ objective aperture, or an RCA EMU 3D microscope with a 30 μ objective aperture equipped with an external bias control to regulate beam current at magnifications of 16,500 to 40,000.

OBSERVATIONS

Visual Observations

As found for E. coli (3), organisms incubated for periods of 7 to 60 minutes in the presence of TNBT, buffer, and sodium succinate evidenced visible reduction of the TNBT within 30 seconds. Dark clumps of organisms slowly settled and formed a brown-black pellet. After approximately 3 minutes, the color remained constant. If sodium malonate was added to the incubation medium before the TNBT was added, there was an obvious decrease in visible reduction of the TNBT. No visible extraction of the TNBT-formazan occurred during the process of washing, fixation, dehydration, and embedding.

Electron Microscopy

#### THE FINE STRUCTURE OF UNTREATED B. SUBTILIS

Micrographs of the sectioned organisms obtained from dye control (see Table I) demonstrated a fine structural pattern which was similar to that already reported in the literature (4–11). A thick cell wall (cw) delimits the organism externally (Figs. 1 to 5). A tripartite plasma membrane (pm) is located subjacent to the cell wall (Figs. 1 to 5). In many of the micrographs there was no obvious separation between the cell wall (cw) and plasma membrane (pm) (see Figs. 1, 3, and 5). The plasma membrane measures ∼80 A in diameter, i.e. the outer dense components ∼25 A, and the middle light zone ∼30 A. Membranous organelles in various forms and locations were evident in the sectioned organisms. Whorls of parallel unit membranes originating from the plasma membrane (Fig. 1) were found in the peripheral cytoplasm. Sometimes these whorls reach considerable size (Fig. 5). These organelles also consist of tubulo-vesicular profiles (Fig. 4) that are associated frequently with cross-wall formation. In these instances, the organelles appeared to originate from the region of the plasma membrane associated with the new cross-wall. Organelles have been located also in nuclear areas (Fig. 3). Here they may assume either a parallel concentric array or a tubulo-vesicular pattern. The cytoplasm consists also of an abundance of closely packed ribonucleoprotein granules (∼140 A) (Figs. 1 to 5). The nuclear areas contain a fibrillar meshwork with elements measuring between 25 and 30 A; no nuclear delimiting membrane was observed (Figs. 1 to 3).

Noteworthy in the preparations is the preservation of flagella protruding from the surface of the organism (Figs. 4 and 6). The flagella measure ∼190 A in diameter and appear to have thorn-like projections. In tangential-oblique sections of the cell wall, many pores (∼220 A) were seen with a center-to-center spacing of ∼450 A (Fig. 6). These pores were assumed to represent the points of emergence of the flagella.

FIGURE 2 Part of a bacterial profile is shown here illustrating portions of two membranous organelles on both sides of a cross-wall. These organelles are continuous with the septal plasma membrane. Other structures identified include the cell wall (cw), plasma membrane (pm), and part of a nucleus (n). × 120,000.

FIGURE 3 This micrograph shows a membranous organelle consisting of a whorl of unit membranes within the nuclear area (n). The cell wall (cw) and plasma membrane (pm) are indicated. × 120,000.

cw

pm

n

②

cw

pm

n

③
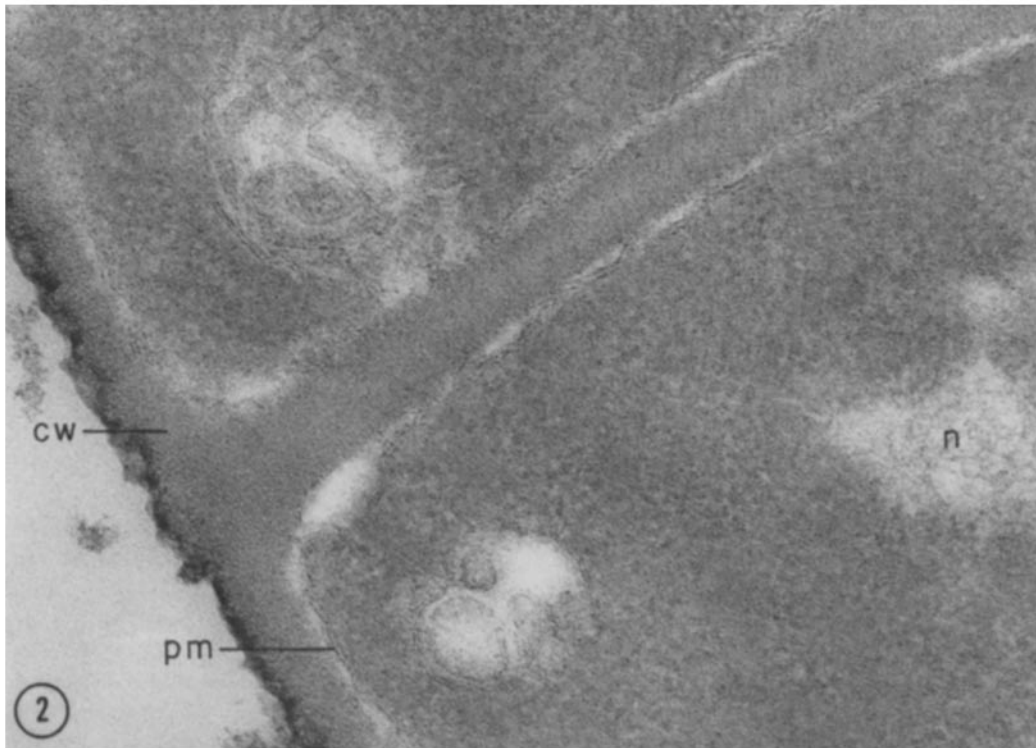
The Fine Structure of Organisms Incubated with TNBT

Enzymatic activity of the SDH system is seen in the electron micrographs as aggregates of TNBT-formazan (TNF) that exhibit electron-scattering properties. These aggregates were found on membranous organelles associated with the cytoplasmic membrane (a, Fig. 7), septal plasma membrane (b, Fig. 7), nuclear area (c, Figs. 7, 8, and 12), and the plasma membrane (d, Figs. 8 to 10). There was no evidence of reduction of TNBT on the flagella (Fig. 7). In general, precipitation of the formazan was heavy and included apparent deposition on non-membranous areas of mesosome as well as a zone of cytoplasm contiguous with the mesosome (Figs. 7, 9, and 10). The distribution of formazan was uniform throughout the entire membranous organelle.

Micrographs demonstrating better resolution provided evidence for deposition of the TNF on the outer dense components of the unit membrane (Fig. 11, arrow). Organelles containing tubulo-vesicular structures showed TNF deposition on profiles that appear to consist of a single dense membrane (e, Figs. 10 and 11). In micrographs showing better resolution, the plasma membrane in continuity with the membranous organelles, for a distance as great as 0.6 μ (d, Fig. 10), exhibited reduction of the TNBT (Figs. 9 and 10). It is interesting to note that in some of these instances the plasma membrane folded back upon itself, showing a parallel arrangement (arrows, Figs. 9 and 10). In rare instances deposition of the reduction product of TNBT appeared as needle-like microcrystals upon organelle structures (Fig. 13).

DISCUSSION

Electron micrographs of B. subtilis presented in this study demonstrated a fine structure very simi-
lar to that already reported in the literature (4–11, 14). The technique, in addition, allowed for the preservation of flagella in the sections of the organisms. These flagella, ∼190 A in diameter with thorn-like projections, were seen to protrude from the cell wall. Tangential-oblique sections of the surface of the organisms showed pores with a constant center-to-center spacing; these pores presumably are the locale for emergence of the flagella. The sections provided no evidence for the existence of a basal body associated with the flagellum. The fine structure of these flagella and their relationship to the organism certainly merit further investigation.

Earlier experiments on the localization of SDH activity in E. coli avoided the use of uranyl acetate staining because of the possibility that it would mask fine deposits of TNBT-formazan (3). The present report demonstrated that the uranyl acetate staining did not appreciably mask reduction products of TNBT. Moreover, the Ryter-Kellenberger technique (19) with uranyl acetate staining resulted in excellent preservation of the fine structural anatomy of B. subtilis, so that the formazan could be identified on a specific structure.

Localization of enzymic sites in bacteria has been studied using biochemical fractionation procedures, cytochemistry, and combinations of cytochemistry and electron microscopy (21–24). Biochemical fractionation procedures identified the site of respiratory enzymes with the Membran fraction (22) that contained both cell wall and plasma membrane fragments as well as possible additional membranous elements (24). Early investigators (25–28) using such dyes as neotetrazolium, triphenyltetrazolium, and tetrazolium salts probably obtained spurious localization of enzymatic activity in bacteria due to the characteristic lipid affinity of these dyes. The formazans accumulated

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Figures 4 and 5   These micrographs show additional examples of membranous organelles in Bacillus subtilis. Fig. 4 illustrates a tubulo-vesicular configuration originating from the plasma membrane (pm). Fig. 5 provides an example of a large whorl of unit membranes in the cytoplasm. The cell wall (cw) and plasma membrane (pm) are labeled in both figures. Flagella (f) are indicated in Fig. 4. × 120,000.

Figure 6   This micrograph was obtained from a tangential-oblique section of Bacillus subtilis. Flagella (f) with thorn-like projections protrude from the cell wall. In areas devoid of flagella, pores are seen in the cell wall; these presumably represent the regions of emergence of flagella. × 120,000.
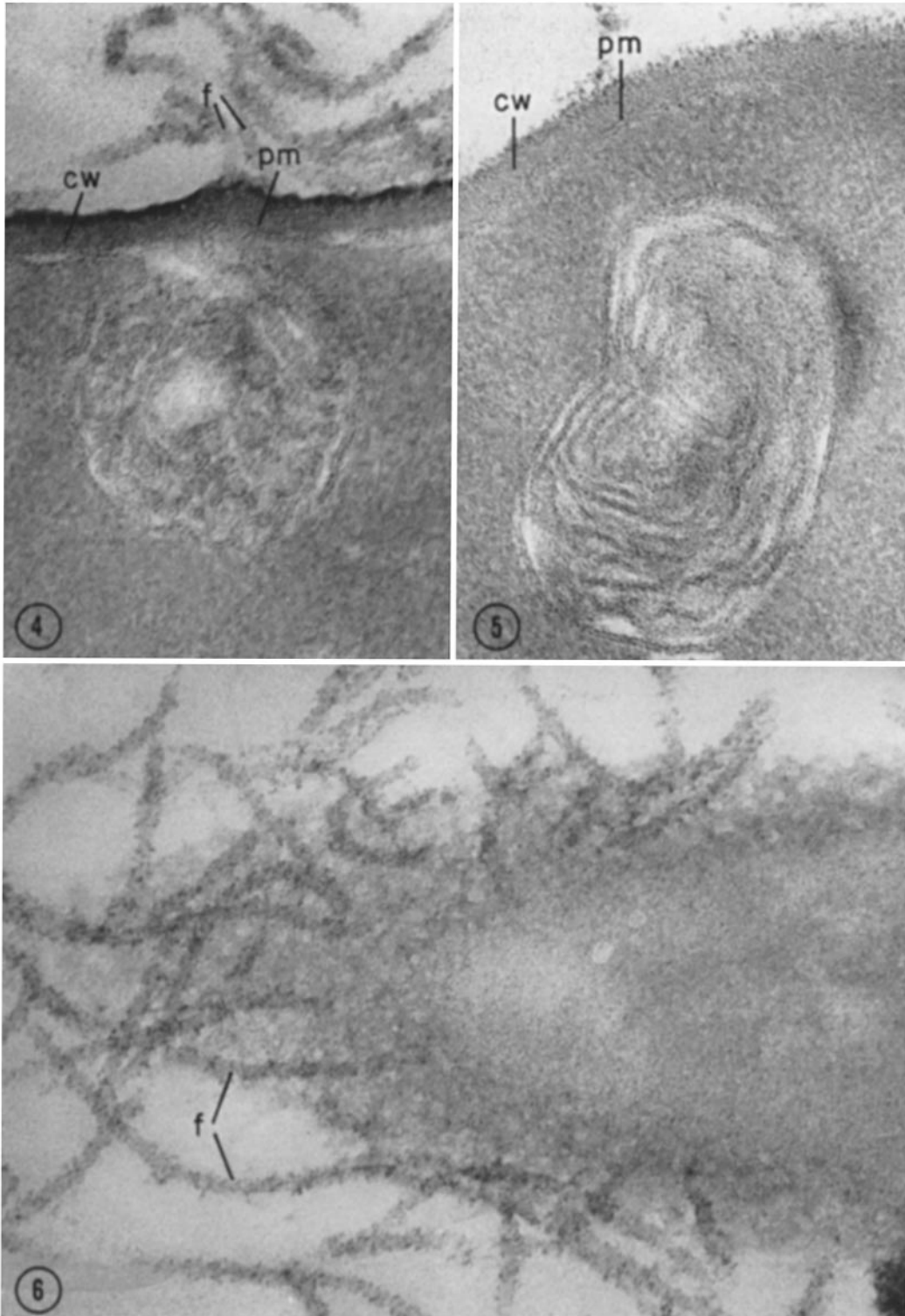
in large lipid bodies which were interpreted as mitochondrion-like elements. Weibull's investigation (27) on *Bacillus megaterium*, using triphenyltetrazolium, showed the possibility of artifact in the deposition of the formazan, since it accumulated first peripherally and later coalesced to form large central aggregates. Kellenberger and Huber's (12) results in *Bacillus cereus* could be interpreted in the same manner.

In more recent studies, Vanderwinkel and Murray (10), using triphenyltetrazolium and electron microscopical techniques, found in *E. coli* large areas corresponding to the formazan that had no relation to "regularly occurring and clearly defined organelles." On the other hand, these same authors showed an association of the formazan reduction site, represented by an acetone-extracted area, with the locale of mesosomes in both *Bacillus subtilis* and *Spirillum serpens*. Takagi, Ueyama, and Ueda (29), using triphenyltetrazolium (TTC) in *E. coli* and *Fusobacterium polymorphum*, found large crystal-shaped deposits in their electron micrographs that "seemed to be formed secondarily by the intracellular aggregation of small granules primarily formed in the cytoplasm, it was almost impossible to localize the initial sites of TTC reduction." These authors, using nitro-blue tetrazolium (NBT) in these same organisms, found "less electron-dense structures" associated with the cytoplasmic membrane in *E. coli* and mesosomal elements in *Fusobacterium polymorphum*. It seems that van Iterson and Leene's results with the use of nitro-blue tetrazolium in *B.*

subtilis were similar, in that the formazan was partially extracted by the acetone used in dehydration (14). Using tetranitro-blue tetrazolium, Sedar and Burde (3) found in electron micrographs of *E. coli* that the SDH system was localized at the site of the plasma membrane.

Conflicting results have been obtained using potassium tellurite as an electron acceptor. Brieger (23) observed tellurium needles randomly dispersed in the cytoplasm of human tubercle bacilli. van Iterson and Leene (14, 30) found reduced tellurite on the mesosomes and peripheral rod-like bodies in *B. subtilis*, and they observed large clusters of tellurium needles subjacent to the plasma membrane in *Proteus vulgaris* but no deposition on the plasma membrane.

The results reported in this paper provide evidence that reduction of TNBT in *B. subtilis* is associated with (*a*) membranous organelles in continuity with the plasma membrane or septal plasma membrane, (*b*) a length of the plasma membrane itself that is in continuity with the mesosome, and (*c*) the nuclear area. In contrast to previous work, using TNBT in *E. coli* (3), reduction of the dye was not spotty, but involved deposition on the membranes of the entire organelle and the length of plasma membrane associated with it. In micrographs of unstained preparations fixed with glutaraldehyde only, deposition of TNF was seen along the entire plasma membrane. However, the density of the TNF deposits in nuclear areas and presumed sites of membranous organelles was greater than that on the plasma membrane. This

FIGURE 7   Bacterial profiles are illustrated here from a preparation incubated in a medium containing TNBT and succinate. Deposition of the formazan of TNBT, indicating activity of the succinic dehydrogenase system, is seen in a cytoplasmic mesosome (*a*), septal mesosome (*b*), a zone of cytoplasm contiguous with the septal mesosome, and nuclear area (*c*). Flagella appear unreactive. The fine granules seen scattered in the cytoplasm of the organism sectioned longitudinally are assumed to represent "beam damage." × 41,500.

FIGURE 8   This micrograph was obtained from a preparation fixed with glutaraldehyde only and unstained. Deposition of the formazan of TNBT is evident both at the site of the plasma membrane (*d*) and in the nuclear area (*c*). × 41,500.

FIGURE 9   A portion of a dividing organism incubated in a medium containing TNBT and succinate is seen in this micrograph. The formazan of TNBT is localized on the tubulo-vesicular organelles in the region of the new cross-wall. The plasma membrane (*d*) in connection with one of these membranous organelles also shows deposition of formazan. This reactive portion of the plasma membrane has folded back on itself (arrows). × 58,000.
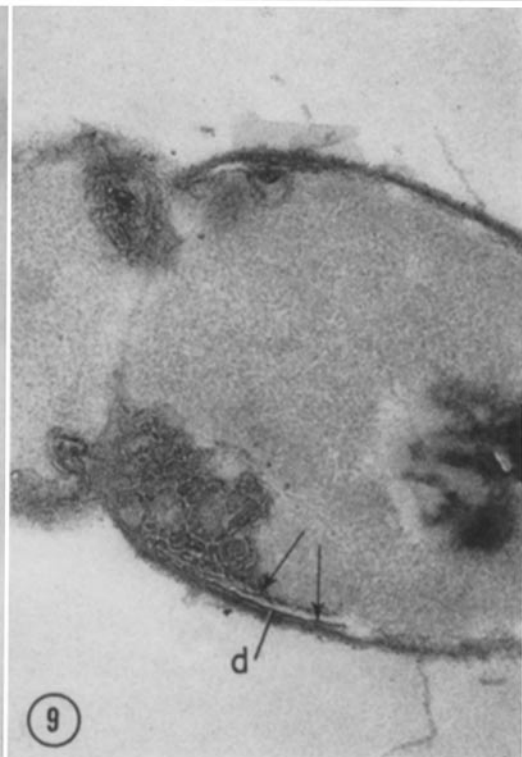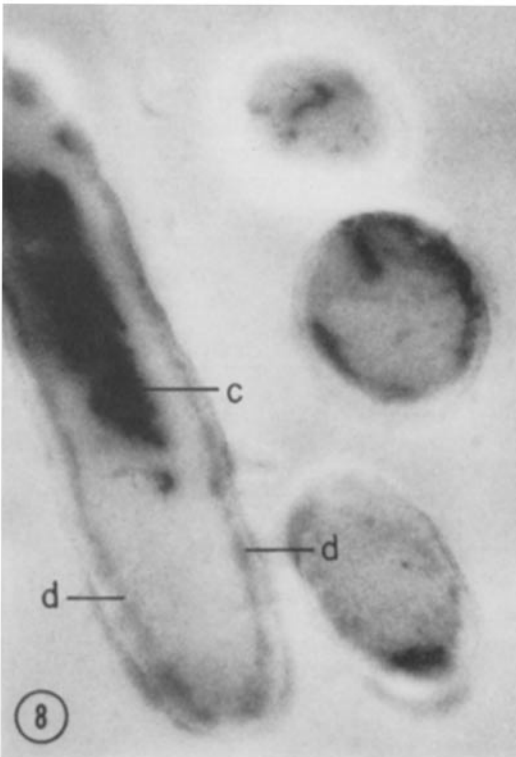
suggests that qualitatively there is more redox activity associated with that portion of the plasma membrane associated and continuous with the mesosome. Micrographs of organisms fixed using the Ryter-Kellenberger technique and stained with uranyl acetate did not show evidence of reduction on the entire plasma membrane. This could be interpreted possibly on the basis that the uranyl acetate staining masked the less dense TNF deposits on the plasma membrane. The reduction of TNBT in nuclear areas is difficult to explain since the deposits were not associated with organelles. The possibility that the structure of nuclear organelles was destroyed during preparation procedures seems unlikely, because in the same bacterial profile cytoplasmic organelles were evident. In some instances, e.g. Fig. 12, TNF deposits were found on the fibrillar elements of the nucleus. The finding of redox activity in the nucleus has been reported in other bacteria (3) and in certain mammalian cell-types (31).

Critical appraisal of some micrographs, e.g., Fig. 7, provided evidence that TNBT-formazan was found on non-membranous areas of the mesosome including an adjacent zone of cytoplasm. The question arises whether the TNBT-formazan was sufficiently confined to warrant the conclusion that the membranous elements of the organelle are indeed the sites of enzymatic activity. One could suggest that transport of formazan occurred either in one of the solvents during the preparation or before when the bacteria were still alive. It seems unlikely that diffusion of formazan does happen, since previous workers using TNBT combined with electron microscopy for localization of redox activity in mitochondria have not reported diffu-

sion or solubility artifacts (17, 18, 32). It is uncertain whether diffusion of formazan can take place in the living cell. Such a criticism might be invoked in appraising a number of histochemical techniques. A more likely explanation for the deposition of formazan in non-membranous areas of and adjacent to the mesosome is that prolonged incubation caused the production of large TNBT-formazan aggregates.

The data obtained using TNBT as an electron acceptor in B. subtilis confirmed the findings of earlier investigators, using different techniques (10, 14, 32), that the membranous organelle (mesosome, chondrioid) is a site of redox activity. On the other hand, the data did not substantiate the existence of enzymatic activity associated with thin rod-like elements at the cell periphery (14). The suggestion that these rod-like elements could possibly represent basal bodies of flagella seems remote, in view of the fact that basal bodies have not been identified in B. subtilis. Another interpretation of these peripheral rod-like bodies is that they could represent reduced tellurite crystals which precipitated near reductive sites in the plasma membrane. The present study showed that it is possible to obtain reduction of TNBT at the site of the plasma membrane, although the amount of reduction appears to be greater in that region of the plasma membrane associated with the membranous organelle. The finding of TNF in the nucleus of B. subtilis was discussed above. Investigators (10, 33) have reported marked differences in the results of experimental incubations carried out under aerobic and anaerobic conditions. They have suggested that oxygen competes for electrons with the tetrazolium dye markers. Although we

FIGURE 10 Part of a dividing cell is shown here. Formazan deposition, demonstrating activity of the succinic dehydrogenase system, is found associated with a tubulo-vesicular organelle (e), a portion of the plasma membrane (d) continuous with these membranous organelles, and the nuclear area (c). It is interesting to observe that on the left side of the figure (arrows) the TNF-positive plasma membrane appears to have folded back upon itself. × 79,500.

FIGURE 11 In this micrograph showing a portion of B. subtilis it can be seen that TNF deposition, indicating activity of the succinic dehydrogenase system, coincides with the outer dense members of the unit membrane (arrow). TNF deposition is associated also with single membranes of the tubulo-vesicular profiles comprising the membranous organelle shown at e. The fine granules observed scattered in the cytoplasm of the organism probably represent "beam damage." × 120,000.
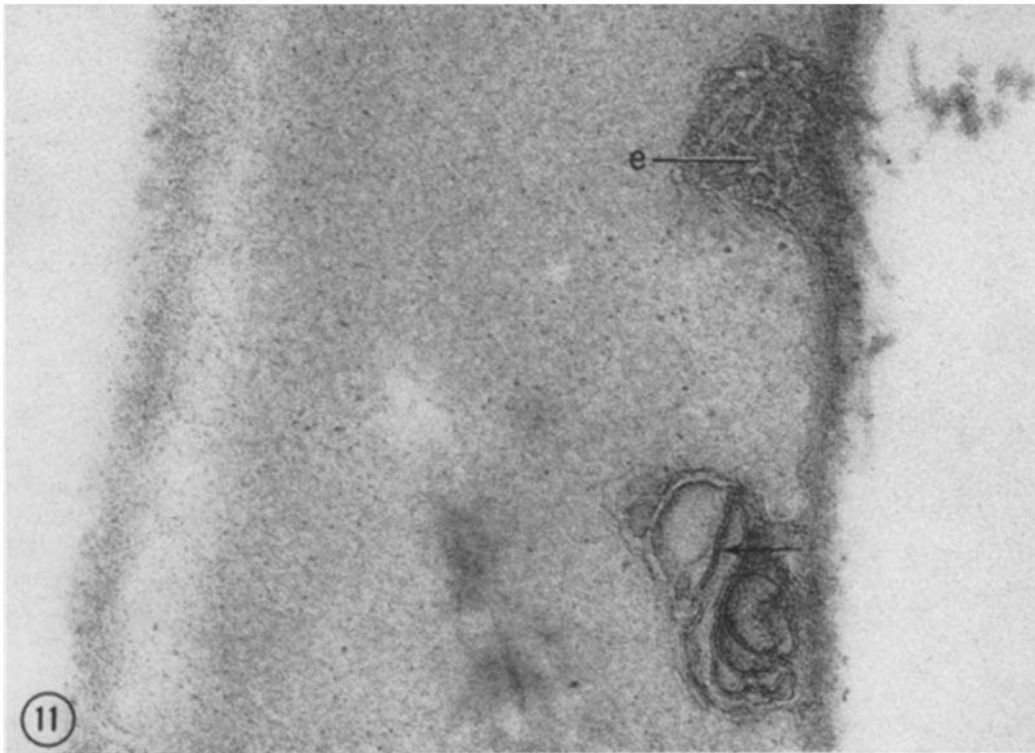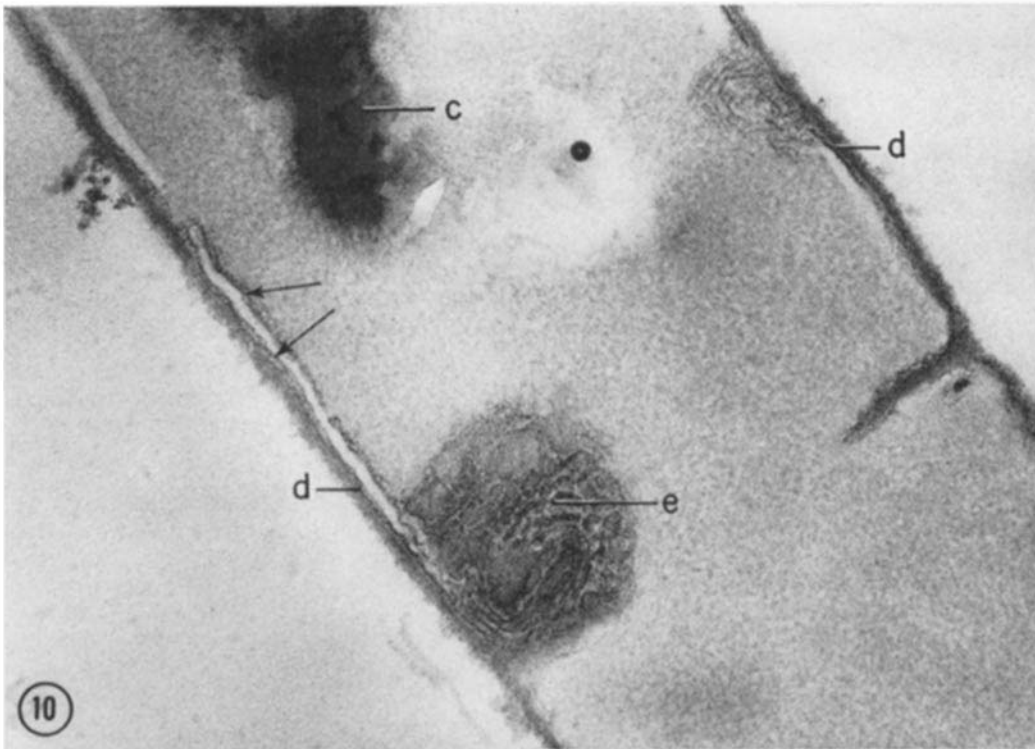
have attempted a comparison of these incubation conditions, preliminary results have not demonstrated any obvious differences.

The membranous organelles, including the cytoplasmic, septal, and nuclear tubulo-vesicular or membranous structures, all originate from the plasma membrane. These elements serve to increase the effective surface area of the organism. Enzymatic activity has been localized in all these configurations, including the plasma membrane. It seems reasonable to suppose that in the course of evolution these bacteria required greater amounts of energy for metabolism that necessitated differentiation of specialized organelles. A

number of authors already have drawn the conclusion that these membranous organelles of bacteria and the mitochondria of higher organisms are analogous structures. Robertson (13) has suggested that mitochondria could arise from the plasma membrane.

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FIGURE 12 This micrograph shows an example of reduction of TNBT within the nuclear area (c) of *B. subtilis*. It appears here as if the formazan is associated with the fibrillar elements within the nucleus. The significance of redox activity associated with the nucleus remains unexplained. × 120,000.

FIGURE 13 A portion of a dividing organism is seen in this micrograph. Note that the deposition of the formazan of TNBT appears in the form of needle-like microcrystals within the membranous organelles associated with the new cross-wall. Similar microcrystals have been found occasionally associated with mitochondria of mammalian cells incubated with TNBT and succinate. The origin and significance of these crystals are unknown. × 79,500.
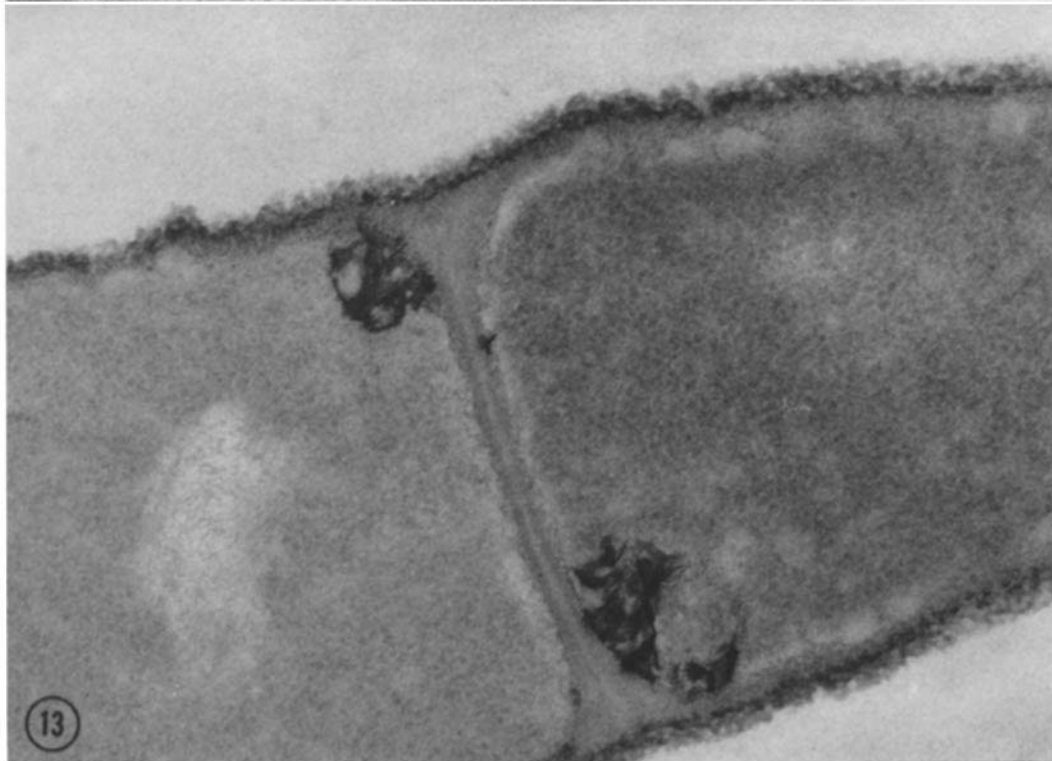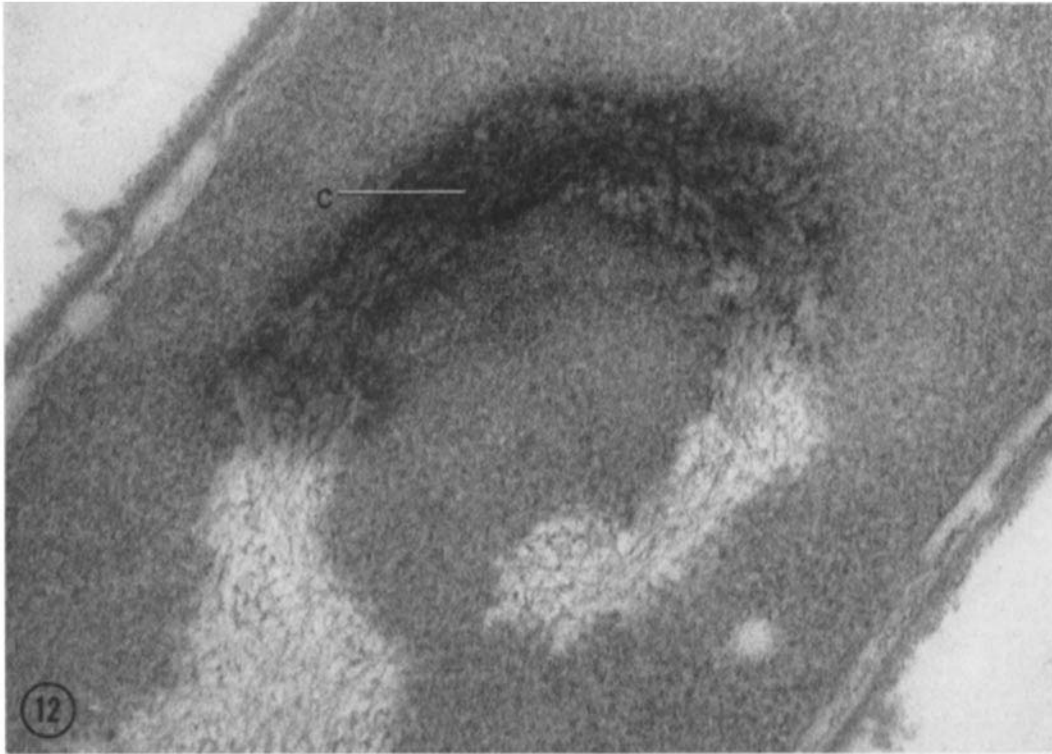
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