

LOCALIZATION OF THE SUCCINIC DEHYDROGENASE SYSTEM IN *ESCHERICHIA* *COLI* USING COMBINED TECHNIQUES OF CYTOCHEMISTRY AND ELECTRON MICROSCOPY

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ABSTRACT

The activity of the succinic dehydrogenase system was studied in *Escherichia coli* 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 the deposition of aggregates of TNBT-formazan, was found associated with the site of the plasma membrane of the bacterium.

INTRODUCTION

Localization of enzyme systems in bacteria has been the subject of intensive research. A large proportion of this type of investigation employed biochemical fractionation procedures. Results of such studies have shown that oxidative enzymes are associated with the cell *Membran* fraction (1) which contains both cell wall and plasma membrane fragments. Early work (2-5) involved visual localization of enzyme sites with the use of tetrazolium salts which became reduced to colored formazans. More recently combined techniques of histochemistry and electron microscopy have been applied to the specific structural localization of respiratory enzyme sites in bacteria (6-8).

Recent reports from these laboratories have demonstrated the advantages of using 2,2',5,5'-tetra-*p*-nitrophenyl-3,3'(3,3'-dimethoxy-4,4' biphenylene)-ditetrazolium chloride (TNBT) for localization at the electron microscope level of the succinic dehydrogenase system (SDH) (9, 10). The enzyme activity, as evidenced by the deposition of the formazan of TNBT (TNF), was found

within the membranous component of individual cristae mitochondriales (cells of rat myocardium). The properties of this formazan which make it extremely desirable include insolubility in common organic solvents used in electron microscopy (11), its stability in methacrylate or Epon sections examined in the electron beam (9), lack of lipid affinity (12) as compared with the formazan of nitro-blue tetrazolium, and the small diameter of formazan aggregates (30 to 40 Å) (9).

The more desirable qualities of this cytochemical procedure provide a means to investigate specific enzyme location in bacteria. *Escherichia coli* was selected because of the large volume of biochemical data available on this species and also because the fine structure of the organism has been well defined (13-16). The following report presents the findings where combined techniques of cytochemistry and electron microscopy were used to localize enzyme activity of the SDH system in *E. coli*.

MATERIALS AND METHODS

A stock culture of *E. coli*¹ was inoculated and maintained in a 3 per cent aqueous Trypticase Soy broth at room temperature. The experiments were performed on organisms 15 to 18 hours postinoculation. The experimental tubes (standard 12 ml centrifuge tubes) were centrifuged at 3300 RPM in a clinical centrifuge for approximately 5 minutes. The supernatant was decanted and 5 to 10 ml of either 0.1 M or 0.2 M Na₂HPO₄/KH₂PO₄ buffer at pH 7.2 were added and the organisms resuspended. The bacteria were washed for periods ranging from 10 to 80 minutes, by repeating this procedure. The average time of washing was approximately 15 minutes. After this washing, the organisms were subjected to the experimental conditions listed in Table I and incubated for periods of 1 to 30 minutes.

Then the blocks were sectioned using either a Porter-Blum microtome or an LKB Ultratome. Thin sections on carbon coated, 150 mesh copper grids were examined with an RCA EMU 3D electron microscope at magnifications from 11,000 to 19,000 at 100 kv. The microscope was equipped with an external bias control to regulate the beam current.

OBSERVATIONS

Visual Observations

Examination of organisms incubated for periods of 1 to 10 minutes in media containing TNBT, buffer, and succinate showed visible reduction of the TNBT within 30 seconds. This reduction was seen as dark aggregates of organisms which slowly

TABLE I
Composition of Incubating Media for Studying Activity of SDH in E. coli

| Constituents | Fixation control | Experimental | Substrate control | Dye control | Competitive inhibition |
|--|------------------|--------------|-------------------|-------------|------------------------|
| 0.1 M Na ₂ HPO ₄ /KH ₂ PO ₄ at pH 7.4 (ml) | 5.00 | 3.75 | 5.00 | 3.75 | 3.75 |
| 0.80 M Na ₂ succinate (ml) | none | 1.25 | none | 1.25 | 1.25 |
| TNBT* (1 mg/ml) | none | 5 mg | 5 mg | none | 5 mg |
| Sodium malonate (mg) | none | none | none | none | 500 |

* TNBT is relatively insoluble in water. It is put into solution by the following: Take 100 ml of N,N-Dimethylformamide (DMF) and add 3 tbs. each of activated charcoal (30 gms) and 5 A molecular sieve 60 gm (Linde). Shake mixture vigorously until an aromatic (pleasant) aroma is obtained. Filter through a hard paper (S & S No. 576 paper). Use 0.1 ml DMF to dissolve 5 mg TNBT. If a precipitate forms after addition of buffer solution, filter (S & S No. 576 paper) before using (25).

The organisms were treated also with lysozyme according to the technique of Respaske (17) using the light microscope to follow the degree of lysis. The lysed organisms were then treated as indicated in Table II for periods of from 1 to 5 minutes.

Following incubation the organisms were concentrated by centrifugation and fixed in either of two ways: (a) 1 per cent osmium tetroxide buffered at pH 7.2 in 0.2 M Na₂HPO₄/KH₂PO₄ for periods of 5 to 30 minutes at 4°C or (b) 5 per cent glutaraldehyde at pH 6.1 with the acetate-Veronal buffer of Michaelis (18) at 4°C for 12 to 15 hours. 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 (19).

¹ Obtained from stock cultures of Dr. R. J. Mandel, Department of Microbiology, Jefferson Medical College.

settled and formed a brown-black pellet. Beyond a period of approximately 3 minutes there was no further change in color of the pellet. The bacteria treated with lysozyme presented similar results with respect to coloration of the formazan after incubation. On the other hand if sodium malonate was added to the incubation medium (before the TNBT was added) there was a marked decrease in visible reduction of the TNBT. During the procedures of washing, fixation, dehydration and embedding, there was no visible extraction of the reduced TNBT.

An experiment was performed to detect the possible diffusion of enzyme away from the organisms. TNBT was added to nutrient agar and poured into Petri dishes. *E. coli* were streaked on

the surface. The visible reduction of TNBT was evident only within the colonies.

Electron Microscopy

THE FINE STRUCTURE OF UNTREATED *E. COLI*

Micrographs of the sectioned organisms provided a fine structural pattern which was similar to that already reported in the literature. The organism is limited externally by a cell wall (*cw*, Fig. 1) that appears trilaminar in structure consisting of two dense layers separated by one of the lesser density (Fig. 2). The plasma membrane (*pm*) is found subjacent to the cell wall delineating the protoplast (Fig. 2), but it is not well defined in our preparations which have not been stained

TABLE II
Composition of Incubating Media for Studying Activity of SDH in Spheroplasts of *E. coli*

| Constituents | Experimental | Dye control |
|--|--------------|-------------|
| 0.2 M Na ₂ HPO ₄ /KH ₂ PO ₄ at pH 7.2 (ml) | 3.75 | 3.75 |
| 0.80 M Na ₂ succinate (ml) | 1.25 | 1.25 |
| TNBT (1 mg/ml) | 5 mg | none |

with lead hydroxide or uranyl acetate. The nuclear material (*n*) includes both fine fibrils (*f*) and clumped material (*c*) connected by filaments contained within an area exhibiting relatively low electron scattering properties (Figs. 1 and 2). The remaining portion of the protoplast is more dense and is composed of ribosomal granules, possibly larger polymetaphosphate granules (*pp*) (20) and light zones (*g*) presumably the locale of glycogen deposition (21).

THE FINE STRUCTURE OF *ESCHERICHIA COLI* EXPOSED TO TNBT

The activity of the SDH system is represented in the micrographs by deposits of the formazan of TNBT that exhibit electron scattering properties. These deposits (arrows) are contained within the bacterial profiles and follow the outer contour of the organism (Figs. 3, 4, and 9). Although the TNF deposits tend to be disposed in linear fashion, at the site of the plasma membrane, the deposits are spaced unequally. In individual

cells enzyme activity varies as demonstrated by the size of the TNF deposits (Figs. 3 and 4). Some dividing cells (Fig. 5) appeared to have more SDH activity when compared with non-dividing cells. In rare instances TNF deposits were seen within the nuclear area (Figs. 6 and 9).

In cases where the deposition of TNF was not heavy, it was possible to localize specifically a site of SDH activity on the plasma membrane (*x*, Fig. 4). In addition, in cells having undergone partial plasmolysis with retraction of the cell membrane from the cell wall, the TNF deposit (*t*, Fig. 7) is always found on the plasma membrane rather than on the cell wall. Under the experimental conditions used in this work fine deposition of TNF on the plasma membrane was found infrequently. However, when such deposition of reaction product is observed, the contrast of the outer members of the unit membrane is enhanced (*z*, Fig. 4). Generally the particle aggregates of the formazan were too large for precise localization on the plasma membrane. However, the TNF deposit is always in the locale of the plasma membrane subjacent to the cell wall.

In earlier observations (7) fine, extremely dense particles (20 to 100 Å) were seen on and within the cell wall of incubated organisms (Fig. 8). Generally they were dispersed uniformly on the free surface of the cell wall. Such deposits could lead to spurious localization of enzyme activity. To rule out this possibility, *E. coli*, lysed previously to alter cell wall structure, were incubated with TNBT (Table II). Micrographs of these preparations (Fig. 10) showed TNF deposits (arrows) associated with membranous elements of the disrupted organisms.

DISCUSSION

In general, the fine structure of *E. coli* reported in this work resembles that already found in the literature (13-16). However, our preparations showed obvious clumping of nuclear material and a poorly defined plasma membrane, in contrast with the fine nuclear filaments (30 Å) and clearly defined plasma membrane usually seen following the Ryter-Kellenberger (13) technique. These differences in results can be explained presumably on the basis of using a procedure for fixation at a pH of 7.2 rather than a pH 6.1 and the omission of heavy metal staining (uranyl acetate). Other factors such as the addition of calcium chloride, tryptone, and duration of fixation and staining

may contribute also to the variations in structural pattern of the organism. The neutral pH was selected for fixation in this report (except for the glutaraldehyde preparations) in order to maintain the pH under which the experimental procedures (culture, incubation for succinic dehydrogenase activity) were carried out. The supplementary staining with uranyl acetate was omitted in order to avoid enhanced membrane contrast which might lead to confusion with deposits of the TNBT-formazan marker. It was noted in some of the micrographs that large granules exhibiting some electron scattering properties were encountered in the peripheral cytoplasm of the bacterians (Fig. 1). These may represent polymetaphosphate granules (20) and showed less electron scattering than the TNBT-formazan marker. Areas presumably associated with glycogen location (21) demonstrated less electron scattering than either the TNBT-formazan or the polymetaphosphate granules.

The findings with direct cytochemical procedures to localize enzymatic activity in bacteria have been reviewed (1, 21, 22). Generally the reviewers are in accord that certain formazans derived from neotetrazolium, triphenyltetrazolium, and tetrazolium salts are limited in value because of their affinity for lipid. This characteristic property of the formazans led to false localization of enzymatic activity in bacteria by accumulating in large lipid bodies which were interpreted as mitochondrion-like elements. Weibull (4), using continuous microscopic observation, followed the sequential reduction of triphenyltetrazolium to its formazan during the incubation of *Bacillus megaterium*. He noted that formazan first accumulated peripherally and then coalesced to form larger secondary bodies located

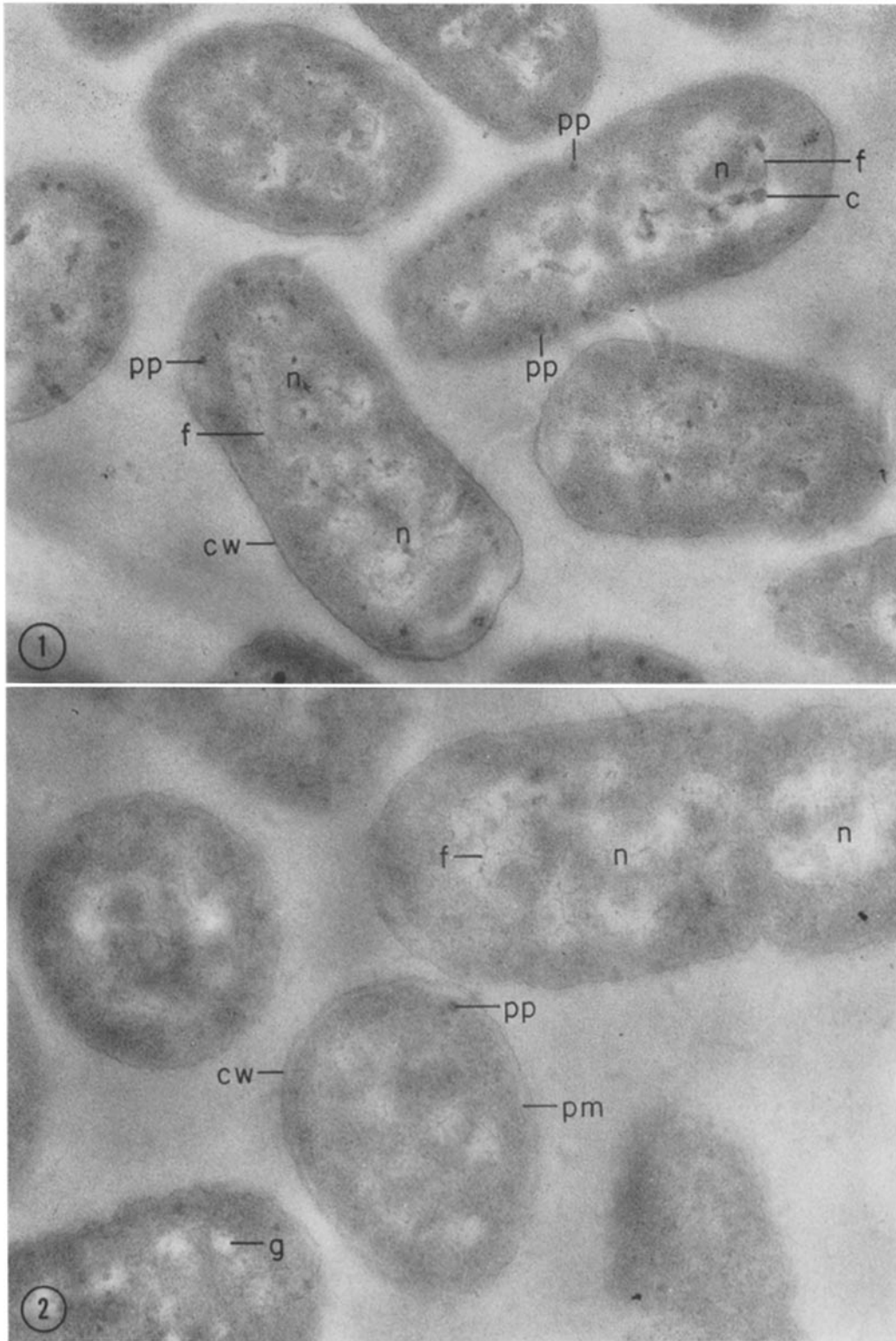
in the cytoplasm of the organism and so concluded that such dyes were unsuitable for localization of true sites of reduction in the cell. Attempts to use potassium tellurite as an electron acceptor to localize enzymatic activity gave results which were difficult to interpret. For example, Brieger (22), using human tubercle bacilli, found in electron micrographs of thin sections that tellurium needles were dispersed randomly in the cytoplasm. More recently, Vanderwinkel and Murray (8), employing triphenyltetrazolium to study localization of cytochrome oxidase in *E. coli* at the levels of both light and electron microscopy, showed deposition of the corresponding formazan in large deposits with no specific localization pattern. On the other hand these authors found localization of formazan near mesosome elements in *Bacillus subtilis* and *Spirillum serpens*. Kellenberger and Kellenberger (23) have made some interesting observations with the use of triphenyltetrazolium to localize oxidation-reduction sites in bacteria. They noted two types of reaction depending on the oxygen content of the medium, (a) the "specific reaction" which involved deposition of two to three large sites of formazan in the cytoplasm of organisms which were incubated in an oxygen-deficient medium and (b) the "aspecific reaction" which resulted in deposition of a variable number of smaller granules on the surface of the cell in organisms incubated in the presence of oxygen. These authors concluded that oxygen and the triphenyltetrazolium compete for electrons.

The results presented in this report demonstrate that a cytochemical technique previously found effective in mammalian tissues (9, 10) can be adapted for localization of enzymatic activity in *E. coli*. The data provide visual evidence that the activity of the SDH system is associated with a

All of the micrographs except Fig. 9 were obtained from preparations of *E. coli* fixed in 1 per cent osmium tetroxide buffered at pH 7.2 with phosphate buffer.

FIGURE 1 Several bacterial profiles are seen here illustrating the fine structure of the organism. These cells were not exposed to TNBT. The cell wall (*cw*) limits the organism externally. Nuclear material (*n*) is often clumped (*c*) and contains some fine filaments (*f*). Presumed polymetaphosphate granules (*pp*) are found in the peripheral cytoplasm. $\times 33,000$

FIGURE 2 A higher magnification micrograph of untreated organisms to show the location of the plasma membrane (*pm*). Other structures identified include cell wall (*cw*), nuclear material (*n*), nuclear filaments (*f*), presumed polymetaphosphate granules (*pp*), and light zones (*g*) representing glycogen. $\times 47,000$



specific structure of the bacterial anatomy, namely, the plasma membrane. In rare instances the reduction product, representing enzymatic activity, was seen to coincide with the outer membranes of the unit membrane structure of the plasma membrane. The pattern of enzymatic activity, as evidenced by formazan deposition, appears to be random. The amount of SDH activity, although variable, seems to be greater in dividing cells which may reflect a quantitative difference in enzymatic activity corresponding to the physiological state of the organism. The occasional finding of a formazan deposit within a nuclear region is difficult to explain although similar findings have been noted in mammalian cells (24).

The biochemical literature already has provided evidence that oxidative enzymes are associated with the Membran fraction of the bacterial cell (1). This fraction contains both cell wall and plasma membrane in addition to possible adsorbed contaminants. The combined cytochemical and electron microscopy techniques offer the obvious advantage in dealing with the whole organism.

The results presented here for localization of enzymatic activity in *E. coli* do not agree with the reports of Vanderwinkel and Murray (8) and Kellenberger and Kellenberger (23) who also used combined cytochemical and electron microscopy techniques. These workers used triphenyl-tetrazolium as an electron acceptor in their experiments. Weibull (4) among others has provided evidence that this dye has an affinity for lipid which could lead to false localization of enzymatic sites. TNBT, on the other hand, used in this report, is known to have little if any affinity for lipid (12, 25).

Future modification in experimental procedure could lead to greater accuracy in locating the

enzyme in studies such as this using combined techniques of cytochemistry and electron microscopy. The culture conditions used in our work were not optimum, *i.e.* oxygenated and controlled number of organisms per unit volume, for demonstrating maximum enzymatic activity. However, the sensitivity of the TNBT to reduction allowed for visual observation of reduced dye within 30 seconds. Other parameters might also play a role in the final representation of the reaction product such as osmolarity, temperature, TNBT concentration, as well as incubation conditions.

Part of this work was presented at the 2nd International Congress of Histochemistry and Cytochemistry, held in Frankfurt, Germany, August, 1964, (28).

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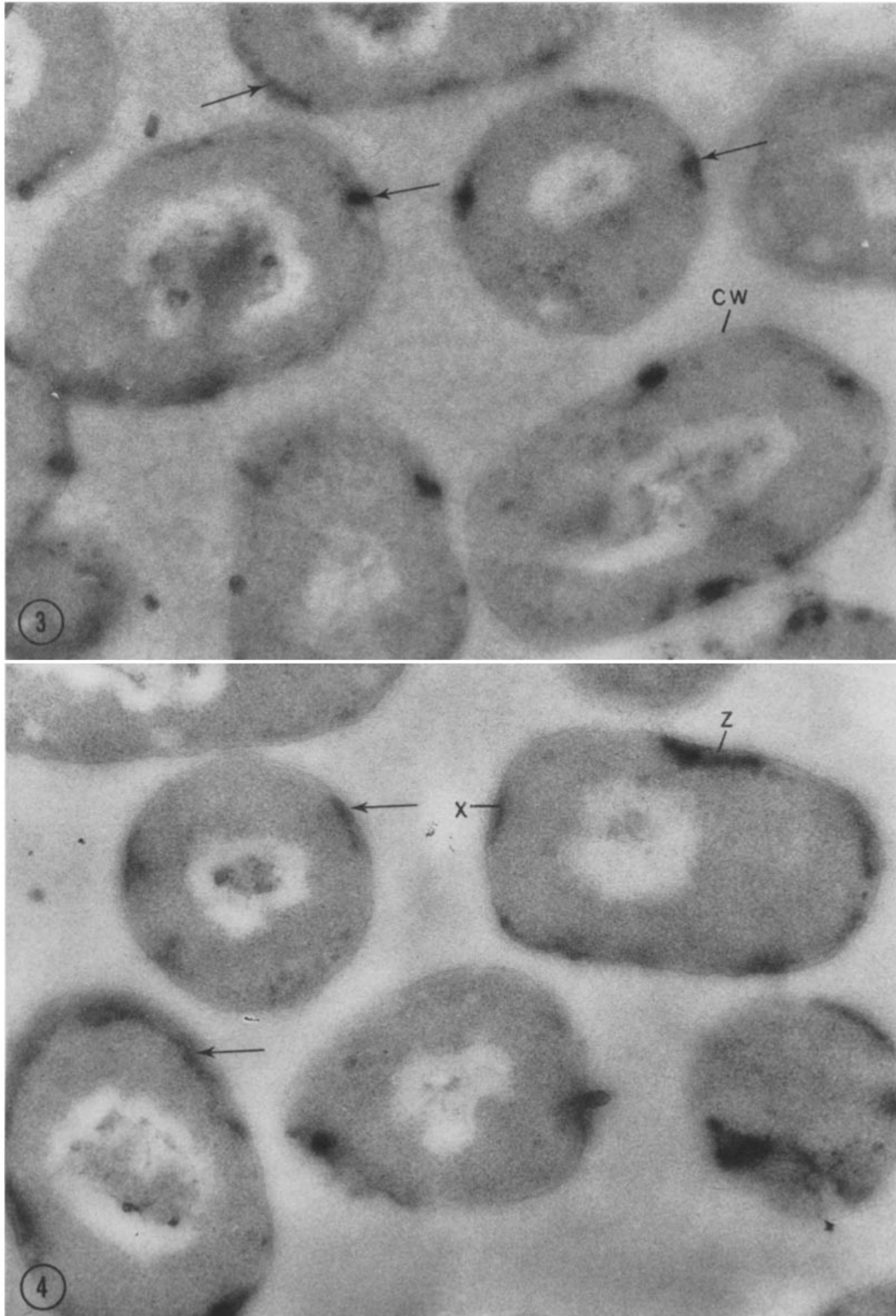
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ADDENDUM

After this paper was submitted for publication, two papers appeared in the literature that provided data on the use of potassium tellurite for localizing reductive sites in both Gram-positive and Gram-negative organisms (26, 27). These authors found in the case of *B. subtilis* a Gram-positive organism, that enzymatic activity of the respiratory system was associated with both membranes of "particular organelles" (chondrioids, mesosomes) and "rod-like elements" at the cell periphery; no obvious reduction product was found associated with the plasma membrane. Similarly in the case of *Proteus vulgaris*, a Gram-negative organism, the "reduced tellurite was not deposited on the plasma membrane to any important degree," but was found deposited in large clusters subjacent to the plasma membrane. These results differ from those previously reported by Bricger (22) who observed tellurium needles dispersed randomly in the cytoplasm of human tubercle bacilli.

FIGURE 3 Several bacterial profiles are illustrated here from a preparation incubated in the medium containing TNBT and succinate. Deposition of the formazan of TNBT (TNF) indicating activity of the succinic dehydrogenase system is seen (arrows) subjacent to the cell wall (*cw*); the deposits are spaced randomly and follow the outer contour of the organism. In most cases the size of the formazan aggregates is too large for precise localization of enzyme activity. $\times 47,000$

FIGURE 4 This micrograph shows that when deposition of the TNBT formazan (indicating activity of the succinic dehydrogenase system) is not excessive (*x*) more precise localization of enzymatic activity is possible. Here the activity is associated with plasma membrane. In some instances, the formazan enhances the contrast of the outer members of the unit membrane (*z*). Arrows in the figure indicate areas of TNF deposits. $\times 47,000$



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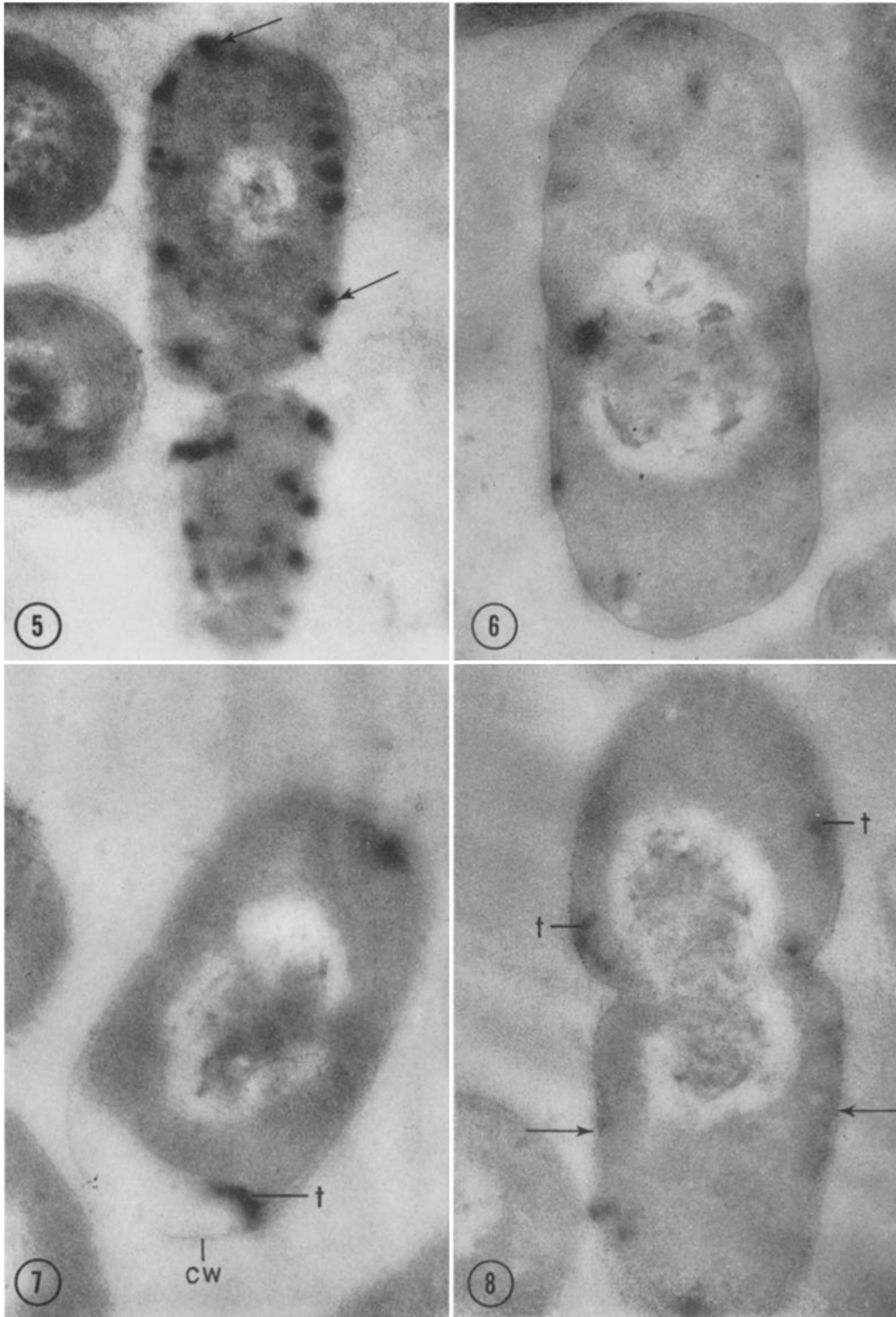
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FIGURE 5 A profile of a dividing cell is shown here. Formazan deposits (arrows), demonstrating activity of the succinic dehydrogenase system, are found in greater numbers than in most non-dividing cells. Here the size of the TNF aggregates precludes specific membrane localization of enzymatic activity. $\times 47,000$

FIGURE 6 This micrograph shows a rare example of TNF deposition within the nuclear area of *E. coli*. $\times 42,000$

FIGURE 7 An example of partial plasmolysis is shown in a profile of *E. coli* in this micrograph. It is evident that the TNF deposit (*t*), in the area of plasmolysis, is associated with site of the plasma membrane rather than cell wall (*cw*). $\times 47,000$

FIGURE 8 In this micrograph fine and extremely dense deposits are seen dispersed within the cell wall of *E. coli* (arrows). In addition TNF deposits (*t*) are found in the locale of the plasma membrane. The deposits on the cell wall could lead to false localization of enzymatic activity. $\times 47,000$



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FIGURE 9 Bacterial profiles are seen here from a preparation incubated in the medium containing TNBT and succinate. This material was fixed in 5 per cent glutaraldehyde in order to eliminate the contribution of osmium tetroxide to the density of the organisms. Although the fine structure of the bacteria is not well preserved here, the deposits of the formazan of TNBT (TNF) are obvious (arrows). These deposits follow the outer contour of the bacterial profile subjacent to the cell wall and are found also within nuclear material (*y*). $\times 29,000$

FIGURE 10 This preparation was obtained from *E. coli* treated with lysozyme to alter cell wall structure. Such specimens after incubation with TNBT and succinate in medium showed TNF deposits associated with membranous elements of the disrupted organisms (arrows). $\times 29,000$

