# A CYTOCHEMICAL LOCALIZATION OF REDUCTIVE SITES IN A GRAM-POSITIVE BACTERIUM

## Tellurite Reduction in *Bacillus subtilis*

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ABSTRACT

In bacteria the exact location of a respiratory enzyme system comparable to that of the mitochondria of other cells has remained uncertain. On the one hand, the existence of particulate "bacterial mitochondria" has been advocated (Mudd); on the other hand, important enzymes of the respiratory chain were recovered in the cytoplasmic membranes associated with some granular material (Weibull). In order to gain insight into this question, sites of reducing activity were localized in thin sections of bacteria using the reduction of potassium tellurite as an indicator. When this salt was added to the culture medium of *Bacillus subtilis*, it turned out that in this Gram-positive organism the reduced product is strictly bound at two sites, and that the plasma membrane does not materially gain in electron opacity through deposition of the reduced product. The reduction product is found on or in the membranes of particular organelles, which may possibly be regarded as the mitochondrial equivalents in Gram-positive bacteria, and which are sometimes seen connected to the plasma membrane. The second location is in thin rod-like elements at the cell periphery, possibly the sites from which the flagella emerge.

## INTRODUCTION

In a previous communication we described the occurrence, location, and structure of a particular organelle in *Bacillus subtilis* (2). Bacteriologists using the light microscope only occasionally drew attention to such adventitious bodies of a size close to the limit of resolution of their instruments (3). In electron microscopy, application of Ryter and Kellenberger's refined fixation technique for bacteria (4) enables similar structures composed of membranes to be detected in a number of bacterial species—although by no means in all. The most elaborate of such membrane systems have been found in the actinomycetes, namely, *Streptomyces coelicolor* (5, 6), *Nocardia* (7), and *Actinomyces* (8). The structures are common also in species of *Mycobacteria* (7, 9–12) and in the bacilli investigated so far (3, 13, 14); in addition, they have been observed, for instance, in *Corynebacterium* (7), in Gram-positive cocci like *Micrococcus* (15, 16) and *Staphylococcus* (17), and in *Lactobacillus* (17). Apparently all the organisms in which membranous structures have been discovered belong to the group of Gram-positive bacteria.

Although the structure of the organelles has been described in some detail, considerable uncertainty still exists, about their functions. There are a few indications: in dividing bacteria the organelles have been observed as so called peripheral bodies (2, 13, 15, 18, 19, 20) in intimate association with the developing cross-wall and,

therefore, it seems reasonable to suppose that they are engaged in the synthesis of cell wall material; furthermore, Fitz-James was tempted to deduce from his micrographs of spore-forming bacilli that the bodies would play some role in the formation of the spore membrane (13, 21). But this cannot be all: these organelles must be endowed with other functional possibilities, too, since their presence is not restricted to cells and to sites at which new walls or spores are being formed. In single sections the organelles can be seen to occur in the nuclear area, in the cytoplasm, or at the periphery of the cell.

The present investigation aims at gaining more information on the functions of these organelles which, in *Bacillus subtilis*, are referred to as "chondrioids" by Ryter and Kellenberger (4). It has long been known that micro-organisms possess respiratory enzyme systems comparable to those in animal or plant cells. But whereas it is understood that in the latter the systems are integrated in the mitochondria, their exact location in the bacterial cell remains a controversial question. Some authors, including Dietrich and Liebermeister (22), Bielig, Kausche, and Haardick (23), and, in particular, Mudd *et al.* (24–31), attempted to locate in the intact cell the sites of "oxidative-reductive" events (*i.e.* "analytical morphology" (32)) and identified these sites as cytoplasmic granules. Stimulated by the well known work of Weibull (33), the biochemical study of cell fractions (*i.e.* "direct cytochemistry" (32)) led to the opposing view that the plasma membrane is the main site of the respiratory enzymes. The latter opinion has recently been formulated characteristically by Hughes (34): "If analogies for bacterial structures are to be sought in the higher cells, it would seem more correct to regard the cytoplasmic membrane as equivalent to the 'cristae mitochondriales' of animal or plant mitochondria".

Because of the uncertainties inherent in the identification of cell fractions with components of the intact cell, we considered it desirable to work out first a procedure that would convincingly reveal the location of the respiratory enzymes in thin sections of the complete bacteria. Barrnett and Palade (35–37), who used small pieces of animal tissues, demonstrated that inside mitochondria small deposits were formed by reduction when potassium tellurite and a substrate were added to an incubation medium, or when the reagent was added intravitally. The phenomenon is explained as indirect acceptance of electrons from respiratory enzyme systems by the tellurite. The advantages of adopting this procedure for bacteria are that the tellurite can conveniently be added to the normal culture medium, that the precipitate of the reduced tellurite is much more opaque to electrons than organic matter, and that this precipitate may be expected to occur close to the site of its formation, since it is insoluble. Tetrazolium salts, more commonly used in analogous experiments, do not offer these advantages to the same extent that tellurite does,

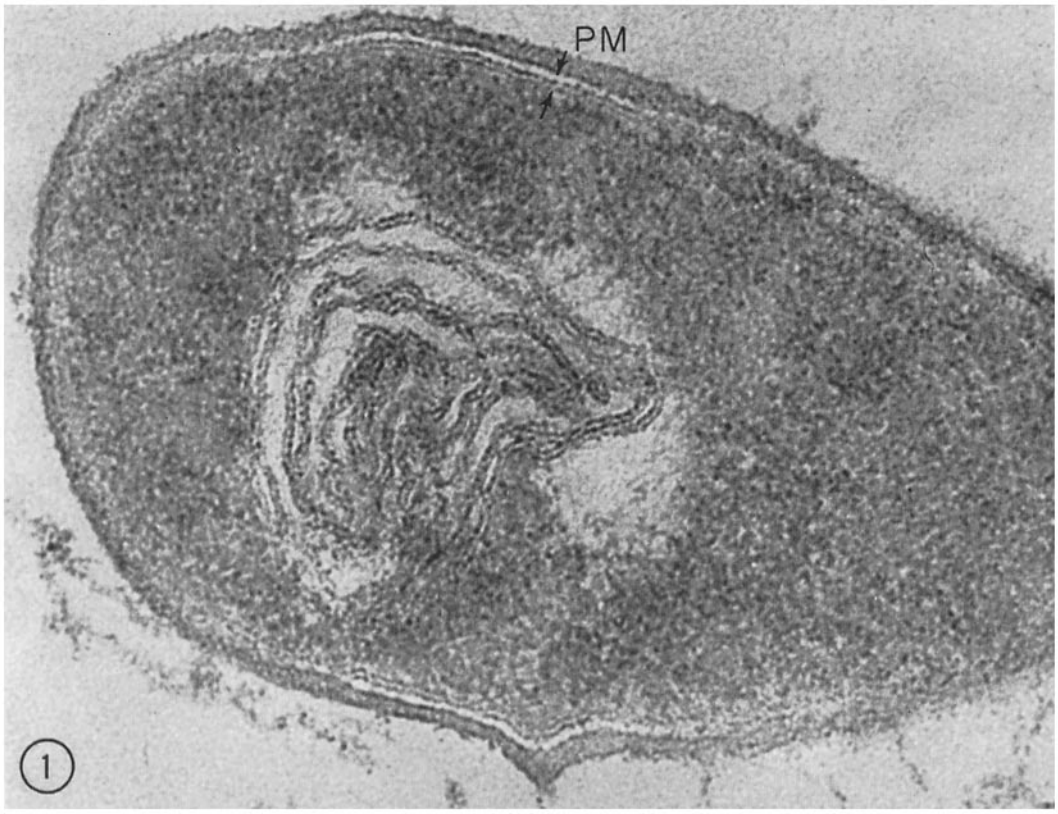
*Abbreviations for Figures*

*N*, nucleoplasm
*PM*, triple-layered plasma membrane
*Br*, "bridges" between cell wall and plasma membrane
*D*, dense line contiguous with the cell wall
*P*, peripheral body
*R*, rod-like structure

FIGURES 1 and 2 Control cells of *Bacillus subtilis* grown in normal cultures without potassium tellurite. In contradistinction to the cells treated with tellurite, in these cells the density of the layers composing the organellar membranes is sufficiently comparable to that of the plasma membrane (*PM*). In both Figs. 1 and 2 the contrast is somewhat enhanced by underfocusing. × 158,000.

FIGURE 1 In the centre of the cell is an organelle consisting of a whorl of unit membranes.

FIGURE 2 A tubulo-vesicular organelle in contact with the fibrillar nucleoplasm (*N*). The organelle is surrounded by a single unit membrane, and its elements frequently show a border of one dense line only. The plasma membrane after retraction may have left the dense line (*D*) and "bridges" (*Br*).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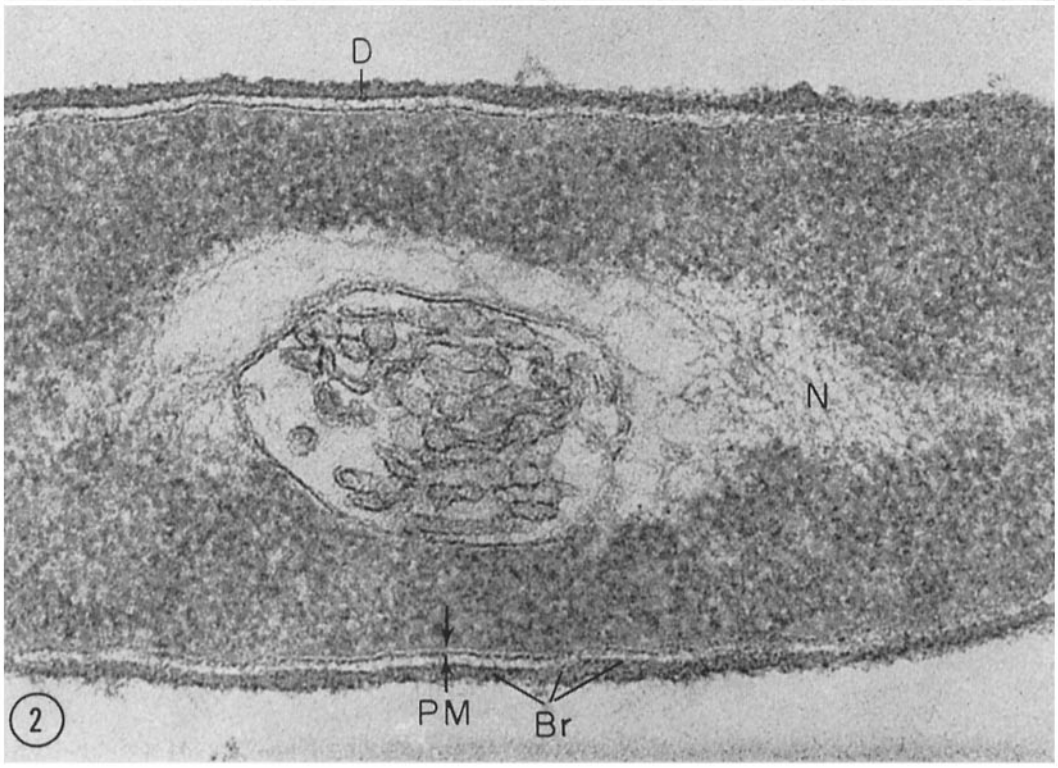
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and, therefore, we concentrated on the use of the latter.

The sites of deposition of the reduced tellurite in a Gram-negative bacterium (*Proteus vulgaris*) forms the subject of a subsequent communication (38).

## MATERIALS AND METHODS

*Bacillus subtilis* strain Marburg, grown in Difco heart infusion broth at 37°C with vigorous agitation, was used in the early logarithmic phase (4 hours). Potassium tellurite was added to a final concentration of 0.05 per cent. Since Kellenberger (39) has found that the reduction of tetrazolium salts in bacteria and their uptake of oxygen are competitive, and as we considered the possibility that this could also be the case with tellurite reduction, we stopped the aeration of the cultures after addition of the tellurite salt. The cultures were kept in the incubator for another hour and then the bacteria were spun down.

The precipitated cells were either suspended in distilled water for direct examination or suspended in the fixative after which they were left undisturbed overnight at room temperature. For the direct examination the suspension was pipetted onto Formvar-coated grids, dried in air, and the residue was shadowed with platinum. The fixation procedure and posttreatment with uranyl acetate of Ryter and Kellenberger (4) were applied in most experiments. In order to avoid the introduction of other heavy metals apart from tellurium, in one experiment 6½ per cent glutaraldehyde (40) was used instead of $OsO_4$, and in some cases the uranyl acetate treatment was omitted. All fixed material was embedded in Vestopal W.

Thin sections were made on an LKB Ultrotome.

The thickness of the various sections was estimated qualitatively in the electron microscope. This was done by taking the supporting film as the reference and by measuring the decrease in amount of electrons passing the fluorescent screen, when the sections were moved into the beam, with a Philips D.E. microvoltmeter G.M. 6020.

Micrographs were taken with a Philips EM 200, using the double condenser lens system and an objective aperture of 25 $\mu$.

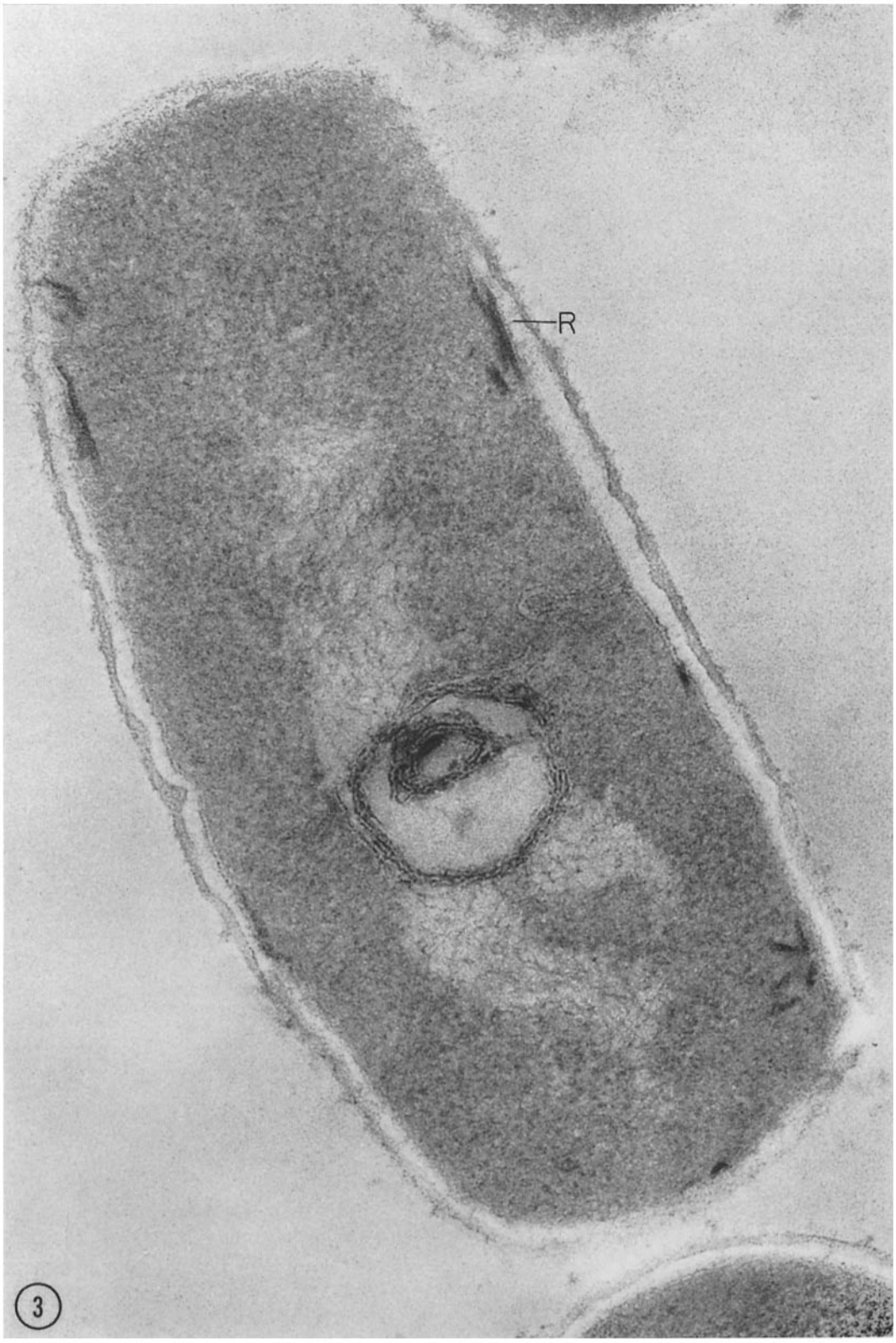
## OBSERVATIONS

After the colourless solution of potassium tellurite had been added, the cultures of *Bacillus subtilis* gradually turned greyish black. The cell centrifugate of these cultures was black, in contradistinction to that of the controls; in both cases the supernatant was clear. When the precipitate of the treated cells was resuspended in distilled water and pipetted in drops onto the supporting film, no reduced tellurite was seen outside the cells in the electron microscope. Therefore, it may be assumed that the bulk of the reduced tellurite is contained within the cells.

In whole cells (Fig. 13) the products of this reduction are visible as thin elongated elements of considerably greater density than the remainder of the cells. The details of the areas in which the reduced product accumulates can be studied to better advantage in thin sections of embedded bacilli.

Comparison of Figs. 3 and 4 of treated cells with Figs. 1 and 2 of control cells clearly reveals the sites of reduced tellurite deposition: in the cell centre some organellar structures (chondrioids) and at the periphery some smaller structures appear conspicuously dense. However, in comparing different cells (Fig. 1 with Fig. 3, and Fig. 2 with Fig. 4), it should be borne in mind that the densities of the various structures are also determined by the section thickness and the deviation from focus of the exposures. The thickness of all four of the sections is sufficiently comparable (see Material and Methods), but the contrast of the membranes in Figs. 1 and 2 is enhanced by a slight underfocus of the exposures, whereas Figs. 3 and 4 were taken close to focus. The best way of appreciating the influence of the tellurite is by comparing in the same cell the density of the membranes of the chondrioids with that of the plasma membrane. In the controls these densities appear comparable (Figs. 1 and 2), whereas in the treated cells the density of the membranes of the chon-

FIGURE 3 Cell grown for an extra hour with potassium tellurite in the medium. The reduced tellurite appears deposited in, or on the membranes of the central organelle, and in slender rod-like structures at the cell periphery. The latter structures have not been noticed before in the untreated cells. In the rod at *R* there is a suggestion of structural details, but it should be realized that this may be influenced by overlapping cytoplasmic structures. × 158,000.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drioids exceeds that of the plasma membrane (Figs. 3 and 4).

Intracellular deposits of reduced tellurite were never encountered in sites other than in the chondrioids and in the small structures at the cell periphery, nor was there a perceptible increase in contrast of the cytoplasm. This observation appears of importance because of the expectation that the deposits would occur close to the site of the reduction. In the electron micrographs the reduction product appears situated exclusively on or in the membranes and in special peripheral elements.

At the periphery of the cells in Figs. 3 and 4, opaque elements appear in a typical configuration: short rods are either apposed to the plasma membrane or situated a short distance away from it. At high magnification a delicate substructure in the rods can be discerned, but the possibility should not be overlooked that this is caused by superposition of the cytoplasmic fine structure. The rods seem to be lighter in the centre and are usually bordered by dense lines, and their greatest width seems to approach 80 A. Rarely are they seen to pierce the plasma membrane. We wonder whether such filamentous structures could be the bases of the flagella. This supposition is substantiated by Fig. 13 of a shadowed preparation, which suggests that the flagella have their origin in the dense rods.

The peripheral configuration of dense elements continues along developing cross-walls (Figs. 5 and 6). There could be two reasons for their presence at these sites: first, genesis of new flagella; secondly, formation of the new wall. In Fig. 7 at *P*, a dense structure can be seen which represents a so called peripheral body, possibly connected with the formation of the new cross-wall (2). This peripheral body is different from the rod-shaped elements and is in fact a membranous organelle.

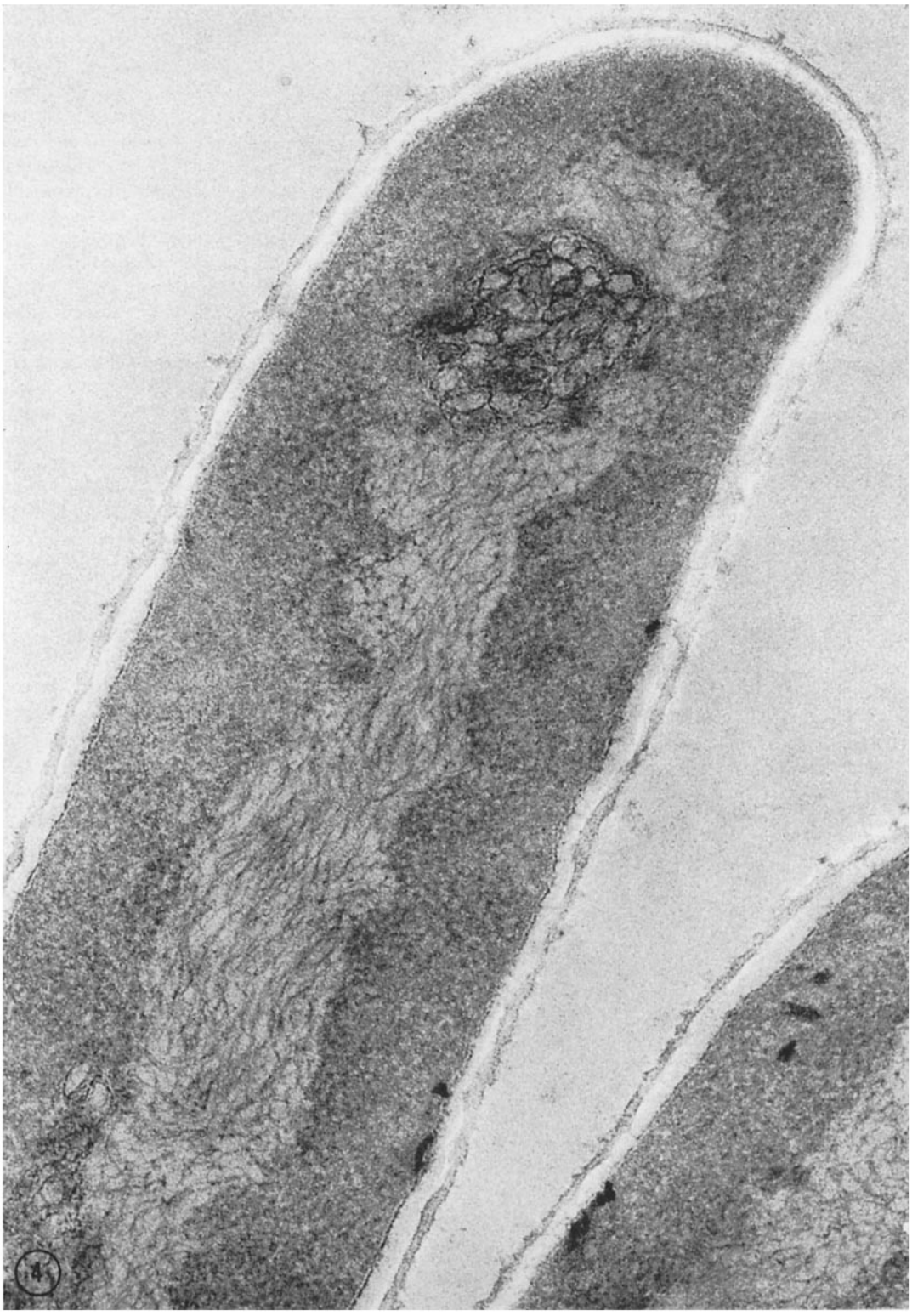
As previously described (2, 15, 1), membranous organelles can appear either as a body of more or less concentric membranes (Figs. 1 and 3) or as a cluster of vesicles and tubules (Figs. 2 and 4) or as combinations of the two types (Fig. 11). The membranes of the first type of chondrioid show a substructure of two dense borders with a width close to 25 A separated by a more transparent intermediary zone. Such a "unit membrane" (41) has a total width of *ca.* 80 A. The vesiculo-tubular chondrioids are composed of elements with very delicate borders often possessing only one dense layer of the unit membrane. The morphogenetic relationship between the membranes of vesiculo-tubular elements and concentric unit-membranes remains an intriguing problem.

It might be suggested that the dense peripheral rod-shaped elements represent the sites of origin of new chondrioids functioning in cross-wall formation. This supposition is contradicted by Fig. 8 which represents the first of a series of sections from part of an organelle. Short dense rods are seen next to the lowest part of a bent tube (arrow) of a chondrioid (Figs. 8 to 11). The series shows the connection of the chondrioid with the cell envelope. In the original print of Fig. 10 the boundary of the chondrioid can be distinguished as the continuation of the plasma membrane.

The interpretation of the limits of the plasma membrane is a controversial question (*cf.* 1). In our opinion, when Gram-positive bacteria are most satisfactorily fixed the plasma membrane appears strictly adherent to the cell wall (*cf.* 15, 2, 1). However, when the cytoplast retracts from the cell wall it may carry most of the plasma membrane with it, leaving only small remnants attached to the wall (Figs. 2 and 10, *PM*). This would explain the frequent presence of a dense line on the inner side of the cell wall and the "bridges" (17) crossing the space between the plasma membrane and the cell wall (Fig. 2, *Br*).

In Fig. 10 the space caused by the retraction of the plasma membrane from the cell wall appears continuous with the space containing the vesicles and tubules of the chondrioid. Within this continuous space the bent tube linking the upper part of the chondrioid to the cell envelope seems to be directly connected to the cell wall (arrow). Therefore, it is suggested that there is direct access from the environment through the cell wall to the interior of the vesicles and tubules of the chondrioid without need to pass the cytoplasm. As observed in several other instances, the density of

FIGURE 4 Tellurite-treated cell. As in Fig. 3, the density of the organellar structures is higher than that of the plasma membrane, owing to the deposition of the reduced product. × 158,000.



most of the membranes of this chondrioid, with the exception of a part of the structures in Fig. 11 (arrow), has not been markedly increased by the tellurite, which perhaps indicates an uneven distribution of the reducing activity along the membranous surfaces. The plasma membrane itself does not visibly increase in opacity through deposition of tellurium compounds.

In order to obtain electron micrographs in which the various density gradations reflect the natural mass distribution free from heavy metals introduced during the $OsO_4$ fixation or the post-treatment with uranyl acetate, specimens were merely fixed in glutaraldehyde. Fig. 12 convincingly reveals the deposits of the reduced tellurite in the cell centre and at the periphery, but the ultrastructural arrangement is disturbed during fixation.

## DISCUSSION

Although, in general, soluble substances entering the bacilli will have to pass the envelope of the cytoplast, in our experiments the plasma membrane did not appreciably gain in electron opacity through uptake of potassium tellurite. Therefore, the plasma membrane will not contain an important quantity of the enzymes causing the reduction of the tellurite. As Barrnett and Palade (35) have made plausible, the dehydrogenase system responsible for this reduction in animal cells is part of the respiratory enzyme chain.[1] Consequently, our results do not support the concept that the plasma membrane would be the main site of the respiratory enzyme chain. In our experiments, reducing activity was found at two typical sites: in the membranes of particular organelles and in thin elements at the periphery of the cell.

Weibull *et al.* recovered important enzymes of the respiratory chain in a "ghost fraction" of *Bacillus megaterium* strain M which, with phase contrast and electron microscopy, was observed to contain the cytoplasmic membranes associated with some granular material (33, 42, 43). In the literature much discussion still goes on about the question of whether the enzymes are located in the lipoprotein plasma membrane or in particulate matter (*cf.* 32, 44–46). In most publications there is no clear correlation between the particulate matter described and the structure of the complete bacterium.

With the aid of tetrazolium compounds in particular, but also with the Nadi reagent, Janus Green B, and potassium tellurite, Mudd *et al.* (24–31) described circumscribed local sites where oxidative-reductive events were found to take place. Weibull (47) pointed out for light microscopy that vital staining of bacteria with tetrazolium cannot be relied upon to reveal the intracellular localization of centres of reducing activity; on the electron microscopic level, there is even less certainty that the formazan is at the site of its formation. According to our experience, when potassium tellurite was substituted by nitro-blue tetrazolium,[2] the red formazan was in part extracted by the acetone used in the dehydration procedure (*cf.* also VanderWinkel and Murray, 48). A critical account of the applicability of tetrazolium salts in solving problems of reductive

[1] So far, the authors have not yet succeeded in proving the connection between tellurite reduction and the activity of the oxidative enzymes in *Bacillus subtilis* by exhaustion of the pool of endogenous substrate and subsequent addition of suitable substrate.

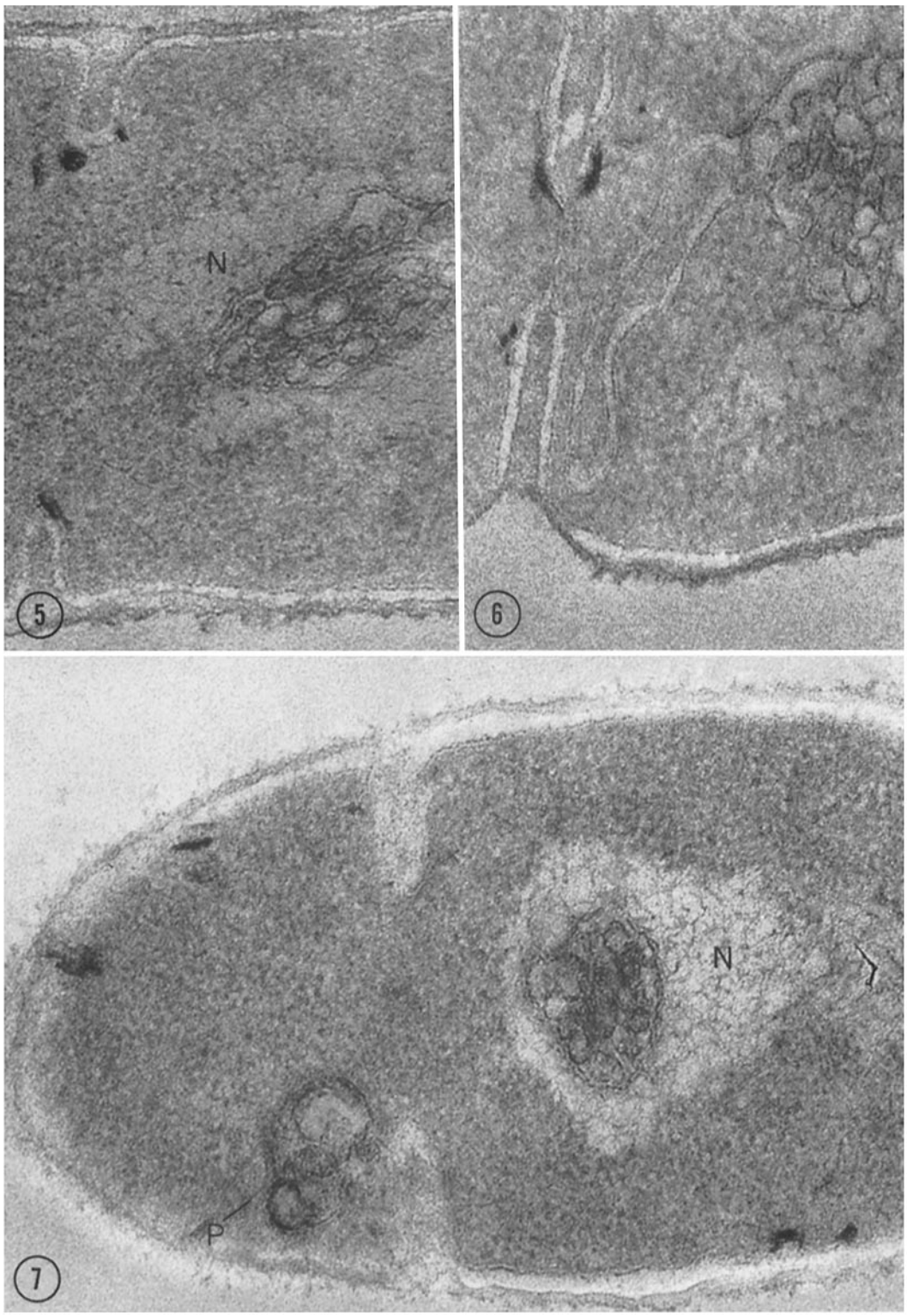
[2] Obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

FIGURES 5 to 7 Examples of cells treated with tellurite. The bacilli are preparing for division. × 158,000.

FIGURE 5 Dense elements close to the plasma membrane bordering the new septum, and at some distance a tubulo-vesicular organelle in the nucleoplasm.

FIGURE 6 In contrast to the rod-like structure near the new septum and the vesiculated part of the organelle, the membranes meandering towards the site of ingrowth of the new septum have not importantly gained in electron opacity owing to deposition of reduced tellurite.

FIGURE 7 At *P* is a peripheral body of the new cross-wall darkened by reduced tellurite. To the right in the nucleoplasm is a vesiculo-tubular organelle which incorporated reduced tellurite.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enzyme location has been given by Novikoff (49) and others. Recently, however, new tetrazolium compounds were introduced with which formazan staining of the cristae mitochondriales in true mitochondria was unequivocally obtained (50–53). Notwithstanding the drawbacks inherent in the use of the older tetrazolium compounds, it is obvious that the primary sites at which formazan first becomes visible must reflect the site of the reduction. Comparing in the light microscope the reduction of triphenyl tetrazolium with that of tellurite in *B. subtilis*, we gained the impression that the sites of the deposits in the two cases corresponded fairly well. Therefore, we believe that, in principle, the particular cytoplasmic granules discussed by Mudd correspond with the chondrioids illustrated in this study. Moreover, Fitz-James (13) demonstrated with Janus Green B that the stained granules were identical with the membranous bodies in electron micrographs of bacilli, and Begerson (54) adduced evidence that granules in *B. megaterium* that can be stained with Janus Green B and Nadi reagent were related to "growing points" (55), which, in turn, often seem to correspond in site with the peripheral bodies visible by electron microscopy. Recently Vander-Winkel and Murray (48), studying in thin sections the effect of 2, 3, 5-triphenyl tetrazolium applied to the living bacteria, observed, in *B. subtilis*, "white spaces," in all likelihood caused by dissolution of the formazan. The authors conclude that the "mesosomes" are the site of the oxidative-reductive system of the cell.

The term "mesosome" was recently introduced by Fitz-James (13) to denote the membranous structures described here. It is based on Robertson's suggestion (41) for electron microscopy "that 'meso' is either a double membrane, leading from some included structure to the outside" or might be used for "cytoplasmic structures connected to the outside by a double membrane". Figs. 8 to 11 indeed confirm that the membranous structures presently in question are wrapped in a unit membrane continuous with the plasma membrane, but information from complete serial sections as to whether all the membranous organelles are always in open connection with the external environment is still lacking. The finding itself is of interest because of the creation of a separate enclosed system within the cytoplasm and nucleoplasm, allowing the secluded passage of solutes (*cf.* discussion on endoplasmic reticulum, *e.g.*, 56). But it remains open to discussion whether for the present organelles a term should be coined that is based on a morphological feature, or whether the proposed name should qualify their function. The above data make it seem likely that the main function of the membranous bodies may be comparable to that of the mitochondria in other cells. Moreover, modern work of Hess and Dietrich (57) on formazan production in *B. megaterium* was interpreted to suggest "that cytoplasmic constituents of bacterial cells form part of highly organized enzyme units related to particulate mitochondrial fractions." All these facts taken together make it seem desirable to denote these organelles by a term

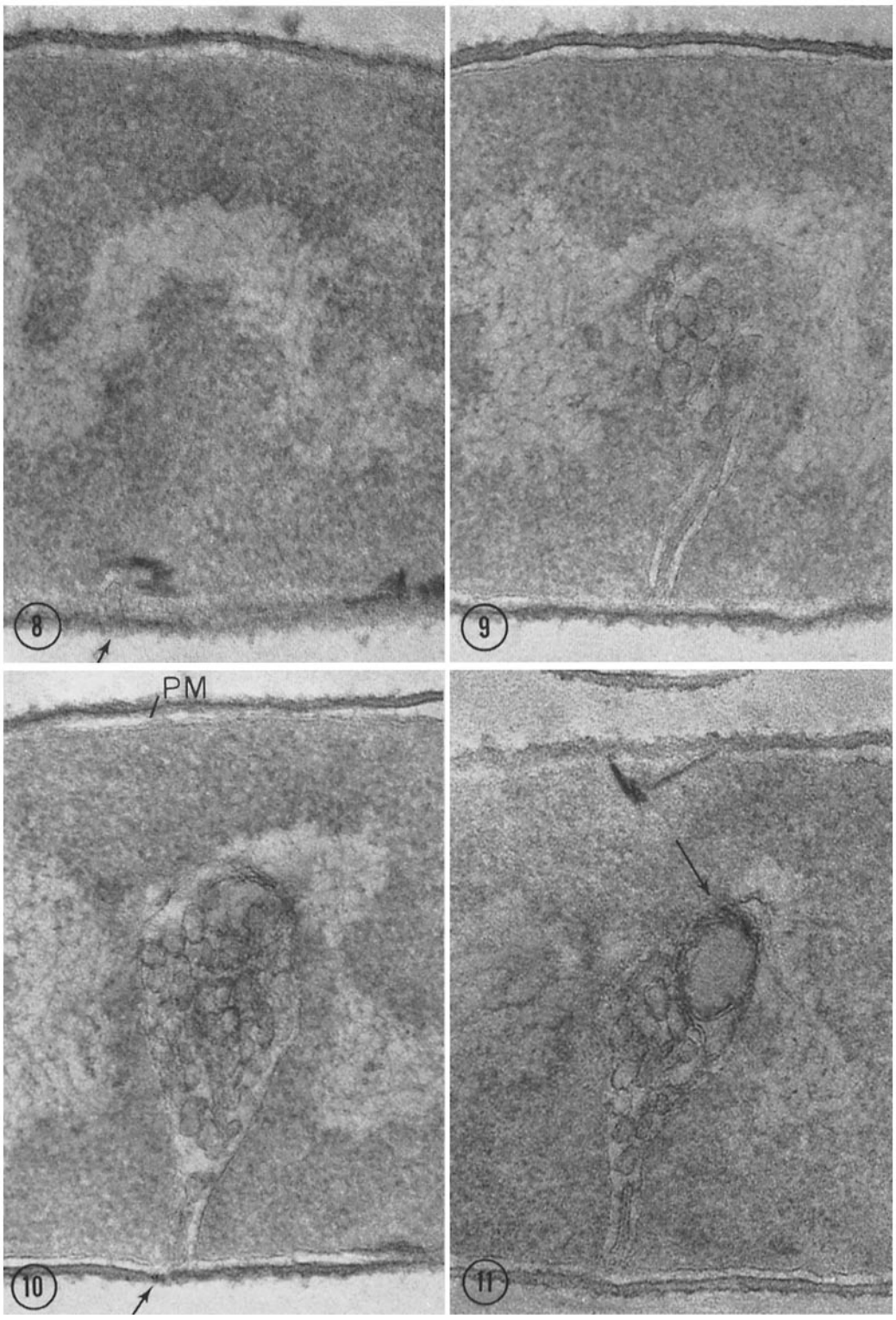
FIGURES 8 to 11 Series of four sections through an organellar structure showing its connection to the cell envelope. × 126,000.

FIGURE 8 At the arrow, to the left of the dense rod-like structure, the lower part of the tube connecting the organelle with the cell envelope.

FIGURE 9 The vesiculo-tubular structures in the cell centre are connected to the cell periphery by a tube.

FIGURE 10 In the original print it can be observed that the organelle is enveloped by a continuation of the plasma membrane, and furthermore, at the arrow, that the organellar tube within the lower space of the organelle protrudes towards the cell wall through the empty space between the cell wall and the plasma membrane, caused by retraction of the latter. The empty-looking space inside the organelle and the space between the plasma membrane and the cell wall are in this way interconnected.

FIGURE 11 The organelle here appears to be a combination of vesicles and concentric membranes. Concentric membranes in the upper part of the organelle blackened by reduced tellurite.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suggesting a mitochondrial function, for which "mitochondrial equivalents" and "chondrioids" (Kellenberger, 58) appear quite satisfactory.

Some authors have already identified the organelles with mitochondria: Chapman, Hanks, and Wallace (59), Drews (9), and Giesbrecht (14) on morphological grounds, and Shinohara *et al.* (10) on physiological grounds also. This appears premature, however, because for morphological identity it should be demonstrated that the organelles are built according to the same pattern and principle as true mitochondria, which appears to be based on double membranes, *i.e.* two unit membranes. The membranous organelles are surrounded by one unit membrane only, and the inside structures are unit membranes or are bordered by an even thinner layer. For physiological identity it must be ascertained biochemically that the pathways of oxidative phosphorylation are similar in both cases.

In addition to the chondrioids in *Bacillus subtilis*, another type of structure shows strong reductive activity: these are thin and rod-shaped structures found not far from the plasma membrane. Possibly these structures function as the cytoplasmic bases of the flagella. However, there is no conclusive proof that these thin rods consist of reduced tellurite on, or in, organic filaments and do not represent completely inorganic material.

The experiments with potassium tellurite seem to suggest that the cytoplasmic membrane may not play an important role in the respiratory chain but may give rise to the membranous organelles functioning as mitochondrial equivalents.

As will be discussed in the following communication (38), the sites of reductive events in the Gram-negative *Proteus vulgaris* are different from the two types of structures illustrated here.

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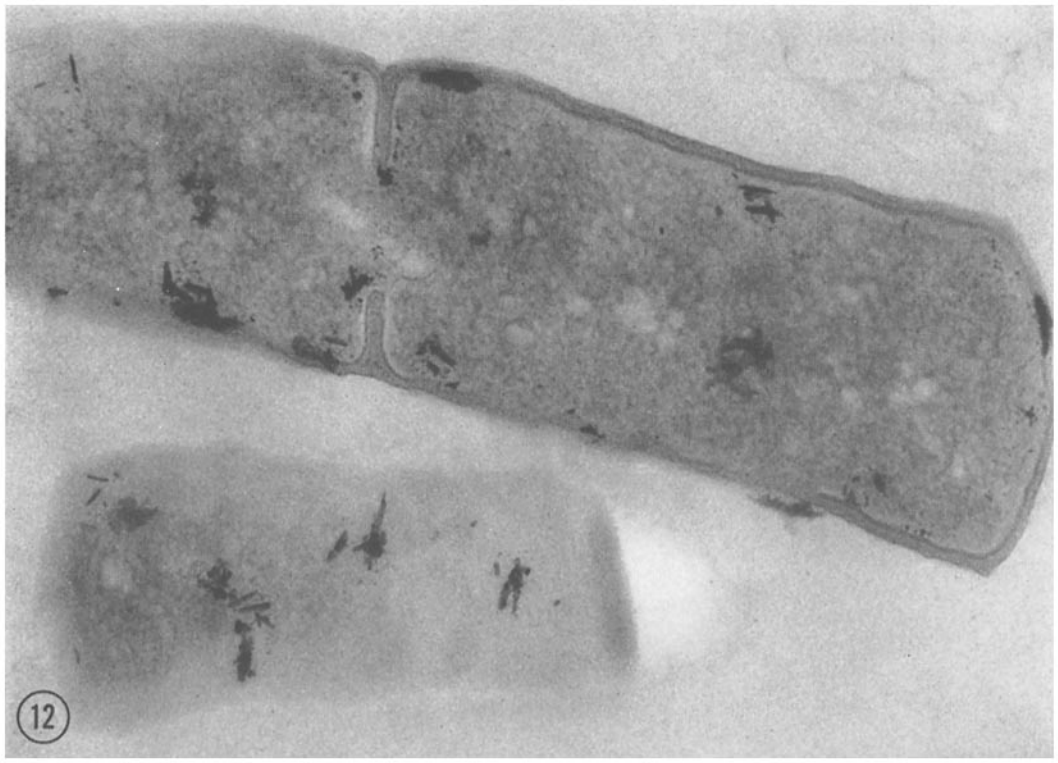
FIGURE 12 In order to give an unequivocal proof of the presence of reduced tellurite inside the *Bacillus subtilis*, introduction of heavy metals during the preparation (uranyl and osmium) have been avoided here. The structural details have not been well preserved but the distribution of the densities is as natural as could possibly be obtained. It can be inferred from this rather thick section that the reduced product is present in the cell centre, at the periphery, and along the new septum. In the lower portion of the picture is a section of another cell cut close to its surface. × 100,000.

FIGURE 13 Shadowed whole cell treated with tellurite, and printed in reverse. A flattened, rather empty cell was chosen in order to demonstrate best the opaque accumulations of the reduced tellurite. At the arrows there are indications that the flagella could emerge from these dense structures. × 40,000.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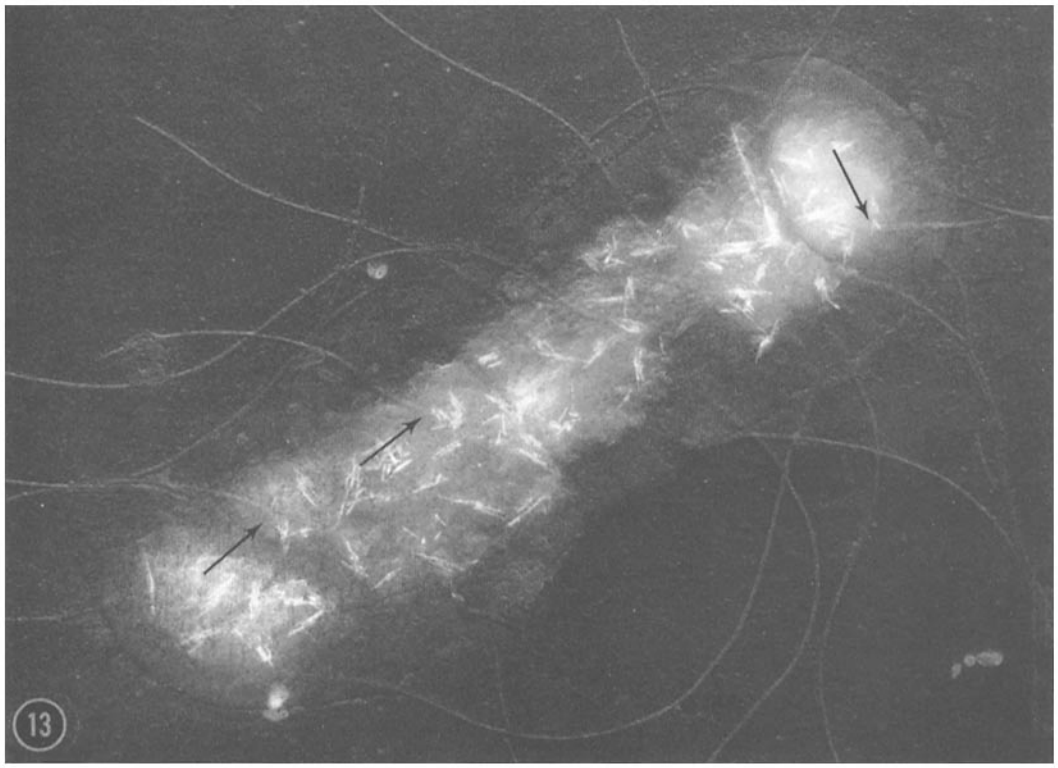
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