TETRANITRO-BLUE TETRAZOLIUM REDUCTION IN *BACILLUS SUBTILIS*

W. LEENE and WOUTERA VAN ITERSON. From the Laboratory of Electron Microscopy, University of Amsterdam, the Netherlands. Mr. Leene's present address is the Laboratory of Histology, Department of Electron Microscopy, Free University, Amsterdam, The Netherlands

In order to decide whether in the bacterial cell the enzymes of the respiratory chain are located in "particulate elements" (1) or in the plasma membrane (2) we investigated the reduction of potassium tellurite in Gram-positive and Gram-negative bacteria. For the first time in 1962 (3), and in more detail in 1964 (4), we reported that with *Bacillus subtilis* we had found that reduction products of tellurite are deposited in or on the membranes of the particular organelles previously described by one of us (5, 6), and called mesosomes by Fitz-James (7). This localization of the reduced tellurite seemed to indicate that in this Gram-positive bacterium the membranous organelles represent the bacterial equivalents of the mitochondria of higher cells. For this reason we now use the term chondrioids (8) to indicate discrete elements possessing reductive activity.

VanderWinkel and Murray in 1962 (9), using 2,3,5-triphenyl tetrazolium, found in *Bacillus subtilis* "white spaces" near mesosomes, probably caused by dissolution of the formazan formed, and concluded that the mesosomes would be the sites of the oxidative-reductive system of the cell. Because it is unsatisfactory that the formazan itself was not visualized, it was deemed desirable to reexamine, with the help of one of the newer tetrazolium compounds, the contention of the mitochondrial nature of the membranous structure in the cell.

At present few attempts have been made to define precisely the step in the respiratory chain that leads directly or indirectly to tellurite reduction. Of old, the reduction of tetrazolium has been considered specific for mitochondrial function. The newer nitro-blue tetrazolium and tetranitro-blue tetrazolium compounds appear to accept electrons directly from the respiratory chain: according to Novikoff (10), possibly at a step before coenzyme Q and cytochrome b. The use of tetranitro-blue tetrazolium (TNBT) would have certain advantages over that of nitro-blue tetrazolium (NBT); it would suffer, for instance, less of the non-specific affinity of the diformazan for lipid (11, 12). With the TNBT method, deposits of diformazan under the electron microscope were seen to occur in or on the cristae mitochondriales of the cells of various tissues (13–15). It seemed important to make use of the new compounds in a renewed search for the sites of enzymes of the respiratory chain in bacteria (16).

MATERIALS AND METHODS

Bacillus subtilis strain Marburg was cultivated under vigorous agitation in Difco heart infusion broth. In the early logarithmic phase the cells were spun down and resuspended in a solution of 0.05 per cent TNBT and 0.1 M sodium succinate in a Sörensen buffer (pH 7.4). The commercial TNBT from Sigma Chemical Company, St. Louis, Missouri, was washed first with ethyl acetate in order to separate it from possibly present monotetrazolium. During the experiment the cultures were kept at room temperature under anaerobic conditions by bubbling nitrogen gas through the tubes. Under the light microscope the blue granules of diformazan became visible after 10 minutes; after incubation for this length of time, therefore, the cells treated with TNBT were spun down and resuspended in the fixative. The fixation, posttreatment with uranyl acetate, and embedding in Vestopal W were carried out according to the

descriptions of Ryter and Kellenberger (17). The control cells were treated similarly, the only difference being that the sodium succinate was left out of the TNBT solution in the Sörensen buffer.

Thin sections were cut on LKB ultrotomes with glass knives, and the electron micrographs were made with a Philips EM 200, using the double condenser lens system and an objective aperture of 25 μ. When making the exposures in the electron microscope, the intensity of the electrons at the site of the photographic material was kept constant.

RESULTS AND COMMENT

The cells in Figs. 1 and 2 were treated for 10 minutes with TNBT in the presence of succinate as substrate. The cells definitely changed in appearance as compared to the *B. subtilis* cells fixed immediately from normal broth culture (3, 4, 6). The cytoplasm in the TNBT-treated cells shows considerable loss in contrast and in fine structural detail as compared with cells from broth cultures. In the normal cells (4, 6) the cytoplasm appears to consist of a mosaic of granules. In the treated cells (Figs. 1 and 2) such granules are very often difficult to discern. Unlike the cytoplasm, the cell wall appears to have preserved its normal electron opacity. Inside the cytoplasm and contrasting with it are two types of structures: membranous organelles (*Ch*) and small scattered particles (*P*). The distinct nuclear areas which, after Ryter-Kellenberger fixation, possess delicate fibrils in various configurations are often missing in our TNBT-treated cells. Another striking fact is that it is nearly always impossible to distinguish the triple-layered plasma membrane. On the other hand, as mentioned above, the involutions of the plasma membrane, the membranous organelles, are quite distinct. These membranous structures are seen to possess electron-opaque thin sheets (*S*) of *ca.* 20 A width, and inbetween these sheets appears minutely granular material (*M*) of somewhat higher electron opacity than the main cytoplasm. Normally the plasma membrane and the sheets which form the membranous organelles are of about equal density (4). That is not so in the micrographs obtained in the present work. In these the plasma membrane is indistinct and only the membranous organelles are clearly visible.

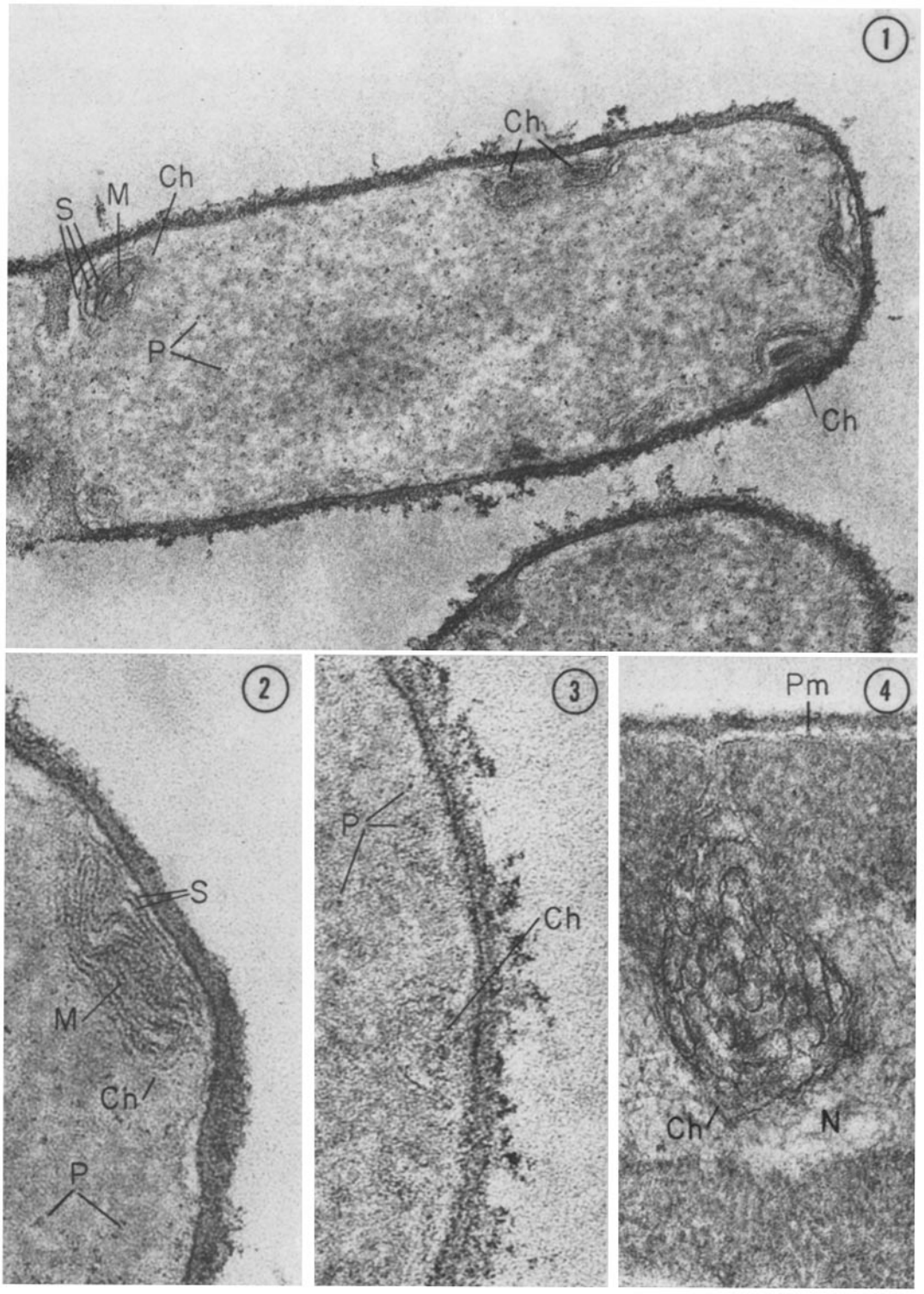
Fig. 3 is from a control treated with TNBT in the absence of succinate substrate. Control cells resemble cells exposed to TNBT and substrate in the normal appearance of their walls and of their cytoplasm, and in the indistinctness of the plasma membrane and the absence of normal fine structure. It proved difficult to find membranous organelles in the control cells, but suggestive arrays of a few dense sheets were seen here and there.

Fig. 4 shows an area of a cell treated with potassium tellurite in the normal broth as described previously (4). In this preparation the cell appears to have preserved its normal nuclear structure (*N*), and the cytoplasm possesses its usual fine structure and shows normal gradations in electron opacities. The plasma membrane structure (*Pm*) is well visible and is of lower electron opacity than the membranes of the structure (*Ch*) which extends from it and invades the cell interior. We have previously suggested (4) that the gain in

FIGURES 1 and 2 *Bacillus subtilis* cells treated with TNBT and succinate. The cytoplasm is unusually electron-transparent, and shows a lack in fine structure. The cell wall is of normal electron opacity. The electron opacity of the chondrioids (*Ch*) appears a little higher than normal (4, 6), presumably owing to the deposition of diformazan. This is true of both the electron-opaque sheets (*S*) and the granular substance (*M*). The structure of the plasma membrane is difficult to distinguish. The cytoplasm contains electron-opaque granules (*P*). Fig. 1. × 27,000; Fig. 2. × 200,000.

FIGURE 3 Part of the periphery of a control cell from a sample exposed to TNBT. There is loss in range of contrast and in structural detail; but the cell wall has preserved its normal electron opacity. The chondrioids and plasma membrane are indistinct. × 200,000.

FIGURE 4 *B. subtilis* treated with potassium tellurite in its normal culture medium. The range of contrast in the cell is normal, apart from the chondrioid (*Ch*) which has gained considerably in electron opacity due to deposition of reduced tellurite. The plasma membrane (*Pm*) is of normal triple-layered structure. The nuclear area (*N*) has the usual appearance. × 200,000.


1
Ch
S
M
Ch
P
Ch
2
S
M
Ch
P
3
P
Ch
Pm
4
Ch
N

electron opacity of these latter membranes is due to the deposition of reduced tellurite.

The results obtained with TNBT and tellurite are comparable. The intracellular membranous structures have gained in electron opacity as compared to the plasma membrane. The sites of the membranous structures, which in the electron micrographs become so well visible after TNBT treatment, must coincide with the blue granules we observe in the light microscope. The higher electron opacity of the membranous organelles as compared to the cytoplasm must be the result of diformazan deposition, comparable to the reactions occurring in the mitochondria of the cells of various tissues (10–15). Therefore the TNBT experiment confirms our previous contention that the membranous organelles represent the chondrioids in the present Gram-positive bacterium. However, there are also remarkable differences in the results of both techniques: TNBT treatment and tellurite treatment; these concern the deposition of the reduction products and the preservation of the normal cell structure. In the case of the diformazan deposition, a certain gain in contrast can be observed (*a*) in what we described above as denser sheets in the membranous organelles, and (*b*) in the granular material (*M*) between sheets. The sheets are presumably identical to those in untreated cells. The granular material, on the other hand, is far more developed than we are finding in normal or in tellurite-treated cells. It seems premature to give an explanation of these appearances in the TNBT-treated cells. The deposition of the reduced tellurite is confined mainly to the dense sheets and is of considerably higher electron opacity than the deposits of diformazan.

The preservation of the normal cell structure is considerably poorer in the TNBT-treated cells than in cells treated with potassium tellurite. Treatment with TNBT leads to loss in contrast and in cellular detail. It was difficult to obtain photographic prints of Figs. 1, 2, and 3 of sufficient range of contrast. The normal structure of the tellurite-treated cells, on the other hand, is so well preserved that the distribution of electron-opacities in the object is much greater than in the TNBT-treated cells. Moreover, the intensity of the "black" of the reduced tellurite is, in the photographic negatives, considerably greater than that of the diformazan.

For the localization of the respiratory enzyme chain in Gram-positive bacteria such as *Bacillus subtilis*, the tellurite appears to have advantage over the TNBT method, in that it gives better preservation of the various cell components and the reduced product is of strikingly higher electron opacity than the diformazan. Moreover, the reduced tellurite appears to be strictly bound to the membranes whereas the TNBT products are (*a*) less specifically precipitated in the chondrioids, and (*b*) granules (*P*) are found scattered everywhere in the cytoplasm. So far, however, the nature of the granules (*P*) is unknown to us, though various suggestions could be made: for example, they may be small formazan deposits formed by reducing enzymes *in situ*, or they may result from diffusion processes. The tetranitro-blue tetrazolium and its reduction product are comparatively large organic molecules, the behavior of which in the "milieu interieur" of the cell or even in the media used for specimen preparation (18) is insufficiently known. But comparing TNBT with the older tetrazolium compounds, the former proved to have the advantage that under our experimental conditions crystal formation did not occur.

The chondrioid in the Gram-positive *B. subtilis* parallels the mitochondrion in tissue cells in that it is built up of membranes. However, unlike the cristae mitochondriales and the outer membrane of the usual mitochondrion, the membranes of the bacterial chondrioid appear single instead of double.

The authors wish to thank the following technical assistants for their valuable help: Mrs. I. G. M. de Groot, Miss E. Nijman van Zanten, Mr. P. J. Barends, Miss E. H. Bon, and Miss N. M. Slikker.

Received for publication, March 18, 1965.

REFERENCES

1. MUDD, S., Cytology of bacteria. Part I. The bacterial cell, *Ann. Rev. Microbiol.*, 1954, **8**, 1.
2. WEIBULL, C., BECKMAN, H., and BERGSTRÖM, L., Localization of enzymes in *Bacillus megaterium*, strain M., *J. Gen. Microbiol.*, 1959, **20**, 519.
3. VAN ITERSON, W., Membranous structures in micro-organisms, *in* Recent Progress in Microbiology, (N. E. Gibbons, editor), University of Toronto Press, 1962, **8**, 14.
4. VAN ITERSON, W., and LEENE, W., A cytochemical localization of reductive sites in a Gram-positive bacterium. Tellurite reduction in *Bacillus subtilis*, *J. Cell Biol.*, 1964, **20**, 361.

5. VAN ITERSON, W., Membranes, particular organelles and peripheral bodies in bacteria, Proceedings European Regional Conference on Electron Microscopy, (A. L. Houwink, B. J. Spit, editors), De Nederlandse Vereniging voor Electronenmicroscopie, Delft, 1960, **2,** 763.
6. VAN ITERSON, W., Some features of a remarkable organelle in *Bacillus subtilis*, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 183.
7. FITZ-JAMES, P. C., Participation of the cytoplasmic membrane in the growth and spore formation of bacilli, *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 507.
8. KELLENBERGER, E., and HUBER, L., Contribution á l'étude des équivalentes des mitochondries dans les bactéries, *Experientia*, 1953, **9,** 289.
9. VANDERWINKEL, E., and MURRAY, R. G. E., Organelles intracytoplasmiques bactériens et site d'activité oxydoréductrice, *J. Ultrastruct. Research*, 1962, **7,** 185.
10. NOVIKOFF, A. B., Electron transport enzymes biochemical and tetrazolium staining studies, *in* First International Congress of Histochemistry and Cytochemistry, Pergamon Press, Oxford, London, New York, Paris, 1963, 465.
11. SEDAR, A. W., ROSA, C. G., TSOU, K.-C., Tetranitro-blue tetrazolium and the electron histochemistry of succinic dehydrogenase, *J. Histochem. and Cytochem.*, 1962, **10,** 506.
12. ROSA, C. G., and TSOU, K.-C., The use of tetranitro-blue tetrazolium for the cytochemical localization of succinic dehydrogenase, *J. Cell Biol.*, 1963, **16,** 445.
13. ROSA, C. G., and TSOU, K.-C., Use of tetrazolium compounds in oxidative enzyme histo- and cytochemistry, *Nature*, 1961, **192,** 990.
14. SEDAR, A. W., ROSA, C. G., and TSOU, K.-C., Intramembranous localization of succinic dehydrogenase using tetranitro-blue tetrazolium, *in* Electron Microscopy, (S. S. Breese, editor), New York, Academic Press, Inc., 1962, **2,** L-7.
15. SCARPELLI, D. G., CRAIG, E. L., and ROSA, C. G., Submicroscopic localization of two dehydrogenase systems, *in* Electron Microscopy, (S. S. Breese, editor), New York, Academic Press, Inc., 1962, **2,** L-6.
16. LEENE, W., VAN ITERSON, W., and KIELICH, J. C., A cytochemical demonstration of bacterial chondrioids, *in* Proceedings of the Third European Regional Conference on Electron Microscopy, (M. Titlbach, editor), Prague, Publishing House of the Czechoslovak Academy of Sciences, 1964, 521.
17. RYTER, A., and KELLENBERGER, E., Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucléiques. I. Les nucléoides des bactéries en croissance active, *Z. Naturforsch.*, 1958, **13b,** 597.
18. LEENE, W., and VAN ITERSON, W., Tetranitro-blue tetrazolium reduction in *Proteus vulgaris*, *J. Cell Biol.*, 1965, **27,** 241.