DNA-MEMBRANE COMPLEXES OF *BACILLUS SUBTILIS*

Contact of Mesosomal Vesicles and Nuclear Fibrils

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INTRODUCTION

The isolation of complexes of membrane-associated DNA from disrupted bacteria has been reported by Ganesan and Lederberg (9), Smith and Hanawalt (26), and several others, and preliminary attempts to make such complexes visible in the electron microscope have been made by Ganesan (8) and Tremblay et al. (30).

The development of satisfactory methods of preservation of bacteria for electron microscopy (Ryter and Kellenberger, 24) was soon followed by the recognition that the membranous structures now called mesosomes (4) are in intimate contact with the bacterial genophore or nucleoplasm (van Iterson, 31). Earlier high-resolution studies from this laboratory (unpublished, and van Iterson et al., 33) had yielded suggestive evidence of connections between DNA fibrils and mesosomes in *Bacillus subtilis*. In the preparation of specimens to be examined for the disposition of DNA in the bacterial cell, drastic methods of disruption are best avoided. In the present renewed attempt to study the presumed relationship between DNA fibrils and mesosomes we have used a technique previously applied by Fitz-James (5) and Ryter and Landman (25), and reviewed by Ryter (22), which achieves the extrusion of mesosomal vesicles beyond the cell surface by exposing bacilli to lysozyme in a hypertonic medium; mesosomal vesicles are then expelled from the interior of the cells (for an exception cf. van Iterson and op den Kamp, 34).

MATERIALS AND METHODS

Two strains of *B. subtilis* were used, Marburg and W23. Some cultures were started from heat-shocked spores and were grown for 112 min in heart infusion broth; others were grown overnight in a medium A as described previously (34), containing 10 g of peptone (Difco Laboratories, Inc., Detroit, Mich.), 10 g of yeast extract (Difco Laboratories, Inc.), 5 g of NaCl, and 400 mg of Na_2HPO_4 per liter of water (pH 7.0).

Pellets of living bacilli obtained by centrifugation were dispersed at approximately 46°C in 2% agar made up with Ryter-Kellenberger (R.-K.) buffer. The agar was allowed to set and was cut into blocks smaller than 1 × 1 mm, which were then permeated with 0.25% lysozyme in R.-K. buffer with 0.3 M sucrose. Applied at room temperature, lysozyme does not visibly affect the integrity of the cell membrane, but particularly in young cells there is loss of membrane over parts of the bacterial surface when digestion with lysozyme is carried out at 37°C. The results about to be described were obtained from specimens treated with lysozyme at 37°C.

The lysozyme-treated material was fixed overnight through the agar with 1% OsO_4 according to the Ryter-Kellenberger procedure and embedded in Vestopal W.

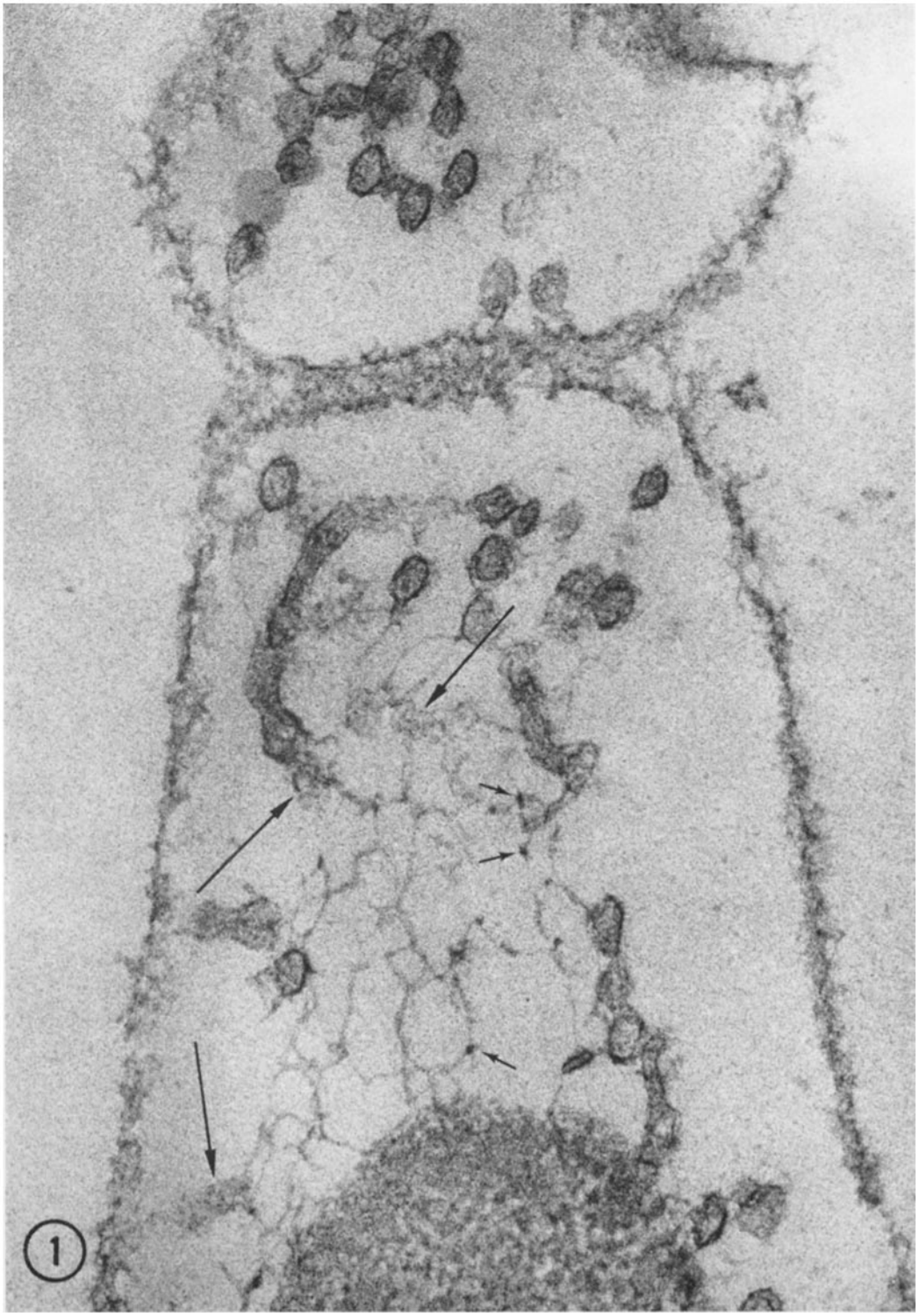
Sections were cut with an LKB ultrotome and stained with either 20% uranyl acetate in methanol for 2 min after Stempak (27), or lead citrate after Reynolds (19).

Electron micrographs were made with a Philips EM 300 at 80 kv and an aperture of 40 μ, with the cooling device in operation.

RESULTS

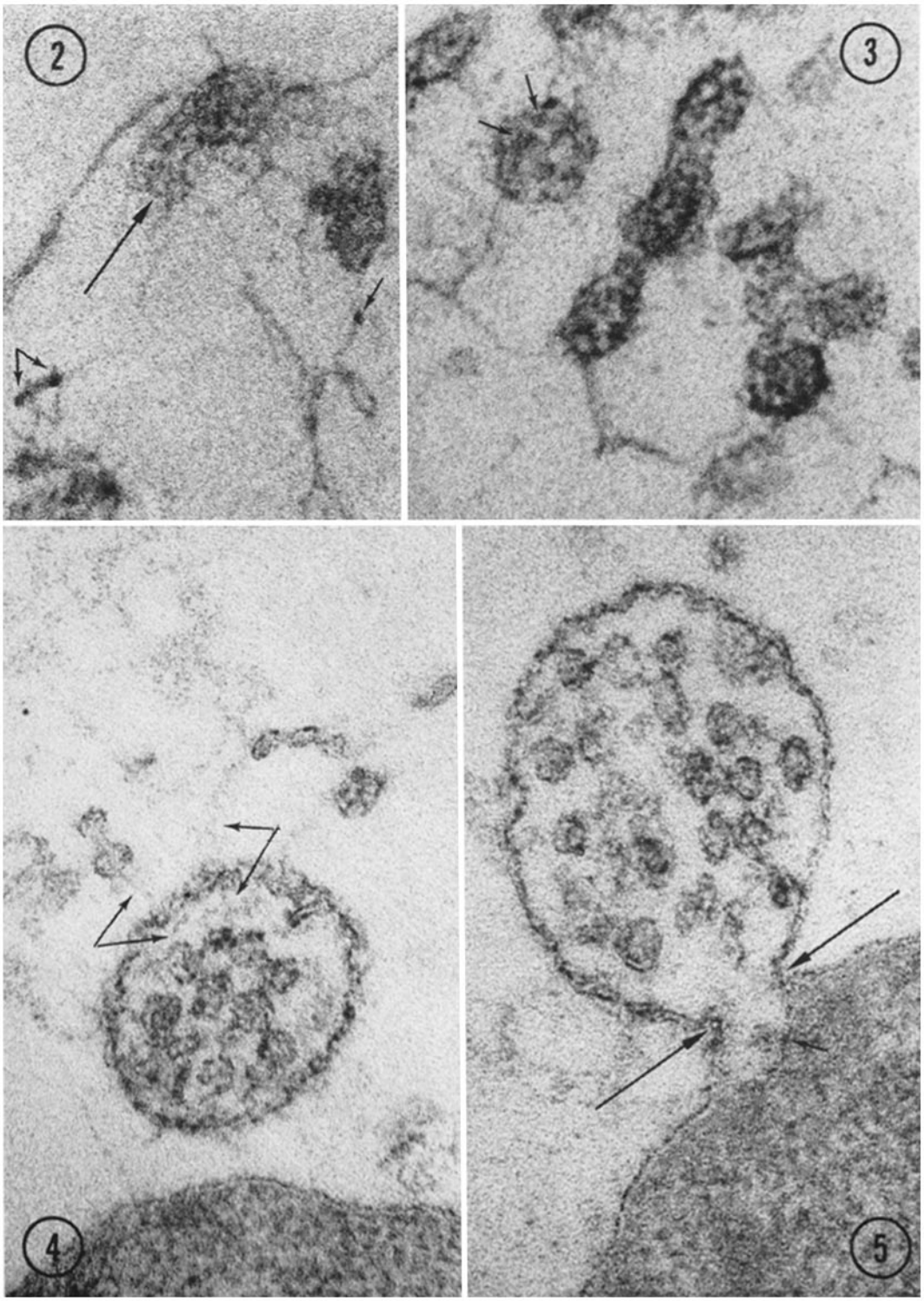
The walls of the bacilli grown for 112 min from spores were by the lysozyme treatment only partly digested (Figs. 1–3). The mesosomal tubules and vesicles, extruded into the space between the partially destroyed plasma membrane and the cell wall (Fig. 1), were found to be in contact with fine dense fibrils at many points. Contrary to what was observed when cells are digested at room temperature, the cytoplasmic membrane of these bacilli treated with lysozyme at 37°C was affected. Notwithstanding absence of the cytoplasmic membrane at several sites, neither fragments of cytoplasm nor ribosomes were found in the infra–cell wall space. The fibrils inside the infra–cell wall space are, in several other electron micrographs (unpublished), seen to extend from the nucleoplasm and may therefore be assumed to represent DNA fibrils. It is of interest that the released fibrils manifest themselves, after the Ryter-Kellenberger fixation, in a similar, though looser, entangled network than those in the nuclear area. In the fibrillar network at some sites opaque dots can be seen (short arrows, Figs. 1–3). The outstanding feature of Figs. 1–3 is the suggestion that the fibrils seem to take part in the organization of the tubules and vesicles (for instance, at large arrows Figs. 1, 2). The tangled structure in Fig. 2 (large arrow) provides a good illustration of the fibrillar nature of vesicular or tubular fragments commonly encountered in our specimens. In Fig. 3 a fibril can be seen passing through what seem to be rounded subunits of a vesicle (arrows).

The cytoplasmic membrane of cells from overnight cultures was preserved better than that in the previous much younger bacilli. In the preparation from which Figs. 4–7 were made the cell walls had been dissolved almost completely. Numerous, rounded mesosomal pockets containing vesicles and indications of fine filaments (Figs. 4–7) were seen outside the protoplasts (gymnoplasts). In Fig. 5 such a pocket is incompletely released from the protoplast, and in Fig. 6 a mesosome remains attached to a membrane extending from the protoplast. In Fig. 4 fibrils from outside the pocket seem to continue into its interior (arrows). Figs. 6 and 7 are sections from the same mesosome in which



FIGURES 1–3 are from *B. subtilis* W23 grown for 112 min from spores and treated at 37°C with lysozyme through agar. The cell walls had not completely dissolved. Preparations poststained with uranyl acetate.

FIGURE 1 From a dividing cell. Within the spaces formed by the incompletely dissolved cell walls and the remaining protoplasts, expelled tubules and vesicles of disrupted mesosomes can be seen with adhering nuclear fibrils. Long arrows point to fibrils which seem to take part in the organization of tubules and vesicles. Short arrows point to opaque dots in the fibrillar network. × 231,000.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vesicles and fibrils are in contact in several places (arrows). The mesosomal envelopes in these latter figures, and also in Figs. 4–5, appear to differ in structure from the cytoplasmic membrane. This difference in appearance may partly be due to tangential sectioning of the small and, therefore, strongly curved, spherical mesosome. In Fig. 7 two vesicles are attached to the outside of the mesosomal envelope, and another one appears to be connected to its inside by a triple-layered "stalk." Some vesicles in the mesosome of Figs. 6–7 seem bordered by a triple-layered structure, whereas the borders of the released tubules and vesicles in Figs. 1–3 appear more delicate.

DISCUSSION

Hypotheses of mesosome organization have been advanced by Fitz-James (4), Koike and Takeya (14), Imaeda and Ogura (11), Pate and Ordal (18), and Ryter (22, 23). In all these representations mesosomes are defined as a pocket formed by the invagination of the cytoplasmic membrane. In the words of Ryter (23): "When bacteria are introduced into a hypertonic medium, this pocket opens and flattens out and a tube (consisting of a chain of small vesicles) is pushed out into the space between membrane and wall." The present results, however, show that the contents of the interior are not necessarily released immediately and that the mesosomal envelope then straightens out and becomes continuous with the cytoplasmic membrane. In the preparation of Figs. 4–7 mesosomal pockets were expelled as complete spheres. In this connection Fig. 5 is of some importance since it may demonstrate a stage in the extrusion of a mesosomal pocket from the cell.

On the basis of freeze etching, Nanninga (16), working in our laboratory, reached the conclusion that the mesosomal envelope cannot be regarded as a mere invagination of the cytoplasmic membrane, and that it is better looked upon as a structural differentiation of the latter. The work of Fitz-James (6, 7) suggests that the mesosomes might be the cell's site for membrane synthesis.

In the current literature there is much discussion of the question at which point or points the bacterial chromosome is attached to the cell membrane. Jacob et al. (12) hypothesized the attachment of the bacterial DNA to membrane at the replication point, whereas it is suggested by Lark (15), for instance, that attachment is at the replication origin, while Sueoka and Quinn (28) believe in attachment at both: origin and replication point. These theories thus consider one or two unique membrane attachment sites. A dissenting conclusion has been reached by Rosenberg and Cavalieri (21) who found evidence of multiple points of membrane attachment of the bacterial DNA.

The method of mesosome extrusion used here is essentially the same as that applied by Ryter et al. (cf. 22, 23). Ryter interprets her results as follows: "During its extrusion, the mesosome seems to pull the nucleus to the membrane which suggests the existence of a real linkage between DNA and mesosome." With this we agree, but we find no evidence that DNA is attached only to the exterior of the mesosomal envelope at one specific point (cf. her

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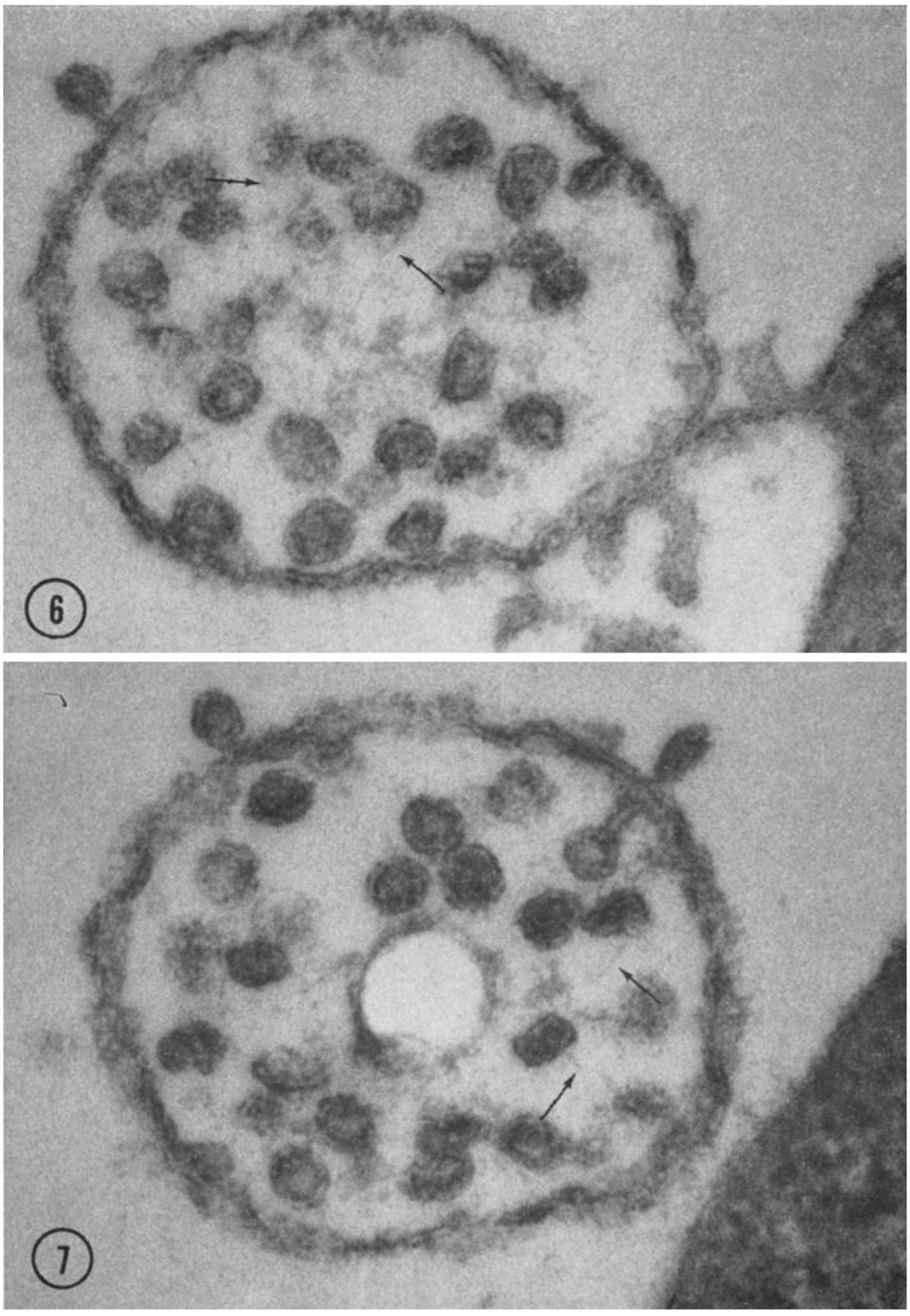
FIGURE 2 Expelled vesicle or tubule fragment of fibrillar construction (large arrow). Short arrows point to opaque dots, probably too electron-opaque to be cross-sections of fibrils. × 390,000.

FIGURE 3 A thin fibril appears to traverse a vesicle with small, rounded substructures (arrows). × 475,000.

FIGURES 4–7 are from *B. subtilis* strain Marburg grown overnight and treated at 37°C with lysozyme through agar, by which complete dissolution of the cell wall had been achieved. Mesosomal pockets are released from naked protoplasts. Preparations poststained with lead citrate.

FIGURE 4 Mesosomal pocket containing vesicles. At arrows, fibrils from outside may continue in the interior of the pocket. Note difference in appearance between the strongly curves mesosomal envelope and the cytoplasmic membrane (at bottom of picture). × 148,500.

FIGURE 5 Section through an incompletely released mesosomal pocket. Perhaps this picture can be explained by assuming that the pocket was pushed through a break in the cytoplasmic membrane marked by large arrows. The tangentially cut membrane at the short arrow may thus belong to the mesomal envelope, and indeed a vesicle can be seen at this arrow. × 175,000.



FIGURES 6 and 7 Two of a series of sections through a mesosomal pocket. The section in Fig. 6 passes through a membrane by which the pocket is attached to the protoplast. Note fibrils inside the pocket (arrows). A few vesicles are situated outside the mesosomal envelope; in Fig. 7, a vesicle appears connected to its inside by a triple-layered stalk. In the center of Fig. 7 part of the mesosome is missing. × 282,000.

schemes Fig. 20 [22] and Fig. 8 [23]). Our results indicate, on the contrary, the existence of many points of contact between fibrils of DNA and mesosomal vesicles.

Our trust in the reliability of the preparation from which Figs. 1–3 were made is based on the fact that vesicles as well as DNA were expelled from the cell interior during digestion at 37°C, that is, long before their fixation. There seems to be no compelling reason to assume that DNA fibrils and vesicles were expelled separately and thereafter coagulated within the infra–cell wall space. Moreover, the existence of contact between DNA and mesosomal vesicles is strengthened by the evidence of the presence of fine fibrils within the mesosomes extruded intact (Figs. 4–7). In this connection it may be noted that Robinow in 1960 (20), working with the light microscope, described small round Feulgen-positive bodies adjacent to bacterial chromatin structures which he identified with the membrane structures found by van Iterson at corresponding sites with the electron microscope (mesosomes).

Highton's suggestion (10) that vesicles are produced by membrane destruction in the mesosome does not materially influence our conclusion of extensive contact between DNA and mesosomal structures.

The advantage of the present preparations of DNA and the mesosomal fragments in sections over those by Ganesan (8) and Tremblay et al. (30) is that here the origin of the membranous material connected with the DNA fibrils is well-known.

Connections between DNA and membranes have also been reported for mitochondria (cf. Nass [17], Swift et al. [29]) and for photosynthetic lamellae (Wookcock and Fernández-Morán (35) and Bisalputra and Burton [1]).

Cell wall–membrane complexes with DNA replicating capacity have recently been isolated from *Escherichia coli* by Knippers and Strätling (13). Large mesosomal pockets comparable to those of *B. subtilis* are lacking in *E. coli*. It would therefore be of great interest to investigate morphologically how the equivalent of the phenomenon described here is achieved in *E. coli*.

Cairns and Denhardt (3) consider that for replication of the bacterial chromosome in vivo a continuous expenditure of energy is required, and Cairns and Davern (2) suggest that one should determine whether the region of replication of the nucleoplasm is close to the mesosome or not. We have demonstrated here the existence of an intimate contact between nucleoplasm and mesosomal structures, but whether mesosomes are sites of DNA replication remains open to further investigation. Formerly, we (32) found that mesosomes have strong reducing capacity for tellurite and tetranitro blue tetrazolium. In view of recent doubt as to whether this is indicative of the location of the respiratory chain in mesosomes, we are at present investigating the presence of cytochrome oxidase in them.

SUMMARY

At high magnification extruded mesosomal tubules and vesicles appear to have an intricate fine structure, and several of our micrographs (Figs. 1–3) suggest that DNA fibrils form part of this fabric. It appears desirable to establish with another technique the existence of the DNA-membrane associations here described. Confirmation of their reality by other procedures would require changes in currently accepted ideas in molecular biology.

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