THE FINE STRUCTURE OF *DIPLOCOCCUS PNEUMONIAE*

ALEXANDER TOMASZ, Ph.D., JAMES D. JAMIESON, M.D., and ELENA OTTOLENGHI, M.D.

From The Rockefeller Institute and the New York University School of Medicine, New York

ABSTRACT

The fine structure of an unencapsulated strain of *Diplococcus pneumoniae* is described. A striking feature of these bacteria is an intracytoplasmic membrane system which appears to be an extension of septa of dividing bacteria. The possible function of these structures and their relationship to the plasma membrane and other types of intracytoplasmic membranes found in pneumococcus is discussed.

INTRODUCTION

Our main interest in the fine structure of *Diplococcus pneumoniae* stems from the fact that these bacteria readily undergo genetic transformation. Prior to undertaking electron microscope studies on this process, the fine structure of pneumococcal cells in thin sections was examined. During the preliminary stage of these studies on a transformable strain, we observed some unique membranous structures which, to the best of our knowledge, have not previously been described in bacteria.

MATERIALS AND METHODS

Unencapsulated strains of *Diplococcus pneumoniae* R6, R1, and some nutritional mutants derived from R6 were used in these studies. These strains were derived from *Diplococcus pneumoniae* R36A (1) which has been used as a laboratory strain for over 20 years. Forty-ml batches of bacteria were grown in a modified Adams-Roe medium (2) (medium A) or in a semisynthetic medium (3) (medium B) at 37°C in 25 x 150 mm tubes, without aeration. Small inocula (10⁶ cells/ml) of cells from the exponential phase of growth were used and the cultures were harvested in the late exponential phase. In these media, stationary phase cultures undergo spontaneous autolysis, the first recognizable stage of which is a quantitative conversion of the cocci to fragile spherical bodies of fairly uniform size which are deficient in their cell walls. Throughout this paper, such preparations will be referred to as "spheroplasts."

The bacteria were fixed and stained according to the method of Ryter and Kellenberger (4), embedded in cross-linked methacrylate, and sectioned with a Porter-Blum microtome using a diamond knife. The sections were stained with lead according to the method of Karnovsky (5) (method B) and were examined in the RCA electron microscopes models 2B, 3F, or in the Siemens Elmiskop I.

RESULTS

The nuclear region of pneumococcus resembles that of other bacteria prepared by the method of Ryter and Kellenberger (4). It is located centrally in the cell, is devoid of a nuclear membrane, and has a density lower than that of the surrounding cytoplasm (Fig. 1). The nuclear region is filled with more or less tightly packed uniform fibrils 25 to 30 Å wide (Fig. 2).

Differences in the internal structure of the nuclear region could be observed from one experiment to another or even in adjacent cells within the same section, in spite of the fact that the preparative procedure was the same throughout.

These variations were essentially of three types: variation in the degree of interpenetration of the nuclear region and the cytoplasm which ranged
from a smooth nuclear profile to one deeply indented by cytoplasmic material; variation in the degree of aggregation of nuclear fibrils; and, finally, variation in the orientation of nuclear fibrils.

These differences were not studied systematically, but it is likely that the variations in the profile of the nuclear region reflect differences in the growth conditions of the cells since the indented nuclear profiles were predominant only in cells grown in the semisynthetic medium (Fig. 2). On the other hand, differences in the degree of aggregation of the nuclear fibrils have been observed in the nuclear regions of adjacent cells within the same preparation irrespective of medium and must, therefore, be the direct or indirect results of some physiological heterogeneity in the cell population, for instance, in the ion-permeabilities of the cells (6). Frequent examples of such morphologic variation can be seen in bacteria taken from the late exponential phase of growth in semisynthetic medium: here the nuclear fibrils of adjacent cells have markedly different morphology (fine fibrils versus coarse bundles) (Fig. 3).

The orientation of the nuclear fibrils within a nuclear region appears to be random in the majority of the cells; frequently, however, the fibrils have a distinct preferential orientation parallel to one another (Fig. 2 a). It is not clear whether this represents remnants of some higher degree of organization which was partly randomized during the cytological procedure (7, 8), or reflects a physiological state of the nucleoplasm, or possibly an artifact.

A similar orientation of the nuclear fibrils after the Ryter-Kellenberger procedure has been observed in other bacteria (9–11).

The cytoplasm of pneumococcus contains numerous dense particles about 150 A in diameter, presumably ribosomes, which may be distributed homogeneously in the cytoplasm (Fig. 2 b) or concentrated around the nuclear region (Fig. 3).

The cytoplasm also contains areas of low density which are generally round, indistinct in outline, and which vary in diameter and in number per cell (Fig. 1). The largest number can be observed in cells grown in semisynthetic medium while few are found in cells grown in broth or neopeptone-containing media. Low density areas are frequently seen at the growing tips of developing septa. The chemical nature of the material within these areas is not known.

The plasma membrane of pneumococcus appears as a laminated structure consisting of two parallel dense bands enclosing a less dense middle band (Fig. 4 a). The appearance of the plasma membrane of intact cells differs somewhat from that seen in pneumococcal "spheroplasts" which have partially or totally lysed. In "spheroplasts," the plasma membrane is composed of two dense bands, each 25 to 30 A wide, which enclose a less dense band of the same dimensions (Fig. 5). It is thus identical with the "unit membrane" (12) observed in many cellular membrane systems. In the intact pneumococcal cell, the structure of the plasma membrane is asymmetrical: while the inner band and the middle band have about the same width as in "spheroplasts," the outer band is wider (about 40 A). Such an asymmetrical appearance characterizes also the intracytoplasmic membranes of pneumococcus (e.g., Figs. 4 a and b).

Arguments in support of the above interpretation of the structure of the pneumococcal plasma membrane will be presented in the discussion.

Intracytoplasmic membranes of varying length, shape, and complexity are observed in pneumococcus. A feature common to all these membranes is that their basic resolvable structure seems to be identical to that of the plasma membrane in dimensions and in staining characteristics, and that in appropriate sections they appear to be continuous with the plasma membrane.

Two types of intracytoplasmic membranes can be distinguished in pneumococcus: (1) globular bodies similar to the membranous organelles described in a large number of Gram-positive bacteria under various names (chondrioids,1 mesosomes, vesiculotubular bodies, etc.) (4, 9, 11, 13, 15), and (2) the lamellar invaginations of the plasma membrane called here the septoid or S membranes. To the best of our knowledge, the S membranes have not been described in other bacteria.

1 Throughout this paper, preference is given to the term chondrioid instead of mesosome for reasons discussed by van Iterson (14).

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**Figure 1** Pneumococcal cells grown in Medium A. Low density areas (lda); S-membranes (s). RCA 2B, × 34,000.
Chondrioids are inpocketings of the plasma membrane and consist of a stalk, in which the layers of the plasma membrane appear as a double membrane (or meso (12)), and a distended portion whose lumen is occupied by a complex system of vesicles (Fig. 4 a). Chondrioids continuous with the plasma membrane seem to be exclusively located at the growing tips of septal membranes in pneumococcus (Figs. 4 a and b).

S membranes are strongly staining lamellae of varying length and curvature and are frequently seen in pneumococcal cells (Fig. 1). Their location within the cytoplasm is variable, but whenever they are continuous with the plasma membrane they appear as terminal extensions of developing septa in dividing bacteria.

The fine structure of these membranes is different from that of the chondrioids: S membranes are composed of two unit-membranes which run parallel throughout their entire length, while the limiting membrane of the chondrioid is always a single unit-membrane. The combination of the two unit-membranes gives rise to the sevenfold structure of the S membranes which can be seen in Fig. 6; also shown is the continuity of these membranes with the plasma membrane.

The spacing between the two central dense bands of the S membranes varies from cases in which the bands are almost fused (e.g., in Fig. 9) to cases in which a considerable "gap" exists between them (Fig. 6), indicating that the association between these bands is not well defined; consequently, we are not dealing with compound membranes of the type found in the myelin sheath (16). Occasionally, short fibrils appear over such gaps and produce a striated appearance similar to that seen between the plasma membrane and the cell wall (Fig. 7) (see below).

Another characteristic feature of the S membranes is that they do not fold upon themselves to form stacks of lamellae or concentric layers but always remain as simple invaginations of the plasma membrane.

The fine structure of the S membrane bears a striking resemblance to that of the pneumococcal septal membrane and to the spore membranes of bacilli (13). The chief difference, however, is that in the two latter structures the spacing between the central dense bands seems fairly constant.

The determination of the 3-dimensional structure of the S membrane will require serial sectioning. It is probable, however, that S membranes are not finger-like invaginations but sheets of infolded plasma membrane, since ring-like structures with a diameter of slightly larger than twice the width of the plasma membrane were not observed in cross-sections of S membranes. Membrane e in Fig. 9 probably represents a tangential section of S membrane.

The cell wall is the outermost structure of pneumococcus. It appears as a trilaminated structure composed of an outer and an inner dense band, each 30 to 40 A wide, enclosing a less dense band 60 to 80 A wide (Figs. 2 and 6).

In these bacteria, the cell wall is generally seen to be separated from the underlying plasma membrane by a low density space. Within a single cell the width of this space can remain constant or show large variations (Fig. 4 a). This space often appears striated due to the presence of short fibrils connecting the outer band of the plasma membrane with the inner dense band of the cell wall (Fig. 7). These fibrils seem to originate from the plasma membrane rather than the cell wall since striations with an identical appearance can be seen within some of the intracytoplasmic membranes (see above). Similar connections between the cell wall and the plasma membrane have been described in other bacteria (11).

**DISCUSSION**

**Structure of the Plasma Membrane**

Thin sections of partially lysed "spheroplasts" of pneumococcus clearly demonstrate that the
plasma membrane of these organisms has the characteristics of a unit membrane (Fig. 5) of the symmetrical type.

The identification of the inner and outer limits of the plasma membrane of intact pneumococcal cells, however, is worthy of comment because of a disagreement in the literature with regard to the structure of the plasma membrane of Gram-positive bacteria (17a, and b). In pneumococcus, as in other Gram-positive bacteria, there are five parallel bands distinguishable interior to the cell wall (Fig. 4a). It is not obvious which of these bands correspond to the osmiophilic bands of the unit membrane visible in "spheroplasts." We identified the dense, 40 to 45 A wide, osmiophilic band (band 3) as the outer layer of the plasma membrane. In the alternative interpretation, the outer layer of the plasma membrane is contiguous with the inner edge of the cell wall (band 5), and the less dense band—which we identify as the inner layer of the plasma membrane—is assumed to be the boundary of the cytoplasm. Our identification of the limits of the plasma membrane rests mainly on the arguments presented below.

If one assumes that the basic structure of the bacterial plasma membrane is essentially a unit membrane, then from knowledge of the characteristics of bimolecular lipid films one would expect the distance between the osmiophilic bands to remain strictly constant throughout. This condition is only satisfied if one defines the limit of the plasma membrane as indicated in Fig. 4a. Measurements made along the plasma membrane often revealed significant variation in the width of band 4, while the dimensions of bands 1, 2, and 3 were constant.

The width of the complete plasma membrane as defined in Fig. 4a is 80 to 100 A; the alternative interpretation yields a much wider (120 to 180 A) membrane.

In cells undergoing partial plasmolysis (Fig. 4b), the cytoplasm retracts from the cell wall along band 4, while there is practically no change in the dimensions of bands 1, 2, and 3. During plasmolysis as well as during autolysis (Fig. 5), band 5 remains with the cell wall without any decrease in density, indicating that this band does not represent remnants of the plasma membrane but is probably part of the cell wall.

An additional difficulty exists in the identification of the outer layer of the plasma membrane with band 5. On the basis of this interpretation, one would be forced to conclude that the two layers of the plasma membrane become separated from each other every time the plasma membrane infolds to form a septal membrane, a chondrioid, or an S membrane (see Fig. 4b). A definition of the limits of the plasma membrane, as indicated in Fig. 4a, avoids these difficulties.

It seems that the arguments presented above for the structure of the plasma membrane in pneumococcus may also be applied to other Gram-positive bacteria; our interpretation agrees well with that of Glauert (11) and is similar to one of the alternative interpretations discussed by Fitz-James (13). However, our interpretation of the plasma membrane differs from that of van Iterson (17b).

One consequence of the present interpretation of the structure of the pneumococcal plasma membrane is the recognition of its asymmetry. Such an asymmetry seems to exist in other Gram-positive bacteria as well (9, 11, 13, 14, 18). The cause of this asymmetry is not clear. The production of more symmetrical membranes during autolysis is not restricted to pneumococcus (13).

Another consequence of the interpretation of the plasma membrane presented here is that in pneumococcus and in other Gram-positive bacteria as well the boundary of the chondrioids is not a single layer of the plasma membrane (9), but rather a complete unit membrane (see Fig. 4a) (14, 17b).

Intracytoplasmic Membranes and Septal Growth

In pneumococcus as in a number of other bacteria (19, 20), the centripetal growth of the septal membrane precedes the formation of the cross-wall, and in sections of this organism one finds the growing tip of the septal membrane either continuous with chondrioids (Figs. 3 and 4) or with S membranes (Figs. 2b and 6), or unassociated with either of these structures (Fig. 1). This lack

Figure 3 Variation in the morphology of the nuclear fibrils. The plasma membrane (m), the cell wall (cw), an S-membrane (s), and a chondrioid (ch) are also well resolved; ribosomes (r) seem to be concentrated around the nuclear region. The nuclear fibrils (nf) are coarse in the upper cell and fine in the lower cell. Medium A. Elmiskop I. X 80,000.
of association may be due to the plane of section, and serial sections would be required to elucidate this point.

Because of their structural identity, it is impossible to distinguish a septal membrane from an S membrane which is continuous with it. The S membrane may thus appear as an abnormal elongation of the septal membrane. However, since cell wall deposition was not observed along the S membranes but only along septal membranes, these two structures, though morphologically similar, must be functionally distinct.

As described above, in pneumococcus the only place in which intracytoplasmic membranes seem to be continuous with the plasma membrane is at the septal membrane of dividing bacteria. This suggests that in pneumococcus these organelles originate from the septal membranes during cell division. In other bacteria, such as *B. subtilis* (9, 11), chondrioids seem to occur also in other locations along the cell membrane.

**Origin of Intracytoplasmic Membranes**

Various schemes can be constructed for the mode of origin of intracytoplasmic membranes, but the number of schemes is restricted because the disposition of the two component layers of the plasma membrane (recognizable by their prevailing asymmetry) must be accounted for. In addition, it is assumed that the membrane growth occurs by the insertion of new material into the existing plasma membrane followed by elongation and folding of the membrane.

One can now construct at least two simple developmental schemes (Fig. 8) as follows: the infoldings of the plasma membrane initiate cell septation, after which one of the two alternative paths can be followed. In Scheme I, the two outer layers of the plasma membrane adhere to one another so that the synthesis of new membrane material results primarily in the elongation of lamellar S membranes. Alternatively (Scheme II), the outer layers separate, and the infolding is distended, forming a sac-like structure. Further synthesis of new membranous materials results in the formation of secondary infoldings towards the lumen of the sac, giving rise to a chondrioid structure.

Although the diagram stresses the septal membrane as the common precursor of the two types of intracytoplasmic membrane, the possibility of more direct interconversions is also illustrated. Thin sections of cells with membranes closely resembling the "intermediate" stage in Fig. 8 are presented (Fig. 9). Although the "intermediate" stages were observed, it was not possible to establish a precise correlation between particular membrane profiles and the division stage of the cell as judged by centripetal septal advance.

**Function of Intracytoplasmic Membranes**

A number of physiological functions for the chondrioids have been proposed (9, 21, 22). Recently, Vanderwinkle and Murray (23) and van Iterson (14) obtained evidence for a mitochondrion-like function of these membrane systems in *B. subtilis*. It may be noted that the chondrioids of pneumococcus (at least of pneumococci grown under the semi-anaerobic growth conditions used here) appear less complex in their intraluminal structure.

Membrane systems resembling somewhat the pneumococcal S membranes have been described in *Streptomyces* (20). However, in these organisms the predominant morphology seems to be a stack of concentric or parallel unit membranes.

If S membranes and chondrioids have a functional similarity, it may be that the S membranes are more primitive forms of the mitochondrial equivalents and would be sufficient, therefore, for cells grown under relatively anaerobic conditions. Anaerobically grown pneumococci are known to be deficient in enzymes involved in electron transport (24). Alternatively, the function of S membranes may be totally different from that of the chondrioids and may be related to some unique feature of the division process (septal membrane development) in pneumococcus, e.g., a temporary imbalance.

![Figure 4 a, b](https://jcb.rupress.org/content/22/4/458/F4.large.jpg) The surface structures of the pneumococcal cell: cell wall (cw), outer band of the plasma membrane (o), and inner band of the plasma membrane (i); chondrioid (ch). For the interpretation of bands 1, 2, 3, 4, and 5, see text. The limiting membrane of the chondrioid is a complete unit membrane continuous with the plasma membrane at the tip of the septal membrane. Note the asymmetrical appearance of the membrane, both on the cell surface and in the limiting membrane of the chondrioid. Medium B. Elmiskop I. Fig. 4 a, × 180,000; Fig. 4 b, × 220,000.

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between the synthesis of membrane and wall material.

It should be pointed out that since both types of intracytoplasmic membranes substantially extend the cytoplasmic surface, they may play a role in the exchange of materials between the cell and its environment. Such a possibility has already been suggested by van Iterson (9, 14). It may be important in this connection that both S membranes and chondrioids can frequently be seen in contact with the nuclear region of the cell. The potential adsorptive capacity offered by these invaginations of the cell membrane may be particularly significant in the absorption of large molecules, such as the uptake of DNA molecules during the process of genetic transformation in pneumococcus. This possibility is presently under investigation.

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FIGURE 5 A partially lysed pneumococcal “spheroplast” from an autolysing culture. Note residual cell wall material (cw); plasma membrane (m); ribosomes (r); the membranous structure in the center (x) may be the remnant of a chondrioid or an S-membrane. Medium B. RCA 2B. X 300,000.

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**FIGURE 6** Fine structure of the S-membrane (s). Cell wall (cw); outer (o) and inner (i) bands of the plasma membrane. Medium B. Elmiskop I. × 300,000.

**FIGURE 7** Fibrillar connections (striations) between the cell wall and the outer band of the plasma membrane. Note striation of identical appearance in the S-membranes (s). Disposition of the outer (o) and the inner (i) bands of the plasma membrane is shown by arrows. Medium A. RCA 3B. × 300,000.

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FIGURES 8 and 9. Fig. 8 presents schemes for the development of chondrioids and S-membranes. Membrane profiles resembling the developmental stages are shown in Fig. 9 and are designated by the same letters used in Fig. 8. Solid lines in Fig. 8 represent the outer band of the plasma membrane; broken lines represent the inner band of the plasma membrane. In Fig. 9, (ch) stands for chondrioid.

Magnifications: a, b, c, × 75,000, RCA 2B; d, × 88,000, RCA 2B (upper part), and × 80,000 (lower part); e, × 66,000, RCA 2B; f, × 80,000, Elmiskop I; g, × 80,000, Elmiskop I; i, × 80,000, Elmiskop I; (ch), Chondrioid. × 300,000, RCA 2B.