ULTRASTRUCTURE OF THE OBLIGATELY ANAEROBIC BACTERIA *CLOSTRIDIUM KLUYVERI* AND *CL. ACETOBUTYLICUM*

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Abstract

The morphology of *Cl. kluyveri* is compared with that of *Cl. acetobutylicum*. The multilayered cell wall of both organisms differ in the number of layers and total thickness. The plasma membrane of *Cl. acetobutylicum* is asymmetric in electron density and can be separated from the rest of the cell by plasmolysis. Tubular or vesicular structures are observed between the wall and the plasma membrane. In contrast, the plasma membrane of *Cl. kluyveri* is more symmetrical, with no structures between the cell wall and the plasma membrane.

Intracytoplasmic membrane systems present in *Cl. acetobutylicum* are morphologically similar to the mesosomes of Gram-positive aerobes. The intracytoplasmic membrane systems of *Cl. kluyveri* are characteristically rough in appearance, and differ in general morphology from systems usually described as mesosomes. Roughness is attributed to membrane-bound ribosomes, these being smaller than those of *Escherichia coli*.

Polygonal structures containing moderately electron-dense material (possibly DNA) were observed in *Cl. kluyveri*. *Cl. acetobutylicum* possesses membrane-bounded inclusions containing electron-dense particles similar in dimensions to *E. coli* ribosomes. Rows of electron-dense bands were also observed.

It is suggested that the polygonal structures and rough membranes of *Cl. kluyveri* are components of a system for the functional expression of genetic information.

I. Introduction

Investigations of the ultrastructure of bacteria have been mainly with aerobic species (Salton 1967). In comparative studies of the ultrastructure of bacteria differing in oxygen requirements (Cho 1968) the obligate anaerobes *Clostridium kluyveri* and *Cl. acetobutylicum* showed unusual structures and differed from each other. Results with vegetative cells of these species are described in the present paper. Other studies with clostridia have emphasized sporulating rather than vegetative cells (Takagi *et al.* 1960*a*, 1960*b*; Fitz-James 1962; Hoeniger and Headley 1968; Pope *et al.* 1968; Betz 1970; Smith 1970).

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II. Materials and Methods

(a) *Organisms and Growth Conditions*

Cultures of *Cl. acetobutylicum* (NCIB 8052) and *Cl. kluyveri* were kindly provided by Dr. D. Ilse. The composition of the growth medium for *Cl. acetobutylicum* was as follows: yeast extract (Difco) 5 g, peptone (Oxoid) 5 g, glucose 10 g, KH_2PO_4 0·5 g, K_2HPO_4 0·5 g, $MnSO_4.4H_2O$ 0·005 g, $FeSO_4.4H_2O$ 0·001 g, biotin 0·001 g, thiamine HCl 0·5 g, sodium thioglycollate 0·5 g, pH adjusted to 6·5 with 1·0N NaOH, distilled water to 1 litre. The growth medium for *Cl. kluyveri* was as described by Stadtman and Burton (1955).

Cultures (750 ml medium per 1-litre flask) were grown at 37°C without shaking. Samples were taken at 36 hr and 60 hr.

(b) *Plasmolysis*

Cells were suspended for 15 min at room temperature in 2M sucrose dissolved in Tris–HCl buffer (0·05M, pH 7·5).

(c) *Negative Staining*

The negative stain was 2% phosphotungstic acid (PTA) neutralized with 1·0N NaOH to pH 7·2. A drop of the cell suspension was mixed with one drop of PTA and a trace of bovine albumin to assist the adhesion of cells to the grid. A drop of the mixture was then placed on a 200-mesh copper grid coated with Parlodion (purified pyroxylin). Excess liquid was removed with filter paper. The grid was stored in a desiccator and examined within 24 hr.

(d) *Electron Microscopy*

Cells were fixed by two methods: (1) a modification of Ryter and Kellenberger's method (1958)—pellets used for embedding had no agar and the period of fixation was reduced to 4 hr; (2) a double-fixation method in which cells were prefixed with 1% glutaraldehyde in sodium cacodylate buffer (0·05M, pH 6·0) supplemented with 0·01M $CaCl_2$ for 2 hr at room temperature (20–22°C). After washing six times with sodium cacodylate buffer the cells were fixed with osmium tetroxide as in method 1.

Fixed cells were treated with 1% uranyl acetate for 2 hr. Dehydration was carried out in a graded series of mixtures of acetone and water. The specimens were infiltrated with Araldite and acetone (1 : 1 v/v) for 2 hr, with Araldite for 24 hr, and with freshly prepared Araldite at 60°C for 1 hr. Embeddings were done in freshly prepared Araldite and were polymerized at 60°C for 2 days.

Sections were cut with glass knives on a Reichert ultramicrotome, collected on unsupported 400-mesh grids, doubly stained with saturated uranyl acetate in 50% (v/v) ethanol for 1 hr, then with lead citrate for 30 min (Reynolds 1963). Electron micrographs were taken with a Siemens Elmiskop model 1A electron microscope with a double condenser system.

III. Results and Discussion

In general, cells fixed by osmium tetroxide or glutaraldehyde–osmium tetroxide showed the same morphology.

(a) *Clostridium kluyveri*

(i) *General Morphology*

The cell envelope consists of a multilayered straight cell wall and an asymmetric plasma membrane (Figs. 1 and 2). DNA fibrils of the nucleoplasm are diffuse in most cells. In a few cells the DNA was more condensed and appeared as electron-dense filaments, 25–30 Å thick, coiled or twisted in long threads (Fig. 5), or as numerous dots in cross-sections (not illustrated). In addition to ribosomes, intracyto-

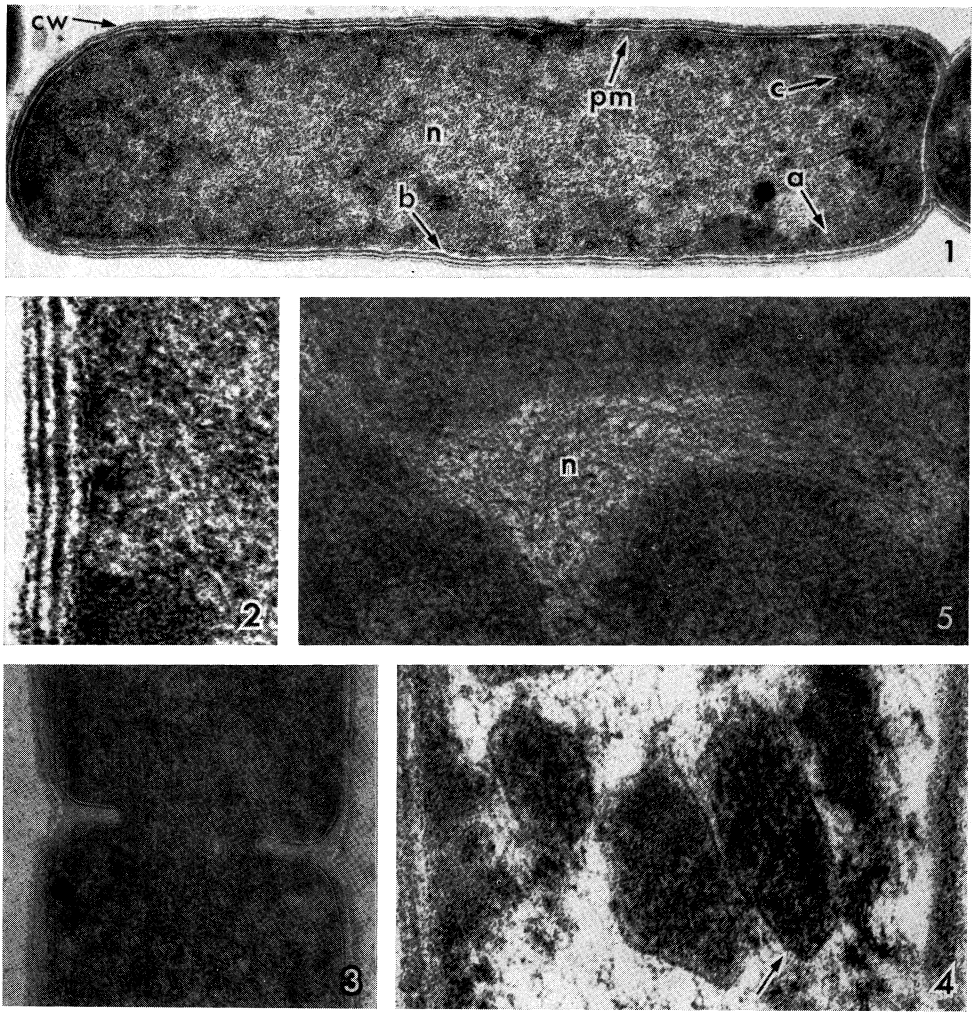

Fig. 1.—Section of *Cl. kluyveri* (36 hr culture) showing a multilayered cell wall (*cw*) and a triple-layered plasma membrane (*pm*). DNA fibrils are diffuse in the nucleoplasm (*n*). *a*, invagination of the plasma membrane; *b*, plasma membrane overlain with dense material; *c*, the boundary of a polygonal structure. Fixation: osmium tetroxide. × 38,000.

Fig. 2.—A higher magnification of part of Figure 1 showing the fine structure of the cell wall and the plasma membrane. × 190,000.

Fig. 3.—Section of *Cl. kluyveri* (36 hr culture) showing cell division. Fixation: glutaraldehyde and osmium tetroxide. × 70,000.

Fig. 4.—Section of a lysed cell of *Cl. kluyveri* (36 hr culture) showing details of polygonal structures (arrow). Fixation: glutaraldehyde and osmium tetroxide. × 115,000.

Fig. 5.—Section of *Cl. kluyveri* (36 hr culture) showing the DNA fibrils of the nucleoplasm (*n*) in a condensed form. Fixation: osmium tetroxide. × 97,000.

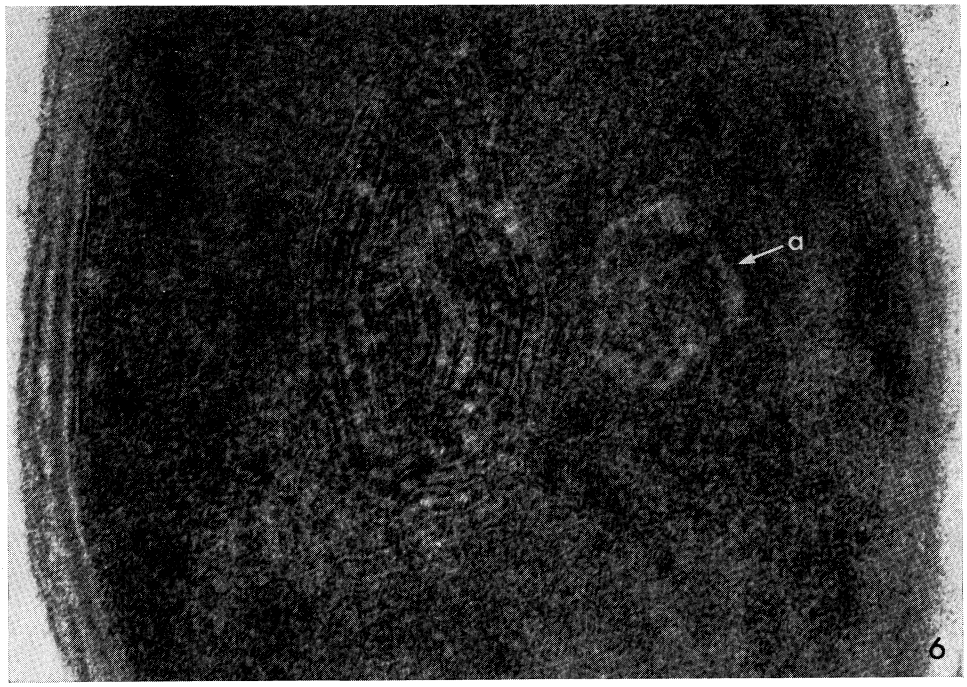

Fig. 6.—Section of *Cl. kluyveri* (36 hr culture) showing an intracytoplasmic rough membrane system at a late stage of development. *a* shows a polygonal structure (see also Fig. 7). Fixation: osmium tetroxide. × 152,000.

Fig. 7.—Section of *Cl. kluyveri* (36 hr culture) similar to Figure 6 but the rough membrane system is cut in a different plane (arrow). Several polygons can be seen. Fixation: osmium tetroxide. × 157,000.

plasmic membranes (Figs. 6 and 7) and polygonal structures (Figs. 4, 6, and 7) are present in the cytoplasm.

Cell division is similar to that of most Gram-positive bacteria in that it is preceded by the formation of a septum in the division plane (Fig. 3). A few spores are present in 60 hr cultures (not illustrated).

(ii) *Cell Wall*

The five alternate electron-dense and light layers of the cell wall have a total thickness varying from 380 to 470 Å. The average thickness of each layer starting from the outermost is 80, 70, 60, 70, and 100 Å. The innermost layer is the most dense and may be thickened at the pole of the cell (Fig. 1). The cell wall is straight by comparison with the undulating cell wall of Gram-negative bacteria such as *E. coli.*

(iii) *Plasma Membrane*

Because of the high electron density of the cytoplasm, the triple-layered plasma membrane is resolved only in very thin sections. Although it shows the dense–light–dense profile and spacing typical of a unit membrane, the outer layer is frequently overlain with dense material (Fig. 1). The outer dense layer of the plasma membrane is straight and thus the light layer separating the cell wall from the plasma membrane is clearly observed in most micrographs.

(iv) *Intracytoplasmic Membranes*

In thin sections, some intracytoplasmic membranes can be seen to blend with the plasma membrane (not illustrated). Intracytoplasmic membranes were not detected by negative staining but this could be due to the failure of phosphotungstate to penetrate the cell wall as for Gram-negative bacteria (Salton 1967). The cells were resistant to plasmolysis. All stages of membrane development were seen, the most highly organized being structures (organelles) like those shown in Figures 6 and 7. Being rough, the membranes resemble the rough endoplasmic reticulum of eukaryotes. The electron-dense particles (80–120 Å) responsible for the rough appearance may represent ribosomes of smaller dimensions (100–200 Å) than is usual for bacterial ribosomes (Petermann 1964). It has been reported that the "70 S" ribosomes of *Cl. kluyveri* are composed of 26 S and 40 S subunits rather than the 30 S and 50 S subunits usually attributed to bacteria (Schachman *et al.* 1952). Our electron micrographs commonly show that the cytoplasm is full of electron-dense particles of similar size and appearance to those associated with the membranes.

The organelles (Figs. 6 and 7) consist of concentric rough membranes (Fig. 6) which may be paired as continuous loops doubled back on each other (Fig. 7). These organelles are associated with an electron-light background resembling the usual appearance of DNA. The periphery of the organelles frequently extends as membranes which eventually blend with the polygonal structures or the plasma membrane or both.

(v) *Polygonal Structures*

These are regions of relatively low electron density, bounded by a single dense layer and found in both intact and lysed cells (Figs. 4, 6, and 7). The internal appearance of these polygons resembles that of DNA which in some examples may be associated with ordered arrays resembling membranes (Fig. 4).

(*b*) *Clostridium acetobutylicum*

(i) *General Morphology*

The envelope consists of a slightly wavy cell wall which is triple-layered and an asymmetric plasma membrane (Figs. 8 and 9). In most electron micrographs the nucleoplasm is diffuse. Micrographs of plasmolysed cells show a condensed nucleoplasm (Figs. 17 and 19). Complex membranous organelles are frequently seen (Figs. 8, 10–12), often in association with the nucleoplasm. Electron-dense particles, presumably ribosomes, are dispersed in the cytoplasm. Some cells have regions of a dense granular appearance (Fig. 8). Cell division is similar to *Cl. kluyveri*. Cells from 60 hr cultures contain membrane-bounded crystalline inclusions (Fig. 13), rows of electron-dense bands (Fig. 14), and spores (not illustrated).

(ii) *Cell Wall*

The triple-layered cell wall is resolved into an undulating outer electron-dense layer (40–60 Å) separated from the inner dense layer (220–240 Å) by a light layer of 40 Å (Fig. 9). In plasmolysed cells, the outer electron-dense layer can be shown to be differentiated into subunits separated by electron-light material (Figs. 19 and 20). The outer electron-dense layer is not always discernible in cells from 60 hr cultures (Fig. 12). This phenomenon is similar to that reported for *Dermatophilus congolensis* (Gordon and Edwards 1963). It is not known whether a progressive dissolution of the cell wall may be related to the production of exoproteinase which is common for clostridia, including this species (Breed *et al.* 1957).

(iii) *Plasma Membrane*

The triple-layered plasma membrane is asymmetric (Figs. 8 and 9). The outer layer is thick (60–80 Å), very electron-dense, and is separated by a light layer of 30–40 Å from the inner dense layer (25–30 Å). The difference in electron density between the layers suggests that these may differ in composition such that the outer layer is more osmiophilic. Asymmetric plasma membranes previously observed in other bacteria (Edwards and Stevens 1963; Murray and Watson 1965) have been attributed either to masking by the cytoplasm of the inner profile (Fitz-James 1965; Glauert *et al.* 1961) or to the presence of heavy metal enzyme complexes in the outer layer (Murray and Watson 1965).

Fig. 9.—A higher magnification of part of Figure 8 showing the fine structure of the cell wall and the plasma membrane. × 190,000.

Figs. 10 and 11.—Section of *Cl. acetobutylicum* with mesosomes (arrow) in different configurations and in association with the nucleoplasm. Arrow *a* of Figure 11 shows a vesicular structure between the cell wall and the plasma membrane. Fixation: osmium tetroxide. × 65,000 and × 51,000 respectively.

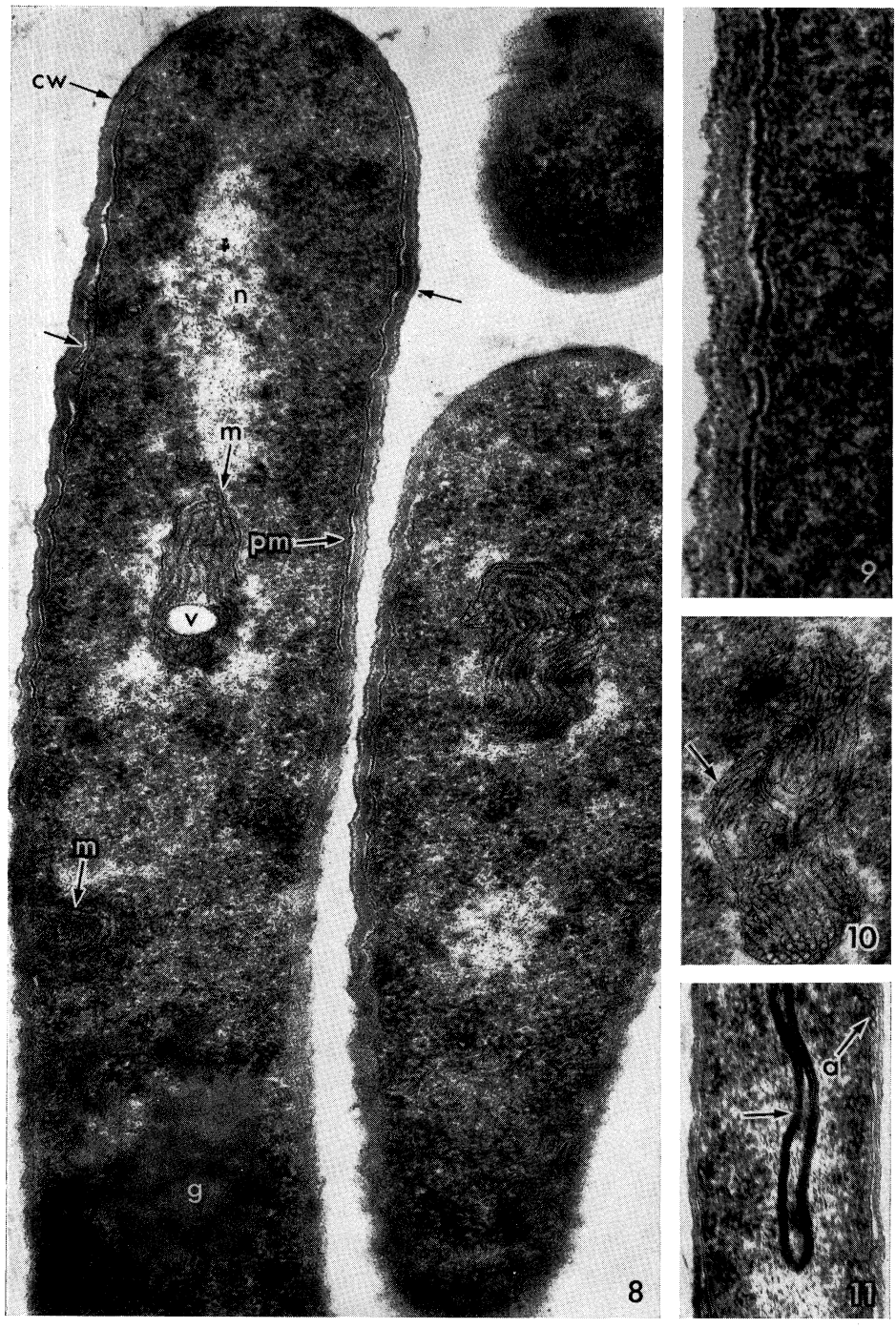

Fig. 8.—Section of *Cl. acetobutylicum* (36 hr culture) showing the triple-layered cell wall (*cw*), the asymmetric triple-layered plasma membrane (*pm*), the granular region (*g*) towards the pole, the nucleoplasm (*n*), and several mesosomes (*m*) in different parts of the cytoplasm. Note the large vesicle (*v*) in one mesosome. Thickening or tubular–vesicular structures (arrow) or both can be seen at some places between the plasma membrane and the cell wall. Fixation: osmium tetroxide. × 57,000.

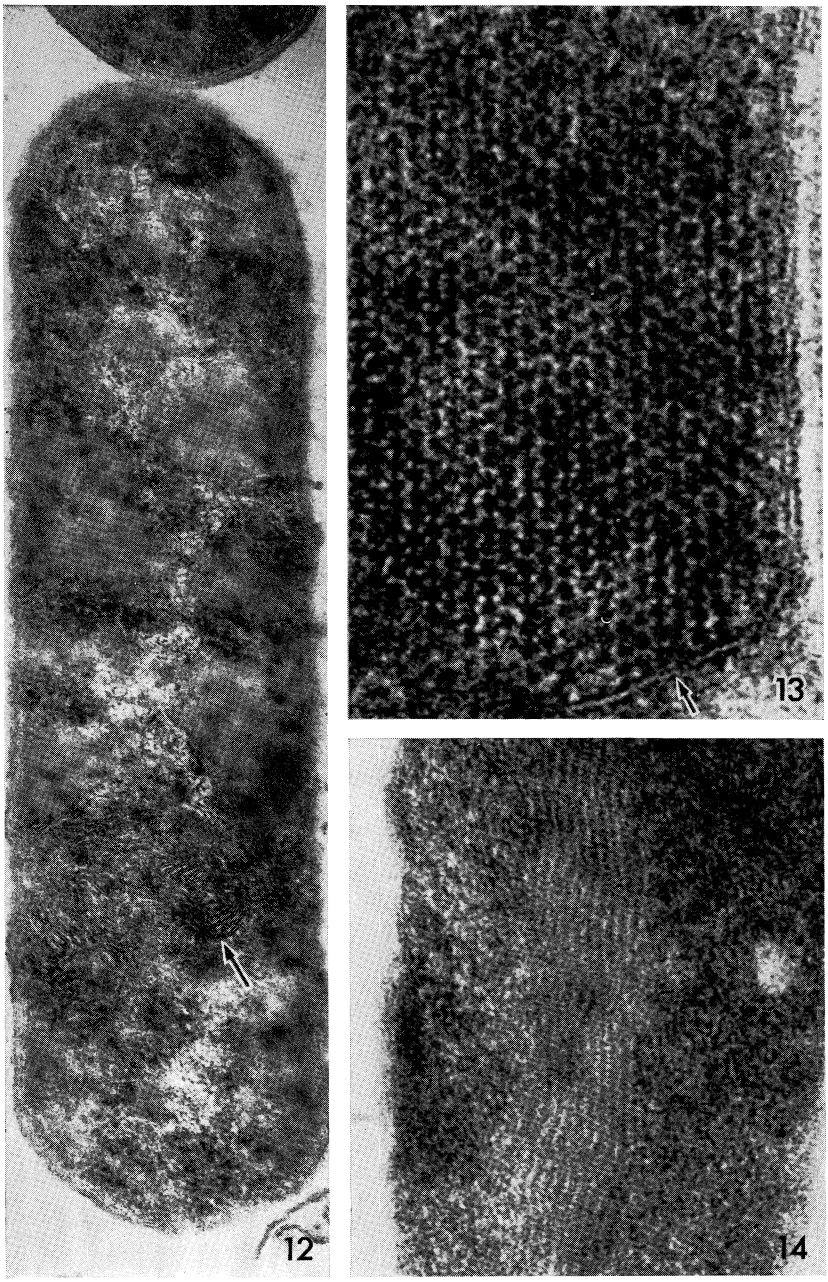

Fig. 12.—Section of *Cl. acetobutylicum* (60 hr culture) showing a reticulated mesosome (arrow). Note also the dissolution of the outer dense layer of the cell wall. Fixation: glutaraldehyde and osmium tetroxide. × 60,000.

Fig. 13.—Section of *Cl. acetobutylicum* (60 hr culture) showing arrays of crystalline inclusion (arrow) bounded partially by a continuation of the plasma membrane. Fixation: glutaraldehyde and osmium tetroxide. × 200,000.

Fig. 14.—Section of *Cl. acetobutylicum* (60 hr culture) showing rows of electron-dense bands. They do not appear to be membrane-bounded. Fixation: glutaraldehyde and osmium tetroxide. × 120,000.

In one micrograph of a plasmolysed cell the outer electron-dense layer of the plasma membrane is clearly separated from the inner (Fig. 20). This suggests that the outer dense layer of the plasma membrane may represent a fusion of both the original layer of the plasma membrane and the innermost component of the cell wall.

(iv) *Intracytoplasmic Membranes*

One or more complex membranous organelles per cell can be seen in most sections, often associated with the nucleoplasm (Fig. 8). Micrographs of plasmolysed cells show that these organelles are bounded by the plasma membrane (Figs. 17–19). In sections of normal cells this membrane is grossly invaginated. Negatively stained cells show that a variable number (8–18) of different-sized organelles are distributed throughout the cytoplasm and may sometimes be associated with cell division (Fig. 16). When appropriately orientated these organelles can be shown to be invaginations of the plasma membrane which is much folded within the cytoplasm (Fig. 15). On this basis they can be termed mesosomes as defined by Fitz-James (1960). In sections the plasma membrane cannot be traced completely round a mesosomal profile and therefore in both plasmolysed and normal cells the membrane is either discontinuous or has variable staining characteristics.

Mesosomes of varied organizations are seen. Their internal structure is often tubular or tubular–vesicular. These tubules form whorls, or complex anastomosing patterns in the cytoplasm (Figs. 8, 10, and 11). They are connected with the thick electron-dense layer of the plasma membrane and contain moderately dense to very dense material, a characteristic seen particularly clearly after plasmolysis (Figs. 19 and 20). In Figure 19 these structures extend completely across the cell.

Large vesicles are common and are bounded by an extremely electron-dense layer frequently with vestiges of an inner, less electron-dense layer. In contrast with the tubular–vesicular structures, the lumen of these vesicles is electron-light (Fig. 8). In 60 hr cultures, mesosomes of a type rarely observed in 36 hr cultures are frequently detected. They form a reticulum very often close to granular regions (Fig. 12).

Unlike *Cl. kluyveri*, structures between the cell wall and the plasma membrane are frequently observed in *Cl. acetobutylicum*. They consist of (1) thickening of the outer electron-dense layer of the plasma membrane (Fig. 8); (2) vesicles (Fig. 11); and (3) tubules (Fig. 8). In plasmolysed cells, they are seen to be connected with both the wall and the thick electron-dense layer of the plasma membrane (not illustrated) and with similar structures within the mesosomes (Fig. 17).

(v) *Membrane-bounded, Electron-dense Inclusions*

Figure 13 shows arrays of interconnected dense particles partially bounded by a continuation of the plasma membrane. The particles (approximately 110 Å in diameter) are separated by regular spacing of 60–70 Å. These structures may themselves consist of subunits. Their dimension and density are similar to the arrays of ribosomes found in higher cells.

Figure 14 shows rows of parallel electron-dense bands, 60–80 Å wide, separated from each other by 60–70 Å. The difference in dimension of the bands as compared with the electron-dense inclusions shown in Figure 13 indicates that these are a different type of inclusion.

IV. General Discussion

These studies and those of others (Takagi *et al.* 1960*a*, 1960*b*; Fitz-James 1962; Hoeniger and Headley 1968; Pope *et al.* 1968; Betz 1970; Smith 1970) show that the morphology of the clostridia varies greatly from species to species. Clearly membranous organelles exist in anaerobes as well as aerobes. In the example of *Cl. acetobutylicum*, the organelles may be appropriately described as mesosomes since they are clearly invaginations of the plasma membrane. The function of the intracytoplasmic membranes remains a matter for speculation except that in the clostridia they cannot be associated with respiration, a function often attributed to the mesosomes (chondrioids) of aerobes. Similarly, since they are found in vegetatively growing cells well before sporulation they are not necessarily confined to the process of sporulation (Fitz-James 1962). As for other organisms, internal membranes may be associated with the process of cell division but the frequency with which they occur within a single cell suggests that they have wider functions. This would be hardly surprising since membranes may provide a basic framework within evolution for the organization of functional units and compartments. The exact form and function would be expected to vary with the environment and the required cellular activity for which there is a selective advantage. Evidence exists in the work of others to support this suggestion (Ellar *et al.* 1967; Beaton 1968).

Of the many structures described the most novel are the rough membranes and polygons of *Cl. kluyveri*. As far as we know rough membranes have not been observed previously in bacteria. In both the clostridia examined the electron-dense particles filling the cytoplasm are smaller than the usual bacterial ribosomes. The closest approximation to the dimensions of *E. coli* ribosomes are the arrays of particles illustrated in Figure 13. Given that the functional ribosomes are probably smaller than those of *E. coli* the roughness of the membranes may be due to ribosomes. Alternatively, or in addition, roughness may represent components of membrane organization necessary for function.

The intracytoplasmic membranes of bacteria are commonly associated with DNA. This has an obvious advantage if genetic information is translated by ribosomes bound or otherwise organized within membranous structures which might also contribute to functional organization. An association between the DNA and mesosomes was frequently observed with *Cl. acetobutylicum*. In the example of *Cl. kluyveri* numerous polygons were seen only in cells containing membranes and lacking regions otherwise identifiable as presumptive nucleic acid.

Fig. 18.—Section of a plasmolysed cell of *Cl. acetobutylicum* (36 hr culture). Tubular–vesicular portions containing electron-dense material have been partly excluded. A discontinuity occurs in the boundary of the mesosome (arrow). Fixation: osmium tetroxide. × 65,000.

Fig. 19.—Section of a plasmolysed cell of *Cl. acetobutylicum* (36 hr culture) showing a tubular–vesicular structure extending across the cell (arrow) without evidence of a cross-wall. These structures extend into the envelope and may be continuous with the mesosome. The nucleoplasm (*n*) is well condensed and, unlike non-plasmolysed cells, is separated from the mesosome. Fixation: osmium tetroxide. × 78,000.

Fig. 20.—Section of a plasmolysed cell of *Cl. acetobutylicum* (36 hr culture). One large and several smaller vesicles (*v*) are seen together with lamellar arrangements of membranes. At one place the outer electron-dense layer of the plasma membrane has detached from the inner electron-dense layer (arrow). Fixation: osmium tetroxide. × 60,000.

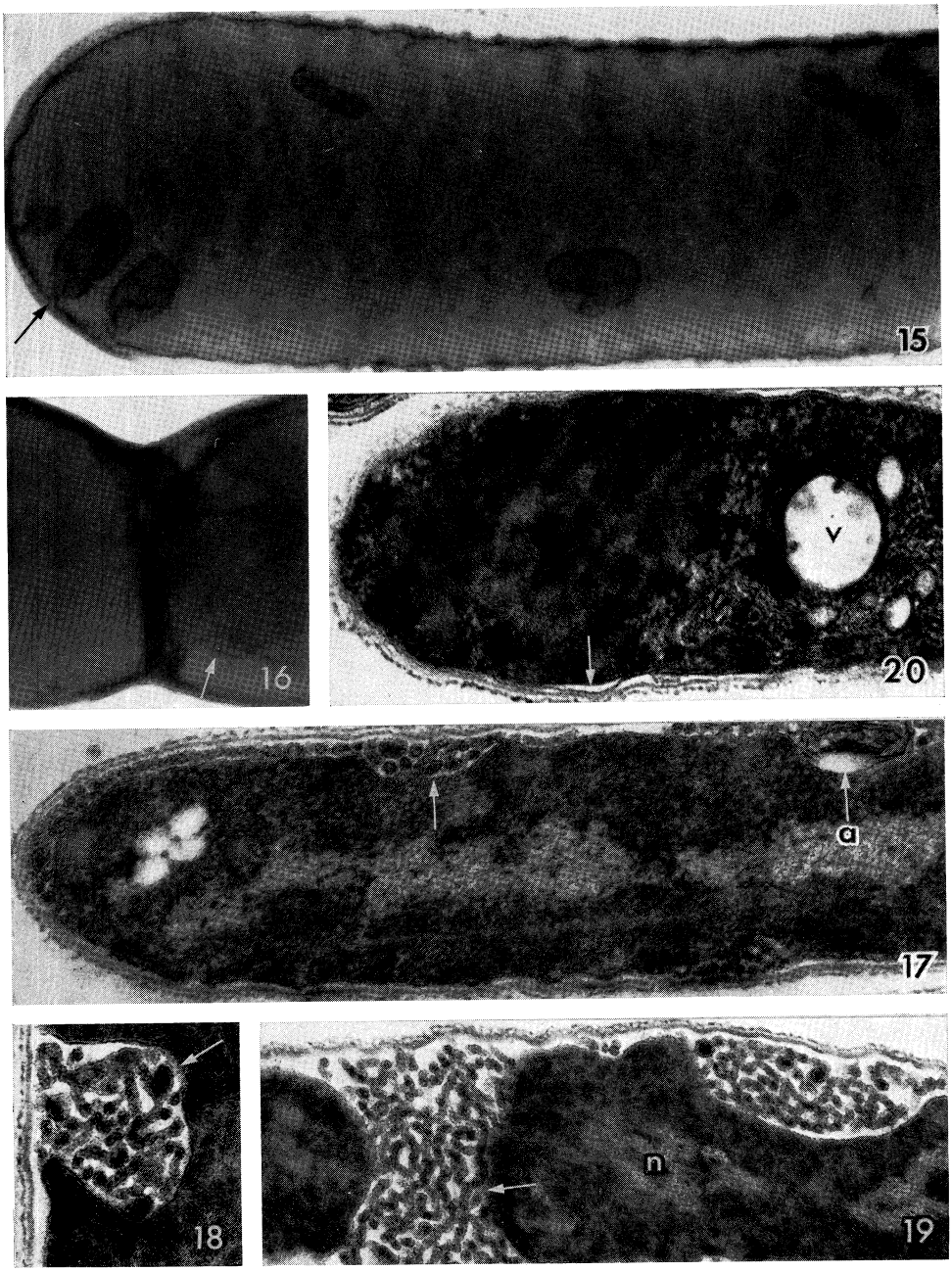

Fig. 15.—Negatively stained preparation of *Cl. acetobutylicum* (36 hr culture) showing many mesosomes of varying size distributed throughout the cytoplasm. One mesosome (arrow) is clearly attached to the plasma membrane by a narrow neck. × 60,000.

Fig. 16.—Negatively stained preparation of *Cl. acetobutylicum* (36 hr culture) showing a mesosome (arrow) connected to the plasma membrane adjacent to the septum. × 60,000.

Fig. 17.—Section of a plasmolysed cell of *Cl. acetobutylicum* (36 hr culture). The tubular–vesicular structure (arrow) extends into the envelope and in at least one place (*a*) a small vesicle has developed and is connected to the envelope by a tube. Fixation: osmium tetroxide. × 60,000.

None of the sections of clostridia revealed polar organelles resembling those we have found in *E. coli* and thought to be concerned with envelope synthesis or the replication and transfer of DNA (Cho and Doy 1972).

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