The Bacterial Cytoplasmic Membrane

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Bacteria are normally regarded as being extremely tolerant to osmotic changes in their external medium (Stuart, Frey & James, 1933). Nevertheless, some particularly Gram-negative organisms harvested in the log phase (Sherman & Albus, 1924) undergo either plasmolysis or plasmoptysis when placed in media containing varying salt concentrations (Winslow & Walker, 1939; Mitchell & Moyle, 1953). Osmotic activity in bacteria is also displayed by their ability to concentrate certain amino acids against concentration gradients (Gale, 1943) and in their selective permeability, particularly towards organic acids (Davis, 1956; Ajl, 1959).

The shrinkage of cytoplasm away from the cell wall, which may be seen by light microscopy during plasmolysis, early led to the prediction that there was a permeability barrier between the cell wall and the inner cytoplasm (Fischer, 1903). From the sensitivity of this barrier to organic solvents and the penetration of lipotrophic material (Eisenberg, 1910) it was also predicted that this barrier consisted of a membrane containing a high lipid content (Overton, 1899). This prediction has been amply confirmed both by the demonstration of a plasma membrane by electron microscopy and from direct chemical analysis of isolated membranes.

Electron microscopy. In ultrathin sections of numerous types of bacteria, a thin osmiumphilic layer is seen sandwiched between the cell wall and the bulk of the cytoplasm (Kellenberger & Ryter, 1958). Where the knife cut is at right angles to this layer it is revealed as a membrane of two dense outer layers each 20–30 Å. wide enclosing a less dense layer 50–80 Å. wide. This double membrane is often more clearly revealed in permanganate- or formalin-fixed material which has been stained with uranyl nitrate after fixation (Pl. 1, fig. 1) (North, 1961; Conti & Gettner, 1962). The dimensions of the cytoplasmic membrane in bacteria are thus similar to membrane structures in animal and plant cells associated with osmotic or secretory functions (Palade, 1956). Measurements of sections of artificially prepared lipoprotein suggest that the clear inner layer is lipid. This is also supported by the heavy staining with uranyl salts. The 50–80 Å. spacing agrees well with that expected from two fatty acid chains each of 15–18 carbons in length (Trurnit & Schidlovsky, 1960; Stockenius, 1960). As seen by electron microscopy dimensions of the bacterial cytoplasmic membrane agree well with the dimensions and structure suggested by Davson & Danielli (1943), for a generalized type of osmotically active cell membrane. That it is the main osmotic barrier is amply confirmed by the osmotic properties of protoplasts and spheroplasts where the cell wall is either wholly or partially removed (Mitchell & Moyle, 1956; Britt & Gerhardt, 1958; McQuillen, 1958).

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Although in most sections the cytoplasmic membrane appears to be firmly attached to, or associated with the cytoplasm, and to separate from the cell wall, this is not always the case. Even in Gram-negative organisms where plasmolysis is more readily demonstrated, the membrane may often remain partly attached to the cell wall and thus detached from the cytoplasm (Chapman, 1959). It is true that cell-wall preparations prepared by prolonged shaking with glass beads (Salton, 1961) are devoid of the enzymic activities and lipoprotein associated with the cytoplasmic membrane. However, when prepared by other methods such as the Hughes press (Hughes, 1951) or French press (Milner, Lawrence & French, 1950) fractions containing both cell wall and the bulk of the membrane are readily obtained both from Gram-negative and Gram-positive organisms, essentially free from cyto-

Fig. 1. Ultrathin section of *Escherichia coli*. An exponentially growing culture of *E. coli* 15T was harvested and fixed 25 hr. by suspension in 10 % formalin in phosphate buffer (pH 7.4). After dehydration in ethanol the cells were placed in 1 % uranyl nitrate in ethanol overnight, washed in ethanol and embedded in butyl methacrylate. Polymerization was carried out at -70° by radiation from a Cobalt-60 source. Electron micrographs were taken in a RCA EMU3B microscope.

plasmic contamination (Hunt, Rodgers & Hughes, 1959). Such preparations when further fragmented, for instance by ultrasonics, yield particulate fractions in which the carbohydrate of the cell wall is still associated with lipoprotein (Hughes, unpublished), suggesting that the membrane may in fact be more firmly associated with the cell wall than the separation during plasmolysis suggests (Mitchell & Moyle, 1956).

Preparation of isolated membranes. Lipoprotein-rich fragments of membranes are commonly prepared by osmotically shocking or bursting by other means sucrose-stabilized protoplasts of organisms such as *Micrococcus lysodeikticus* and *Bacillus megaterium* (Wiebull, 1953). In these organisms, lysozyme removes the bulk of the cell wall (Wiebull, 1958). The isolated fragments (so-called 'ghosts') appear in metal-shadowed specimens to be approximately $500\text{ m}\mu$ in cross section, about $100\text{ \AA}$. thick, and to be free from adhering cell wall and cytoplasm; they nevertheless may contain up to 20 % of their dry weight as carbohydrate, the rest being

mainly lipoprotein. Fractions rich in lipoprotein but containing various amounts of cell wall may also be prepared by similar methods from spheroplasts prepared by lysozyme treatment in the presence of versene (Repaske, 1956). The action of auto-catalytic enzymes (Mitchell & Moyle, 1956), unbalanced growth produced by the action of penicillin or other antibiotics (Lederberg & St Clair, 1958; McQuillen, 1958) or the omission of an essential cell-wall constituent such as lysine or diaminopimelic acid (McQuillen, 1958) have also been used to prepare spheroplasts from which membrane-enriched fractions may be isolated. Protoplast preparations of certain yeasts have been prepared by the action of carbohydrases from *Helix pomatia* (Eddy & Williamson, 1957).

In addition to these chemical and enzymic methods for isolating cytoplasmic membranes, cell fractions can be prepared by mechanical rupture of the cell wall followed by procedures to remove the bulk of the cytoplasm; these contain the bulk of the cell wall and cytoplasmic membrane (Hunt, Rodgers & Hughes, 1959). Such cell-wall membranes prepared from *Pseudomonas fluorescens* were made by rupture of the cells in the Hughes press, treatment with deoxyribonuclease, subsequent emptying by shaking with glass beads and extraction with phosphate buffer. The present author has used this procedure more recently with a wide range of micro-organisms to yield preparations which typically contain the bulk of the cell wall and have an enzymic constitution similar to membrane preparations from protoplasts. Similar preparations may be obtained from cells ruptured in the 'x' press (Edebo, 1960) or the French press (Milner *et al.* 1950) although these presses appear to give greater fragmentation of the wall (Hughes, unpublished). In some cases varying amounts of cell-wall material may be removed from cell-wall membrane preparations by subsequent treatment with lysozyme (Hunt *et al.* 1959) or other carbohydrases (Hughes, unpublished); usually this leads to fragmentation and yields preparations which appear similar to 'ghosts' by electron microscopy but which still consist of particles which contain both lipoprotein and cell-wall carbohydrates.

Although it is obviously of advantage especially for chemical analysis to prepare the cytoplasmic membrane free from cell wall, it is often also advantageous to be able to isolate cell-wall membranes. The membrane in these appears to be structurally intact and easily freed from supernatant fractions containing cytoplasm particles such as ribosomes. These in turn are relatively free from enzymes and other material associated with particles, for instance so-called 'oxidosomes', which are now generally assumed to be comminuted cytoplasmic membrane.

Chemical analysis of cytoplasmic membrane. The most constant feature of cytoplasmic membrane and cell-wall membrane fractions is their high concentration of lipid, up to 30 % of dry weight, which is mostly present as phospholipid. Carbohydrate is also generally present but this depends on the proportion of adhering cell wall. Where the cell wall is absent, amino sugars are absent and the carbohydrate is probably not derived from the cell wall. For instance, in *Micrococcus lysodeikticus*, mannose in contrast to glucose is found in the membrane ghosts. The carbohydrate of cell-wall membrane preparations appears indistinguishable from that of purified cell walls (Hughes, unpublished). The amino acid composition of purified membranes appears similar to cell protein and is unlike that of wall polypeptide in that it contains all the common L-amino acids and no D-amino acids

(Gilbey, Few & McQuillen, 1958). The lipid composition of membranes from *M. lysodeikticus* has recently been analysed more fully by McFarlane (1961*a, b*). About 80 % of the total lipids is phospholipid, the remaining 20 % being neutral lipid, mainly a diglyceride. Of the phospholipid, about 80 % is diphosphatidyl glycerol, the remainder consisting of phosphatidyl inositol and a complex which yields glycerol, mannitol and fatty acids on hydrolysis. The fatty acids in all these fractions were mainly C₁₅ anteiso and iso-acids, an observation which fits well with the 50–60 Å. spacing seen in the electron micrographs. The lipid content of these preparations represented approximately 80 % of that of the intact cell. Cell-wall membranes from *Pseudomonas fluorescens* and *Lactobacillus arabinosus* contain at least 90–95 % of the total cell lipid. The main fatty acids from these organisms are similar to those from *M. lysodeikticus*, and staining reactions suggest they are also present mainly as phospholipid (Hunt *et al.* 1959). In *L. arabinosus* lactobacillic acid can be detected in the membrane extracts and in *P. pseudomonas* an unidentified C₁₇ branched acid was detected (Hughes, unpublished).

Table 1. *Enzymic activities of cytoplasmic membranes and particulate fractions from bacteria*

Organism	Cell fraction	Enzyme activity	Reference
<i>Bacillus megaterium</i>	Membrane and particles	Succinic, malic, lactic oxo-glutarate dehydrogenases, DPNH oxidase	Storck & Wachsmann (1957); Wiebull (1956)
<i>B. stearothermophilus</i>	Membrane and particles	—	Georgi, Miltzer & Decker (1955)
<i>Staphylococcus aureus</i>	Membranes	Succinic, lactic, formic and α -glycerophosphate dehydrogenases, acid phosphatase	Mitchell & Moyle (1956)
<i>Pseudomonas fluorescens</i>	Cell-wall membrane and particles	Nicotinic hydroxylase, succinic dehydrogenase, DPNH oxidase, ATPase, and malic oxidase	Hunt <i>et al.</i> (1959), Hughes (unpublished)
<i>Azotobacter vinlandii</i>	Membrane and particles	Hydrogenase, DPN oxidase, oxidative phosphorylation	Cota-Robles, Marr & Nilson (1958)
<i>Mycobacteria</i> sp.	Particles	TPNH and DPNH oxidase, oxidative phosphorylation, succinic dehydrogenase	Brodie & Ballantyne (1960)
<i>Escherichia coli</i>	Cell-wall membranes	Numerous dehydrogenases, ATPase	Hughes (unpublished)
<i>Lactobacillus arabinosus</i> and other lactobacilli	Cell-wall membranes	ATPase? Hexokinase	Hughes (unpublished)
<i>Alkaligenes faecalis</i>	Membrane and particles	Oxidative phosphorylation, ATPase and dehydrogenases	Shibko & Pinchot (1961); Hughes (unpublished)

The enzymatic function of the cytoplasmic membrane. The cytoplasmic membrane was first established as the site of respiratory enzymes and cytochrome-linked electron transport by studies on highly purified 'ghost' preparations from *Bacillus megaterium* (Storck & Wachsmann, 1957; Wiebull, 1958). Since then a similar localization of enzymes concerned with respiration has been found in many other aerobic organisms (Table 1). In addition to these enzymes in membranes, numerous particu-

late fractions which have a similar enzymic constitution to the membranes have been isolated from mechanically broken cells (Table 1). These particulate enzyme fractions usually consist mainly of lipoprotein granules, are often heterogeneous in size and contain ribonucleoprotein, which, when it can be separated, is usually without enzymic activity (Hunt *et al.* 1959). The enzymic activity associated with the lipoprotein particles and membranes is often difficult to solubilize without destroying their structure. However, by treating them with detergents or other surface-active reagents such as deoxycholic acid (Hunt, 1959), which disassociate lipid and protein, the enzymes sometimes may be brought into solution and further purified. Granules which reduce tetrazolium in whole cells have been regarded as equivalent to animal and plant mitochondria. These have been identified with the isolated lipoprotein granules as 'bacterial mitochondria' (Mudd, Kawata, Payne, Sall & Takagi, 1961). There seems little doubt now, however, that most if not all of such preparations have been derived by comminution of the cytoplasmic membrane from which they can be readily prepared by, for instance, treatment with ultra-sound (Hunt *et al.* 1959). 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 'christae mitochondriales' of animal or plant mitochondria (Green, 1961; Marr, 1960). It would be expected in this case that intact membrane preparations or cell-wall membranes would carry out oxidative phosphorylation. So far this has only been demonstrated in particulate fractions from micro-organisms (Brodie & Ballantyne, 1961; Shibko & Pinchot, 1961). It is possible that this failure to phosphorylate oxidatively is due to either the lack of essential factors present in supernatant fractions. Crude supernatants from disrupted bacteria cannot always be added to the particulate fractions because they contain material and enzymes such as adenosine triphosphatase (ATPase) which interfere with the measurement of oxidative phosphorylation. In the case of cell-wall membranes, the effect of freezing and thawing may also be deleterious.

In addition to enzymes associated with energy production many others have been shown to occur in the cytoplasmic membrane or in particles derived from it. These include enzymes such as formic hydrogenlyase, nicotinic acid and other aromatic hydroxylases and many polyol-dehydrogenases which occur exclusively in microorganisms (Table 1).

Most of the work on membrane and particulate-bound enzymes has so far been done with aerobic organisms, and apart from the demonstration of particulate enzymes such as hydrogenase (Gest, 1954) in *Clostridium pasteurianum*, little work has been reported on the membrane of facultative or obligate anaerobes. Recently cell-wall membranes have been prepared from a number of lactobacilli. These organisms, although obtaining the bulk of their energy by anaerobic glycolysis, nevertheless can utilize oxygen to oxidize substrates such as pyruvate and lactate at low rates. This oxidation is mediated through riboflavin electron-transport systems; cytochromes are normally completely absent. Neither the glycolytic enzymes, with the exception of some weak hexokinase activity, nor the flavo-enzyme systems were found in the cell-wall membranes. The only activity so far found consistently is ATPase. The ATPase in these cell-wall membranes has an unusually low pH optimum (5.2–5.3), is Mg^{2+} -activated but is not activated by *o*-dinitrophenol. It has not so far been found possible to free it from the cell-wall

membrane nor to show definitely that it is associated with the lipoprotein portion of this fraction. However, similar ATPase activity has been demonstrated in membranes prepared from *Pseudomonas fluorescens*, *Escherichia coli*, and *Aerobacter aerogenes*. The enzyme from all these organisms does not liberate on orthophosphate from a wide range of organic phosphates including AMP; a low rate of hydrolysis is found with other nucleoside triphosphates and ADP. The consistent occurrence of an ATPase in the bacteria is of interest in connexion with the suggested role of this enzyme in ion transport and permeability (Whittam, 1961).

The possible role of the cytoplasmic membrane in protein synthesis is discussed by Hunter in this symposium. It is of significance in this connexion that most purified membrane preparations contain about 2 % ribonucleic acid (Hunter, Brookes, Crathorn & Butler, 1959; Nisman & Fukuhora, 1961), and that this also forms a constant feature of cell-wall membrane preparations (Hunt *et al.* 1959). It is difficult to remove this without chemical treatment or disruption of the membrane (Hunt *et al.* 1959).

It seems possible that the cytoplasmic membrane is the site of synthesis as well as of activity of permeases (Kepes, 1960) and other inducible membrane-bound enzymes such as the nicotinic hydroxylase system (Hunt *et al.* 1959). Also of interest in this connexion is the apparent lability of respiratory cytochromes which appear to be firmly bound and almost exclusively in the membrane but yet alter concentration rapidly when oxygen tension in the growth medium is changed (Lenhoff & Kaplan, 1956). Such problems, which are connected with the synthesis of the membrane itself, are open to an experimental approach now that methods for isolating the membrane from a wide range of microorganisms are available.

SUMMARY

A membrane sited between cytoplasm and cell wall forms the permeability barrier in bacteria. It is rich in lipoprotein and in the electron microscope is seen to consist of two parallel outer protein layers between which there is a lipid layer. In aerobic organisms the membrane is the site of cytochrome-mediated substrate oxidation. Oxidative phosphorylation has not been shown to take place in the membrane. In lactobacilli, neither anaerobic glycolysis nor flavine-linked oxidation takes place in the membrane. The membranes from most organisms so far examined have a firmly bound characteristic ATPase. The intact cytoplasmic membrane may thus be considered as similar in function, structure and chemical composition to the cristae of animal and plant mitochondria. Particulate fractions isolated from mechanically disrupted cells often mislabelled 'bacterial mitochondria' are probably comminuted membrane.

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