THE FORMATION OF MULTIPLE LAYERS OF MEMBRANE-LIKE STRUCTURES IN *ESCHERICHIA COLI* B

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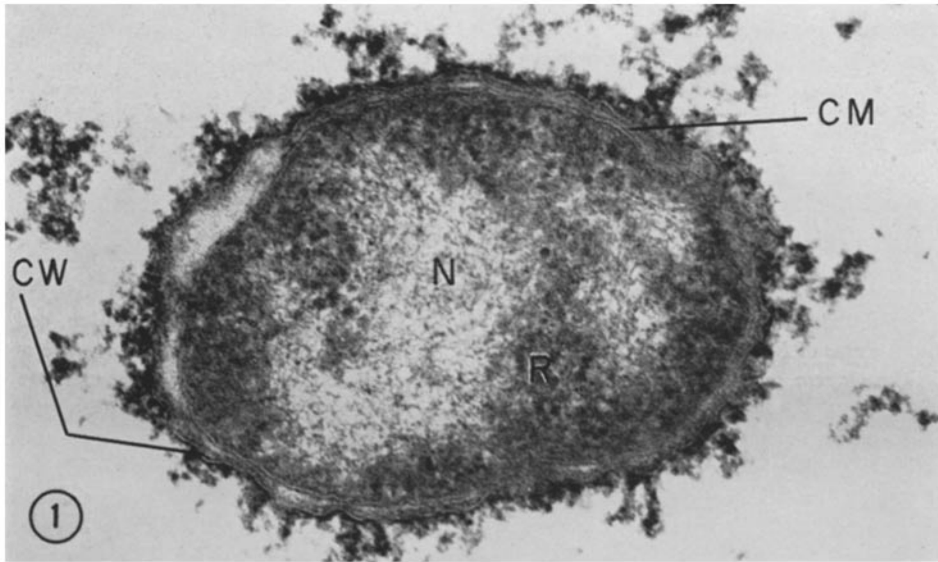
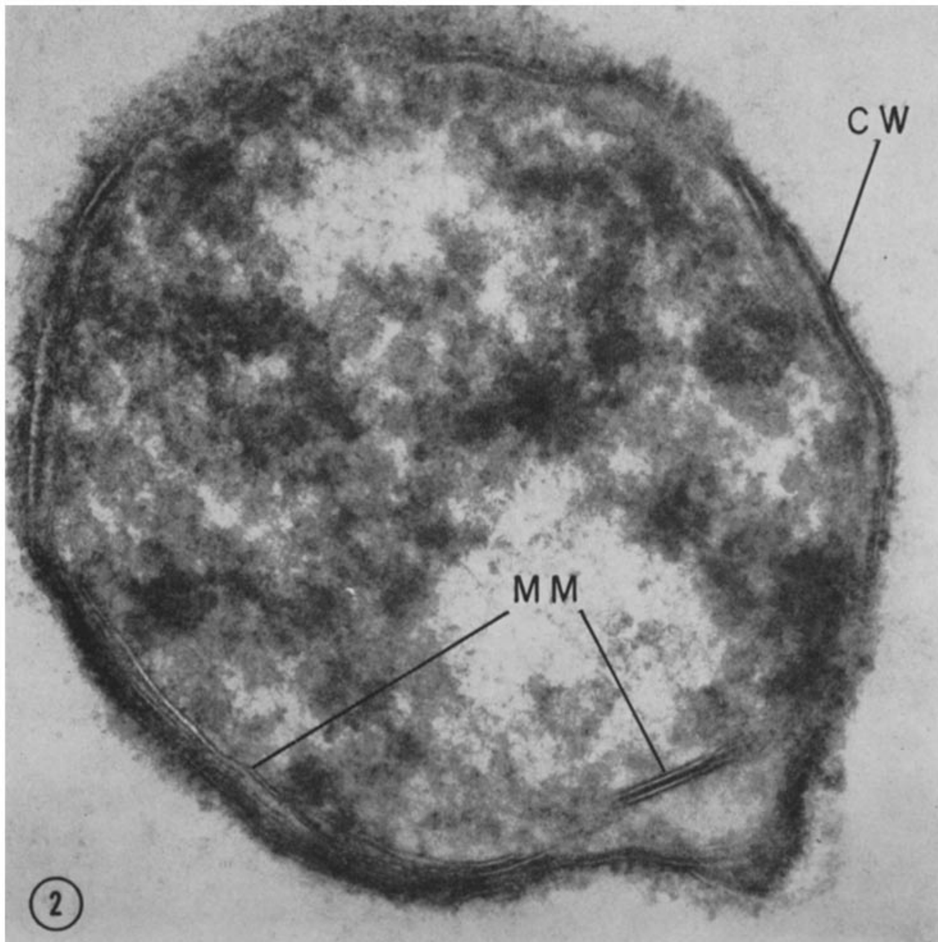
Intracellular membranes in both plants and animals have been recognized for a long time as playing an important role in cellular metabolism. Membranes have been implicated clearly in ion transport (1), protein synthesis (2), and oxidative phosphorylation (3). Recently, increasing attention has been drawn to the presence and possible functions for membrane systems in bacteria (4–7). Although intracellular membranes, termed mesosomes, have been documented well in most Gram-positive organisms, and have been seen in some Gram-negative bacteria (6), their presence in *Escherichia coli* has not been well established (8–14). There are available biochemical and electron microscopic data linking bacterial membranes to DNA replication (15, 16), protein synthesis (17, 18), cell division (5, 19), and oxidative phosphorylation (6, 11, 20–22). To better understand the synthesis, assembly, structure, and function of bacterial membranes, it would be useful to have a system in which the formation of membranes along with membrane-associated enzymes could be reversibly induced and correlated by biochemical and electron microscopic methods. It is the purpose of this report to present evidence for such a system, using *Escherichia coli.*

Stock cultures of *E. coli* B were grown in a minimal salt-glucose medium (synthetic medium). As

FIGURE 1 An electron micrograph of *Escherichia coli* B after growth in the synthetic medium (minimal medium) which demonstrates the typical morphology of this organism. The triple-layered cell wall (*CW*) and cell membrane (*CM*) can be clearly resolved. Nucleoplasm (*N*) and ribonucleoprotein (*R*) areas are labeled. Ryter-Kellenberger fixation; Araldite embedding; phosphotungstic acid, uranyl acetate, and lead citrate staining. ×100,000.

FIGURE 2 The same strain of *E. coli* following growth in the enriched medium. This bacterium has been magnified to the same degree as that in Fig. 1; thus, the large size differential can be readily appreciated. In addition to the increase in cell diameter, numerous triple-layered, membrane-like structures (*MM*) are seen beneath the cell wall (*CW*). Same electron microscope techniques as used for the cell in Fig. 1. ×100,000.





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previously described (23), when these bacteria are transferred to an enriched medium containing 0.75% (w/v) NaCl, 1% (w/v) L-lysine hydrochloride, 2% (w/v) glucose, 2.4% (w/v) nutrient broth, and 5% (w/v) Hycase (Sheffield Chemical Company, Norwich, N.Y.), their characteristically short, rodlike shape is markedly altered and the bacteria grow into long (50 μ or more) filaments which are often branched. Preliminary information on the fine structure of these modified cells, as revealed by electron microscopy, has been presented (24, 25); and the evidence is clear that the long, filamentous forms of *E. coli* B are single, large cells in which transverse septal formation apparently is inhibited.

For electron microscopic examination the bacteria in late log–phase growth were fixed by the method of Ryter and Kellenberger (26), and embedded in Araldite. Thin sections were stained sequentially with phosphotungstic acid, uranyl acetate, and lead citrate; specimens were examined in an AEI EM 6B electron microscope at an instrumental magnification of 20,000.

A representative bacterial cell grown on synthetic medium is presented in Fig. 1. This bacterium illustrates the characteristic morphology of *E. coli* as reported by previous authors (8, 9, 28, 29). Moving inward from outside the cell, the following structural features are encountered: (*a*) a densely staining, granular coating which may represent a combination of absorbed, precipitated medium and the outermost layer of the cell; (*b*) a triple-layered structure, which has been shown to be part of the cell wall (8, 27–29); (*c*) a pale staining, variably spaced layer which contains the innermost part of the cell wall (27–29) and a space produced by cell shrinkage during preparation for electron microscopy; (*d*) a triple-layered, unit membrane, which is the cell or plasma membrane (8, 28, 29); (*e*) cytoplasm containing densely stained and tightly packed material rich in ribonucleoprotein which irregularly encloses the nucleoplasm network or nucleoid (30).

In Fig. 2, a bacterium grown in the enriched medium is presented. The major morphological differences with respect to the cell grown in synthetic medium are its large size, multiplicity of internal membrane-like structures, clumped ribonucleoprotein material, and more loosely woven nucleoplasm. In certain areas, at least four or five lamellae of triple-layered structures are visible, giving a myelin-like appearance beneath the cell wall. Although the intracellular content of the large, filamentous cells appears less densely packed, biochemical assays of these bacteria indicate that the DNA: protein ratio is essentially identical with that of normal *E. coli* B, whereas the RNA content relative to protein or DNA is higher than in normal cells (25). This is expected for cells growing in enriched medium (32). Perhaps some of the intracellular material within the filamentous forms is lost or extracted during the preparative techniques for electron microscopy.

In Gram-negative cell wall isolation, cell walls and cell membranes usually adhere and are isolated together (31). These wall-membrane complexes, hereafter termed cell envelopes, have been isolated during late log–phase growth from *E. coli* B grown in both synthetic and enriched media. Envelopes have been isolated by vigorous shaking with glass beads using a Shockman head in a refrigerated International Centrifuge (International Equipment Company, Boston), and separated from soluble components by differential centrifugation (23). Following 12 washes in distilled water the enzyme profile of the envelopes was compared to the supernatant solution to see whether the altered morphology is paralleled by a modified enzyme constitution. The data are presented in Table I. It is apparent that a five-fold

TABLE I

Specific Activity of Enzymes Isolated from Escherichia coli B

Enzyme assayed	Normal form		Filamentous form	
	Envelope	Supernatant	Envelope	Supernatant
$NADH_2$ oxidase*	12.0	4.0	65.0	5.5
ATPase‡	0.73	0.42	0.85	0.31
Glucose-6-P dehydrogenase*	0.97	42.4	9.6	19.8
Aldolase*	0.00	9.8	0.00	7.6
Alkaline phosphatase§	0.0	0.0	0.0	0.0
Acid phosphatase§	0.82	3.2	0.21	2.1
Creatine phosphokinase*	2.0	7.9	3.6	9.2
α-Hydroxybutyrate dehydrogenase*	0.00	2.7	0.00	0.61

* mμmoles $NAD(H_2)$ reduced (oxidized)/min/mg protein.

‡ μmoles P_i liberated/hr/mg protein.

§ Bessey-Lowry Units/hr/mg protein.

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higher specific activity occurs in the filamentous forms of $NADH_2$ oxidase, an enzyme known to be membrane bound (6), than occurs in those envelopes isolated from normal *E. coli* B. In contrast, the specific activity and distribution of most soluble enzymes are not changed during growth in the enriched medium. An exception is glucose-6-PO_4 dehydrogenase; only 2% of the specific activity of this enzyme is envelope-bound in normal cells, while in the filamentous forms 30% is associated with the envelope fraction. Whether this is a reflection of altered membrane structure in the abnormal cells is presently being investigated. The alkaline phosphatase appears to be completely repressed in both media. We have additional evidence that the "extra" membranes in the filamentous forms may be defective or at least different from normal *E. coli* membranes. In this regard the phospholipid distribution and fatty acid composition (33) of the filamentous form is appreciably different from the composition in the normal cell form. The significance of the altered concentrations and/or distributions of the acid phosphatase and alpha-hydroxybutyrate dehydrogenase in the normal and filamentous cells is of interest to us. The fact that aldolase and alpha-hydroxybutyrate dehydrogenase are distributed 100% in the cell supernatant fraction suggests that the soluble enzymes have been removed from the envelopes, and those still adhering to the particulate fraction may be so bound in vivo.

On the basis of both the biochemical and the electron microscopic data presented above, it is suggested that *E. coli* B, when grown in the enriched medium, synthesized both an increased amount of intracellular membrane and an increased amount of certain membrane-associated enzymes. The fact that filamentous *E. coli* B revert to their normal morphology when placed back in synthetic medium (23) suggests that the metabolic alterations also are reversible. An electron micrographic study of these reverted cells is planned, for it would be interesting to know whether the additional membranes in the filamentous forms are resorbed or reutilized when *E. coli* returned to their normal shape.

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