STUDIES ON THE OSMOTIC PROPERTIES OF *Escherichia coli* AND *Azotobacter vinelandii*

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MOHAMMAD MEHDI ALEMOHAMMAD M.Sc.

BIOLOGICAL LABORATORIES

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PUBLICATIONS

Osmotically Induced Volume and Turbidity Changes of *Escherichia coli* due to Salts, Sucrose and Glycerol, with Particular Reference to the Rapid Permeation of Glycerol into the Cell.

Alemohammad, M. M. and Knowles, C. J. (1974)
J. Gen. Microbiol. *82*, 125 - 142

Summary

Increases in turbidity of suspensions of *Escherichia coli* strain K12 in dilute buffer due to added non-permeant salts (NaCl and $MgCl_2$) and sucrose are strictly dependent on the medium osmotic pressure, when correction is made for changes in medium refractive index. The volume of the whole cell and the fraction of the intact cell bounded by the cytoplasmic membrane have been measured by dextran and (^{14}C) sucrose exclusion spaces respectively. Increases in medium osmotic pressure due to non-penetrant medium solutes (salts and sucrose) cause outflow of water across the cytoplasmic membrane and contraction of it from the cell wall (plasmolysis). The increases in turbidity exactly correspond to the volume changes of the fraction of the cell bounded by the cytoplasmic membrane. In addition salts (NaCl and $MgCl_2$) but not non-electrolytes (sucrose) cause appreciable contraction in volume of the whole cells. Experiments on isolated cell walls of *E. coli* shows that this contraction in volume of the whole cells is due to binding of the ions to wall polymers, including the peptidoglycan layer, and the concomitant release of protons. The peptidoglycan layer of the cell wall is thus an elastic rather than a rigid layer. On the other hand, sucrose causes only marginal decreases in whole cell volume. Electron micrographs of *E. coli* plasmolysed by NaCl or $MgCl_2$ but not sucrose, show numerous points of adhesion between the wall and the cytoplasmic membrane.

Glycerol penetrates *E. coli* to the same extent as water but, because of its slower rate of penetration, transient

decreases in volume and hence corresponding transient increases in turbidity occur, on addition of high concentrations of glycerol to the medium, which can be measured in a stopped-flow spectrophotometer. In cells grown on a glucose-containing medium the rate of glycerol penetration is non-saturating. In cells grown on glycerol an additional saturating facilitated diffusion system is induced. Mutants deleted in the glycerol facilitator protein (F^-) are available (see ref. 135). These mutants do not show facilitated diffusion when grown on glycerol.

Both water exit and glycerol penetration in glucose-grown cells of *E. coli* show transition points in Arrhenius plots corresponding to phase changes of the membrane lipids. The rate of penetration of glycerol and the transition temperature depend on the fatty acid composition of the membrane. Increases in the degree of unsaturation of the membrane fatty acids cause decreases in the observed transition temperatures of water exit and glycerol penetration.

In bacteria pre-loaded with glycerol, which are then diluted into media containing no glycerol there is a rapid loss of the glycerol into the medium. However since the rate of glycerol loss is less than the rate of water uptake, there is a transient increase in intracellular osmotic pressure. Thus massive water uptake could occur, resulting in cell lysis.

Azotobacter vinelandii, which has a less rigid and presumably less strong cell wall than *E. coli* can be lysed by such glycerol osmotic shock treatment, causing release of the soluble cytoplasmic components together with a crude envelope fraction which can be easily separated at low centrifugal forces.

The sidedness of the relatively undamaged membrane vesicles derived by glycerol osmotic shock of *A. vinelandii* has been studied by (1) the effect of ion-translocating antibiotics on the rate of respiration; (2) glucose transport; (3) NADH-ferricyanide reductase activity. These data show that vesicles are mainly inverted because, (a) addition of ion-translocating antibiotics causes increases in the rate of respiration in the presence of K^+, indicating that K^+ was changed for H^+ which had been pumped into the inverted vesicles, (b) the rate of glucose uptake was relatively low compared to vesicles prepared by lysozyme-EDTA, which are reported to be mainly right side out [66,119,161], and (c) addition of organic solvents (e.g. toluene) causes only a slightly stimulatory effect on the rate of NADH-ferricyanide reductase or NADH oxidase activities, indicating that enzymes which are normally asssociated with the inner surface of membrane in intact cells, are located on the outer surface. After glycerol osmotic shock treatment a number of membrane fragments appear to remain bound to fragments of the cell wall.

After further degradation by sonication, crude envelopes of *A. vinelandii* prepared by osmotic shock treatment can be separated by sucrose density gradient centrifugation to give two distinctive fractions. The heavier band has the characteristic morphology and chemical composition of the cell wall and the lighter band consists of uniform closed vesicles with the characteristic morphology and composition of cytoplasmic membrane. There is very little cross contamination of the wall and cytoplasmic membrane fractions.

ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
CoQ	Coenzyme Q
Cyt	Cytochrome
DAP	Diaminopimelic acid
DCPIP	Dichlorophenol indophenol
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
DNP	2,4-Dinitrophenol
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FP	Flavoprotein
KDO	2-Keto-3-deoxyoctonic acid
LPS	Lipopolysaccharide
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
PMS	Phenazine methosulphate
RNA	Ribonucleic acid
RNAase	Ribonuclease
TCA	Tricarboxylic acid cycle
TPN(H)	Triphosphopyridine nucleotide (reduced)
Tris	Tris (hydroxymethyl) aminomethane

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CHAPTER 1

INTRODUCTION

Section 1

The Cell Envelope of Gram-negative Bacteria

The cell envelope of Gram-negative bacteria is complex both in morphology and chemical composition, though in early studies on the envelope of Gram-negative bacteria only a single dense surface layer was observed[1]. The first demonstration that the plasma membrane and the cell wall exist as two separate layers was given by Kellenberger and Ryter[2].

When sectioned preparations of Gram-negative bacteria are examined by electron microscopy the morphology of each layer can be identified[3,4]. (Figs. 1 and 2). The basic structure of the cell envelope of Gram-negative bacteria consists of, an inner membrane (cytoplasmic membrane), an outer membrane and an intermediate dense layer between the two (murein) whose chemical composition and physical nature differ very markedly[3,5-7].

The outer membrane is 60Å-100Å thick, the intermediate layer has a thickness ranging from 30Å-80Å, and the inner membrane has a thickness of 75Å-80Å. The region between the outer membrane and the intermediate layer contains protein granules, and this region together with the peptidoglycan layer (murein), according to Costerton *et al* [8], is usually called the periplasmic area (Fig. 3).

The structure, components and morphology of Gram-negative bacterial cell envelopes differ from those of Gram-positive bacteria. In Gram-positive bacteria, cell walls appear to be fairly homogenous with thickness ranging from 150Å-800Å [1].

The walls of Gram-positive bacteria are thus apparently not as morphologically complex as those of Gram-negative bacteria. They

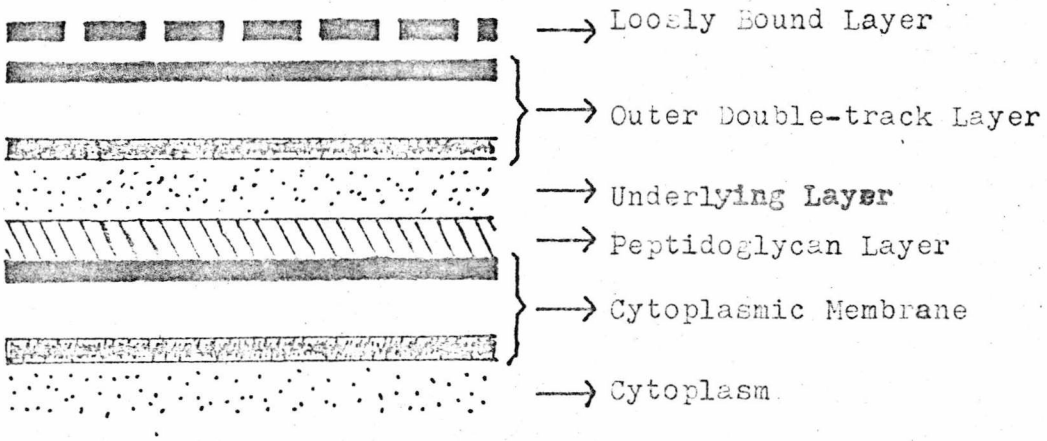


Fig.1. Model of the cell envelope of marine *Pseudomonad B-16* (Taken from ref.3)

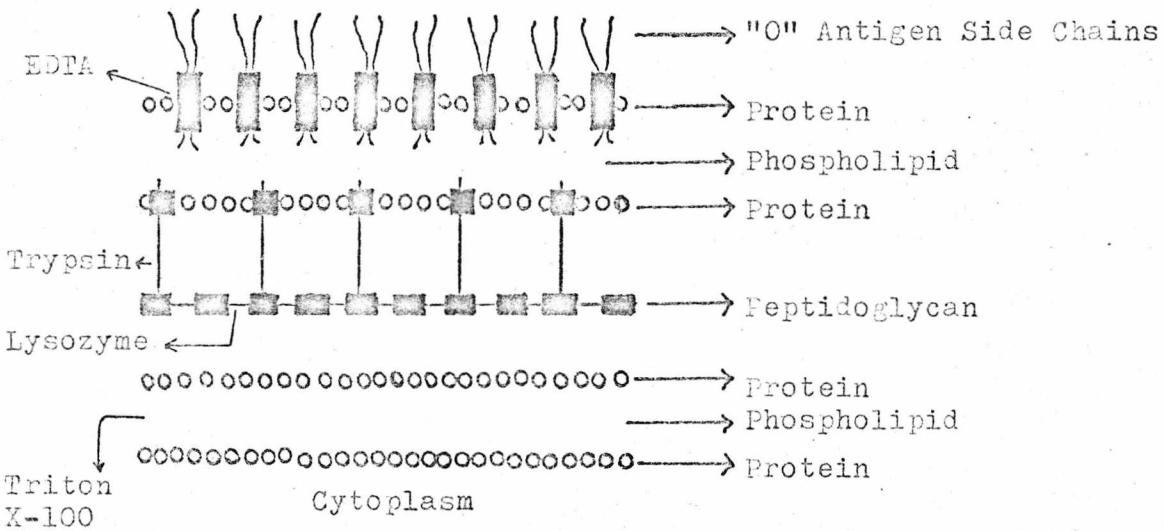


Fig.2. Simplified model of the cell envelope of *Escherichia coli*, various components of the envelope are listed on the right; probable sites of attack of various disruptive agent are indicated on the left.
(Taken from ref.4)

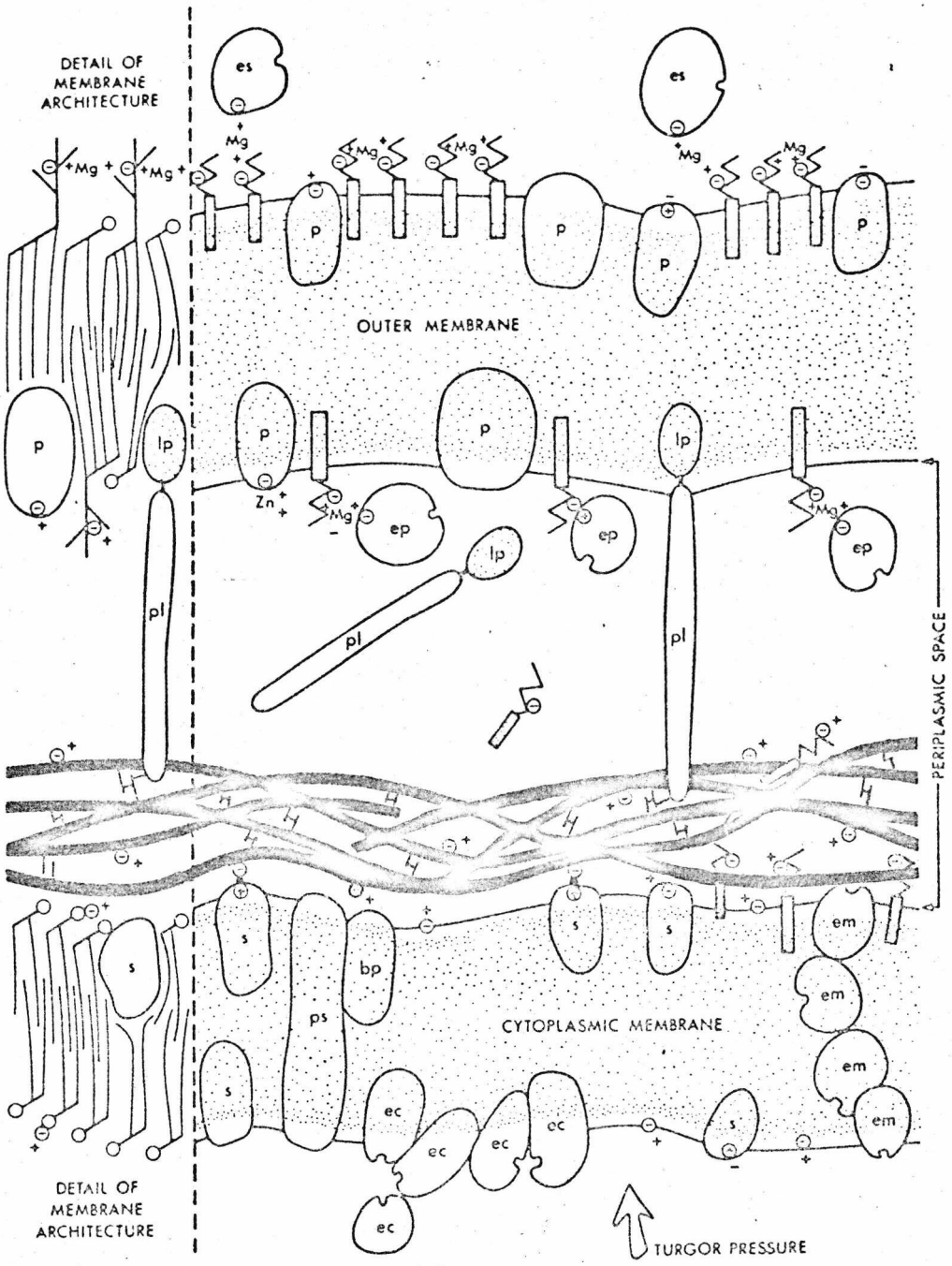


Fig. 3. Schematic diagram of Gram-negative cell envelope. +, Free cation; −, free anion; ⊕ bound cation; ⊖ bound anion; adhesion point produced by ionic bonding; hydrophobic zone; covalent bond; cross-linking polypeptide in the peptidoglycan; polysaccharide portion of peptidoglycan; enzymatically active protein; phospholipid; lipopolysaccharide; lipopolysaccharide (schematic); bp, binding protein; ec, enzymes associated with the cytoplasmic membrane whose function is directed to the cytoplasm; em, enzymes associated with the cytoplasmic membrane which synthesize macromolecular components of the cell wall; ep, enzymes localized in the periplasmic zone; es, enzymes localized at the cell surface; lp, lipid portion of Braun's lipoprotein; p, structural and enzymatic proteins of the outer membrane; pl, protein portion of Braun's lipoprotein; ps, permease; s. structural protein of cytoplasmic membrane. (Taken from ref.8.)

contain a small variety of amino sugars, peptidoglycan which may comprise up to 90% of the dry weight of the walls, protein, polysaccharide, lipid and teichoic acids[1,5,8,9].

A number of the essential features of the comparative anatomy of the surface structures of Gram-positive and Gram-negative bacteria are summarized in Fig. 4.

The cell envelopes of Gram-negative bacteria can generally be studied as three distinct entities :-

1 - Cytoplasmic Membrane

Biological membranes have been the subject of many reviews and articles [8,10-13], and will be discussed briefly here.

Biological membranes are not only barriers between aqueous phases, but also, in bacteria, involved in many biological processes including cell wall synthesis, respiration, transport and phosphorylation. The membrane consists of protein, lipid, nucleic acid and bound carbohydrate as glycolipid and glycoprotein.

Electron micrographs of biological membranes after fixing with osmium tetroxide show that they are trilaminar in structure, i.e. two dark lines, in which the fixing medium has deposited, separated by central light zone[12].

In 1935 Danielli and Davson[14] proposed a membrane model. According to their model a central lipid layer is separated on both sides from the surrounding medium and the intracellular space by protein layers. This proposal was then modified in the "unit membrane" hypothesis of Robertson [15], who suggested that the polar groups of lipid molecules are

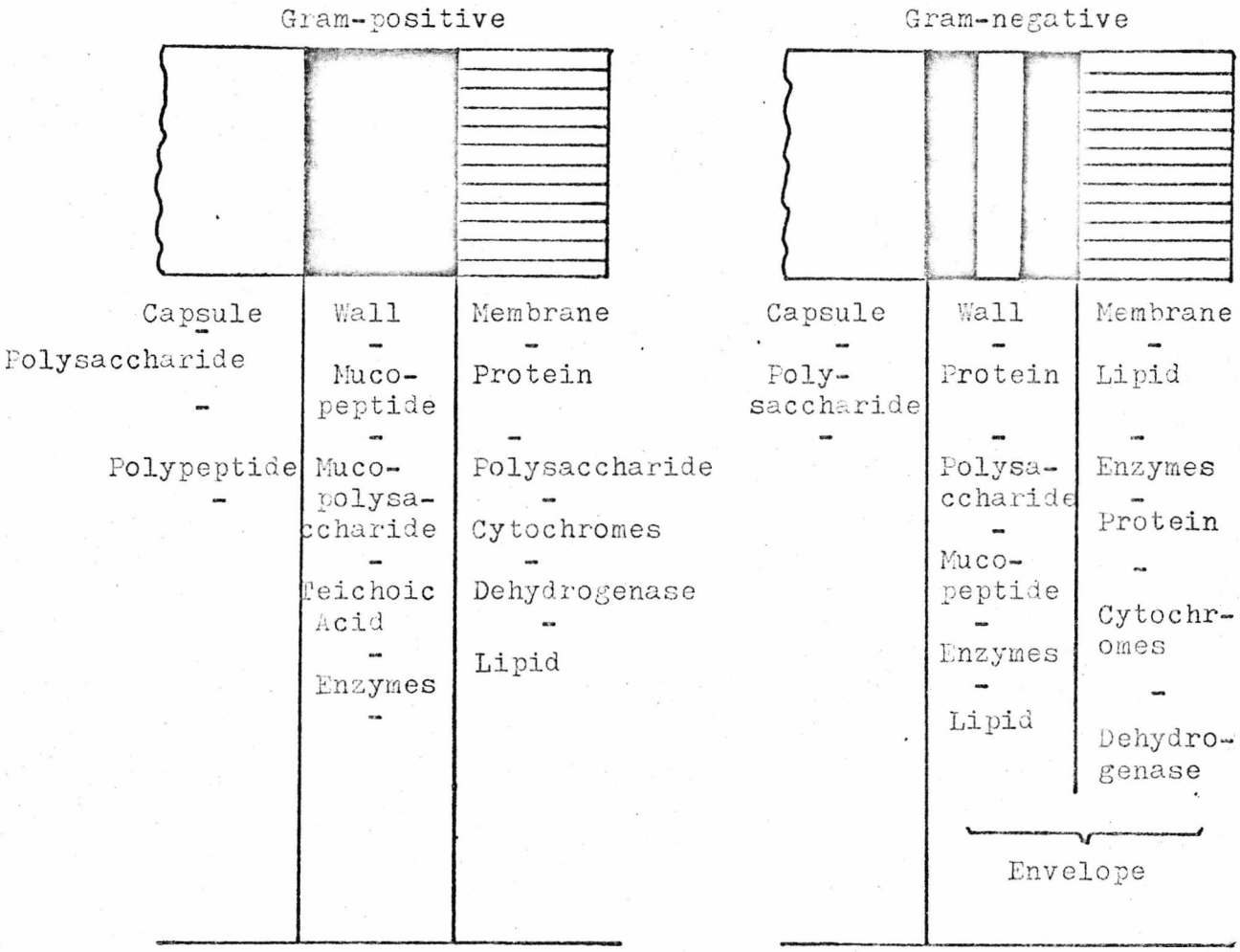


Fig. 4. Comparative anatomy of the surface structures of Gram-positive and Gram-negative bacteria. (Taken from ref. 5).

directed outward and the nonpolar parts of the molecules are turned toward the inside of the membrane, with each surface coated by protein molecules (Fig. 5).

In 1966 Lenard and Singer[16] proposed the lipid-globular protein mosaic model of membrane structure, summarized in Fig. 6. This theory was later modified, and Singer and Nicolson[13] and Singer[17], suggested the fluid mosaic model. According to this model, globular molecules of the integral protein are postulated to be asymmetric with one highly polar end and one nonpolar end, the high polar region is believed to be in contact with aqueous phase in intact membranes, and the nonpolar region is embedded in the hydrophobic interior of the membrane. The globular proteins thus float ("icebergs") semi-submerged, in a matrix of phospholipid. The bulk of phospholipid is organized as a discontinuous fluid bilayer. The membrane-associated proteins are divided into two categories[13]. (A) peripheral proteins, which can easily be dissociated molecularly intact from the membrane, e.g. by chelating agents, and in the dissociated state they are relatively soluble in neutral aqueous buffers. These proteins are thought to be held to the membrane lipid by rather weak interactions, e.g. cytochrome c which can be dissociated free from mitochondrial membrane lipids by high salt concentrations. (B) integral proteins, usually present as more than 70% of the total membrane proteins, and which require much more drastic treatment for dissociation from the membrane. If isolated, they remain associated with lipid. Integral proteins are usually insoluble in neutral aqueous buffer (if completely free of lipid), and it is assumed that they are structural proteins. Structural proteins are heterogenous with respect to molecular weight and are involved with membrane lipid.

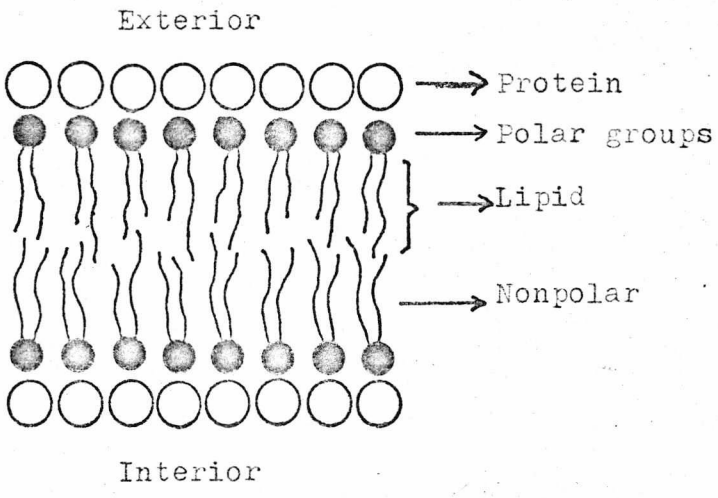


Fig. 5. Schematic cross-sectional view of membrane, the filled circles represent the ionic and polar head groups which make contact with water; the wavy lines represent the fatty acid chains. (Taken from ref.12,13).

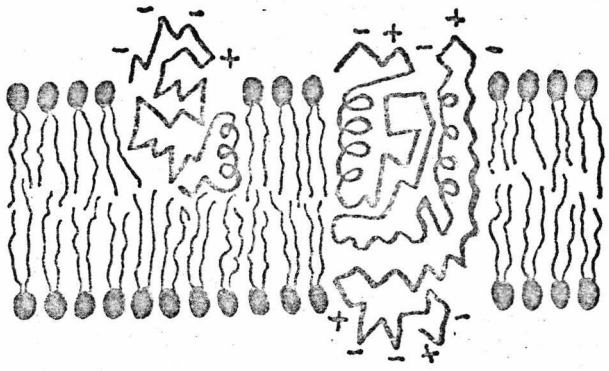


Fig. 6. The lipid-globular protein mosaic model of membrane structure: schematic cross-sectional view. The phospholipids are depicted as in Fig. 5, the integral proteins with the heavy line representing the folded polypeptide chains are shown partially embedded in, and partially protruding from the membrane. The protruding parts have on their surfaces the ionic residues (- and +) of the protein, while the nonpolar residues are largely in the embedded parts (Taken from ref. 13).

It has been postulated[13] that integral proteins are amphipathic like phospholipids i.e. with polar and nonpolar groups (Fig. 6).

Bacterial cytoplasmic membranes have a similar gross chemical composition to that of other biological membranes[18,19].

2 - Cell Wall

The cell walls of Gram-negative bacteria have a mechanically strong structure which provides physical protection for the cells.

Since bacteria are often exposed to different conditions of osmotic pressure and the cytoplasmic membrane is an osmotic barrier, movement of water across the cytoplasmic membrane in hypertonic conditions causes shrinkage of the cytoplasmic membrane away from the cell wall (plasmolysis) (cf. Chapter 1 Section 3). Conversely, excessive uptake of water under hypotonic conditions should lead to explosion (lysis) of the cell. However the mechanically strong cell wall prevents this from occurring, rather in the manner of a car tyre plus inner tube.

Electron micrographs of sectioned *E. coli* cell walls show the presence of the two distinctive layers, (1) the outer double-track layer and, (2) the shape maintaining inner part (murein). These two distinct layers are separated by protein granules[8].

A - The Outer Membrane

The outer layer of Gram-negative bacteria consists of phospholipid, protein and lipopolysaccharide (LPS). Studies on this layer have shown that the proteins and phospholipids of this layer form a bilayer which produce a double track identical in appearance with other biological membranes[20]. The basic structure of this layer is formed by protein and phospholipid, and the oligosaccharide part of the LPS appears to be associated with both the outer and inner surfaces[4,21], (Fig. 3).

This layer is a barrier which acts as molecular sieve[8,11,22-25], e.g. lysozyme (MW 13,930) cannot penetrate the cell walls of intact Gram-negative bacteria and can only affect the murein when the outer layer is breached by EDTA[23], and actinomycin D (MW 1,250) is excluded by intact cell walls of wild-type *E. coli*[25].

The complex LPS, present in the outer layer region of the cell envelope have been intensively studied[9,26-30]. Generally LPS can be divided into three regions (Fig. 7).

(1) - The "o" specific side chains which are responsible for serological specificity. The "o" antigenic side chains consist of oligosaccharide repeating units, and the type of linkage involved determines the serological specificity of LPS.

(2) - The "core" region, consists of sugars together with ethanolamine and phosphates. Among the many species of Gram-negative bacteria that have been studied, the LPS core structures are very similar; but the structures of polysaccharide side chains differ between species and between some strains of a single species. The core structure contains two chemical compounds that do not occur elsewhere in the wall; that is 2-Keto-3-deoxyoctonic acid (KDO) and heptose. Biosynthesis of the core takes place by the sequential addition of basal sugars[9].

(3) - A covalently bound lipid, termed lipid A joined to the core structure part of LPS. Lipid A appears to be very similar in many Gram-negative bacteria, and consist of glucosamine, fatty acids and phosphate. The glucosamine is replaced by galactosamine in some organisms[9].

LPS contains both hydrophilic and hydrophobic regions. Electron microscopy of purified LPS showed a uniform population of hollow spheres[27], ribbon shaped or discs[9,29], (Fig. 8).

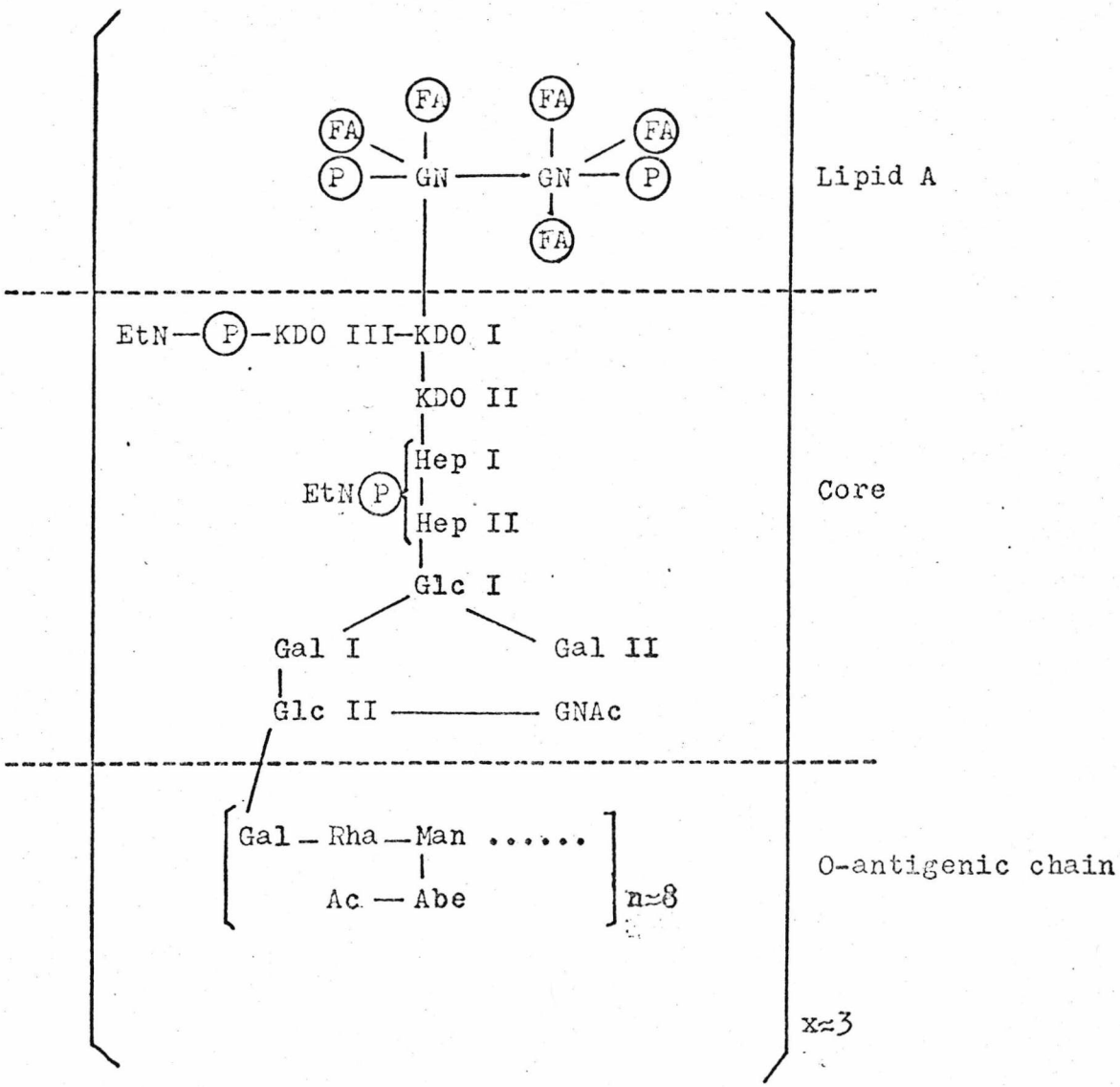


Fig. 7. Diagrammatic representation of the structure of LPS of *Salmonella typhimurium*. Rha, indicates a ramnose residue; Gal, galactose; Abe, abequose; Man, mannose; Glc, glucose; Hep, heptose; KDO, 2-keto-3-deoxyoctonic acid; GN, glucosamine; FA, fatty acid; EtN, ethanolamine; Ac, acetyl; P, phosphate Molecular weight estimations suggest that x has a volume of three. The subunit are linked through phosphodiester bridges involving Lipid A or heptose. (Taken from ref.9).

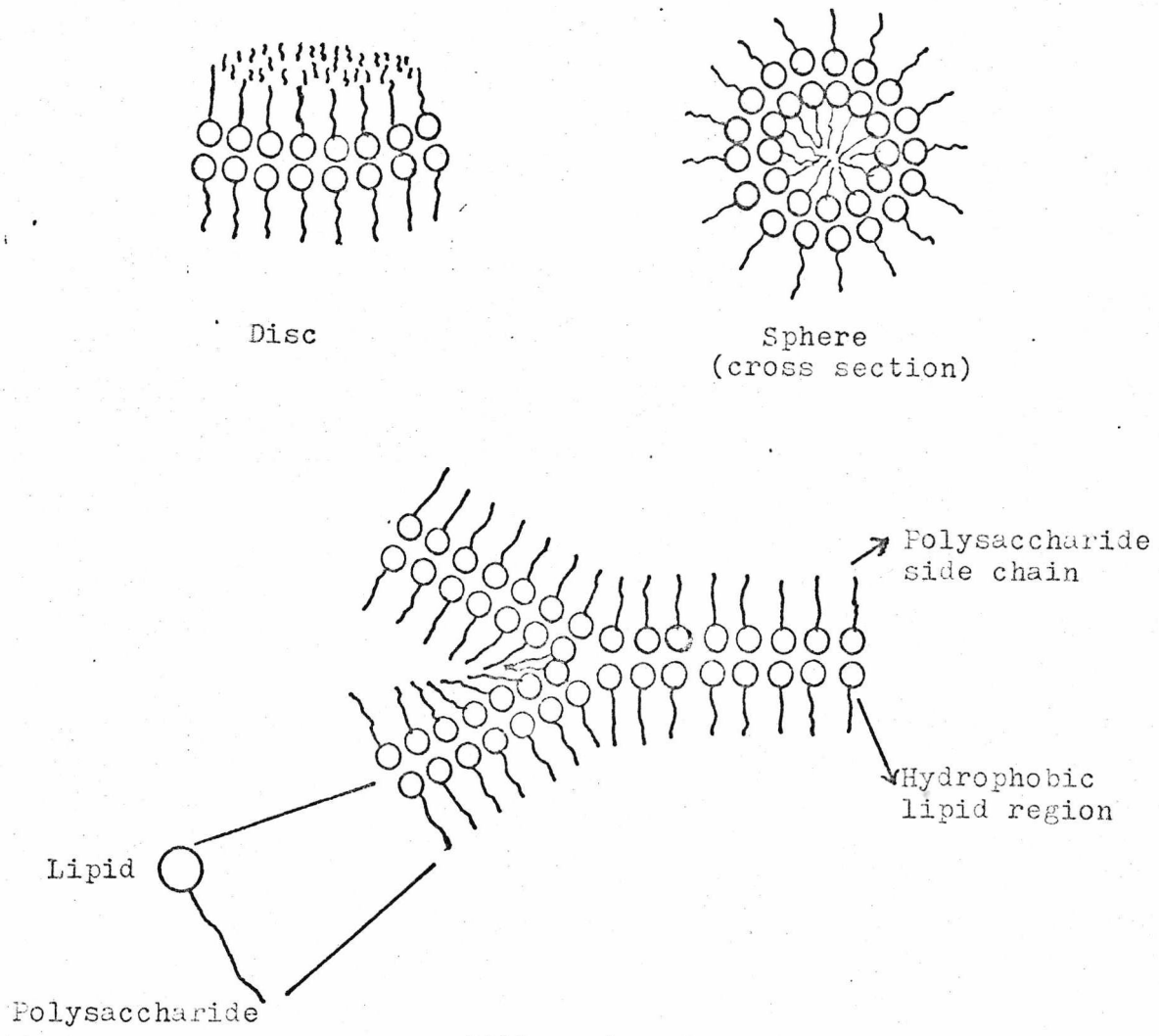


Fig. 8. Proposed assembly of Lipopolysaccharide units of Gram-negative bacteria (Taken from ref. 9, 29).

B - The Inner Layer

(murein, mucopeptide, glycosaminopeptide, peptidoglycan)

The inner layer consists of peptidoglycan. This layer is mechanically strong and confers shape to bacterial cells, preventing them from bursting in hypotonic environments.

The chemical composition of this layer has been discussed in many articles[1,3,5,6,8,9,31-35], and will only be described briefly here.

This layer consists of polysaccharide chains cross-linked by short peptide chains to give a bag-shape, fish-net macromolecular structure[6]. The basic unit of the polysaccharide chain is the muco-peptide which is a disaccharide of muramic acid and D-glucosamine (The amino groups are frequently actylated.), which are joined by the β (1 → 4) glycosidic linkage. In some species galactosamine also occurs[6].

A tetra-peptide side chain containing L-alanine, D-glutamic acid, diaminopimelic acid (DAP) or L-lysine and D-alanine is attached to the hydroxyl group of the lactic acid substituent of muramic acid. DAP is usually present as the *meso* isomer[36].

The long parallel polysaccharide chains are crossed-linked by their peptide side chain to the side chain of a neighbouring polysaccharide (Fig.9).

The enzyme lysozyme affects the peptidoglycan by hydrolysing the β (1 → 4) linkage between sugar monomers of polysaccharide chain. However, in Gram-negative bacteria, the wall material usually remains partially attached to the cell, which is then termed a spheroplast (cf. Chapter 1 Section 2).

The intact peptidoglycan is actually one large saclike molecule termed a sacculus. In different bacterial species sacculii occur as

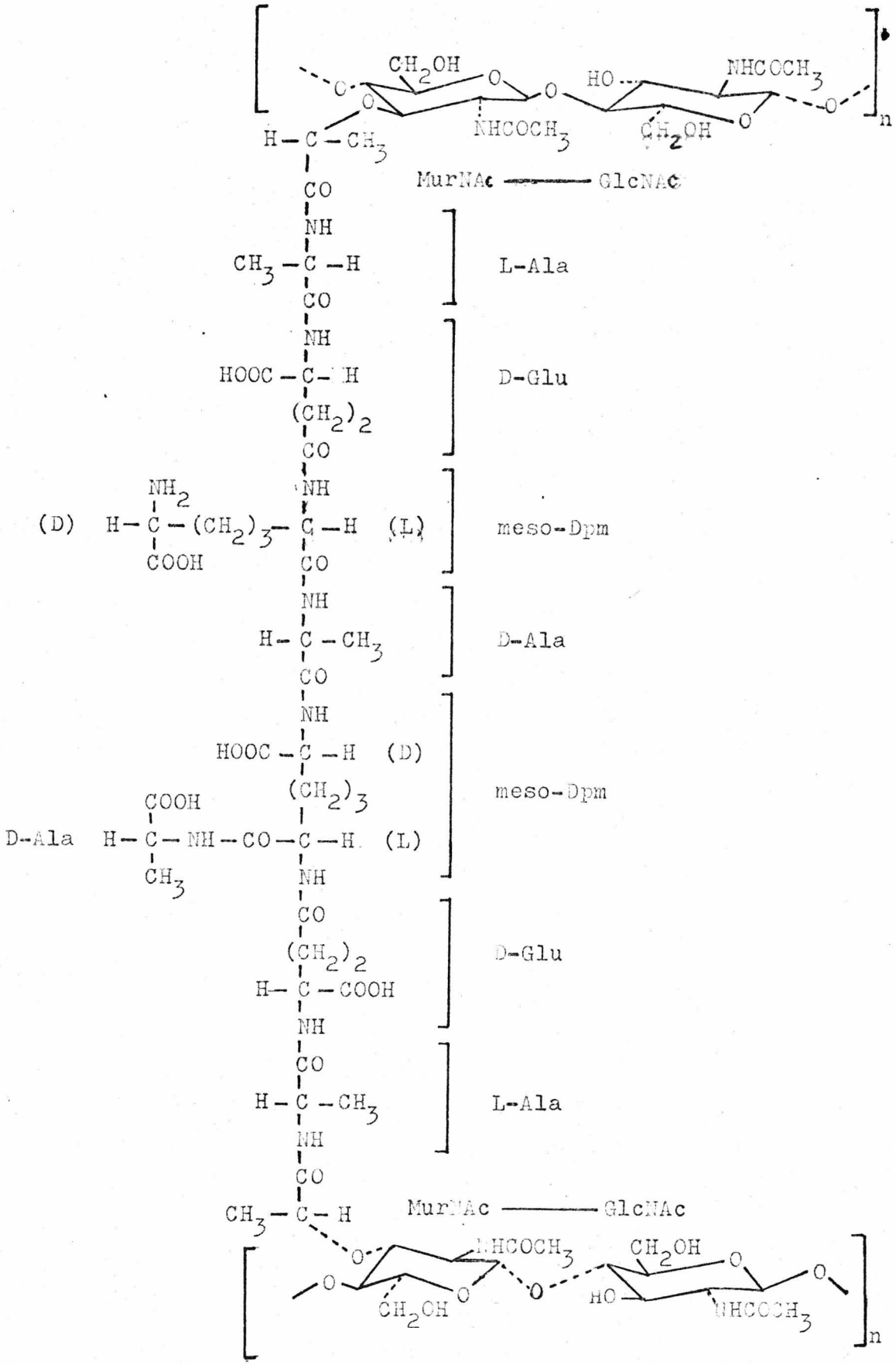


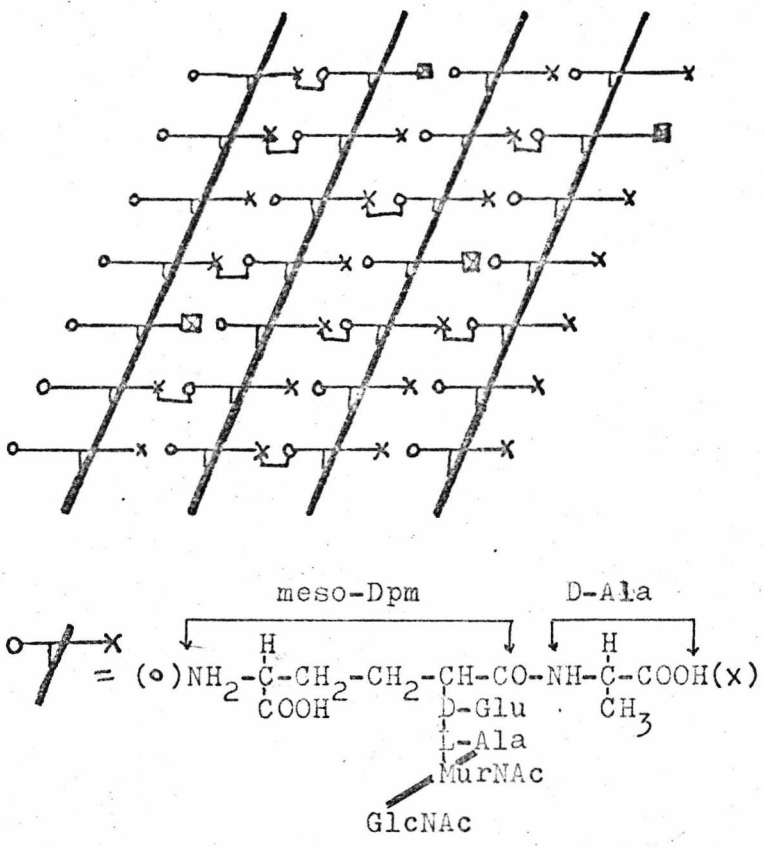
Fig. 9. Basic building block of *E. coli* murein (Taken from ref.35)

either tight nets or as loose nets, depending on the number of cross-linkage. Braun *et al*[35], studied the repeating murein unit of *E. coli* and proposed a three-dimensional model for murein (Fig.10).

In *E. coli*, it has been shown that about 2 x 10^5 lipoprotein molecules[32] are bound to the murein of each cell[37]. The linkage between the lipoprotein and murein is the terminal lysine of lipoprotein and DAP of murein[32], (Fig.10). Braun and Bosch[38] reported the amino acid sequence of this murein lipoprotein, and Hantke and Braun[39] reported the structure of lipid and its linkage to protein of lipoprotein. Twice this amount of the bound form lipoproteins were found to be free in the cell envelope[40], and Bosch and Braun[41], found that bound lipoprotein molecules project from the murein toward the outer and not the cytoplasmic membrane or both directions ((a) in Fig. 11, suggested to be correct).

Schnaitman[4] and Burman *et al*[42] have suggested that the periplasmic protein of enteric Gram-negative bacteria is associated with the inner part of outer membrane (Fig.3). This would have the effect of linking the outer membrane to the peptidoglycan layer via the lipoprotein, so that this mechanically strong inner layer would support the outer membrane.

Only murein is necessary for the maintenance of shape and mechanical strength of the cell walls and none of the other polymers e.g. LPS or lipoprotein seem to be necessary or involved. In marine *Pseudomonad* B-16, Forsberg *et al* [43] showed that cells which have only the peptidoglycan layer around the cytoplasmic membrane (which they termed mureinoplasts) still remained rod shaped and when mureinoplasts have been treated with lysozyme rod forms were converted to protoplasts.



⊠ Attachment sites of Lipoprotein replacing D-alanine

Fig. 10. Model of one-layer *E. coli* murein. A murein section consisting of four parallel polysaccharide chains (heavy lines) all running in the same direction (1 → 4) with T-shaped peptide side chain (faint lines). The extent of crosslinkage between the amino group (o) of diaminopimelate residues and the carboxyl group (x) of D-alanine residues depends on the growth phase. (Taken from ref. 35)

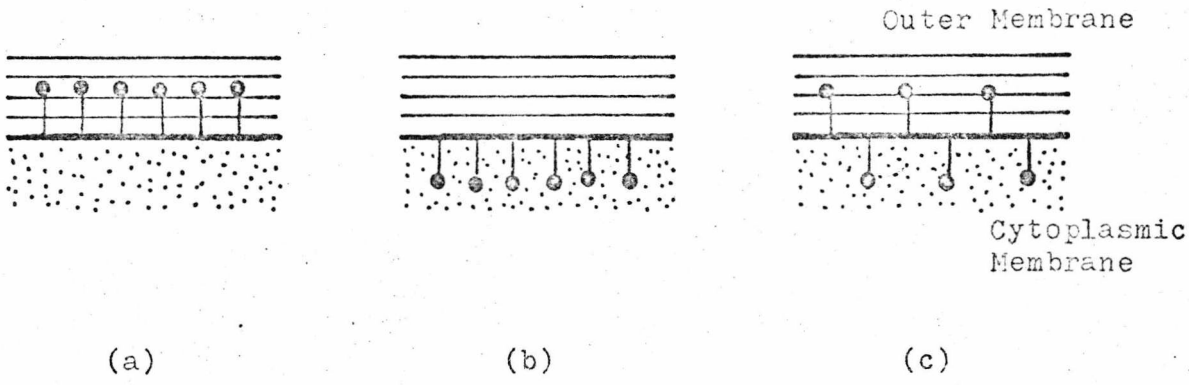


Fig. 11. Schematic representation of the possibilities concerning the direction of projection of lipoprotein from the murein. The spotted area represents cytoplasmic membrane, the lined area outer membrane, and the heavy line between them represents the murein. The three possibilities (a), (b) and (c) show possible directions in which the murein-bound lipoprotein could project. (Taken from ref. 41).

This finding confirms that the peptidoglycan layer of the cell wall is the layer which confers the shape to bacteria.

Teichoic acids consisting of long chains of glycerol or ribitol phosphate polymers which have antigenic activity in Gram-positive bacteria, have not yet been isolated from Gram-negative bacteria, however Salton[44] referred to Lilly[45] who reported the presence of arabitol teichoic acid in the cell wall of *E. coli* strain 26-26.

There are also some ions bound to the protein components of cell wall [46-49]. In Gram-positive bacterial Cutinelli and Galdiero[46], showed by chemical analysis that isolated *Staphylococcus aureus* walls bind a variety of cations e.g. K, Na, Mg and Ca. They also suggested that ions of the same valance bound through the same mechanism.

The components of the cell wall layer are synthesised at the level of the cytoplasmic membrane or within the cell, and transported to the wall. Peptidoglycan components and lipoprotein molecules are built into the preexisting peptidoglycan lipoprotein complex[50]. Studies on peptidoglycan synthesis have shown that uridine diphosphate-muco-peptides serve as a subunit-precursor of murein synthesis[51].

During cell division, cell wall synthesis occurs by centripetal growth of the wall[6].

Walls of bacteria change under different conditions of growth environment e.g. limitation in amino acids of the growth medium affects the composition of the "bridge" unit of murein; temperature affects the content and degree of the lipid fatty acid: the lower temperature the greater proportion of unsaturated fatty acids in the bacterial lipid[47].

C - Periplasmic Space.

The original definition of "periplasmic space" was made by

Mitchell[22]. The periplasmic space is an enzyme-containing compartment bounded on the inside by the cytoplasmic membrane and on the outside by the outer layer of the cell wall.

When the continuity of the outer layer of the cell wall is interrupted by changes in the LPS, e.g. by lysozyme-EDTA action, periplasmic enzymes[52] and binding proteins[53,54] are released.

The distribution of enzymes within the periplasmic space of the cell wall has been examined[55-57]. It has been suggested that some of these enzymes are not firmly bound to structural wall components, and that these enzymes together with some transport factors can be released upon osmotic shock without loss of the cells' viability[58-60].

Enzymes which are released by osmotic shock are degrative enzymes e.g. ribonuclease, and are thought to be confined near the cell surface[59].

3 - External Layers

There are a number of Gram-negative bacteria which have layers external to the LPS zone. These layers are typically thicker and less dense than the cell wall i.e. capsules or slimes. Capsule , are a part of the cell and are polymeric substances of either polysaccharide or polypeptide[5]. Slimes are secretions which are produced by many bacteria under certain environmental conditions and consist of a polysaccharide of sucrose disaccharide units. Hence cells are often surrounded by a slime layer when growing at the expense of sucrose but not other sugars[61].

There are also some appendages related to the envelope of bacteria including :-

Flagella, delicate organs which are used for motion and can be

easily detached from the cell. Their origin is not the wall, but probably the cytoplasmic membrane[62-64]. Studies on bacterial flagella have shown that they are attached to both cell wall and cytoplasmic membrane[62-64]. Flagella consist largely of protein (flagellin) associated with a small amount of sugar and lipid. The number and arrangement of flagella varies with different bacteria, but they are generally constant for each species.

Pili, which are mostly thinner than flagella and can also be mechanically removed from cells. They consist of protein and act as a part of the transfer apparatus for genetic donors in enteric bacteria[61].

Fimbriae, observed as surface appendages on some bacteria e.g. *E. coli*, are believed to be organs of attachment to solid surface and are again protein in nature[61].

Section 2

Preparation of Envelope Fractions

A - Disruption

There are several mechanical and chemical methods available for bacterial cell disruption. These include :-

1 - French pressure cell[18,33,65-68]

This method is originally based on the method described by Milner *et al* [69]. In this technique cells pass under pressure though an adjustable needle valve; the pressure difference causes cell disruption.

2 - Sonication[65,66]

In this method energy for disruption is supplied by sonic or ultra sonic waves to suspensions of micro-organisms. The period of sonication and applied power depend on the micro-organisms.

3 - Lysozyme - EDTA (osmotic lysis)[66,68,70-74]

By this method the bacteria are incubated at 20-37°C in an iso - or hypertonic sucrose or NaCl solution and the bacterial cell walls are then dissolved after addition of lysozyme-EDTA (Gram-negative bacteria) or lysozyme (Gram-positive bacteria), which results in formation of spheroplasts of Gram-negative bacteria (term used for osmotically sensitive spheres (oss) derived from Gram-negative bacteria after treatment with lysozyme-EDTA or penicillin with remaining wall materials. (Fig. 12)) or protoplasts of Gram-positive bacteria.

When the suspending medium osmolarity of the protoplasts or spheroplasts is subsequently reduced they burst and the broken membranes, which coil upon themselves or form vesicles, can be isolated[72].

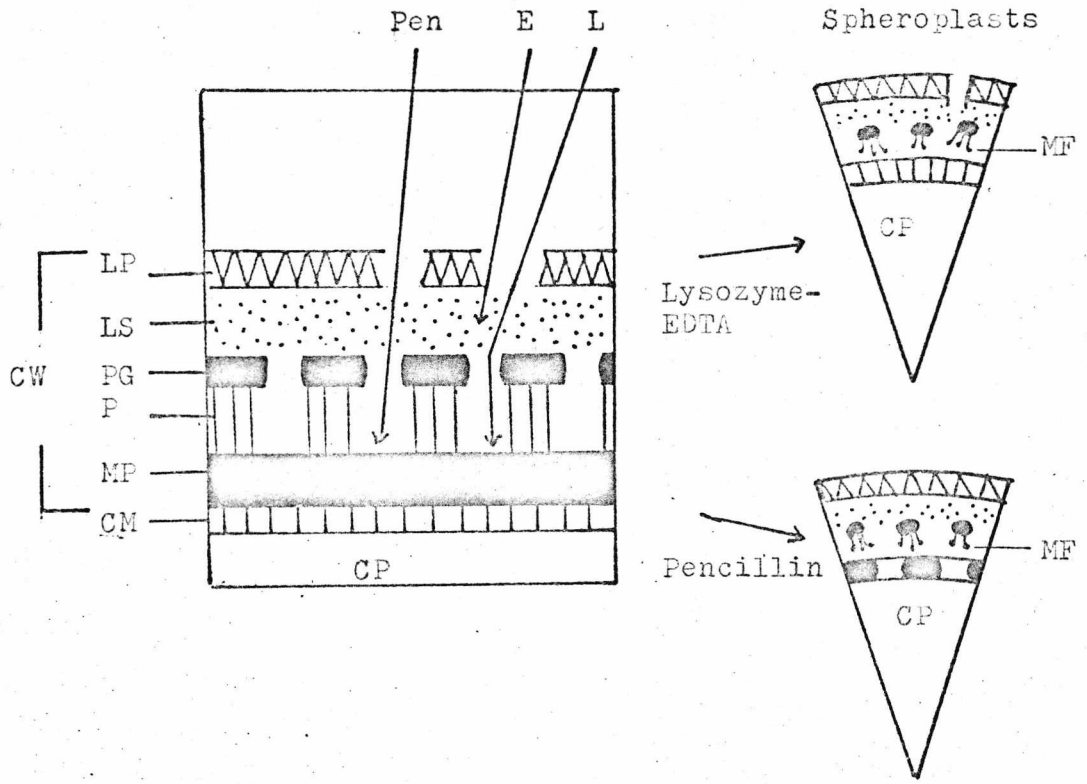


Fig. 12. Formation of spheroplasts. (Taken from ref. 81)

P	=	Proteolytic enzyme
L	=	Lysozyme
E	=	EDTA
Pen	=	Penicillin
MP	=	Mucopolymer
MF	=	Small mucopolymer fragments
LP	=	Lipoprotein layer
LS	=	Lipopolysaccharide layer
PG	=	Protein granula
CM	=	Cytoplasmic membrane
CP	=	Cytoplasm
CW	=	Cell wall

Lysozyme acts upon the peptidoglycan layer of bacteria by attacking the glycosidic bands between the acetyl amino sugars. In Gram-positive bacteria the peptidoglycan layer is the outermost part of the cell wall, and lysozyme directly hydrolyses the peptidoglycan to form protoplasts (term used for osmotically sensitive forms derived from Gram-positive bacteria, which denotes that all the cell wall materials has been removed). In Gram-negative bacteria the peptidoglycan component is not exposed to the external medium (Chapter 1, Section 1). Furthermore, it has been shown that divalant cations have an important role in the maintenance of the LPS of the outer membrane of Gram-negative bacteria[23,75], so that, certain manipulations are necessary in order to expose the peptidoglycan layer to lysozyme. The presence of EDTA and Tris-HCl[9] (alkaline) buffer enables lysozyme to attack the peptidoglycan of many Gram-negative bacteria[9,76]. EDTA acts on LPS by chelating cations involved in cross linkage of LPS in the cell wall of Gram-negative bacteria[9,23,72,73,75], and causes release of 30-50% of surface LPS[9,75].

Following lysis of bacteria with lysozyme internal DNA is released[72], which makes the preparation difficult to handle. Therefore, DNAase must be added and, since DNAase activity needs the presence of magnesium, magnesium must also be present[72].

4 - Penicillin method[72,77]

Penicillin specifically inhibits the biosynthesis of peptidoglycan by removing the terminal D-alanine molecule from the mucopeptide and affecting the terminal cross-linkage in murein biosynthesis[6]. Addition of penicillin, to rapidly growing cells, therefore affects peptidoglycan synthesis. As a result the wall loses its rigidity and the cells lyse in hypotonic environments[72] (Fig. 12).

5 - Osmotic shock (internal pressure disruption)[78,79]

In this method bacteria are suspended in a high concentration of a permeable solute e.g. glycerol, and then ejected into rapidly stirred solutions of low osmotic pressure.

Due to lower rate of glycerol exit than water entry, an osmotically induced massive uptake of water occurs followed by lysis. Cells lyse by internal osmotic shock only when the concentration of applied permeable solute is sufficient[78,79] (see also results).

B - Isolation of the envelope fractions

Since the different cell envelope fractions frequently have different density and size, it is possible to isolate the fractions on the basis of their density by differential or density gradient centrifugation.

The methods so far used for separation of envelope fractions include the splitting of the envelope preparations into several more or less defined fractions using density gradients[18,30,33,70,71,80]. The composition of the resulting fractions depends largely on the lysis procedure that has been used.

Miura and Mizushima[70,71] were the first workers to separate envelope fractions of *E. coli* by sucrose density gradients after lysozyme-EDTA treatment. In their work, bacterial suspensions, containing an extensive amount of lysozyme, were incubated at 30°C until most of murein layer had been degraded (60min).

The method of Kaback and Stadtman[77] and Kaback[72] of preparing membrane vesicles has been extensively used to study transport mechanisms. They removed the murein layer by penicillin[72,77] or lysozyme-EDTA[72] treatments, and separated the membrane fractions by differential centrifugation.

Schnaitman[18,33] disrupted *E. coli* by French pressure treatment which

of course does not cause degradation of the murein like lysozyme or penicillin treatments. On sucrose density gradients he obtained a fair degree of separation of the wall and membrane fractions.

Osborn *et al*[30] obtained envelope fractions of *Salmonella typhimurium* by sucrose density gradients centrifugation after treatment with low concentrations of lysozyme and a strictly limited time of incubation.

Robrish and Marr[78] and Pangborn *et al*[79] prepared *Azotobacter agilis* crude envelope by glycerol osmotic shock and Robrish and Marr[78] separated disrupted particles by differential centrifugation.

Oltmann and Stouthamer[80] studied the enzymes of the cytoplasmic membrane of *Proteus mirabilis*. They based their procedure on osmotic lysis by using penicillin and separated fractions by sucrose density gradient centrifugation.

C - Estimations of purity of isolated cell envelope fractions.

There are many specific components in the different layers of cell envelopes which can be used as specific biochemical markers to enable estimation of the purity of the separated cell envelope fractions. For example, KDO and DAP for LPS[30,80] and murein[80] layers of cell wall respectively and succinate dehydrogenase[18,30] or cytochromes[18,70] for membrane. The purity and, as well as the morphology, of the fractions can be more crudely estimated by electron microscopy[18,30,70-72].

Section 3

Osmotic Properties of Cytoplasmic Membrane and Osmoregulation

The mechanically strong, shape maintaining murein layer acts as a coarse molecular seive[6,82] but it is not the site of osmotic function of the bacterial cell since protoplasts of Gram-positive and spheroplasts of Gram-negative bacteria (Chapter 1 Section 2) are osmotically sensitive.

The osmotic relationship between the cytoplasm of bacteria and the external medium depends upon the cytoplasmic membrane, i.e. this layer is the permeability barrier of the cell. The cytoplasmic membrane is highly permeable to water, yet impermeable to most medium and intracellular solutes.

Permeability of cell to water

Cells will swell or shrink due to uptake or loss of water through the cell membrane. Therefore, factors that affect water movement into or out of the cell are also those which will tend to affect the cell volume. There is, as yet, no evidence of active transport of water into or out of living cells[83], so that, unlike many electrolytes, water appears to play a passive role in the life of the living cells. Furthermore, the rate of water movement into and out of cells can be studied in relation to applied gradients of osmotic pressure across the cytoplasmic membrane.

Terminology

If two solutions of different concentrations are separated by a membrane water permeable but solute impermeable, water migrates from the more dilute into the more concentrated solution, diluting the latter and concentrating the former (*Osmosis*)[84]. The two solutions will eventually have the same concentration, but if osmosis cannot take place there will nevertheless be a tendency for osmosis to occur, i.e. there will be a

positive *osmotic pressure*.

Bacterial cells suspended in water shrink on raising the medium osmotic pressure as water leaves the cells to regain osmotic equilibrium between the cytoplasm and the environment. Since the cytoplasmic membrane is the osmotic barrier, addition of non-permeant solutes to bacterial suspensions causes water exit and shrinkage of the cytoplasmic membrane away from the cell wall *(plasmolysis)*.

Permeability of cells to solutes

Substances may be placed in two classes with respect to the behaviour of their aqueous solutions; (1) non-permeants, which do not enter bacterial cells and if present in a high enough concentration cause water exit (plasmolysis), e.g. NaCl, sucrose etc., and (2) those which enter the cells, e.g. glycerol, urea etc. (permeants)[84-92].

Changes in osmotic pressure of the medium elicit corresponding changes in the volume[86,87] and also turbidity of suspension of Gram-negative bacteria[86-92]. The optical effects can be attributed to changes in volume of the cells (degree of plasmolysis) and the state of the bacterial cytoplasm.

Thus, increasing amounts of non-permeant electrolytes (e.g. NaCl) or non-electrolytes (e.g. sucrose) added to suspensions of Gram-negative bacteria in distilled water cause the turbidity of suspensions increase, due to changes in light scattering and refractive index. Permeants such as glycerol or urea, which are highly diffusible through biological membranes, and high molecular substances such as albumin or inulin (which do not significantly alter the medium osmotic pressure), are ineffective in causing stable volume and turbidity changes[85,90,91].

Studies on diffusion of permeant molecules into bacteria have shown[85,91,92] that osmotic changes across the semi-permeable cytoplasmic

membrane (in hypertonic medium) are the resultant of two concurrent processes, (a) diffusion of water in the direction opposite to that of the concentration gradient (causing increase in turbidity due to water exit and cell shrinkage), (b) diffusion of solute through the membrane in the direction of the gradient (re gain in turbidity).

Therefore measurements of the rate of reswelling by following transient turbidity changes can be used to determine the properties of entry of permeant molecules. The rate of entry of many permeants cannot be measured by concomitant turbidity changes using conventional spectrophotometers because the rate of water exit and substrate entry is too rapid[88,89], and hence special techniques must be used[88,89].

Regulation of intracellular osmotic pressure

Increases or decreases in medium osmotic pressure cause movements of water across the cytoplasmic membrane, resulting in decreases or increases in concentration of soluble intracellular components. In order to maintain the metabolic patterns, cells must maintain a constant osmotic gradient across the membrane in environments of varying osmotic pressure, thus preventing drastic water movements into or out of the cell.

The osmotic behaviour of the erythrocyte has probably been studied in more detail than other cells. Despite differences in osmotic pressure between the cytoplasm and the environment the volume of erythrocytes changes only to the small degree. Studies on shape and water movements across the plasma membrane of erythrocytes have shown that the volumes of cells in a hypertonic medium is more than that expected[84], indicating that there are essential mechanisms operating to control changes of volume and affecting osmoregulation of the erythrocytes. The general explanation is based on changes in the internal salt concentration; this possibility has been investigated and it has been shown that regulation of size is largely a matter of the regulation of intracellular electrolyte concentration.

Studies on total osmotic content of erythrocytes has shown that the cations K^+ and Na^+, are not at equilibrium distribution and there is uptake of K^+ into the cell when placed in hypertonic media, which would affect the final volume. Since increases or decreases in internal osmotic pressure affect internal water

content and cell volume, erythrocytes therefore prevent large movements of water across the plasma membrane by a potassium pump.

It is not yet understood how erythrocytes regulate the potassium pump, but it is clear that active transport of ions into the cell is mediated by an ATPase system and energy for the process is available from ATP[84,93,94].

In bacterial cells studies on ion transport have shown that transport of cations across the bacterial cell membranes is a major factor in regulating the internal osmotic pressure. In *E. coli* measurements of cellular K^+ and Na^+ concentration in growing cells indicate that the osmolarity of the medium is a factor affecting the cell K^+ concentration and changes in osmolarity of the medium results in rapid changes in K^+ concentration within the cell. In contrast, the Na^+ concentration is not dependent on the medium osmolarity[95].

Thus, the dependence of intracellular K^+ content on the osmolarity of the medium suggests that the intracellular K^+ content is the major osmoregulatory factor. Cells appear to change K^+ for either H^+ or Na^+ in order to increase or decrease the internal osmotic pressure in response to changes of external osmotic pressure.[94-96].

Section 4.

The Function of the Bacterial Cytoplasmic Membrane.

As has already been discussed (Chapter 1 Section 3) the cytoplasmic membrane of bacteria is an osmotic barrier which is impermeable to many molecules, but permits the passive diffusion of water and some small molecules. In addition, the cytoplasmic membrane is involved in many essential biochemical processes. e.g. DNA and cell wall synthesis, respiration, oxidative phosphorylation and substrate transport. Since an understanding of respiration, oxidative phosphorylation and substrate transport is relevant to the work given in this thesis, they will be discussed briefly.

A - Respiration and Oxidative phosphorylation

Respiration and oxidative phosphorylation have been the subject of many books and reviews[97-104]. In mammalian mitochondria and bacteria reducing equivalents derived from TCA cycle etc., are transferred to oxygen or other acceptor molecules by the various electron carriers of the respiratory system (Fig. 13). The components that transfer protons and electrons from the reduced substrates to molecular oxygen are located in the inner membrane of mitochondria of eucaryotic cells and the cytoplasmic membrane of bacteria. Oxidation of reduced substrates by molecular oxygen is accompanied by a large decline in free energy, some of which is conserved as ATP synthesised from ADP and inorganic phosphate. There are several hypothesis available to describe the mechanism of energy conservation across membranes[97,99,102], two of which will be discussed here.

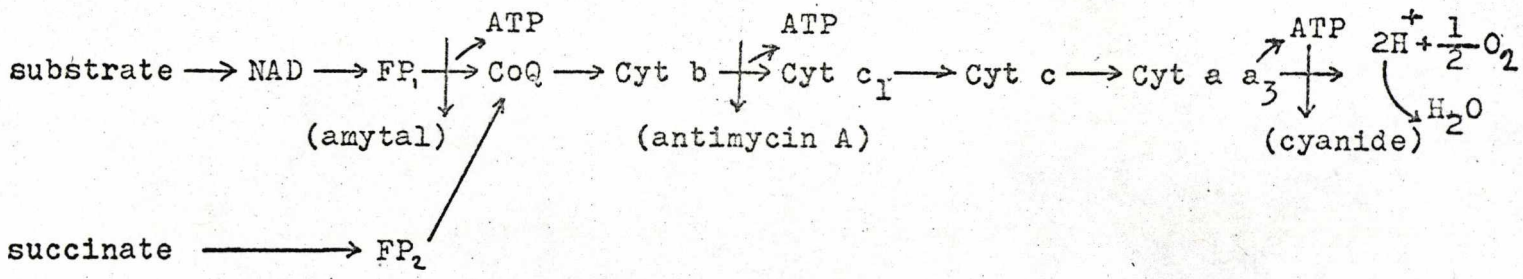


Fig. 13. The respiratory chain in mammalian mitochondria and probable sites of energy conservation. Also shown are the points of inhibition of electron transport by specific inhibitors (taken from ref. 34)

1 - Chemiosmotic hypotheses

By this hypothesis [97,99,102,105,106] the mitochondrial respiratory chain is presumed to be an alternating sequence of proton plus electron and electron carriers, arranged across the membrane in loops. For each pair of electrons passing down the respiratory chain two protons are pumped out of the mitochondria per respiratory loop, making six in total per molecule of NADH[97, 102, 106] and four per molecule of succinate oxidased[97,102,106]. As a result the external proton concentration increases and its internal concentration decreases, causing changes in pH (internal alkaline) and electrical potential (internal negative). The chimiosmotic hypothesis thus requires a membrane which is topologically closed, i.e. vesicular and impermeable to protons to maintain the osmotic equilibrium and the gradient of pH and electrical potential.

The combined force tends to drive the translocated protons back into the inner compartment. This force has been termed proton-motive force (PMF)[97,99,102,106], and it is the sum of two components :

$$\Delta p = \Delta\Psi - Z\Delta pH$$

where

Δp = Proton-motive force

$\Delta\Psi$ = Electrical potential

ΔpH = pH gradient

Z = 2.3 RT/F ≈ 60 at 25°C when proton-motive force is expressed in millivolts.

The $ATP + H_2O \rightleftharpoons ADP + Pi$ equilibrium is in the direction of ATP hydrolysis (the standard free-energy change ($\Delta G'^{o}$) of ATP hydrolysis is -7.3 Kcal [34] at pH 7.0 and 37°C in the presence of

Mg^{++} , and special mechanisms are required to drive the reaction in direction of ATP synthesis. In the chemiosmotic hypothesis the suggested mechanism for driving ATP synthesis consists of coupling the ATPase reaction with protons which have been translocated out of the mitochondria by respiration.

$$2H^{+}_{(in)} + ATP + H_2O \xrightleftharpoons{\text{ATPase II}} ADP + Pi + 2H^{+}_{(out)} \quad (1)$$

$$K_{eq(1)} = \frac{(ADP)\ (Pi)}{(ATP)} \times \frac{(H^{+}_{(out)})^2}{(H^{+}_{(in)})^2} \quad \text{or}$$

$$\frac{(ADP)\ (Pi)}{(ATP)} = \frac{(H^{+}_{(in)})^2}{(H^{+}_{(out)})^2} \times K_{eq(1)}$$

Therefore increase in external proton concentration could in principle reverse the direction of ATPase. Two protons are considered to be re-translocated (reaction (1)) into the mitochondria per mole ATP synthesised [97,106].

2 - The chemical coupling hypothesis

According to this hypothesis, when reducing equivalents pass from an energy conserving electron carrier (Ared) to the next (oxidised) carrier in the chain energy is conserved by linkage with a third component (x) to form (Aox ∿ x). The high energy bond (∿) between Aox and x becomes the precursor of the high energy phosphate bond of ATP after a series of reactions [99].

The main difference between the chemical coupling hypothesis and chemiosmotic hypothesis is the way in which energy is conserved; in the chemical coupling hypothesis the free energy of oxidation-reduction is conserved as a "high energy" intermediate whereas in chemiosmotic hypothesis the energy is conserved as the pH gradient plus electrical potential acorss the membrane [99-102].

Uncouplers

Uncouplers are lipid soluble substances which dissolve in membranes and act as circulating carriers, conducting H^+ across the membrane. Despite the general acceptance of the phenomenon of proton conduction, it is not universally accepted that uncoupling is the consequence of diffusion of protons across membranes, but rather is due to general acid base catalysis of a hydrolytic reaction taking place in membrane (see ref. 102). An understanding of the mechanisms of action of uncouplers has been an essential feature of the development of the chemiosmotic hypothesis with respect to both phosphorylation and transport. Typically uncouplers prevent phosphorylation but stimulate respiration. Some antibiotics (ionophores) act as uncouplers by permitting conduction of ions across the membrane e.g. valinomycin which conducts K^+ and nigericin which catalyses H^+/K^+ antiport.

Bacterial Respiration

In bacteria, classical intracellular organelles such as mitochondria, existing in the cytoplasm of eucaryotic cells and carrying out specific cellular functions are not present, and such functions are performed by the cytoplasmic membrane. Studies of bacterial respiratory chains show that respiration of bacterial cells varies drastically from species to species and from those of mitochondria [102,107], but the coupling of respiration to phosphorylation is in principle the same in bacteria as in mitochondria. In general bacteria are able to respire a wider range of substrates than mitochondria. They are, in addition, also frequently able to utilize other compounds than oxygen as terminal electron acceptors e.g. nitrate [108,109].

Some bacterial respiratory chains to oxygen appear to be terminally branched[110] for example *Azotobacter vinelandii*, which was used for the research in this thesis, has a branched respiratory chain [111-113], and it has been shown that the branches differ in their sensitivity to cyanide [111,112] (Fig. 14).

Sidedness of membranes

Membranes are asymmetrical i.e. they possess a specific orientation. The asymmetry is due to chemically different proteins on either side of the membrane. Asymmetry of the membrane is an important element in the mechanism of directional or vectorical transport process across membranes. For example ATPases capable of forming ATP from ADP and inorganic orthophosphate and enzymes which oxidize NADH and succinate (NADH and succinate dehydrogenases) are associated with the inner part of bacterial cytoplasmic membranes [66,102].

One of the most interesting properties of bacterial membranes is their ability to reseal, after mechanical injury, to form enclosed vesicles. The sealed vesicles may have the same orientation as intact cells or they may be inverted. Fig. 15 illustrates the various orientations that resealed membrane vesicles may be expected to exhibit following disruption of the cytoplasmic membrane of intact cells/protoplasts or spheroplasts. The stalked particles mark the cytoplasmic side.

So far studies on bacterial membrane particles have shown that membrane particles obtained by different methods, e.g. lysozyme-EDTA [72,73,114,115], sonication, French pressure cell [18,33,66], and osmotic shock (refer to results) can retain vesicular properties.

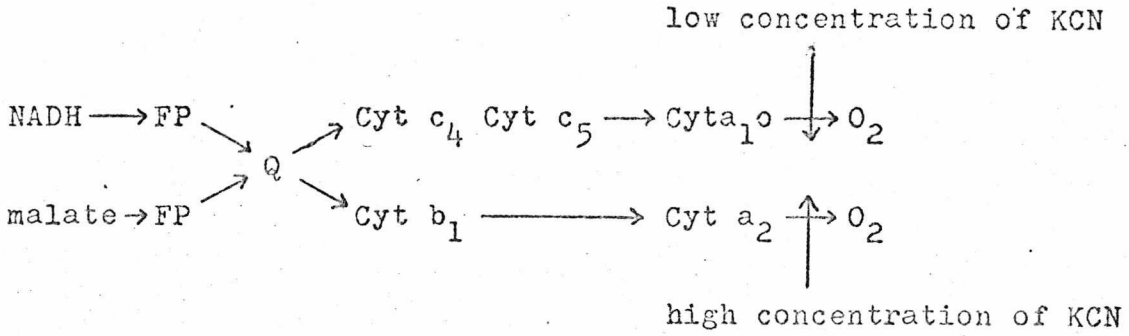


Fig. 14. Diagram for respiratory chain of *Azotobacter vinelandii* FP, flavoprotein; Q, coenzyme Q. (taken from ref.111,112)

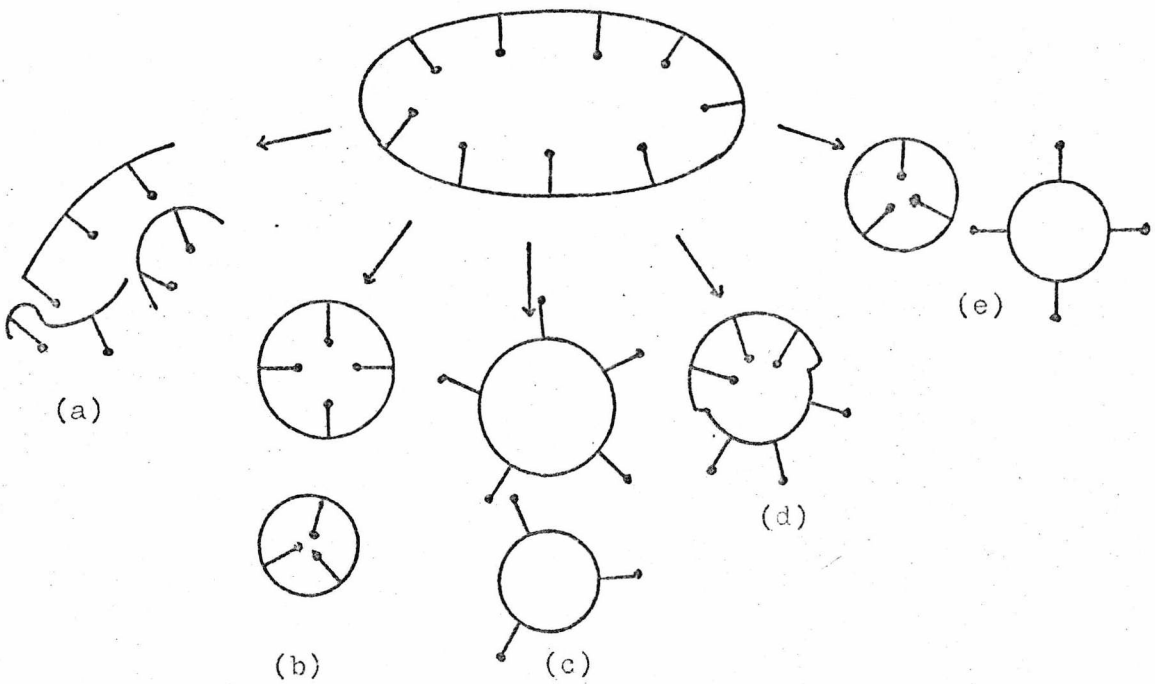


Fig. 15. Formation and orientation of membrane vesicles, (a), fragments, (b) rightside out, (c) inverted, (d), hybride or patchwork, (e), mixture of (b) and (c). The stalked parts represent the ATPase, located on the cytoplasmic side of the membrane. (Taken from ref. 102)

Furthermore investigations of orientation and transport in membrane vesicles prepared by the different procedures have shown that

vesicles prepared by gentle methods, e.g. lysozyme-EDTA tend to be relatively large and possess, partially at least, the same orientation as intact cells i.e. right side out (protons are pumped outwards) whereas membrane vesicles prepared by French pressure cells or sonication are smaller and are inverted i.e. inside out (protons taken up)[66,68]. After resealing hybrid vesicles also may arise[116]. (see also results).

Studies on the orientation of vesicles prepared by different methods and the techniques available for studying orientation have been the subject of many articles [66-68,74,117-120]. John and Hamilton[117] have studied the orientation of membrane vesicles using ionophores; they suggest that ionophores which exchange protons for ions (e.g. K^+) can stimulate the rate of oxygen uptake if vesicles are inverted. Therefore the permeability inducing antibiotics (ionophores) can be used to investigate the sidedness of vesicles. Futai[66] has studied the orientation of membrane vesicles by assaying for the location of NADH dehydrogenase and ATPase activities. These enzymes are located on the inner side of correctly orientated membrane vesicles, and hence should not be available (cryptic) to exogenously added substrates. He therefore suggested that addition of toluene (which permeabilises the vesicles[121,122]) to right side out vesicles should cause an increase in observed enzymic activity; for inverted vesicles such a stimulation of activity should not occur.

Orientation of membrane vesicles can also be investigated by freeze-etching electron microscopy[74] or other methods discussed in

Chapter three.

B - Transport across the cytoplasmic membranes

As already pointed out, bacterial cell membranes are impermeable to most molecules. However, cells must obtain nutrients from their environments and therefore there must be specific mechanisms available to permit entry of many molecules which could not otherwise traverse the cell membrane. There are several mechanisms by which substances are known to enter bacterial cells :-

(a) Passive diffusion. By this process substances cross the cytoplasmic membrane as a result of random molecular motion. This has already been discussed (Chapter 1 Section 3).

(b) Facilitated diffusion. In facilitated diffusion systems a transported solute is presumed to combine reversibly with a specific carrier in the membrane. Facilitated diffusion is energy-independent, saturating and pH and temperature dependent. The rate of substrate entry by this system is more rapid than passive diffusion (refer to results).

(c) Active transport. Transport up a gradient in the direction of increasing concentration. Active transport is therefore vectorial and requires an energy supply. This system is saturable, pH and temperature dependent and can be observed in isolated membrane vesicles. In intact Gram-negative bacteria active transport is sensitive to osmotic shock because components of these systems are frequently associated with periplasmic substrate-binding proteins which are released by osmotic shock treatment[54,123].

(d) Group translocation. Another transport mechanism is group translocation. In this process covalant change is exerted upon the translocated molecule. One such group translocation mechanism

i.e. vectorial phosphorylation, leads to translocation and accumulation of molecules within the cell by phosphorylation during passage through the membrane[124]. This system consists of a soluble cytoplasmic part, enzyme I and HPr (heat-stable protein) and a membrane bound enzyme II complex[125]. The reaction can be summarized by the following equations :

$$\text{P -enolpyruvate + HPr} \xrightarrow{\text{Enzyme I, } Mg^{++}} \text{pyruvate + P - HPr} \quad (1)$$

$$\text{P - HPr + sugar} \xrightarrow{\text{Enzyme II, } Mg^{++}} \text{sugar - P + HPr} \quad (2)$$

$$\text{P - enolpyruvate + sugar} \xrightarrow[\text{Enzyme II, } Mg^{++}]{\text{HPr, Enzyme I}} \text{sugar - P + pyruvate} \quad (1 + 2)$$

Group translocation is also saturable, energy, pH and temperature dependent.

In addition, there are mechanisms such as phagocytosis and pinocytosis[6] which are only involved in transport processes in cells of higher organisms[73].

Bacterial transport mechanisms are summarized in Fig. 16.

The cytoplasmic membrane is the site of control and passage of molecules into cells and transport events are believed to occur in this part of the envelope. Cell-free bacterial membrane vesicles of correct orientation which are largely free from cytoplasmic constituents and cell wall materials have been isolated, and have been shown to be capable of exhibiting active transport[72,73,115].

Cytoplasmic membranes prepared from cells subjected to different procedures usually consist of sacs of varying diameter, and electron microscopy preparations showed that the sacs (vesicles) appear to be empty and without internal structures[72,73].

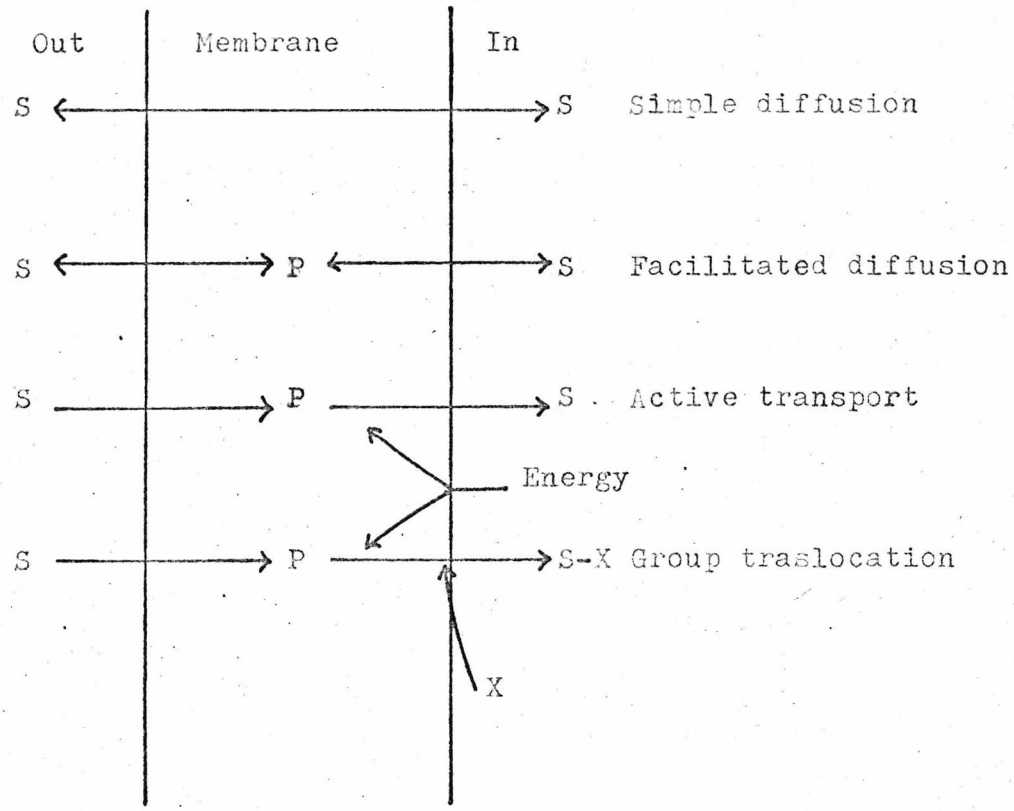


Fig. 16. Basic mechanisms of transport, P, permease or carrier, S, substrate, X, bound component.

Energy coupling of active transport

As mentioned above, metabolic processes provide the energy for active transport or group translocation of many molecules across the cytoplasmic membrane. In *E. coli* it has been proposed that the primary respiratory substrate for coupling to active transport is D-lactate [126-129]. The site of energy coupling by D-lactate in *E. coli* has been placed between the dehydrogenase and cytochrome b1[73]. Different organisms appear to have different primary electron donors e.g. malate in *A. vinelandii*[114]. However, this contention has been criticised and several points have been presented which suggest that the coupling of transport is not directly linked to respiration e.g. uncouplers of oxidative phosphorylation such as DNP strongly interfere with respiratory-driven transport without affecting respiration.

Recent reports suggest that ion and nutrient transport can be explained in terms of Mitchell's chemiosmotic hypothesis[102, 130]. Transport of solute against the concentration gradient is thought to be a result of the proton-motive force (PMF) and transport occurs via a H^+ symport or OH^- antiport. One of the features of the proton-motive force model is that uncouplers, which are believed to function as lipid soluble proton conductors, inhibit active transport by collapsing the proton-motive force[102, 130-132]. Transport of anions ($H_2PO_4^-$) depends only on the pH gradient across the isolated membrane and are translocated on a proton symport. Cation (e.g. K^+) transport depends on the electrical potential across the membrane. Neutral molecules (e.g. sugar) cross the membrane on a proton symport and transport depends on both the pH gradient and the electrical potential.

Aim of Study

As I have pointed out above, the cytoplasmic membrane is the osmotic barrier of bacterial cells, which is freely permeable to water but impermeable to most medium solutes. Therefore, increases or decreases in osmotic pressure of the medium tend to cause water movements across the cytoplasmic membrane and affect the concentration of intracellular intermediates.

At the outset of this project the aim was to investigate water movements across the cytoplasmic membrane of Gram-negative bacteria, particularly *E. coli*. These studies should provide basic data for a longer term investigation of osmoregulation of bacteria exposed to various changes in medium osmotic pressure and osmotic stress.

Because hypertonicity leads to plasmolysis (shrinkage of the cytoplasmic membrane away from the peptidoglycan layer of the cell wall), another objective of this study was to quantify the degree of osmotically induced plasmolysis and to see whether the cytoplasmic membrane is free to shrink away from the cell wall or whether there are some points of physical connection between them.

During the course of these studies I have observed that addition of salts to bacterial suspensions, in order to increase the osmotic pressure of the medium, caused, in addition to plasmolysis, cell wall shrinkage (refer to results). In general changes in composition of the medium affect both its osmotic pressure and its ionic strength. Since it had previously been reported[133,134] that isolated cell walls of

Gram-positive bacteria contract in response to increases in ionic strength of the medium, it was possible that contraction of the whole cell after addition of salts to *E. coli* might be due to changes of ionic state of the medium. I have therefore further studied the effect of salts on isolated wall fractions and also attempted to see more directly if the cell wall of Gram-negative bacteria, like Gram-positive bacteria, respond to the ionic state of the medium.

Osmotically induced changes in 'protoplast'* volume are in some way related to changes in turbidity of bacterial suspensions [86,88,90]. As a result of the studies of changes in 'protoplast' volume I realised that the turbidity change technique could be used to study substrate entry of permeant molecules. Accordingly, a further aim of this study was to investigate the utility of this technique using glycerol, the permeant compound, as a model permeant.

The final aim of this study was investigation of a new method for separation of Gram-negative bacterial cell envelope fractions. The permeability of bacterial cells to glycerol was used as the basis of a technique which was developed to lyse the Gram-negative bacterium *A. vinelandii* by osmotic shock. Membrane vesicles obtained by this method were relatively undamaged and I have investigated some of their biochemical properties i.e. orientation and transport. Lastly I have cleanly separated the envelope fraction into cell wall and cytoplasmic membrane vesicle fractions, which should permit further studies of their ionic and osmotic properties respectively.

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* In this thesis, the term 'protoplast' will be used to refer to that part of the cell bounded by the cytoplasmic membrane and does not imply that protoplasts, devoid of cell wall components, are being used.

CHAPTER 2

MATERIALS AND METHODS

1 - Bacterial strains

The organisms used were *Azotobacter vinelandii* strain N.C.I.B. 8660 (obtained from National Collection of Industrial Bacteria) and a wild type of *Escherichia coli* strain K12 (F^- University of Warwick). In addition various mutant strains of *E. coli* (obtained from Dr. E. C. C. Lin) defective in the glycerol 3-phosphate (glp) regulon were used; the properties of these strains are given in Table 3 using the nomenclature of Richey and Lin[135]. *E. coli* 30E (gift from Dr. C. F. Fox) was used in some experiments (A mutant which requires oleic or elaidic acid for growth. This mutant can synthesize saturated fatty acids but has lost the ability to synthesize unsaturated fatty acids[136].).

2 - Medium and Bacterial growth condition

Unless otherwise stated, the wild type of *E. coli* was grown on 30 mM glucose plus M-9 salts[137], which consist of, per litre; Na_2HPO_4 (anhydrous) 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $CaCl_2$ 22 mg and $MgSO_4 \cdot 7H_2O$ 247 mg. A 5 ml inoculum of an overnight culture was added to 100 ml fresh medium in a 250 ml flask, and the culture was grown to the end of the log phase (4-5 h) at 35°C on a gyrotatory shaker at 200 rpm. Large quantities of cells were grown in eight 600 ml quantities in 2 l flask.

Alternative media, where stated were 30 mM glycerol plus M-9 salts, 1% casamino acids and 1% casamino acids plus 10 mM glycerol 3-phosphate. Growth conditions were exactly as given above.

E. coli 30E was grown on medium consisting of, 30 mM glucose, M-9 salts, 0.5% tween 40, 0.02% oleic or elaidic acid and 1 mg per litre Thiamin-HCl (vitamin B1). Cells were grown on a gyrotatory shaker (200 rpm) at 37°C[136] and fresh medium was incubated with a 5% inoculum of an overnight culture. Cells were harvested at the

end of the log phase (about 20 h).

A. vinelandii was grown at 30°C on 100 ml of Burk's medium (as described by Roberish and Marr[78]), containing per litre; KH_2PO_4 200 mg, K_2HPO_4 800 mg, $MgSO_4.7H_2O$ 200 mg, $CaSO_4.2H_2O$ 25 mg, $FeNH_4(SO_4)_2.12H_2O$ 8.6 mg, $Na_2MoO_4.2H_2O$ 0.25 mg and sucrose 20 g. 25 ml of overnight culture was added to 500 ml of fresh medium in 2 l flasks. The culture was grown to the end of log phase (24 h) on a gyrotatory shaker at 200 rpm.

In some experiments *A. vinelandii* was grown of medium containing per litre; KH_2PO_4 200 mg, K_2HPO_4 800 mg, $MgSO_4.7H_2O$ 200 mg, $CaCl_2$ 65 mg, $FeCl_3.6H_2O$ 1 mg, $NaMoO_4.2H_2O$ 0.1 mg, tryptone 5 g, yeast extract 0.5 g and D-glucose 10 g [114]. Growth conditions were exactly as given above.

When glucose was used as a carbon source, it was sterilized, 5 lbs for 10 min, separately and was mixed with the rest of medium sterilized, 15 lbs for 15 min, before inoculation. Burk's medium was sterilized at 10 lbs for 10 min.

3 - Cell harvesting

E. coli was harvested by centrifugation at 12,000 x *g* for 10 min at 4°, washed once in 10 mM imidazole - HCl buffer (pH 7.0) and resuspended in the same buffer. *A. vinelandii* was harvested by centrifugation at 4° for 15 min at 10,000 x *g*, washed free of slime by two successive centrifugations with distilled water at 2,000 x *g* for 15 min. and washed cells were resuspended in 50 mM tris (hydroxymethyl) aminomethane chloride (Tris-HCl) (pH 7.5).

4 - Measurement of total turbidity changes

These were measured in a conventional spectrophotometer at

700 nm. Cells were suspended in 2.8 ml 10 mM imidazole buffer (pH 7.0) in a 1 cm cuvette at room temperature (21 ± 1°C) at absorbance of about 0.2 absorbance units. Buffer (0.2 ml), of buffer plus NaCl, $MgCl_2$, sucrose, glycerol or Ficoll to give the desired final solute concentration were added and the cuvette contents thoroughly mixed. The absorbance was read 2 min after mixing. Turbidity effects were measured at 700 nm as this wavelength had been used in previous studies[86].

5 - Kinetics of turbidity changes

The kinetics of turbidity changes were measured in a stopped-flow spectrophotometer. This instrument differs from the classical design of Gibson and Milnes[138] in that there was no terminating syringe. The pair of driving syringes were arrested by hitting a rigid block which incorporated a microswitch to activate the recording system. Outflow of solution from the observation tube was partially restricted by having a limited diameter exit tube.

The apparatus was operated in the transmission mode, using a 2 mm path-length observation tube. Changes in transmittance were measured at 550 nm since the output from the optical system was optimal at this wavelength. *E. coli* suspended in 10 mM imidazole buffer (pH 7.0) to an absorbance of 2.0 (in a 1 cm cuvette) was mixed with an equal volume of NaCl, $MgCl_2$, glycerol in buffer, and the change in transmittance displayed on a storage oscilloscope. The traces were photographed, enlarged onto graph paper and converted to absorbance using the Beer-Lambert law[139,140].

6 - Measurement of cell spaces

Cell spaces were measured by the exclusion space method

based on the studies of Scherrer and Gerhardt[82] with *Bacillus megaterium*.

An 18 ml portion of *E. coli* suspended in 10 mM imidazole buffer (pH 7.0) (± medium solute) was added to a tared centrifuge tube and centrifuged at 38,000 x *g* for 20 min at 4°C. The supernatant was discarded and the inside of the tube carefully wiped with a tissue and then re-weighed. The initial concentration of cells was such that the pellet weight was 1.2 - 1.8 g. 0.5 ml of 2% (w/v) marker blue dextran (Sephadex marker blue dextran 2000 with an average molecular weight of 2×10^6 (cf. leaflet, Blue dextran 2000, from Pharmacia Ltd.)) in 10 mM imidazole buffer (pH 7.0), 0.1 ml of uniformly labelled (U - ^{14}C) sucrose (to give a final concentration of 3 mM and 1000 to 2000 cpm) and 0.3 ml of imidazole buffer (pH 7.0), (± medium solute to give final concentration equivalent to that initially used) were added and the solution carefully mixed. After 10 min at 4°C the mixture was centrifuged at 38,000 x *g* for 20 min at 4°C. The dextran concentration was assayed by measuring the absorbance of a suitable dilution at 620 nm. The (^{14}C) sucrose concentration was measured by addition of 0.1 ml of the supernatant to 10 ml of scintillation fluid [141], and counting in a scintillation counter (Packard Tri Carb model 3375). The experiment was done in triplicate for each condition and the average taken. Control dilutions of the (^{14}C) sucrose and dextran were done exactly as above but using 18 ml of buffer.

Black and Gerhardt[142], have shown that solute uptake into the pellet, the solute "space", can be evaluated using the equation:

$$S^W = \frac{W_s}{W_p} \left(\frac{C_o}{C_f} - 1 \right)$$

Where S^W is the space or fraction of the pellet penetrated by solute. This includes the extracellular (interstitial) space as well as the fraction of the cell penetrated. The superscript "w" in S^W refers to the fact that the weight (W_p) rather than volume of the pellet is measured. Since the density of *E. coli* is 1.07 to 1.08 g/ml[142] weight measurements are essentially an index of volume. W_s is the volume of added (^{14}C) sucrose and dextran of initial and final concentration C_o and C_f respectively.

The fraction of the cell penetrated by the solute, R^W, is given by :

$$R^W = \frac{S^W_{sol} - S^W_{in}}{1 - S^W_{in}}$$

Where S^W_{sol} is the fraction of the pellet space penetrated by a particular solute and S^W_{in} is the interstitial space. As dextran 2000 does not penetrate the cell, $S^W_{in} = S^W_{dex}$ and :

$$R^W = \frac{S^W_{sol} - S^W_{dex}}{1 - S^W_{dex}}$$

If S^W_{sol} for sucrose (S^W sucrose) is measured the value R^W (sucrose) can be obtained, which is a measure of the wall volume plus plasmolytic space.

The weight of the cell ($\approx$ volume) can be obtained from the equation :

$$W \text{ (cell)} = W_p \left(1 - S^W_{dextran}\right)$$

and for the protoplast :

$$W \text{ ('protoplast')} = W_p \left(1 - S^W_{sucrose}\right)$$

7 - Electron microscopy

Samples (4 ml, 10 mg dry wt/ml) of *E. coli* suspended in 10 mM imidazole buffer (pH 7.0) at room temperature were mixed with 1 ml buffer or buffer plus NaCl (final concentration 0.2 M) $MgCl_2$ (0.14 M), glycerol (0.34 M) or sucrose (0.3 M). After 1 min incubation they were fixed by injection of 5 ml of 3% glutaraldehyde (made up in the appropriate solute solution). Samples were centrifuged at 4000 x *g* for 10 min at room temperature, washed four times in solute solution and finally suspended in 0.5 ml 1% OsO_4. After 1 h the solutions were washed three times in the correct solute solution and finally suspended in 1 ml solute solution. This was mixed with 1 ml 3% agar at 60°C. The solidified agar was cut into 1 mm cubes, washed in distilled water and dehydrated through a gradual series of ethanolic solutions. Final dehydration was in absolute ethanol. After overnight incubation in a 30% solution of Spurr's[143] resin in ethanol, they were taken through three changes of resin (8 h each) and finally embedded in fresh resin by overnight polymerization at 70°C. Silver sections were cut on an LKB Ultratom III, mounted on Formvar coated copper grids and stained with 5% uranyl acetate in 1% acetic acid at 60°C for 30 min, followed by alkaline lead citrate[144] for 5 min at room temperature. Sections were examined in an AEI EM 801A electron microscope operating at 60KV.

When envelope fractions were studied by electron microscope, pellets were suspended directly in 1% OsO_4 in Kellenberger buffer[145] (overnight).

The fixed pellets were then washed in Kellenberger buffer and resuspended in 0.5% uranyl acetate in Kellenberger buffer at room temperature. Dehydration, polymerization etc. were followed as mentioned above.

Intact *E. coli* was dried on grids and examined directly in the electron microscope.

8 - Refractive indices measurements

Refractive indices were measured in degree using Abbé refractometer (High accuracy, degree scale model 60/ED, Bellingham and Stanley, London) using a sodium lamp (D, line, 589.6 nm) as the light source.

9 - Osmotic pressure measurements

Osmotic pressures were measured in a freezing-point osmometer (model 3L, Advanced, Instruments, Newton Highlands, Massachusetts, U.S.A.). Media of high osmotic pressure (365 m-osM) routinely used were 0.2 M NaCl, 0.14 M $MgCl_2$, 0.3 M sucrose and 0.34 M glycerol in 10 mM imidazole buffer (pH 7.0). Low osmotic pressure medium (21 m-osM) was provided by buffer alone.

10 - Osmotic shock and Preparation of crude envelopes of *A. vinelandii*

A centrifuged pellet of *A. vinelandii* cells suspended in 50 mM Tris-HCl (pH 7.5) was mixed with an equal volume of 3 M glycerol (In some experiments 3.5 M, see Chapter 2 part 17). After 5 min the mixture was drawn into a syringe and slowly ejected into 10 volumes of mechanically stirred 50 mM Tris-HCl buffer (pH 7.5), containing 1 mM $MgSO_4$ at 4°C [74,75]. After disruption, the

preparation was treated with 0.5 µg/ml crystalline DNAase for 20 min at room temperature. The preparation was then centrifuged at 1500 x *g* for 10 min to remove the intact cells, and supernatant was centrifuged at 23,000 x *g* for 30 min at 4°C. Pellet, which was free of intact cells (crude envelope), was resuspended in 50 mM Tris-HCl (pH 7.5) and kept in ice for use.

11 - Cell counts

Cells were counted in 5 µl of a suitably diluted bacterial suspension, using a Thoma slide, uder a phase contrast microscope.

12 - Preparation of cell wall of *E. coli*

Cell walls of *E. coli* K12 were prepared by the Schaitman method[18,33]. Harvested cells were resuspended in 100 ml of 50 mM Tris-HCl (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA). This suspension was placed in a MSE blender at speed of 12,000 rpm for 1 min. This procedure did not result in any cell breakage as determined by phase contrast microscopy but removed the flagella, pili and capsular material. The preparation was then centrifuged at 6,000 x *g* for 15 min and the pellet resuspended to 25 ml in the same buffer solution (Tris-HCl). 1 mg each of pancreatic ribonuclease and deoxyribonuclease were stirred into the suspension and cells were broken twice through a French pressure cell (American Instrument Co. Silver Springs, Maryland) at maximum working pressure (22,000 lbs on a $2\frac{1}{8}$" DIA, RAM). $MgCl_2$ was added to the final concentration of 2 mM and cell suspension was then centrifuged at 3,000 x *g* for 5 min to remove intact cells and debris. The supernatant fluid was centrifuged for 45 min at 200,000 x *g* and the pellet, which contained particulate fractions,

was suspended to a final concentration of 10-30 mg/ml in 10 mM imidazole buffer (pH 7.0). Portions (2 ml) of crude envelope suspension were then layered on 28 ml discontinuous sucrose gradients (8 ml of 2.02 M, 10 ml of 1.75 M and 10 ml of 1.44 M sucrose) prepared in 10 mM imidazole buffer (pH 7.0). The gradients were centrifuged at 45,000 x *g* for 16 h (due to large quantities of samples angle head was used instead of swing out head, and, good separation was achieved (refer to results).) and were fractionated by pumping. The bottom band which was white in colour and contained the wall fractions (see results) was diluted into 5 volumes of 10 mM imidazole buffer (pH 7.0) and was then dialysed against 10 mM imidazole buffer (pH 7.0) for 24 h. Wall particles were obtained after high speed centrifugation.

13 - Preparation of membrane vesicles

Preparation of *A. vinelandii* membrane vesicles was followed by method described by Kaback[72] and Barnes[114,115]. Cells grown in glucose containing medium [114] were harvested, and washed with 10 mM Tris-HCl buffer (pH 8.0) at 4°C. Washed cells were resuspended at room temperature to 1 g wet weight per 80 ml in 30 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and the suspension swirled by means of a magnetic stirrer. EDTA (pH 7.0) and lysozyme were added to final concentration of 10 mM and 0.5 mg/ml respectively, and the suspension incubated for 30 min at room temperature. The spheroplast suspension was centrifuged at 16,000 x *g* for 20 min and the pellet suspended in a small volume (5 ml) of 0.1 M potassium phosphate buffer (pH 6.6)containing 0.5 M NaCl and 20 mM $MgSO_4$, using Teflon-glass homogenizer. Homogenization was facilitated by addition of DNAase to final

concentration of 10 µg/ml in the total lysate. Homogenization was carried out until the suspension was uniformly dispersed. The spheroplast suspension was poured directly into 470 ml of 50 mM potassium phosphate (pH 6.6) which had been equilibrated to 30°C. The lysate was incubated for 15 min at 30°C with vigorous swirling. EDTA (pH 7.0) was then added to 10 mM final concentration and the incubation was continued for a further 15 min. Finally $MgSO_4$ was added to a final concentration of 15 mM and the incubation continued for another 15 min. The lysate was centrifuged for 30 min at 16,000 x *g* and the pellet resuspended by vigorous homogenization in a solution of 0.1 M potassium phosphate buffer (pH 6.6) containing 10 mM EDTA (at 0°C).

Occasionally the membrane pellet was viscous and therefore $MgSO_4$ and DNAase were added to give 20 mM and 100 µg/ml final concentration, respectively and suspension was incubated at 30°C for 30 min with shaking. The preparation was then centrifuged at 40,000 x *g* for 30 min and the pellet resuspended in 0.1 M potassium phosphate buffer (pH 6.6) 10 mM EDTA. The sample was then centrifuged at 800 x *g* for 30 min and yellowish, milky supernatant fluid was centrifuged at 40,000 x *g* for 30 min. The high speed pellet was washed 4 times by resuspending in 0.1 M potassium phosphate buffer (pH 6.6) - 10 mM EDTA, followed by centrifugation at 40,000 x *g* for 30 min. After the last wash the membrane was resuspended in the appropriate buffer.

14 - Cations induced proton release from wall fractions

To *E. coli* wall fractions prepared as mentioned above (Chapter 2 part 12) suspended in distilled water (10 mg/ml wet weight), successive additions of solution containing different volumes of $MgCl_2$, NaCl, sucrose or glycerol were followed and

the pH of the suspension was read after each addition on a sensitive pH meter.

15 - Turbidity changes of *E. coli* wall fractions

These were measured in a spectrophotometer at 700 nm. Wall fractions were suspended in 0.9 ml 10 mM imidazole buffer (pH 7.0) in 1 ml cuvettes at room temperature at an absorbance of about 0.2 absorbance units. 0.1 ml of buffer or salts ($MgCl_2$ or NaCl) in buffer to give the desired final concentration were added and the mixture shaken. The absorbance was read after 1 min.

16 - Glucose uptake assay

Glucose uptake was measured in crude envelopes of *A. vinelandii* prepared by osmotic shock or in membrane vesicles prepared by method given above (Chapter 2 part 13). The assay for glucose uptake was similar to method described by Barnes[115]. The assay mixture (100 µl final volume) contained 50 mM potassium phosphate buffer (pH 7.0), 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 50 µM FAD and 50 µg of membrane protein. These mixtures were incubated at 25°C for 5 min in a test tubes (12 x 75 mm) which were continuously gassed with a stream of water-saturated oxygen. Sodium L-malate (pH 7.0) was added to a final concentration of 20 mM (other electron donors i.e. NADH and DL-lactate were present at 10 and 20 mM final concentration respectively), followed immediately by addition of D-(U-^{14}C) glucose at 40 µM final concentration. Incubation was continued under oxygen at 25°C for an appropriate time i.e. 1 min and then glucose uptake was terminated by addition of 1 ml of 0.1 M LiCl. The mixture was immediately filtered through a millipore filter paper (HA 0.45 µ, 25 mm, HAWP 025 00,25 ea.). The assay tube was filled immediately with another 1 ml of 0.1 M LiCl and this rinse passed through the same filter. The filters were dried and were

counted in 0.4% 2,5-Diphenyloxazol (PPO), made up in toluene, in a scintillation counter Tri Carb model 3375.

17 - Separation of *A. vinelandii* envelope fractions on sucrose density gradients

A suspension of crude envelope in buffer (6 ml), prepared as given above (Chapter 2 part 10) was treated at full power output in a MSE sonicator at 4°C for 4 - 5 min. This suspension was then mixed with 2.5 ml of glass beads (0.17 - 0.18 mm diam, Kat, Nr. 54150 (2884) Glas perlen) and the cup shaken (4.5 mm peak to peak) with a Mickle disintegrator (The Mickle Laboratory Engineering Co., Gomshall, Surrey) for 8 - 10 min. The cup was chilled periodically in an ice bath so that the temperature was below 20°C during treatment[79]. Portions (2 ml) of treated envelope suspension were layered on 16 ml discontinuous sucrose gradients (3 ml of 2.02 M, 6.5 ml of 1.75 and 6.5 ml of 1.44 M sucrose prepared in 50 mM Tris-HCl (pH 7.5))and tubes were centrifuged for 16 h at 20,000 x *g* in a superspeed 65 swing out rotor. Gradients were fractionated by pumping and were diluted 3-5 times in Tris-HCl buffer (pH 7.5) and were centrifuged at 100,000 x *g* for 90 min. Envelope fractions (walls and membranes) were then suspended in small volumes of desired buffer.

18 - Protein assay

Protein was assayed by Buiret method[146] using bovine serum albumin as standard.

19 - Cytochrome assay

Cytochrome spectra were measured at room temperature in a Hitachi-Perkin Elmer 356 Spectrophotometer. Oxidised minus reduced difference spectra were obtained by reducing one cuvette with a few grains of sodium dithionate ($Na_2S_2O_4$) and oxidising the

other cuvette by potassium ferricyanide ($K_3Fe(CN)_6$) [147,148].

20 - Succinate dehydrogenase assay

Succinate dehydrogenase activity was measured aerobically at room temperature by the decrease in absorbance of 2,6-dichlorophenol indophenol (DCPIP) at 600 nm in Unicam SP 1800 Spectrophotometer. The reaction mixture contained 50 mM phosphate buffer (pH 7.8) 0.1 mM DCPIP, 1.65 mM phenazine methosulphate (PMS), 5 mM KCN, and suitable concentration of the extract. The reaction was started by 30 mM succinate as substrate [149].

21 - Oxidase activities

Respiration was measured at 30°C (unless otherwise indicated) with an oxygen electrode (Rank Bros. Bottisham, Cambridge) in a volume of 3 ml containing desired buffers and cell fractions. The reactions were started by addition of substrate. Oxidase rates were calculated using data of Chapell[150] for the dissolved oxygen concentrations.

22 - Isocitrate dehydrogenase assay

Isocitrate dehydrogenase was measured by Unicam SP 1800 Ultraviolet Spectrophotometer. Increase of OD in 3 ml solution containing 1.55 ml distilled water, 1 ml of 100 mM Tris-HCl (pH 7.7), 0.2 ml of 20 mM $MnSO_4.2H_2O$, 0.05 ml of Triphosphopyridine nucleotide (TPN), (12 mg in 3 ml Tris buffer initial concentration), and 0.1 ml of DL-isocitric acid (150 mM in Tris) at room temperature, and 340 nm[151].

23 - RNAase assay

RNAase was assayed by method described by Neu and Heppel[152]. The ribosomal endogenous RNA was first degraded by incubation of samples at 37°C in 100 mM phosphate buffer (pH 7.0 containing 10 mM EDTA for 40 min. To 4.5 ml of phosphate buffer plus EDTA containing 0.3 ml of 10 mg per ml RNA type XI 1.2 ml of fraction was added and

after 0.25, 0.5, 1, 2, 3, 5, 7, 10 and 15 min, 0.5 ml of each sample was mixed with 0. 5 ml of 6% perchloric acid (3% final) and samples were then centrifuged at 2000 x *g* for 10 min at room temperature. 0.2 ml of supernatant was mixed with 2.8 ml of distilled water and the absorbance was read at 260 nm. RNAase activity of each sample was measured per unit absorbance change per min per mg protein after plotting absorbance against time.

24 - Lipopolysaccharide content

This was estimated by determination of 2-Keto-3-deoxyctonate (KDO)[30]. Cell fractions (0.2 to 2 mg of protein) were precipitated with cold 10% trichloroacetic acid (5 ml) collected by centrifugation at 4°C for 10 min at 20,000 x *g*. The precipitate was suspended in 0.7 ml of 0.018 N H_2SO_4 and hydrolysed at 100°C for 20 min to liberate KDO from LPS. The KDO was then determined directly on the hydrolysate by the thiobarbituric acid method[153]. As saturated N-acetylnuraminic acid was used[80].

A solution of sample, blank or standard (containing 5-40 µg of N-acetylnuraminic acid) in 0.5 ml water was treated with 0.25 ml of the periodate agent (25 mM periodic acid in 0.125 N H_2SO_4 pH 1.2) for 30 min in a water bath at 37°C. The excess of periodate was the reduced with 0.2 ml of sodium arsenite (2% solution of sodium arsenite in 0.5 N HCl). As soon as the yellow colour of the liberated iodine disappeared (1 - 2 min) 2 ml of the thiobarbituric acid reagent (0.1 M of 2-thiobarbituric acid in water pH 9.0) was added and the test samples were covered and heated in a boiling water bath. The coloured solutions were then cooled in ice water and shaken with 5 ml of acid butanol (butan-1-ol containing 5% (V/V)

of 11 N HCl). Separation of the two phases was facilitated by rapid centrifugation (1 min at 500 x *g*). The intensity of the colour in butanol layer were compared at 549 nm.

25 - Assay for diaminopimelic acid

Diaminopimelic acid was assayed by paper chromatography[154]. Dried fractions (about 10 mg) were hydrolysed for 18 h with 1 ml of 6 N HCl in an oven/in sealed tubes. After cooling, the tubes were opened and the contents were filtered. The solid material on the paper was washed with 3 drops of distilled water. The liquid hydrolysate was dried three consecutive times on a steam bath to remove most of the HCl. The residue was taken up in 0.3 ml of distilled water and 40 µlitres of the liquid were spotted on Whatman No. 1 paper. Descending chromatography was carried out overnight with methanol-water-10 N HCl-pyridine (80 -17.5 - 2.5 -10 by volume). Amino acids were detected by spraying acetonic ninhydrin (0.1% W/V) on paper, followed by heating for 2 min at 100°C. Diaminopimelic acid spots were olive-green fading to yellow. Other amino acids of the hydrolysate gave purple spots; with this reagent and had greater Rf values. For quantitative determinations, the spots were cut out and were eluted with 0.5 N HCl and extinctions measured at 353 nm[155]. As a reference standard 1,2,3,5 and 10 µlitres of 0.01 M DL-α-ε-diaminopimelic acid were put on the paper and run alongside the samples.

26 - NADH-ferricyanide oxireductase activity

The reduction of ferricyanide was measured in the presence of 0.08 M Tris-HCl (pH 7.4), 1 mM KCN, 0.7 mM ferricyanide and 100 µl of suitably diluted particles. Reaction was started by

addition of 1 mM NADH and substrate-dependent reduction of ferricyanide was followed at 420 nm in Unicam SP 1800 Ultraviolet Spectrophotometer. Toluene was added to the final concentration of 0.1% (3 µl/ 3 ml) followed by 10 min incubation at 30°C [66].

27 - The effect of ion-translocating uncouplers on the rate of respiration

The effects of the ion-translocating uncouplers on the respiration rate of *A. vinelandii* crude envelopes was followed by the method of John and Hamilton[117]. The basic reaction medium in a total volume of 3 ml consisted of 50 mM Tris-acetate (pH 7.3), 5 mM Mg-acetate, 15 µl ethanol, 12 units dialysed alcohol dehydrogenase and 0.1 ml of particles suspension containing 1.0 mg protein. The reaction was started by addition of 0.2 mM NAD^+ at 30°C. Valinomycin (1 µg), nigericin (1 µg), potassium acetate (10 mM) and ammonium acetate (10 mM) were added to basic reaction medium as indicated in table 8. (Antibiotics were added as small volumes (5 µl) of acetone solutions).

28 - Chemicals

All chemicals were the finest grade available. DNAase 1, FAD grade III, DAP, RNAase type 1-A, N-acetylnuraminic acid type II, RNA type XI, oleic acid, elaidic acid, NADH grade III, lysozyme grade I, NAD grade III, TPN and vitamin B1 were obtained from Sigma Chemical Co., PMS from BDH Chemicals Ltd., Valinomycin A grade from Calbiochem (Los Angeles, California), Ficoll and dextran were obtained from Pharmacia Ltd., Nigericin (X-464) from Roche Products Ltd. (Manchester Sq., London).

All solutions were made up in distilled water.

CHAPTER 3

R E S U L T S

1 - Turbidity increases of *E. coli*

It has previously been shown that increases in medium osmotic pressure due to non-permeant solutes cause decreases in volume of *E. coli* and that these decreases in volume can be measured as increases in turbidity[86]. The turbidity changes were found to be strictly dependent on medium osmolality, despite the fact that ions also caused a contraction in volume of whole cells including the wall, whilst non-electrolytes caused only a decrease in the 'protoplast' volume (plasmolysis only). Furthermore, since some divalent cations are bound to bacterial cell walls[46,48,49], it might be expected that there would be small differences in the effects caused by monovalent and divalent ions.

As most solutes cause small changes in medium refractive index , the osmotically-induced turbidity changes in *E. coli* should be corrected for changes in medium refractive index, to see if there are in fact slight differences in the turbidity increments induced by different solutes.

Since Ficoll has a high refractive index but, due to its high molecular weight, has little effect on the medium osmotic pressure (cf. leaflet 'Ficoll for Cell Research' from Pharmacia Ltd.), corrections can be made for changes in medium refractive index. The decrease in turbidity of a cell suspension on addition of Ficoll, giving an equivalent refractive index change to that of a particular concentration of a given solute, can be added to the increase in turbidity observed on addition of the solute to the cells.

Fig. 17 shows the turbidity changes of an *E. coli* suspension

Fig. 17. The observed changes in turbidity of *E. coli*, caused by sucrose (▲), $MgCl_2$ (○), NaCl (□), glycerol (■) and Ficoll (●). *E. coli* suspended in 10 mM imidazole buffer (pH 7.0) was mixed with solutes and the absorbance measured at 700nm in 1 cm cuvettes 2 min after mixing (room temperature).

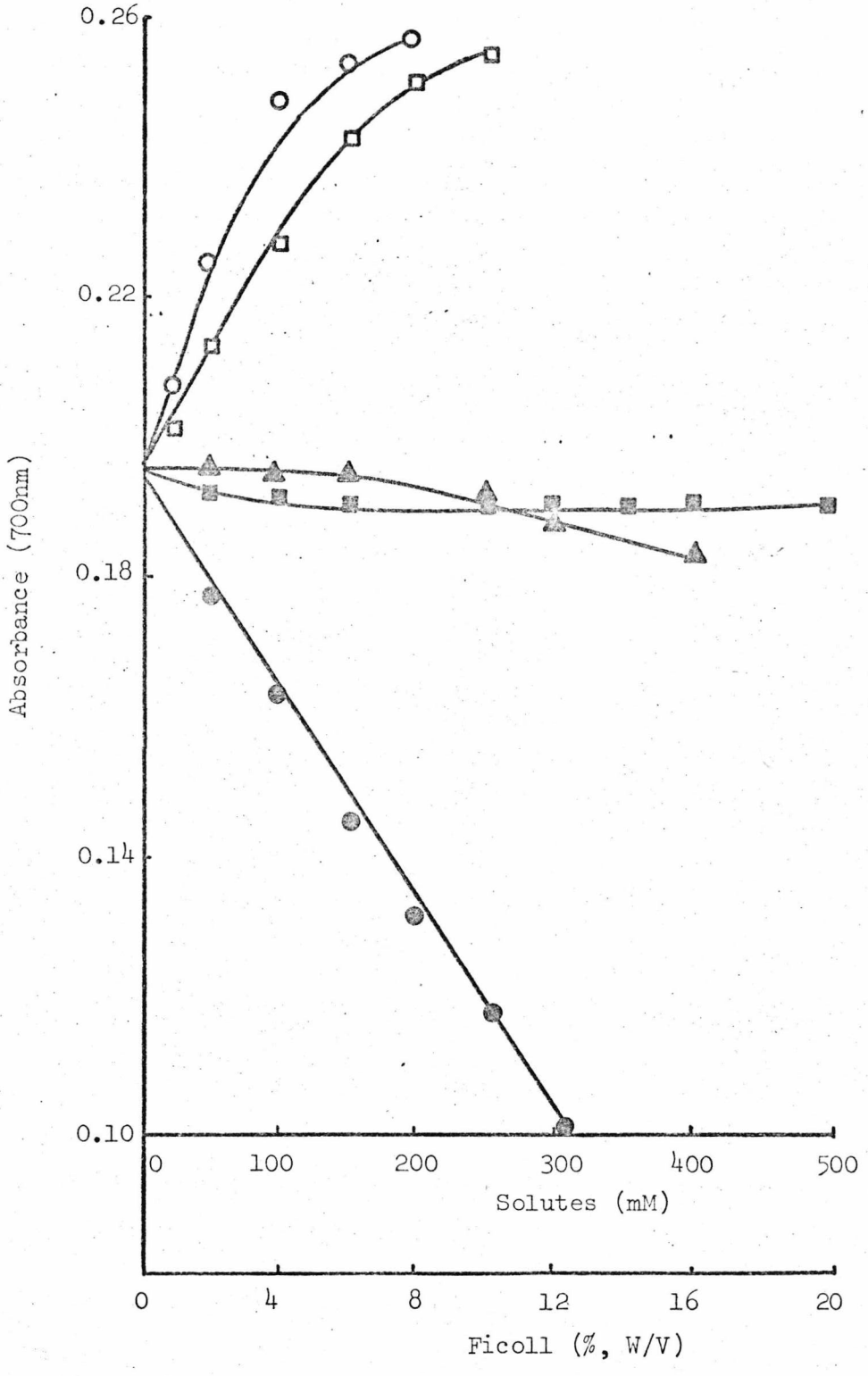

0.26
0.22
0.18
0.14
0.10
Absorbance (700nm)
0
100
200
300
400
500
Solutes (mM)
0
4
8
12
16
20
Ficoll (%, W/V)

Fig. 17

in 10 mM imidazole buffer obtained on raising the medium osmotic pressure by addition of $MgCl_2$, NaCl, sucrose, glycerol and Ficoll. Fig. 18 shows the refractive indices of sucrose, $MgCl_2$, NaCl, and glycerol in buffer and the corresponding refractive index of Ficoll, whilst Fig. 19 shows the osmolarities of the various solutes. Fig. 20 shows the data given in Fig. 17, redrawn with correction for the refractive index changes (from the information given in Fig. 18). Finally, Fig 21 is redrawn from Fig. 20, giving the turbidity changes in terms of the osmolarities of the solutes (from Fig. 19) and also corrected for the changes in refractive index caused by the solutes.

It is evident from Fig. 21, that the increases in turbidity are strictly dependent on the medium osmotic pressure and the curve obtained does not markedly differ from the uncorrected curves due to added salts[86]. The refractive index increments due to NaCl, $MgCl_2$ and glycerol are small and were not expected to have a large effect. On the other hand, the refractive index increment due to sucrose is much larger; the corrected curve due to sucrose exactly fits the curve corresponding to the salts.

The curve obtained for glycerol does not fit the curve obtained for salts and sucrose because glycerol is highly permeable, as will be shown below, and does not effect the 'protoplast' volume (cells are not plasmolysed).

2 - Electron microscopy of *E. coli*

Under a phase contrast microscope it can be seen that addition of non-penetrant salts (NaCl or $MgCl_2$) or sucrose to suspensions of *E. coli* in dilute (10mM) buffer, cause plasmolysis (contraction

Fig. 18. Refractive indices of sucrose (○), glycerol (□), $MgCl_2$ (△), NaCl (▲) and Ficoll (●) in 10 mM imidazole buffer (pH 7.0).

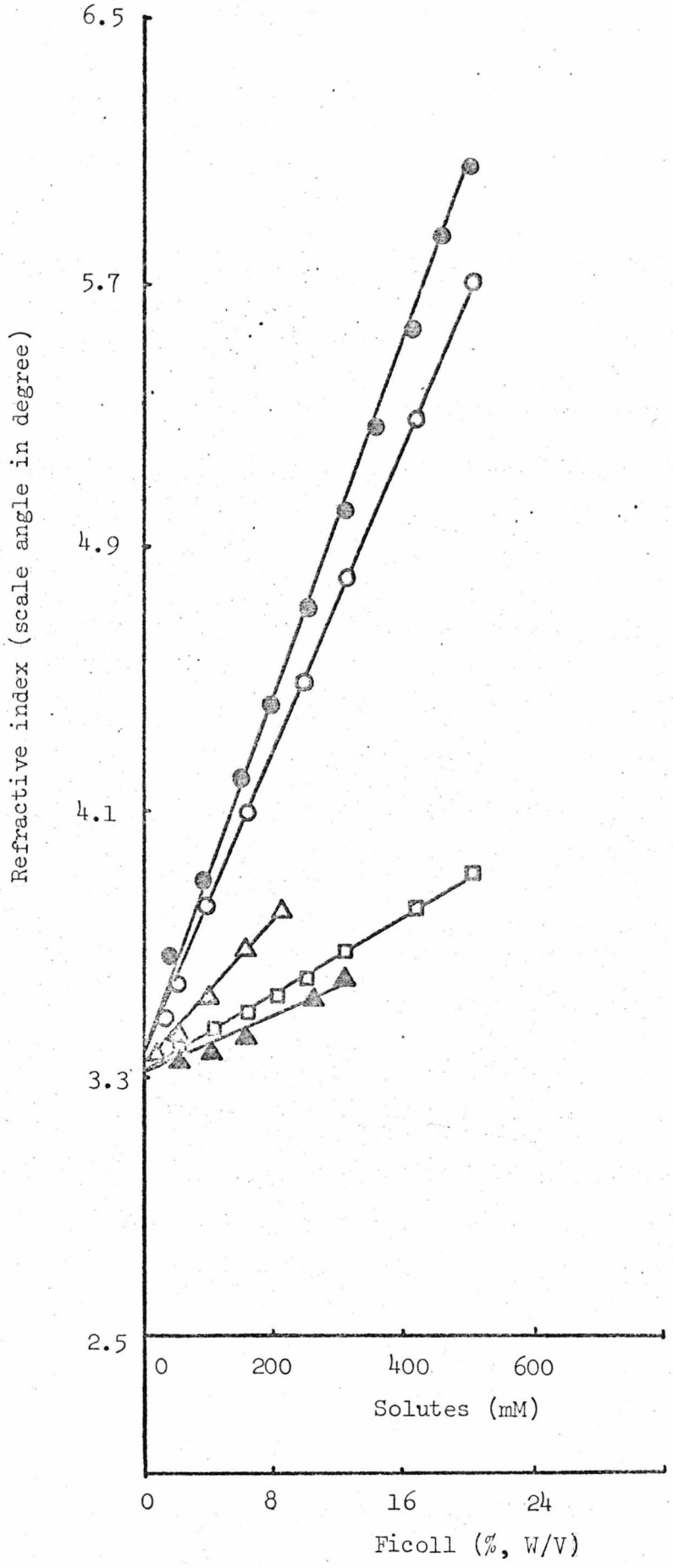

6.5
5.7
4.9
4.1
3.3
2.5
Refractive index (scale angle in degree)
0
200
400
600
Solutes (mM)
0
8
16
24
Ficoll (%, W/V)

Fig. 18

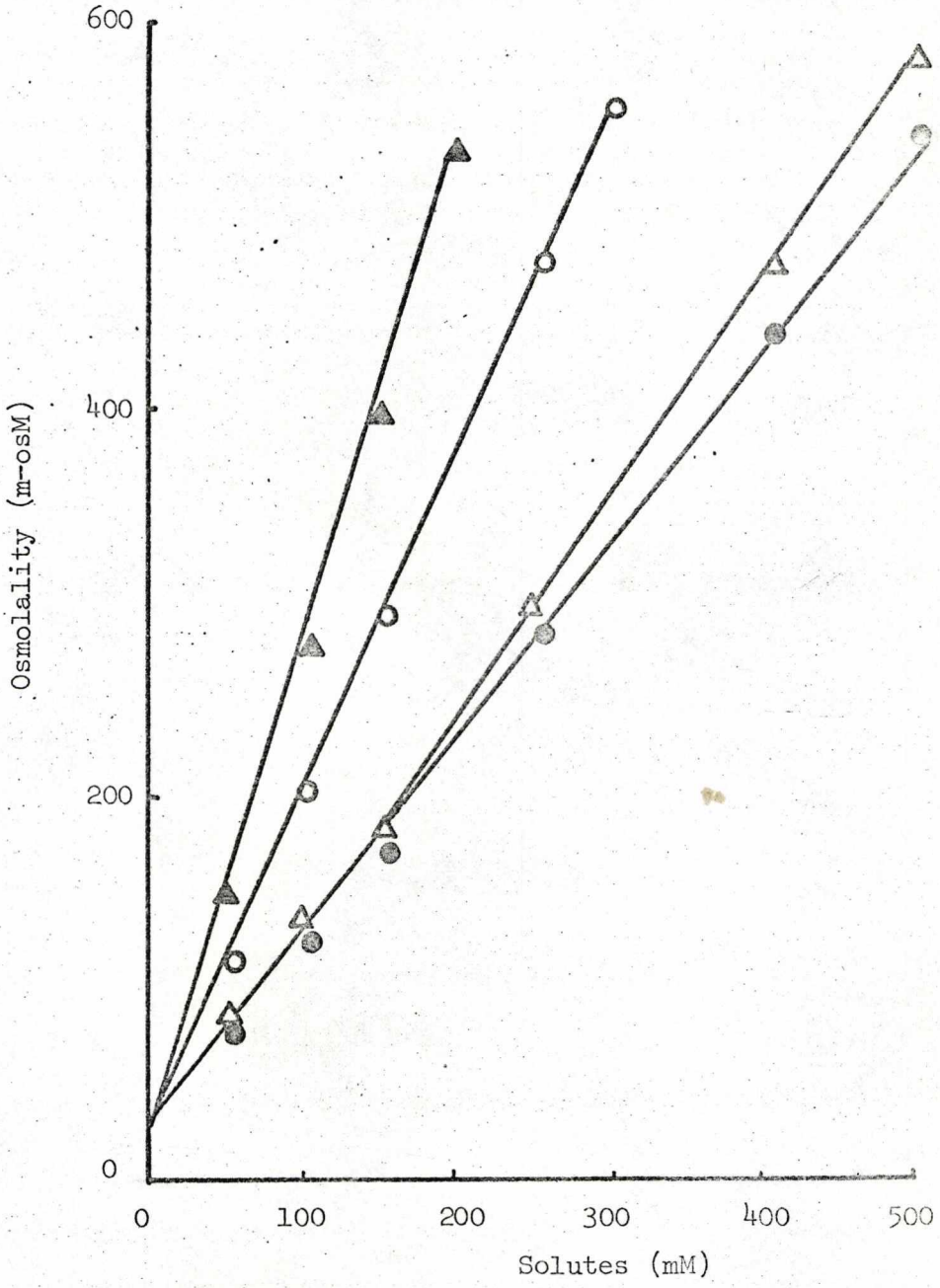


Fig. 19. Osmolality of various solutes suspended in 10 mM imidazole buffer (pH 7.0); (▲) $MgCl_2$, (○)NaCl, (△) sucrose, (●) glycerol. The osmolality of the buffer alone was 21 m-osM.

Fig. 20. Curves of changes in turbidity of *E. coli* corrected for changes in medium refractive index by the addition of the decrease in absorbance seen on addition of Ficoll giving a similar change in refractive index of the medium to that caused by the particular solute. This was found by reference to calibration curves (Fig. 18); (△) $MgCl_2$, (●) NaCl, (□) sucrose, (○) glycerol.

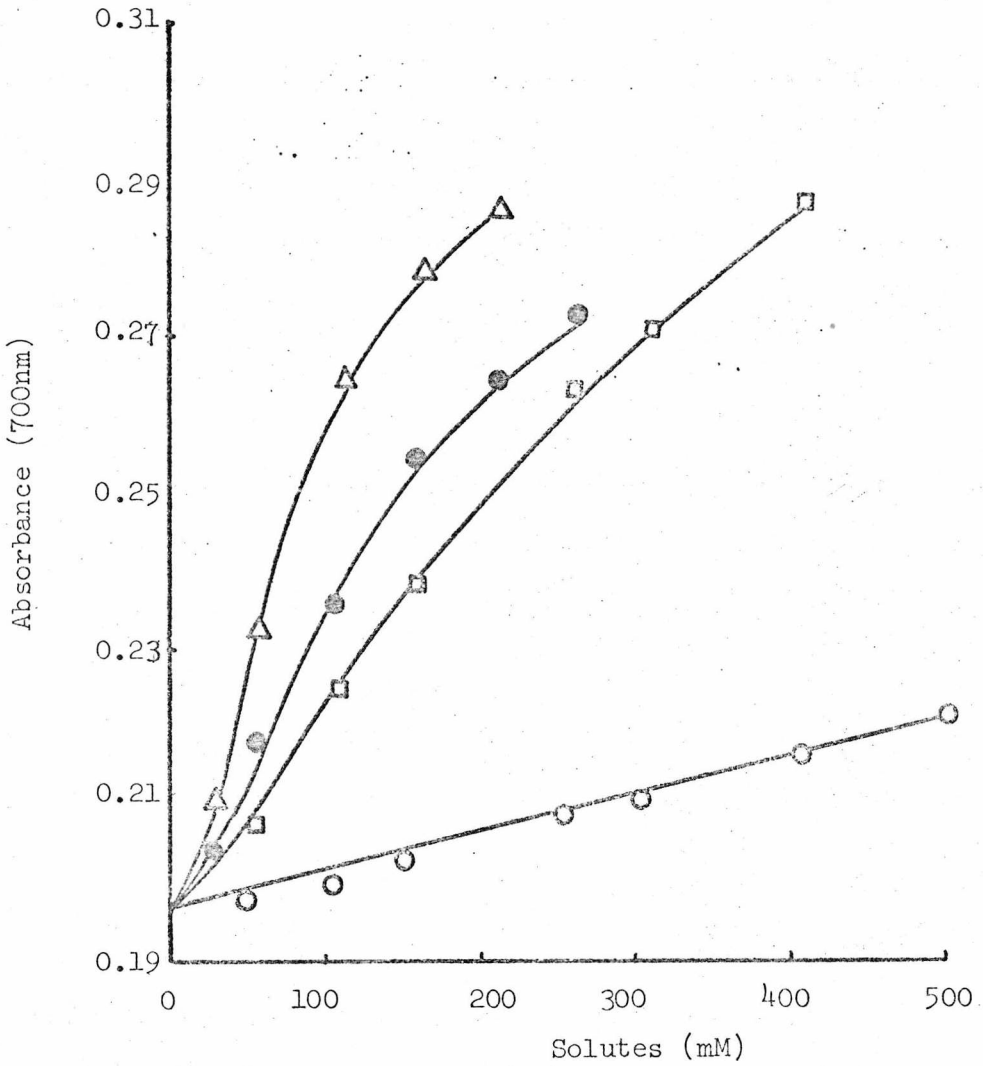

0.31
0.29
0.27
0.25
0.23
0.21
0.19
Absorbance (700nm)
0
100
200
300
400
500
Solutes (mM)

Fig. 20

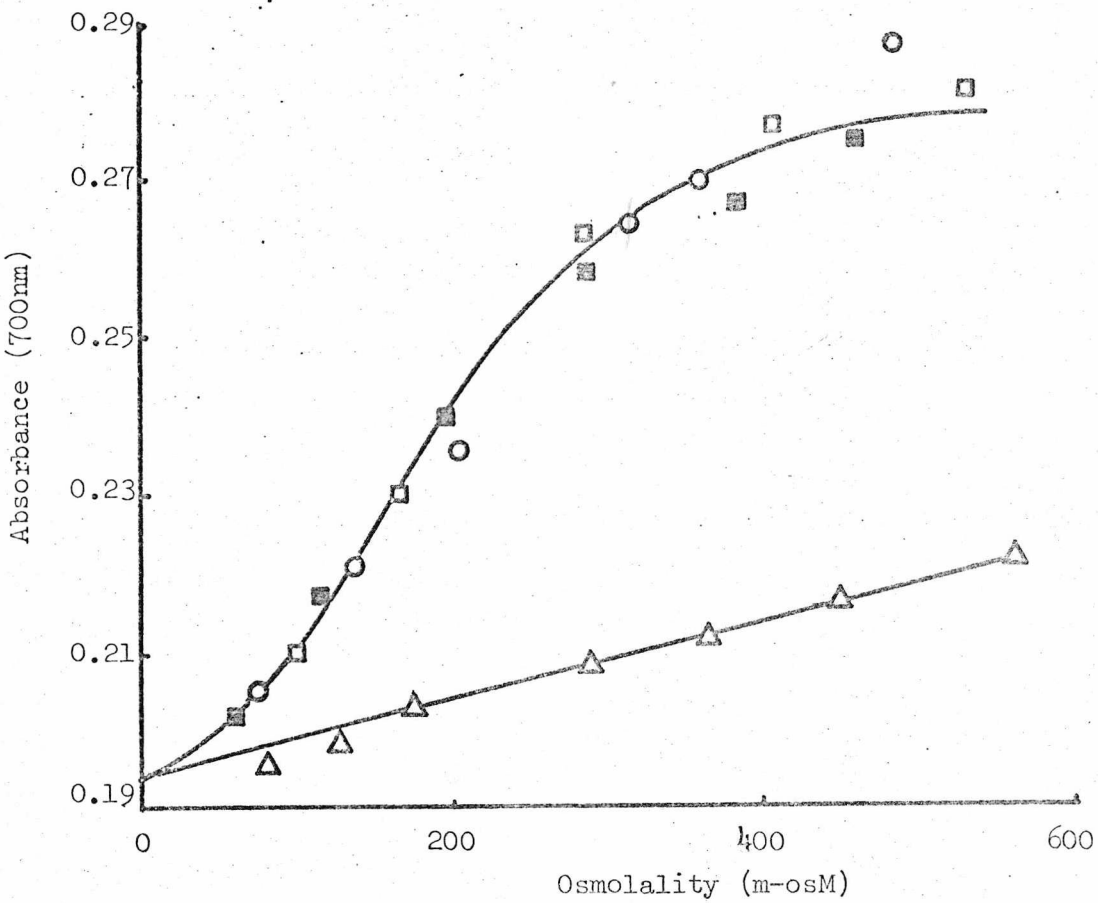
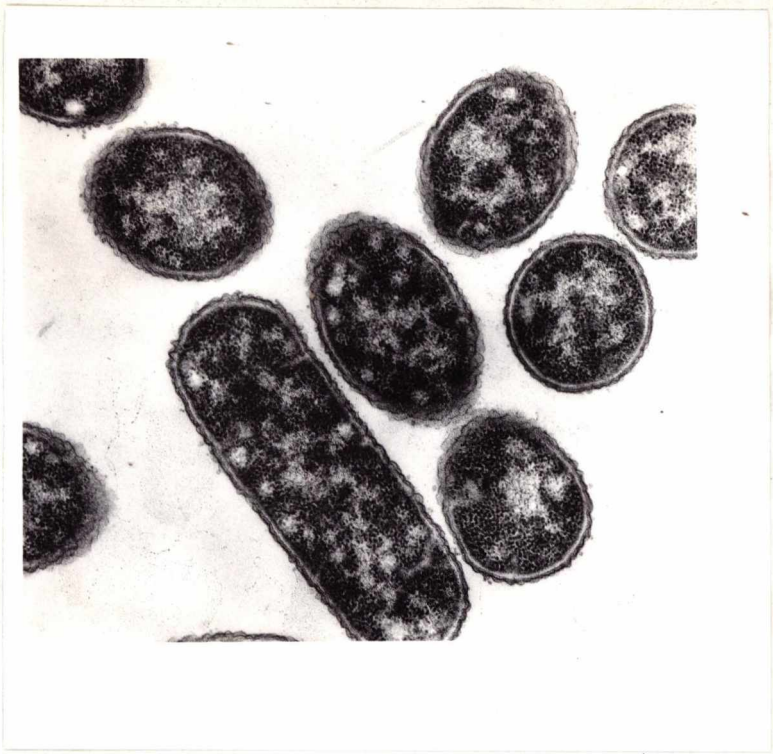


Fig. 21. The increase in turbidity of a suspension of *E. coli* in 10 mM imidazole buffer (pH 7.0) on addition of NaCl (■), $MgCl_2$ (□), sucrose (○), or glycerol (△) after correction for changes in medium refractive index and expressed in terms of medium osmolality. Redrawn from Fig. 17, using the data given in Figs. 18 - 20.

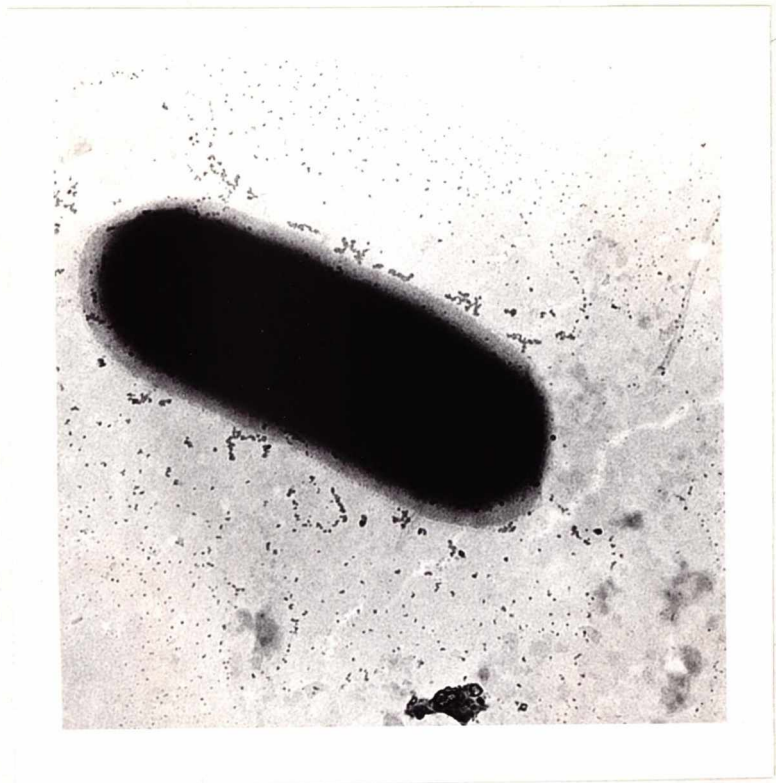
of the cytoplasmic membrane away from the cell wall). In the case of the permeant glycerol no plasmolysis can be seen. If, however, a drop of the concentrated glycerol solution is added to the side of the slide whilst looking down the microscope, the cells appear to "shudder". Due to the rapidity of the effect it is difficult to tell whether or not this is due to transient plasmolysis.

Electron micrographs of stained sections of *E. coli* cells fixed under different osmotic conditions are shown in Figs. 22 to 26. Fig. 22A shows an electron micrograph obtained from cells fixed when suspended in 10 mM imidazole (pH 7.0); Fig.23A, cells fixed when suspended in 0.2 M NaCl, Fig. 24A, cells fixed when suspended in 0.14 M $MgCl_2$; Fig. 25A, cells fixed when suspended in 0.34 M glycerol. In the case of glycerol no plasmolysis can be seen and the cytoplasmic membrane is in continuous close contact with the cell wall. In the cells fixed in the presence of $MgCl_2$ and NaCl plasmolysis occurs. The cytoplasmic membrane is contracted away from the cell wall, and the murein layer remains associated with the wall. Numerous points of contact remain between the wall and the membrane giving a 'loop' effect. Sucrose also causes plasmolysis with contraction from either both sides (Fig.26A) or the end(s) of the cell[156]. The occurrence of the numerous points of contact between the cell wall and the cytoplasmic membrane seen by plasmolysis with NaCl and $MgCl_2$ do not occur in plasmolysis with sucrose; they appear to depend on the ionic strength of the medium.

Figs. 22B, 23B, 24B, 25B and 26B, show the corresponding

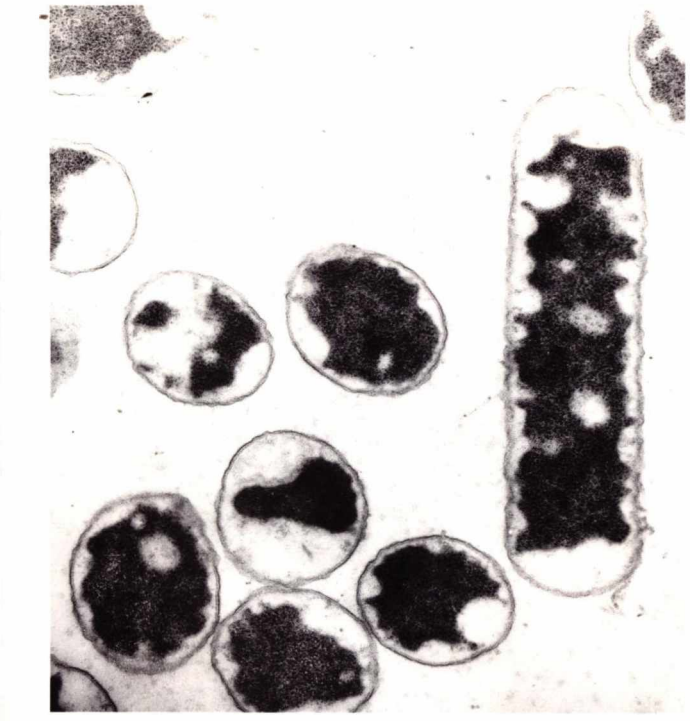


(A)

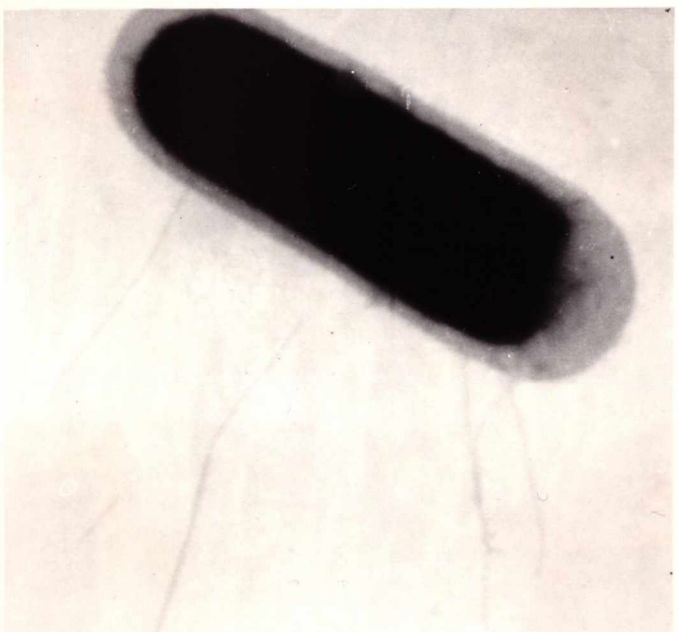


(B)

Fig. 22. Electron micrographs of *E. coli* in 10 mM imidazole buffer (pH 7.0). (A) section: x 29750, (B) intact cells: x 31250.



(A)



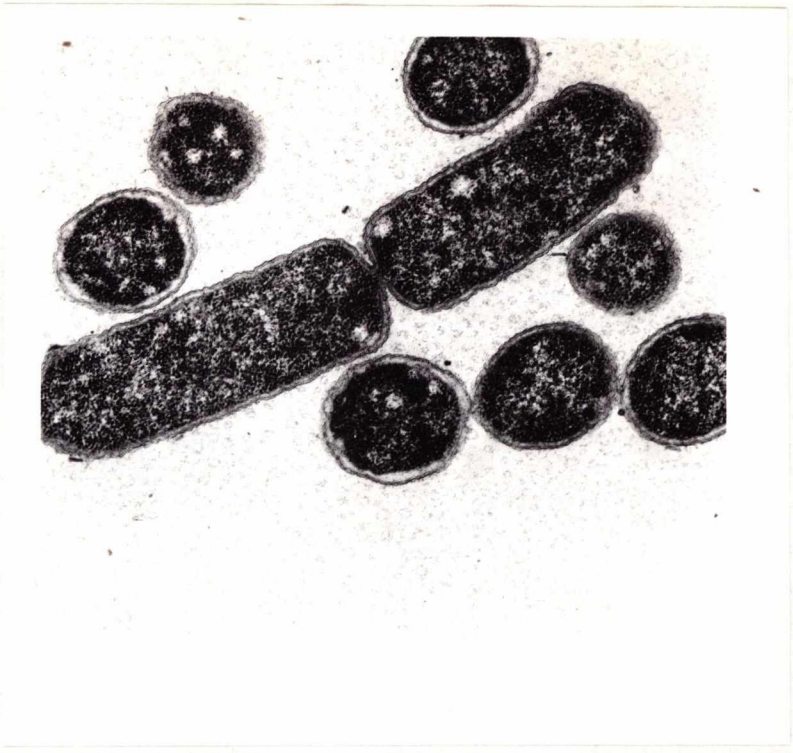
(B)

Fig 23. Electron micrographs of *E. coli* in 10 mM imidazole buffer (pH 7.0) containing 0.2 M NaCl.
(A) section: x 29750, (B) intact cells: x 31250.

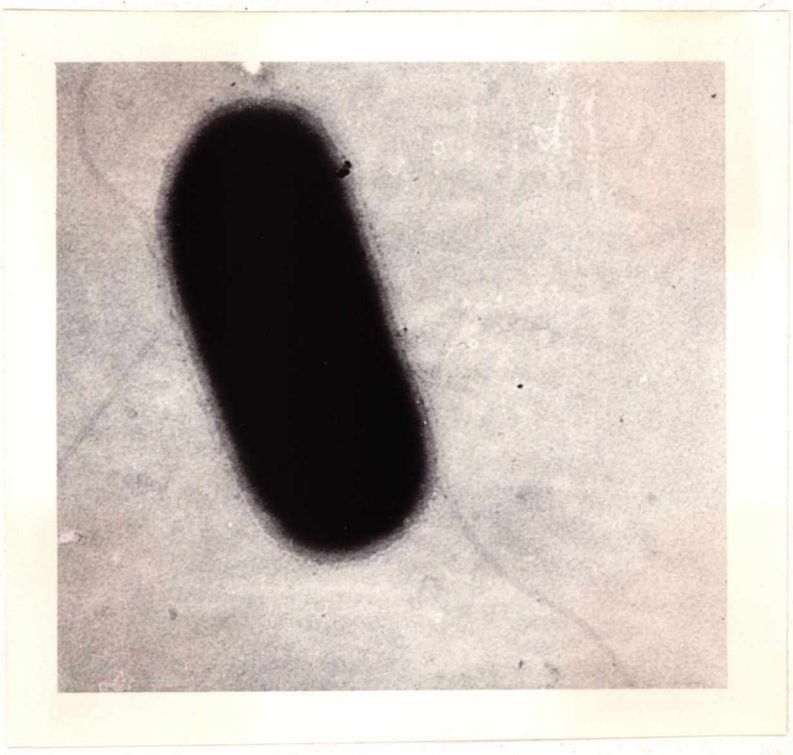
(A)

(B)

Fig. 24. Electron micrographs of *E. coli* in 10 mM imidazole buffer (pH 7.0) containing 0.14 M $MgCl_2$.
(A) section: x 29750, (B) intact cells: x 31250.

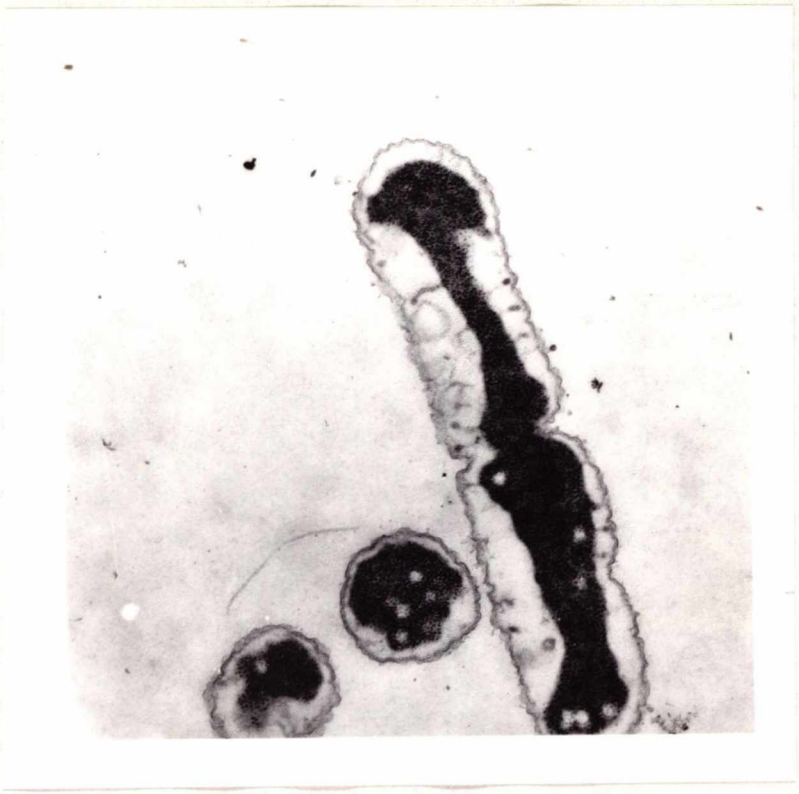


(A)

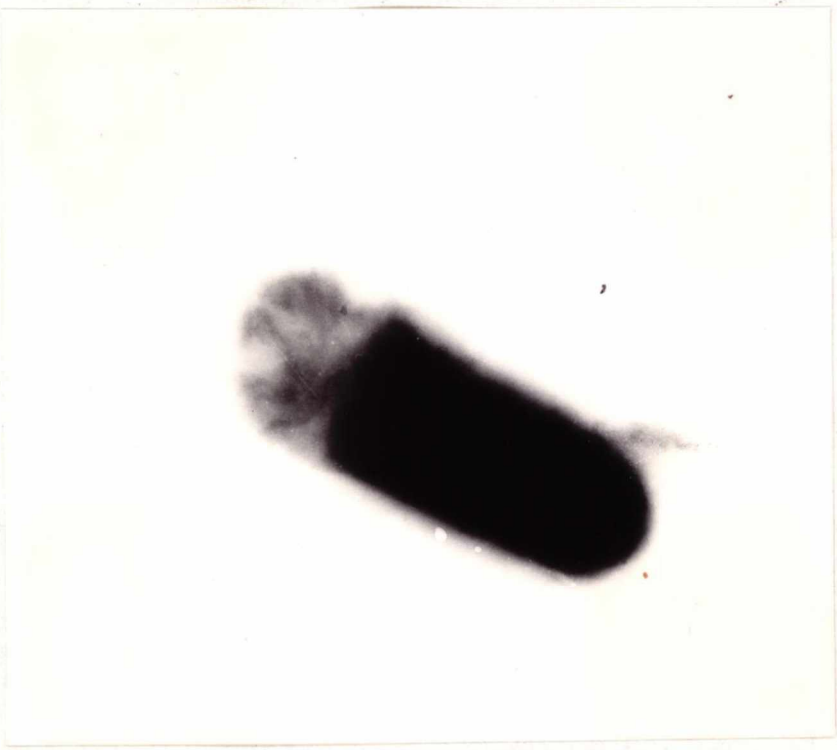


(B)

Fig. 25. Electron micrographs of *E. coli* in 10 mM imidazole buffer (pH 7.0) containing 0.34 M glycerol.
(A) section: x 29750, (B) intact cells: x 31250.



(A)



(B)

Fig. 26. Electron micrographs of *E. coli* in 10 mM imidazole buffer (pH 7.0) containing 0.3 M sucrose.
(A) section: x 29750, (B) intact cells: x 31250.

electron micrographs of intact *E. coli* suspended in 10 mM imidazole buffer (pH 7.0), 0.2 M NaCl, 0.14 M $MgCl_2$, 0.34 M glycerol and 0.3 M sucrose respectively.

All solutes were made up in 10 mM imidazole buffer (pH 7.0).

3 - Volume measurements of *E. coli*

Measurements of the cell volume of *E. coli* were done by the exclusion space method. Scherrer and Gerhardt[82] have shown that dextrans of molecular weight greater than about 10^5 are unable to penetrate the cell wall of *Bacillus megaterium*, but with reducing size, sugars are increasingly able to penetrate the cell wall. I have used blue dextran 2000 and sucrose to determine the whole cell and 'protoplast' volumes of *E. coli* respectively, and measurements of the effect of changes of medium osmotic pressure on cell size (dextran space) and 'protoplast' size (sucrose space), were followed by the method of Black and Gerhardt[142], (Chapter 2 part 6). The results of a typical experiment are given in Table 1, and the results of the average of six experiments of the type detailed in Table 1, are given in Table 2.

Table 2 shows that increasing the medium osmotic pressure by equiosmolal concentrations of NaCl, $MgCl_2$ or sucrose caused a 30 to 35% shrinkage in 'protoplast' volume. In addition the salts caused a 15 to 20% shrinkage in volume of the whole cell, whilst the non-electrolyte, sucrose, caused only a small (5 to 10%) decrease in volume. The fraction of the cell penetrated by (^{14}C) sucrose (R^W for sucrose) was 26 to 34% in cells suspended in buffer (the molarity of (^{14}C) sucrose was only 3 mM and, therefore, it would not itself cause plasmolysis.). This space represents the volume of

TABLE 1. EXPERIMENTAL RESULTS OF EXCLUSION SPACE OF *E. coli* SUSPENDED IN DILUTE BUFFER, BUFFER PLUS NaCl OR BUFFER PLUS GLYCEROL.

	Suspension medium		
	10 mM imidazole buffer (pH 7.0)	+0.2 M NaCl	+ 0.34 M glycerol
Pellet weight, W_p (g)	1.504	1.293	1.571
(^{14}C) Sucrose concentration, C_f (cpm)	12120	11820	12210
Dextran 2000 concentration C_f(A620)	0.261	0.266	0.259
Sucrose space, S^W sucrose	0.424	0.523	0.399
Dextran space, S^W dextran	0.149	0.157	0.148
Cell volume, W (cells)	1.280	1.092	1.339
'Protoplast' volume, W ('protoplast')	0.867	0.617	0.944
W (cells) (%)*	100	85.4	104.8
W ('protoplast') (%)*	100	71.3	109.0
R^W (sucrose) as % cell volume	32.3	43.4	29.5

Initial (^{14}C) sucrose concentration was 3 mM and 20705 cpm. Initial dextran 2000 concentration (C_o) was equivalent to an absorbance of 0.326 at 620 nm.

* Compared to the volume of the cell and 'protoplast' when suspended in buffer only.

TABLE 2. THE VARIATION IN CELL VOLUME AND 'PROTOPLAST' VOLUME OF *E. coli* SUSPENDED IN 10 mM IMIDAZOLE BUFFER (pH 7.0) WITH ADDED SALTS, SUCROSE AND GLYCEROL.

Addition (365 m-osM)	'Protoplast' volume (%)	Cell volume (%)
No addition	100	100
0.2 M NaCl	67	81
0.14 M $MgCl_2$	68	84
0.30 M Sucrose	70	91
0.34 M glycerol	105	101

The 'protoplast' volumes were measured by the (^{14}C) sucrose space and the cell volumes by the dextran space. The results given are each the average of six experiments of the type detailed in Table 1.

the cell wall. In cells suspended in salts (0.2 M NaCl or 0.14 M $MgCl_2$) this space increased to 40 to 45% of the cell volume and in 0.3 M sucrose it was 43 to 47% of the cell volume. This increase in wall space in the presence of NaCl, $MgCl_2$ or sucrose is due to contraction of the cytoplasmic membrane away from the cell wall and the presence of the plasmolytic space.

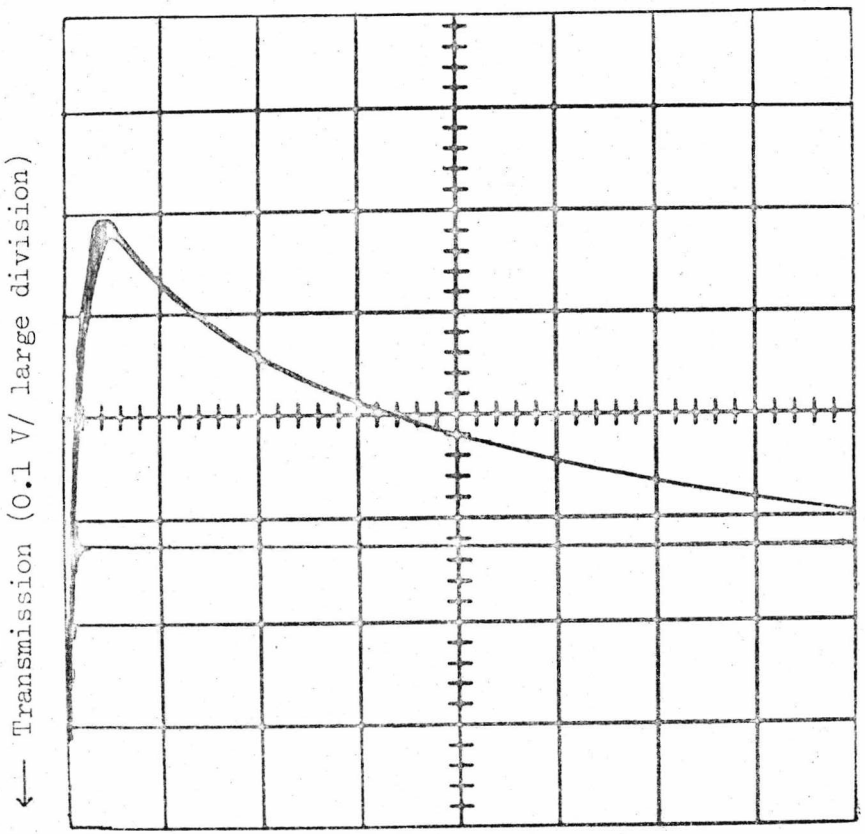
The penetrant glycerol caused no shrinkage in volume (Table 2) or increase in turbidity (Fig.21) though small but consistent (2 to 10%) increases in both 'protoplast' and cell volume were observed.

4 - Turbidity changes of *E. coli* due to glycerol entry

The kinetics of the turbidity changes of *E. coli* as well as the total increase in turbidity, are dependent on osmolarity of the added solutes (Fig. 21)[88]. When the added solute is glycerol there is little increase in turbidity if measured in the period 2 to 60 min after glycerol addition. If, however, the rate of entry into the cell is slower than the osmotically induced outflow of water across the cytoplasmic membrane, then a transient decrease in cell size should occur. Since this effect is over in less than 2 min it is too rapid to measure by direct means, but it should be possible to measure the concomitant turbidity increase.

Fig. 27 shows such a transient increase in turbidity on addition of 0.5 M glycerol to cells suspended in weak buffer. There is an initial rapid increase in turbidity as water leaves the cell, followed by re-swelling (decrease in turbidity) as glycerol enters the cell. The rate of water exit is over 10 times faster than the rate of the subsequent glycerol entry and the

Fig. 27. The transient increase in turbidity of *E. coli* due to 0.5 M glycerol. *E. coli* suspended in 10 mM imidazole buffer (pH 7.0) was mixed in a stopped-flow spectrophotometer with an equal volume of 1 M glycerol in 10 mM imidazole buffer. The change in transmission was measured at 550 nm and the reaction temperature was 25°C. In the right-hand trace the oscilloscope sweep rate was 50 ms/division and in the left-hand trace it was 1 s/division. The lower line in the left hand trace was a re-sweep after a 15 s interval.


← Transmission (0.1 V/ large division)
Time (1.0 s / large division)

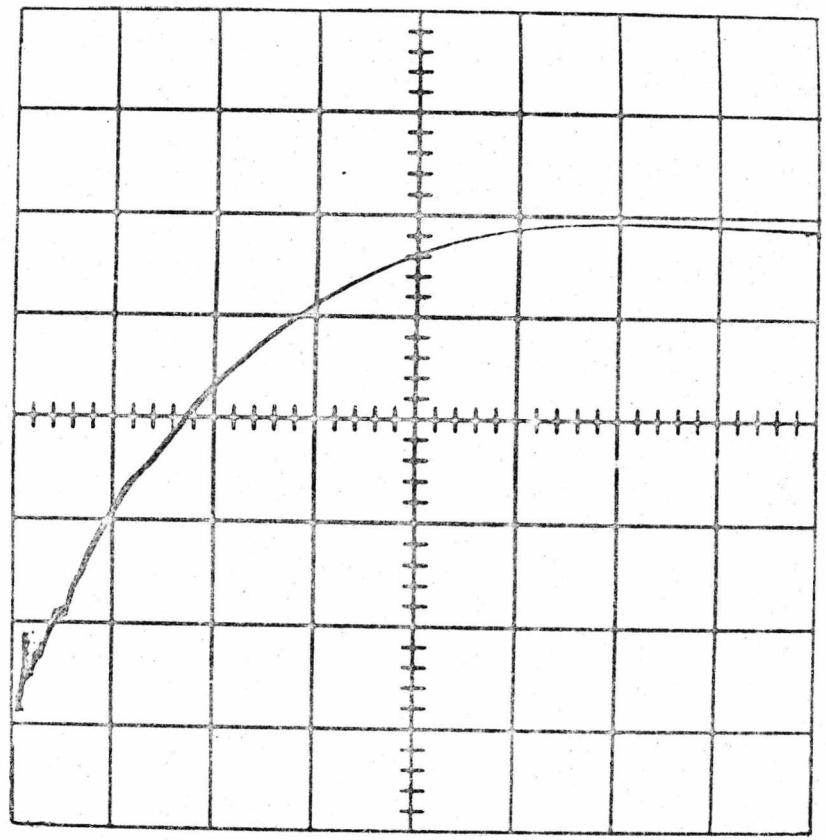

Time (50 ms / large division)

Fig. 27

contribution of each effect to the other is small; they can therefore be considered separately.

The rate of increase in turbidity, due to water exit and cell shrinkage, is dependent on the applied osmolality (Fig. 28), confirming that this is an osmotically induced effect. The turbidity changes are not corrected for the small changes in medium refractive index due to the added NaCl, $MgCl_2$ or glycerol. It is not possible to do this experiment with sucrose as the osmotic agent owing to the large increase in medium refractive index. The effect due to glycerol is not corrected for the second phase glycerol penetration, accounting for the slightly slower apparent rates observed for this solute than for NaCl or $MgCl_2$.

For cells grown on glucose-minimal salts medium, glycerol entry, as might be expected, has first order kinetics (the kinetics of water exit is zero order using a given concentration of added solute [88]). For each applied glycerol concentration a graph of the logarithm of the relative decrease in absorbance against time yields a straight line plot (Fig.29.). The rate of glycerol entry is non-saturating and proportional to the applied glycerol concentration (Fig. 30). The apparent rate constant for glycerol entry is essentially constant up to a glycerol concentration of 400 mM (Fig.29) above that it decreases slightly; this is probably due to the increase in medium refractive index rather than indicating any saturation of the rate of glycerol entry.

The rate of glycerol entry is independent of pH in the range of pH 5.0 to pH 9.0, and independent of the buffer used (provided that

Fig. 28. The rate of increase in turbidity due to osmotically-induced water outflow from *E.coli*. Bacteria suspended in 10 mM imidazole buffer (pH 7.0) were mixed with NaCl (○), $MgCl_2$ (□), or glycerol (△) solutions (in 10 mM imidazole buffer pH 7.0) in a stopped-flow spectrophotometer.

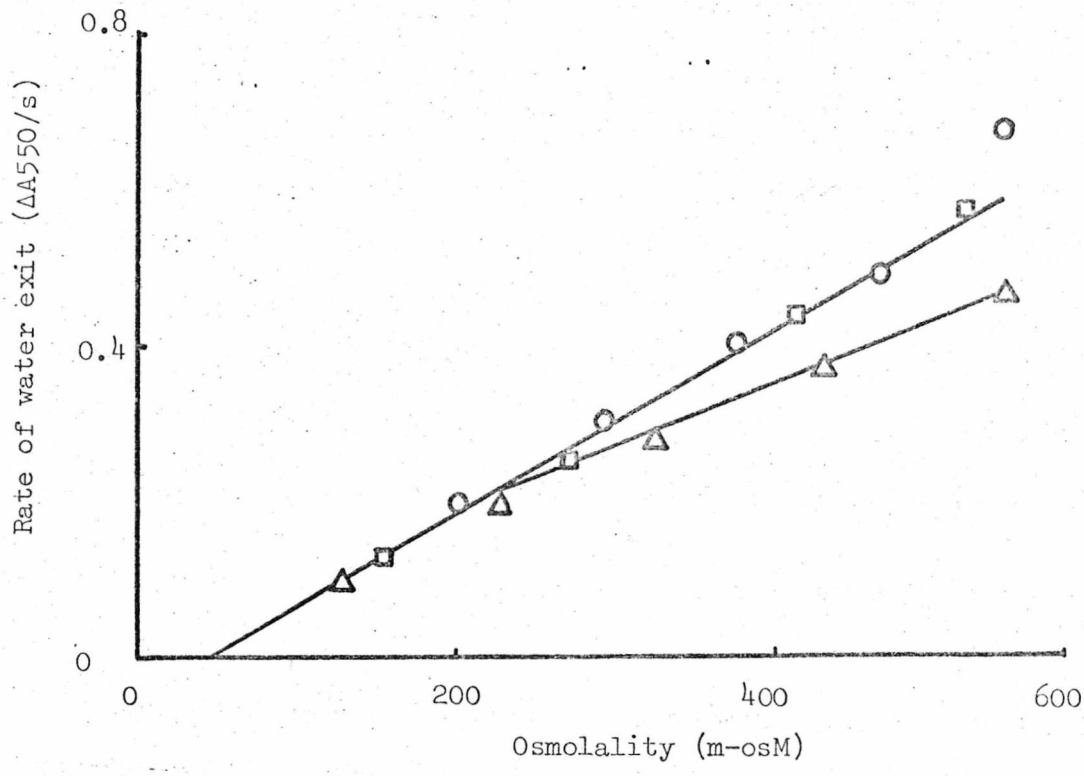

0.8
0.4
0
Rate of water exit (ΔA550/s)
0
200
400
600
Osmolality (m-osM)

Fig. 28

Fig. 29. The first-order kinetics of glycerol-entry into *E. coli*. Bacteria suspended in 10 mM imidazole buffer (pH 7.0) were mixed with glycerol (in 10 mM buffer) to give final concentrations of 150 mM (●), 200 mM (△), 300 mM (○) or 500 mM (▲) glycerol. The transient increases in turbidity were measured at 550nm and at 25°C (cf. Fig. 27). The results of the second phase (decrease in turbidity) are plotted as log $(A_t - A_\infty)$ against time, where A_t is the absorbance at time (t) and A_∞ is the final steady-state absorbance measured by a re-sweep of the signal 2 min after mixing. The first order rate constants were 0.44, 0.41, 0.4 and 0.28 s^{-1} respectively.

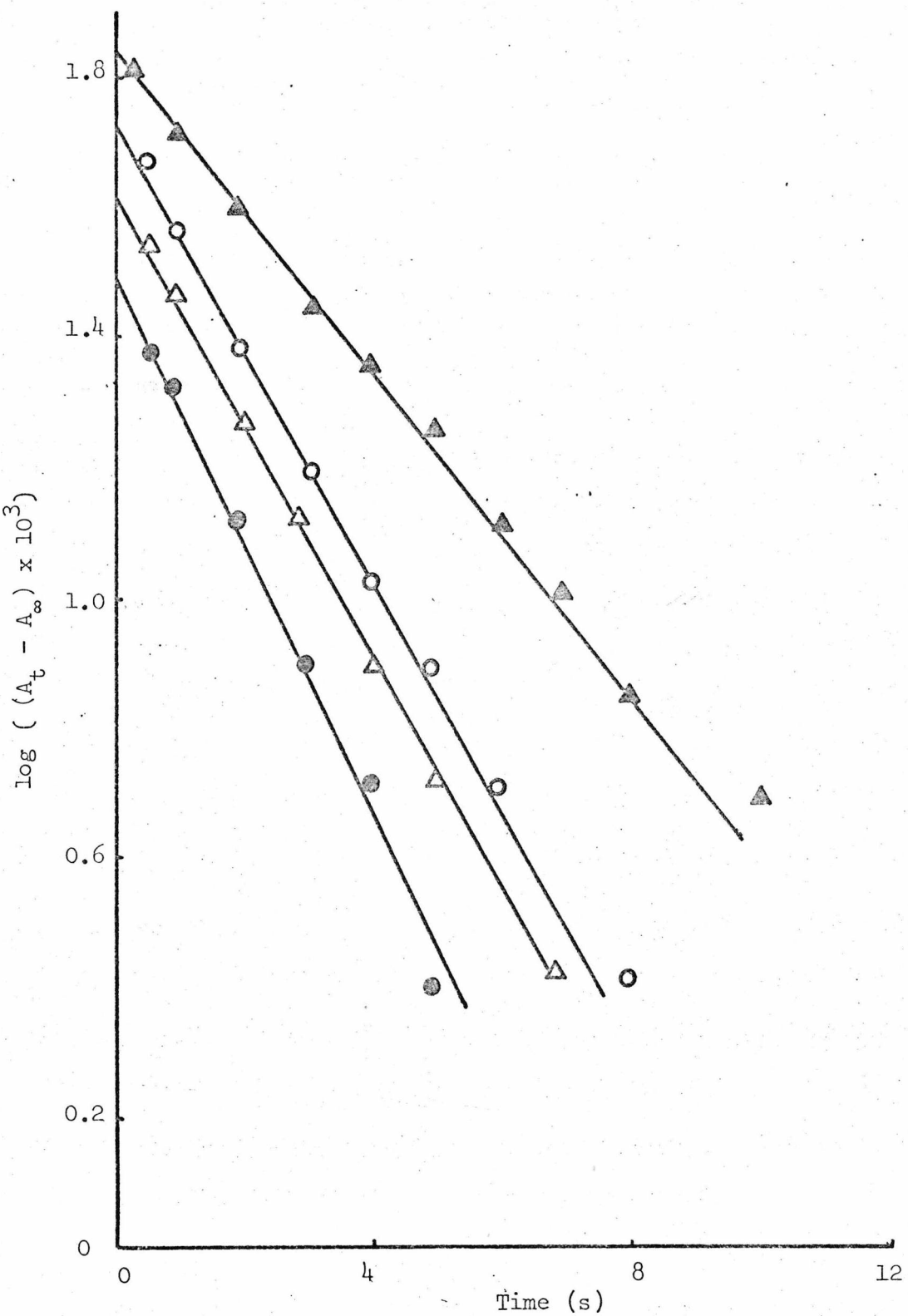


Fig. 29

it is used at a low concentration, 10 mM or less). The rate of glycerol entry is also independent of the growth cycle up to stationary phase (Fig. 31), (the rate of water exit due to glycerol was also independent of the factors given above). The effect of temperature is shown in Fig. 32a. Depending on the temperature range there are two different activation energies, with a distinct transition point at 21°C. Below that there is a low activation energy of 5.0 Kcal/mole, whereas at high temperature it is greater (13.8 Kcal/mole). Measurements of the rate of water exit show a similar transition point (Fig.32b), but the activation energy below 21°C is greater than above (18.3 and 8.4 Kcal/mole, respectively).

Differences in membrane fatty acid composition influence the temperature characteristics of the diffusion of water and glycerol, and cells grown in the presence of a given essential fatty acid supplement have unique temperature characteristics for transport distinguishable from that of cells grown on other fatty acid supplements[136].

E. coli 30E, a mutant which requires unsaturated fatty acids for growth (refer to Chapter 2), was used to determine the transition point for glycerol entry. As shown in Fig. 33, glycerol entry has a transition point of 15°C (Fig. 33a) when cells are grown in oleic acid containing medium, and 28°C when cells are grown in medium containing elaidic acid (Fig. 33b). (Elaidic acid is the trans isomer of oleic acid and has a much higher melting point, as well as being the more stable form.)

The rate of glycerol entry has also been studied in cells

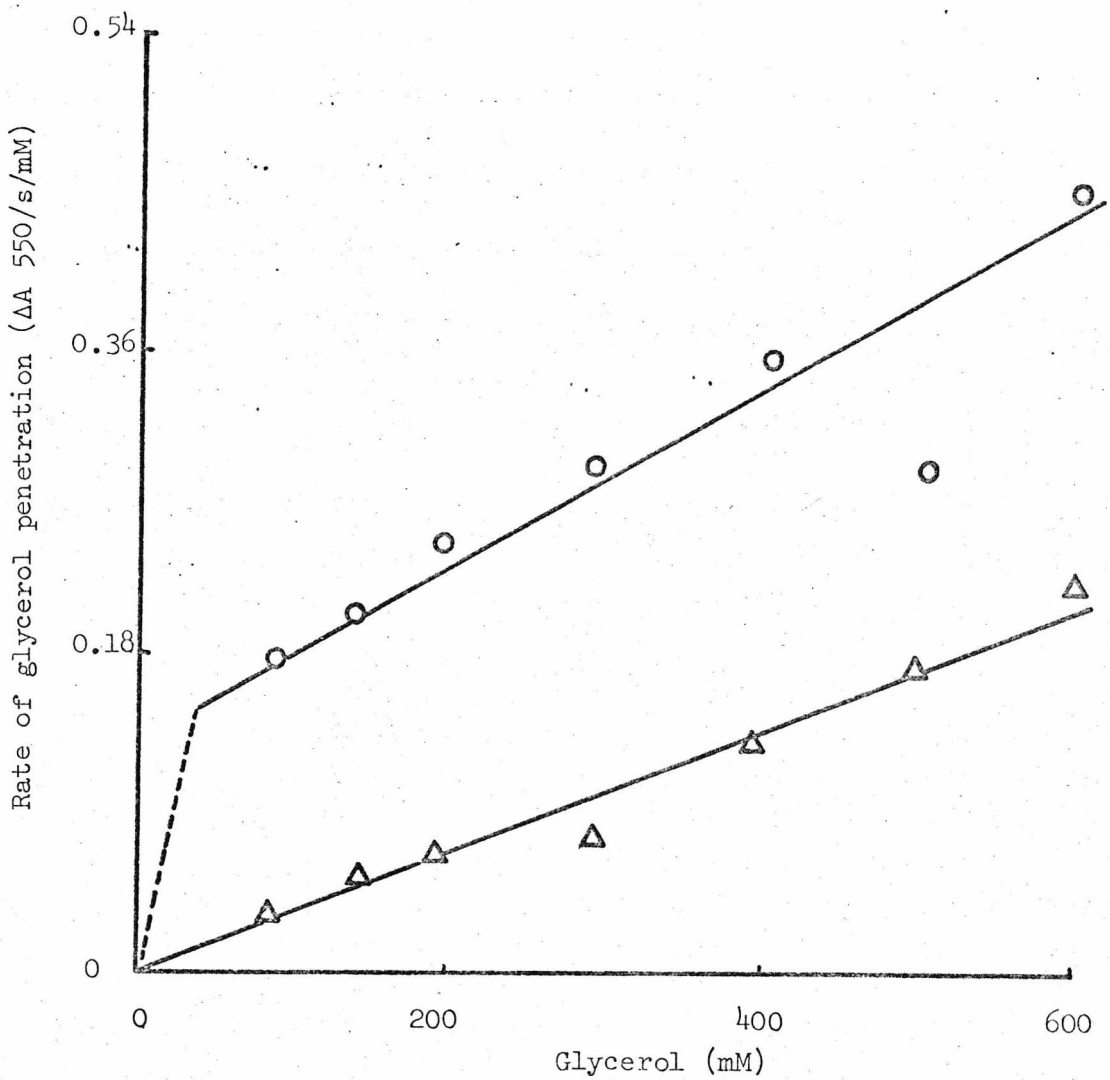
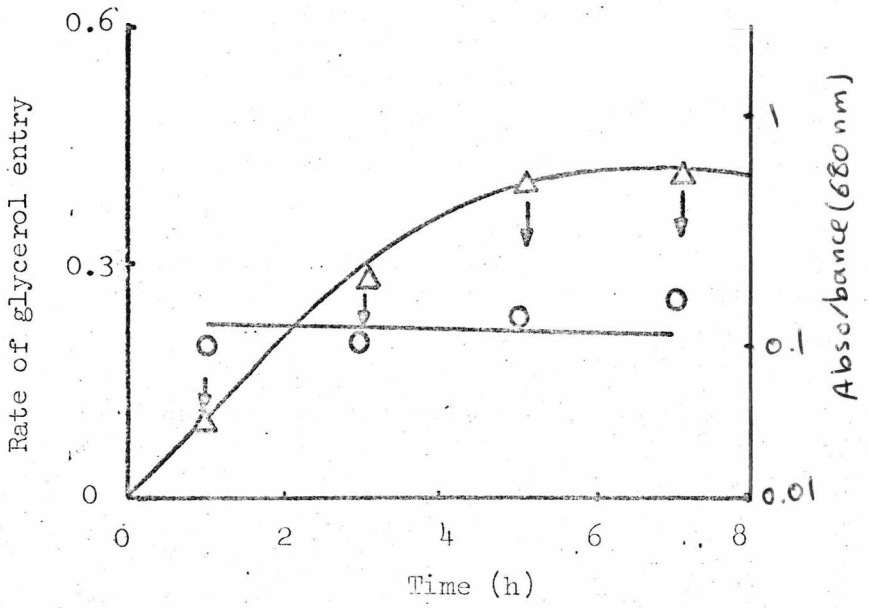
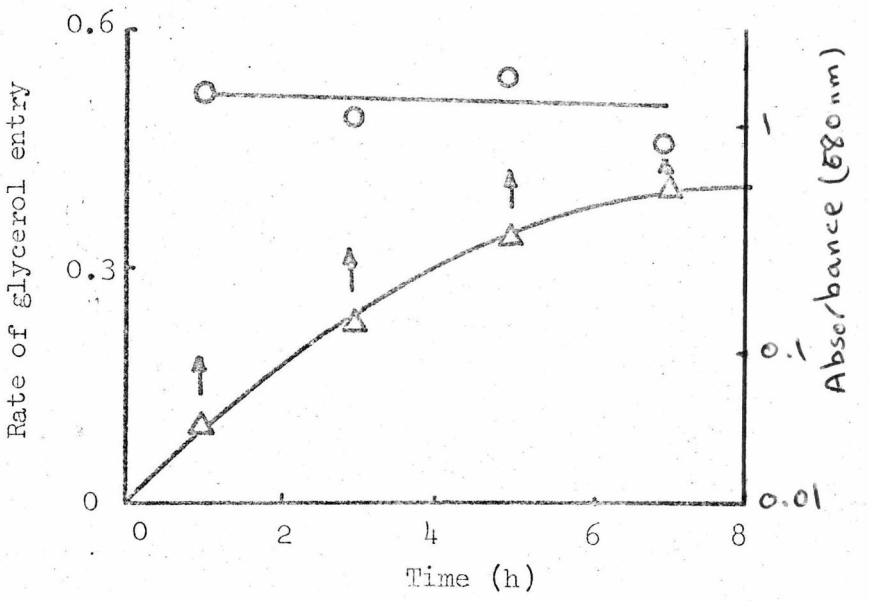


Fig. 30. The effect of concentration on the rate of glycerol entry into wild-type cells grown on media containing glucose (△) or glycerol (○). The rates of glycerol entry were measured at 25°C in a stopped-flow spectrophotometer. The values for the rate of entry were obtained by deriving the first order rate constant (K) as given in Fig. 29.

Fig. 31. Effect of growth cycle on the rate of glycerol entry. *E. coli* suspended in 10 mM imidazole buffer (pH 7.0) was mixed in stopped-flow with an equal volume of 1 M glycerol in buffer at 25°C. (A) cells grown on glucose, (B) cells grown on glycerol. The rate of glycerol entry is faster when cells are grown on glycerol but the rate does not vary with growth phase in both cases. (△) growth curve, (O) rate of glycerol entry. The growth curves are log graph units/absorbance.


Rate of glycerol entry
0.6
0.3
0
0
2
4
6
8
Time (h)
1
0.1
0.01
Absorbance (680 nm)

A


Rate of glycerol entry
0.6
0.3
0
0
2
4
6
8
Time (h)
1
0.1
0.01
Absorbance (680 nm)

B

Fig. 31

Fig. 32. Temperature dependency of turbidity changes due to glycerol entry (a) and water exit (b) from *E. coli*. An applied glycerol concentration of 0.5 M was used in both cases. Cells were grown on glucose.

Fig. 32.

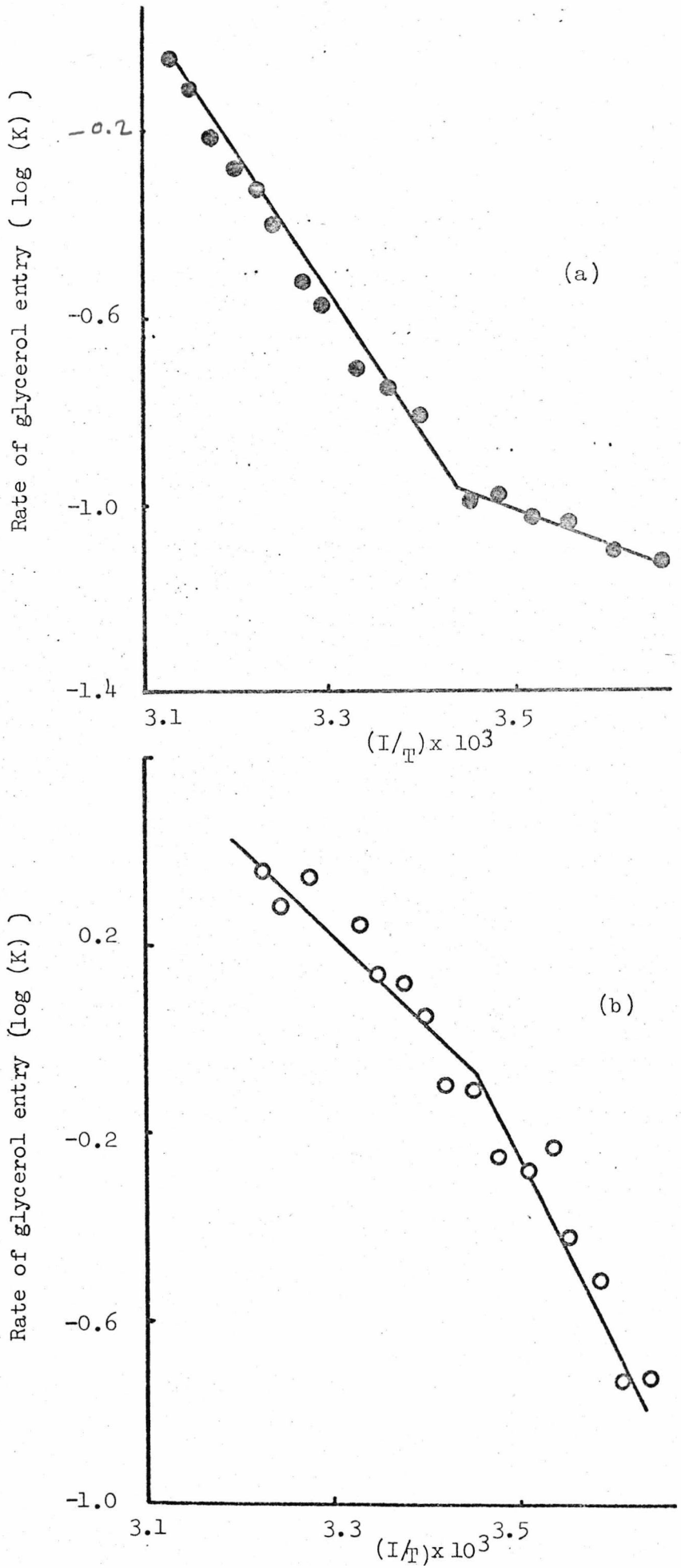

Rate of glycerol entry (log (K))
-0.2
-0.6
-1.0
-1.4
(a)
3.1
3.3
3.5
(I/T) x 10³
Rate of glycerol entry (log (K))
0.2
-0.2
-0.6
-1.0
(b)
3.1
3.3
3.5
(I/T) x 10³

Fig. 33. Temperature and fatty acid dependence of glycerol entry. (a) cells grown on oleic acid, (b) cells grown on elaidic acid. Due to difficulties concerning the measurement of glycerol entry into the cells grown on oleic acid with the stopped-flow spectrophotometer i.e. low rate of glycerol entry at low temperature in *E. coli* 30E, the data given in '(a)' part are probably not very accurate.

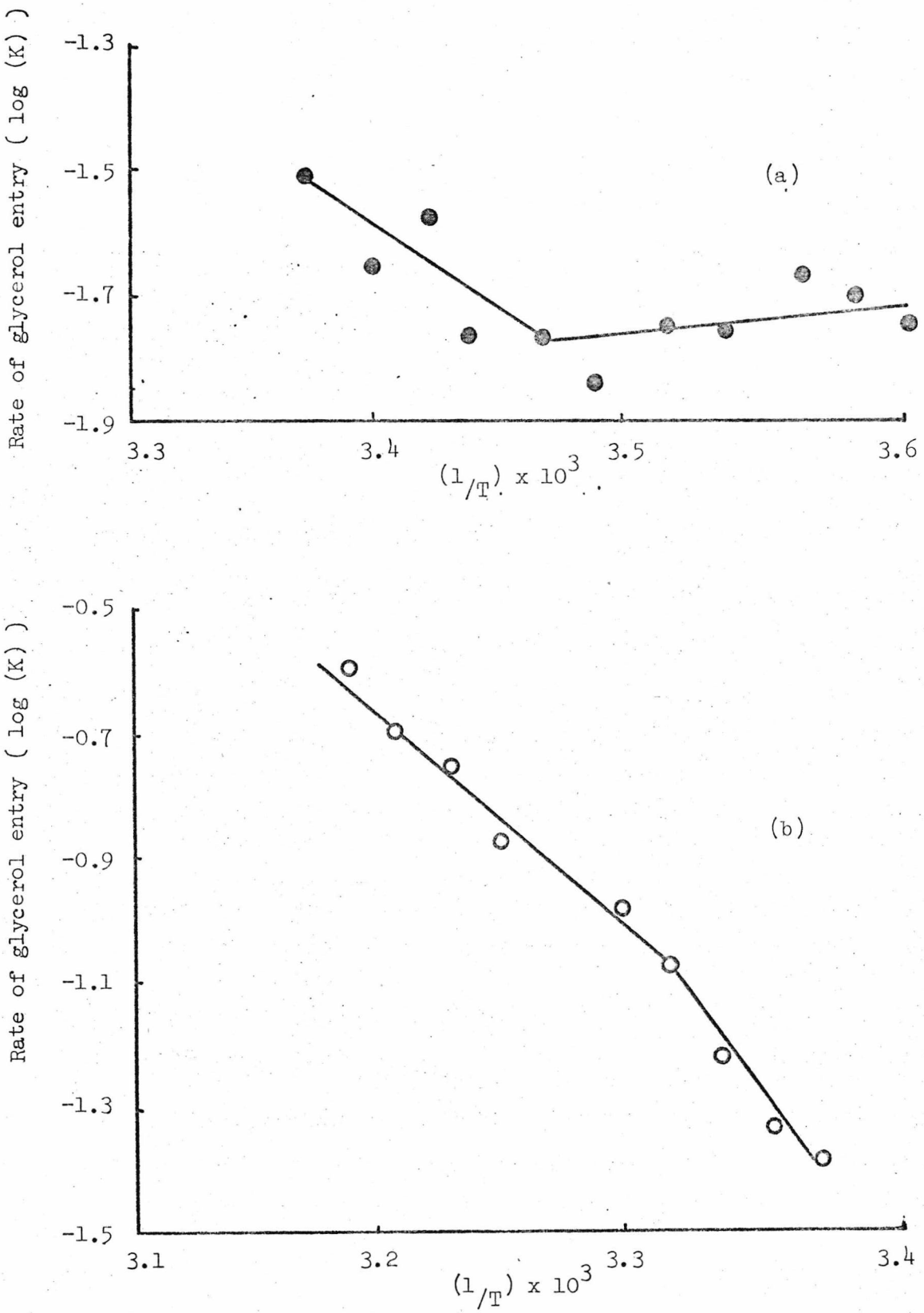

Rate of glycerol entry (log (K))
-1.3
-1.5
-1.7
-1.9
(a)
3.3
3.4
3.5
3.6
(1/T) x 10^3
Rate of glycerol entry (log (K))
-0.5
-0.7
-0.9
-1.1
-1.3
-1.5
(b)
3.1
3.2
3.3
3.4
(1/T) x 10^3

Fig. 33

grown on glycerol-minimal salts medium (Fig. 30). The straight line plot of the rate of glycerol entry versus concentration has a similar slope to that for cells grown on a glucose-minimal salts medium. However, the lines does not pass through zero on the *y*-axis, and at any particular concentration of glycerol the rate of entry is much more rapid than for glucose-grown cells. Unfortunately it is not possible to measure turbidity changes at glycerol concentrations of less than about 50 mM.

If it is assumed that, on glycerol containing media, there are two components for glycerol entry, namely non-saturating simple diffusion as found for glucose grown cells and a saturating, facilitated diffusion system, then the observed rate of glycerol entry will be the sum of these two effects. The facilitated diffusion system will have a carrier protein(s), and is unlikely to have a Km for glycerol greater than 10 mM (as the Km of proteins for their substrates are rarely, if ever, greater than this, and usually very much lower). It will therefore be saturated and acting at its maximal rate at glycerol concentrations of about 50 mM or greater. In the region of 50 to 600 mM glycerol, the observed rate will be due to a constant, saturated rate for the facilitated diffusion system plus an increasing rate due to simple diffusion. The line noticed should be approximately parallel to the line for the uninduced rate (Fig. 30).

The fact that the above effect is inducible is tentative evidence for a facilitated diffusion or active transport system for glycerol. If this is the case, then it should be possible to obtain mutants defective in the carrier system.

Richey and Lin[135] have obtained and characterized mutants deficient in the glycercl 3-phosphate (glp) operon.

Table 3 lists the properties of some of those mutants and gives apparent rate constants for glycerol entry under various growth conditions. In induced cells the rate of glycerol entry approaches the rate of water exit, and it is not therefore possible to consider the effects (water exit and glycerol entry plus water re-entry) as being separable. Thus, in induced cells the apparent rate constant is less than the real rate constant. In uninduced cells it should be equivalent to the real rate constant.

If the rates of water exit and glycerol entry hardly overlap, and water exit is essentially completed before glycerol entry (and water re-entry)occurs, then the maximal increase in turbidity should approach that observed using an equivalent osmalality of a non-penetrant (e.g. NaCl). In cells where the effects overlap appreciably the maximal increase in turbidity will be less than that observed for an iso-osmolal non-penetrant. The maximal increase in turbidity observed can therefore be used as a rough guide to the overlap of the rates.

The results are exactly as one would predict (Table 3). In glucose-grown (catabolite repressed) cells the rate of glycerol entry (expressed as the apparent first order rate constant K) are low except in the repressor-negative strain (7 and 227). However, in the R^- but also F^- strain (229) the rate is low. In Casamino acids-grown (basal level) cells the rates are higher except in the F^- strains (229 and 282) where values are observed that are similar to those for cells grown on glucose. In cells

TABLE 3. THE RATE OF GLYCEROL ENTRY INTO VARIOUS MUTANTS OF *E. coli* DEFECTIVE IN THE glp GENE

Strain*	Genotype † (glp alleles)				Glucose		Glycerol		Casamino acids		Casamino acids plus glycerol 3-phosphate	
	F	K	D	R	K**	%‡	K	%	K	%	K	%
Wild type	+	+	+	+	0.44	84	1.40	68	1.13	46	1.47	42
1	+	+	+	+	0.29	79	1.27	54	0.97	54	1.31	41
7	+	+	+	-	0.71	40	1.93	53	1.36	51	1.80	31
227	+	-	+	-	0.74	51	-	-	0.53	40	0.69	41
229	-	-	+	-	0.28	90	-	-	0.21	87	0.23	81
282	-	-	+	+	0.23	87	0.28	93	0.21	82	0.21	84

* Strains 1, 7, 227, 229, 282 were obtained from Dr. E. C. C. Lin; the numbers of the mutants are those given by Richey and Lin [135].

† The glycerol regulon (glp). F, K, D and R refer to the genes for the glycerol facilitator, glycerol kinase, glycerol 3-phosphate dehydrogenase and the repressor respectively.

** The rate of glycerol entry is expressed in terms of the apparent rate constant (K) (s^{-1}) at 25°C. The experiment was performed using 250 mM glycerol.

‡ The maximal increase in turbidity expressed as a percentage of the increase observed with an equi-osmolal concentration of NaCl.

induced for the metabolism of glycerol (glycerol or Casamino acids plus glycerol 3-phosphate-grown) the rates of entry are higher, particularly in the R^- strain (7). Cells deficient in the facilitator (F^-) will still grow on glycerol (due to entry by simple diffusion) (cf. Richey and Lin[135]); clearly the rate of glycerol entry is low. Glycerol kinase negative (K^-) cells do not grow on glycerol, but on Casamino acids plus glycerol 3-phosphate the facilitator system is only partially induced in the K^- strain (227). Further study of the results suggests that there is a requirement for the presence of glycerol kinase for full induction of the facilitator.

The maximal increase in turbidity for uninduced cells, with rate constants of glycerol entry of 0.5/s or less, is 80% or more of that caused by iso-osmolal NaCl, indicating that the processes of water exit and glycerol entry do not significantly overlap. At higher rates of glycerol entry the maximal relative increase in turbidity is proportionately lower, in good agreement with the figures for the rate constant.

5 - *E. coli* wall contraction

As shown in Table 2 addition of salts caused a 15 - 20% shrinkage in volume of whole cells whereas the non-electrolyte, sucrose, caused only a small (5 - 10%) decrease in volume.

It has been found that isolated cell walls of Gram-positive bacteria contract in response to changes in environmental pH and ionic strength. These changes in volume were related to changes in electrostatic interactions among fixed ionized group in wall polymers including peptidoglycan[133, 134].

Cell walls of Gram-negative bacterium *E. coli* were prepared by French pressure cell disruption, followed by sucrose density gradient centrifugation (Schnaitman method[18,33]).

Fig. 34, shows the distribution of proteins on the gradient. The lower band was almost white in colour and did not contain any measureable cytochromes (Fig.35), but retained a little succinate dehydrogenase activity (Table 4) indicating the relatively low concentration of the cytoplasmic membrane fraction in this band. The purity of lower band (wall) was examined by electron microscopy and measuring the LPS and DAP concentration in each layer. Fig.36 shows an electron micrograph of the cell wall enriched fraction. This fraction consists almost entirely of large, open fragments with the characteristic morphology of the cell wall of intact cells.

The distribution of LPS and DAP in the two bands are given in Table 5. The lower band contained most of LPS and DAP indicating that the lower band was derived primarily from the cell wall.

TABLE 4. ACTIVITY OF SUCCINATE DEHYDROGENASE IN WALLS AND MEMBRANES OF *E. coli*

Fraction	Decrease in absorbance of DCPIP at 600 nm (Abs. units/mg protein/min)
Membrane	0.85
Wall	0.10

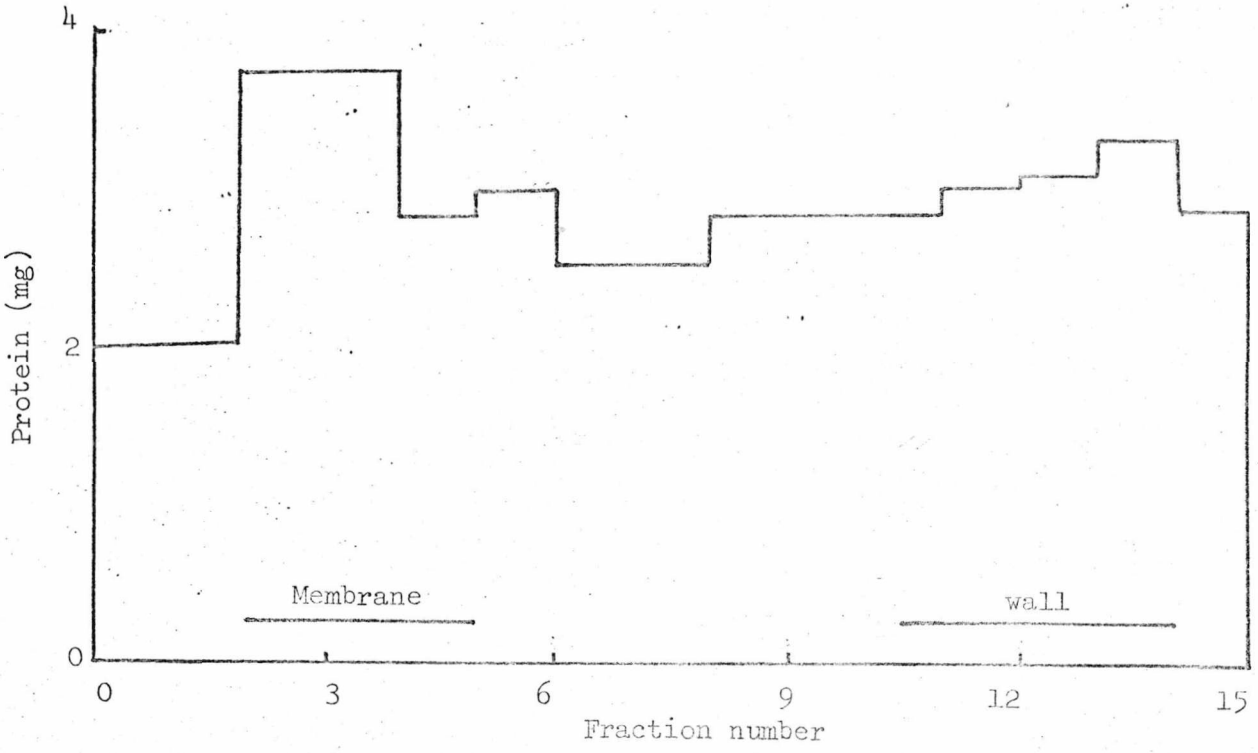


Fig. 34. Protein distribution on gradient. *E. coli* was disrupted by French pressure cell and disrupted cells were layered on sucrose density gradient. The gradients were centrifuged at 45,000 x *g* for 16 h and were fractionated by pumping. Total protein in each fraction was measured by Biuret method[146].

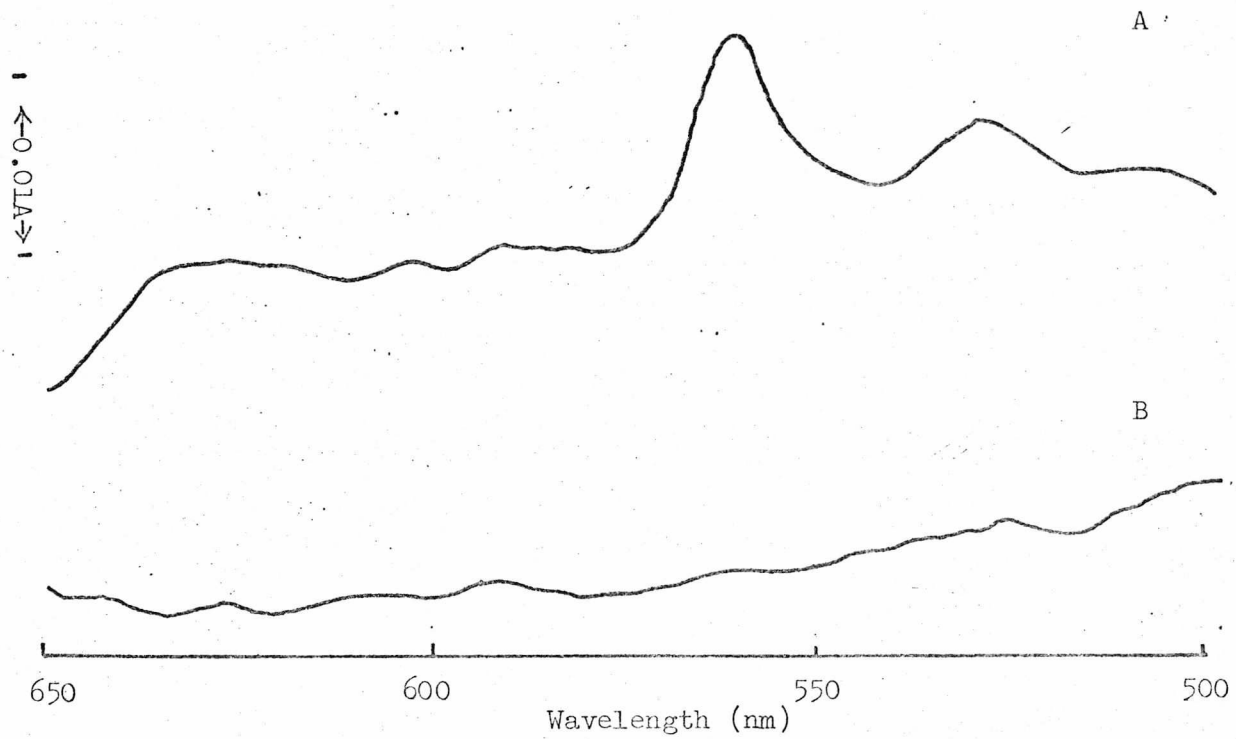


Fig. 35. Difference spectra of cytochromes of *E. coli*; (A) cytoplasmic membrane fractions, (B) cell wall fractions. In each case the final protein concentration was 1 mg/ml.

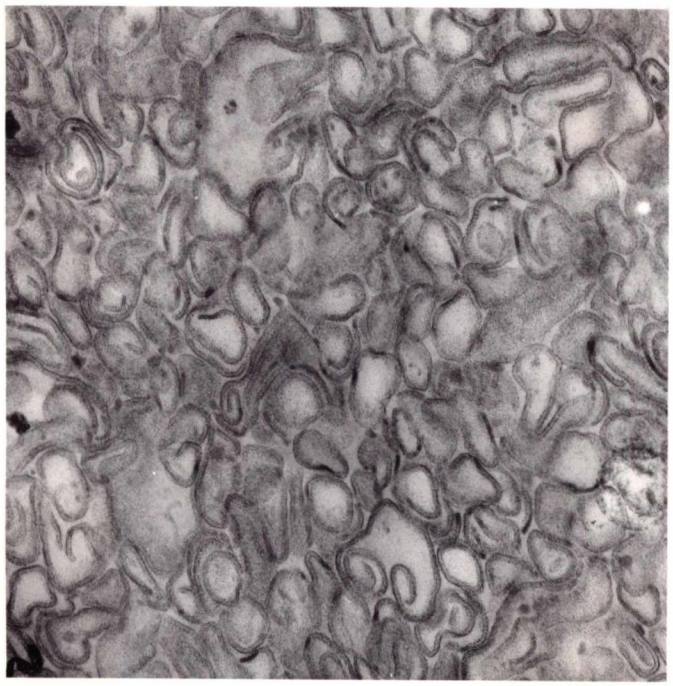


Fig. 36. Electron micrograph of section cell wall of *E. coli* prepared by French pressure cell and sucrose density gradients. Fractions were suspended directly in 1% OsO_4 in Kellenberger buffer and fixed pellets were dehydrated and polymerized after washing in Kellenberger buffer and 0.5% uranyl acetate in room temperature (cf. Chapter 2 part 7) : x 47600.

TABLE 5. DISTRIBUTION OF LIPOPOLYSACCHARIDE AND DIAMINOPIMELIC ACID AMONG WALL AND MEMBRANE OF *E. coli*

Fraction	LPS (%)	DAP (%)
Wall	86.6	75.96
Membrane	13.4	24.03

The addition of salts (not sucrose or glycerol) to isolated cell wall suspensions of *E. coli* in distilled water caused a rapid drop in suspension pH (Fig.37) as well as contraction of the wall (Fig. 38).

Salt-induced pH changes were interpreted in terms of ion binding by the protein in exchange for protons. It has been shown by direct chemical analysis that isolated *Staphylococcus aereus* walls bind a variety of cations[46], and therefore proton loss from *E. coli* cell walls might be due to cation binding, resulting in wall contraction.

As shown in Fig. 39, the proton release depended more on environmental ionic strength than on the particular cation present.

The overall shrinkage of cells (Table 2), therefore appeared to be primarily due to electrostatic wall contraction rather than being due to an osmotic response.

Fig. 37. Effect of glycerol (●), sucrose (□), NaCl (■), KCl (○), $CaCl_2$ (△) and $MgCl_2$ (▲) on proton release from isolated *E. coli* cell walls suspended in distilled water. To cell wall suspensions, successive additions of solutes in distilled water were followed and the pH was read 5-10 seconds after each addition at room temperature. Control experiments were done by addition of salts, sucrose or glycerol to distilled water. The maximum decrease of pH of distilled water was 0.25 (in the case of $MgCl_2$) and minimum of 0.1 (in the case of glycerol) pH units at the highest solutes concentration (100 mM).

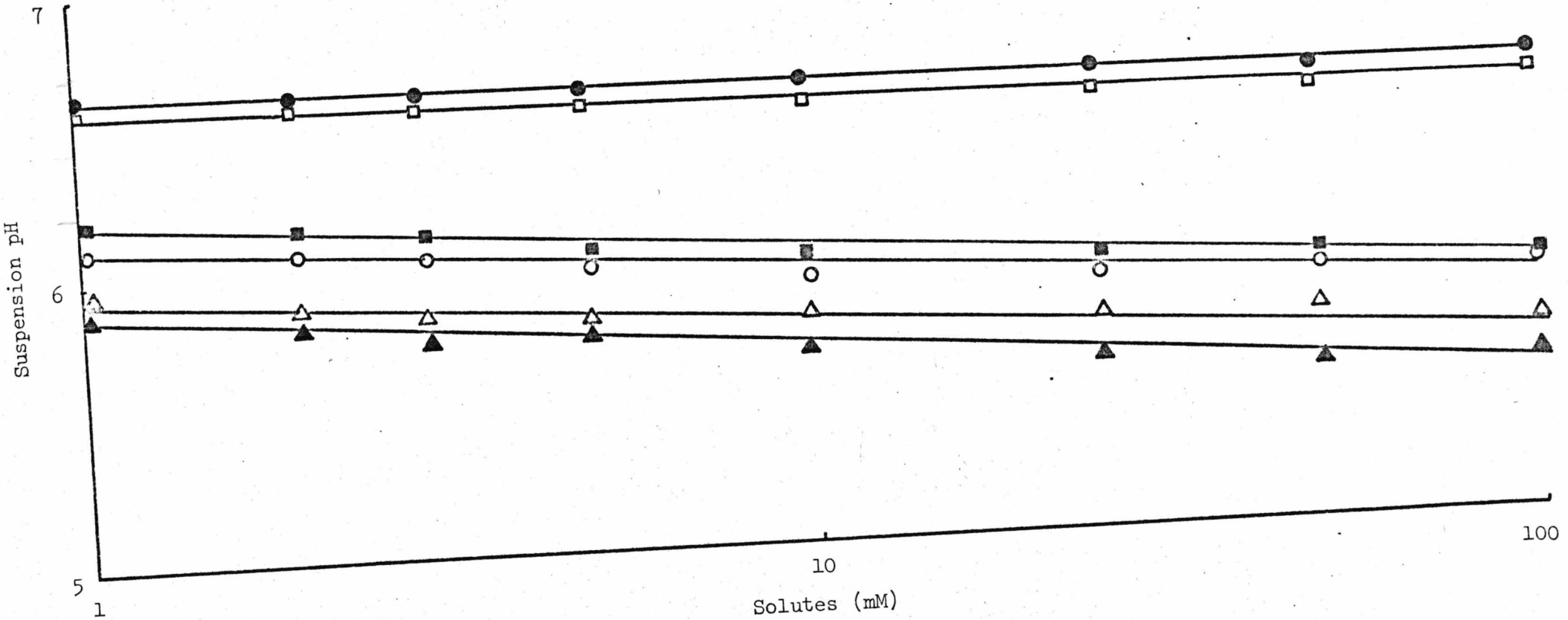


Fig. 37

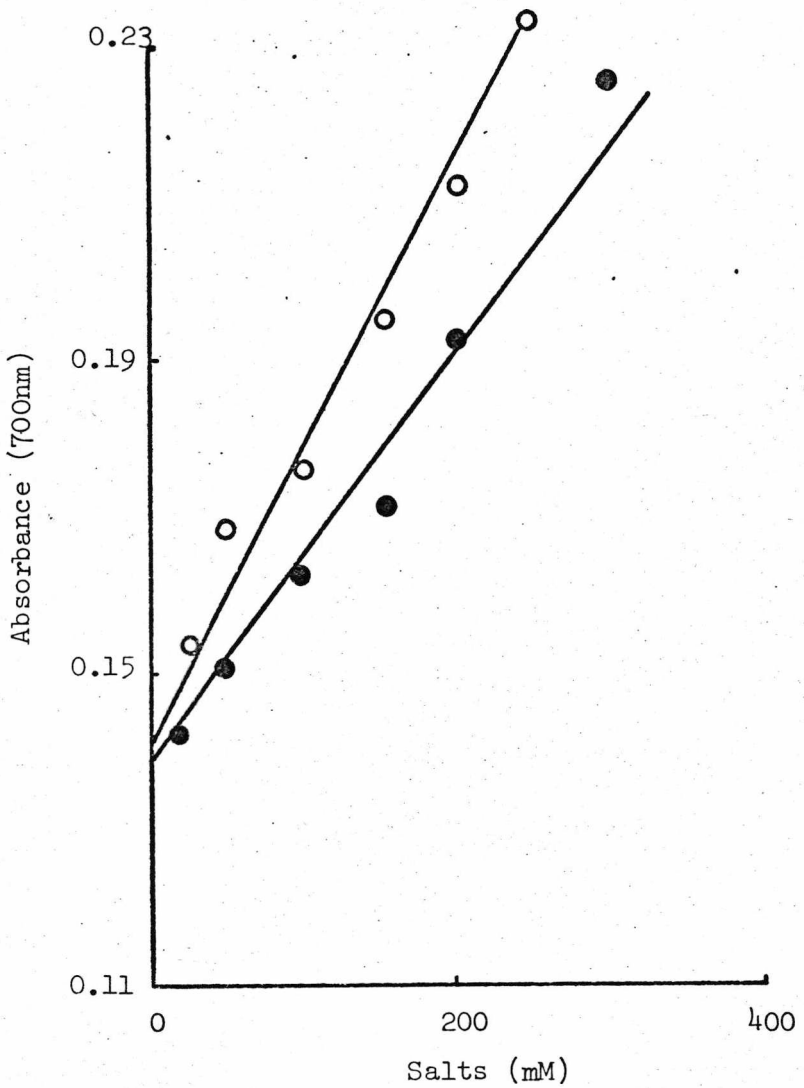


Fig. 38. Effect of salts on contraction of *E. coli* cell walls. Data are not corrected for any refractive index changes; (O) $MgCl_2$, (●) NaCl.

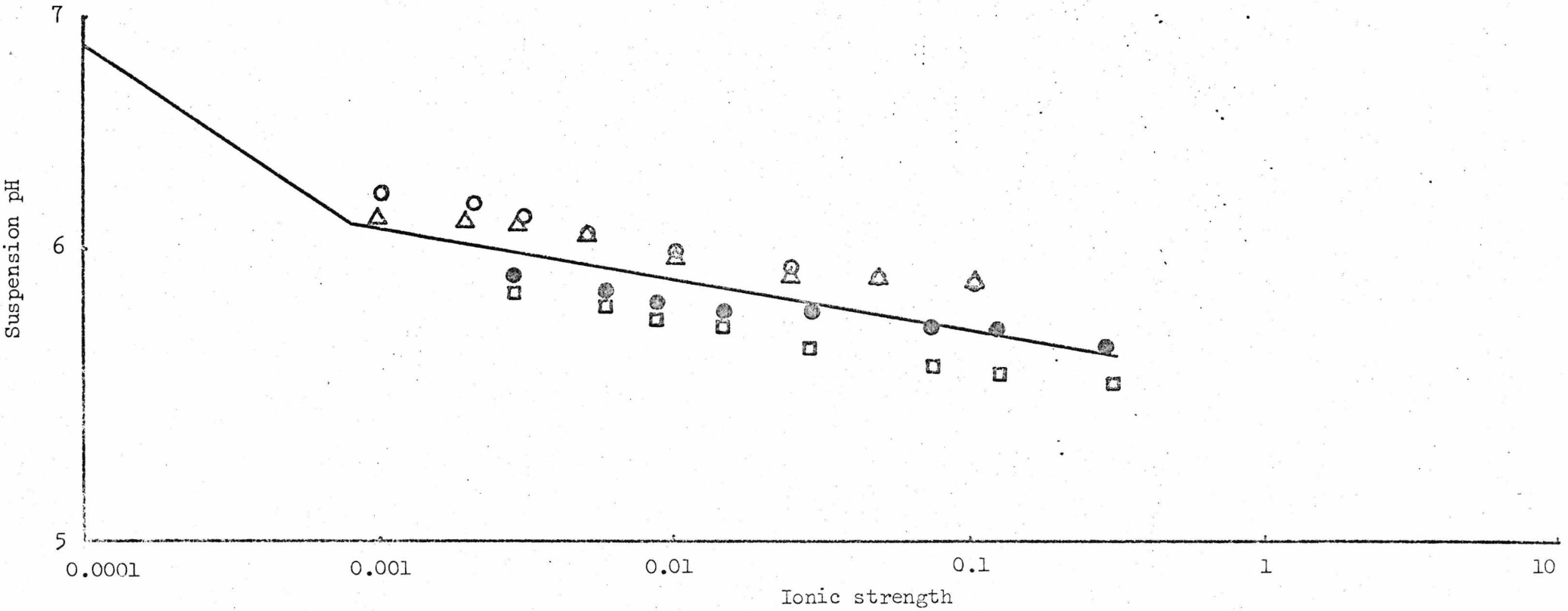


Fig. 39. Salt-induced release of protons from *E. coli* cell wall. Proton release (Fig. 37) is replotted against ionic strength of medium. A single curve was drawn to fit pH values of suspensions caused by NaCl (○), KCl (△), $CaCl_2$ (●) and $MgCl_2$ (□).

6 - Breakage of *A. vinelandii* by glycerol osmotic shock

As already shown for *E. coli*, bacterial cells are highly permeable to glycerol. However, the rate of glycerol penetration is considerably lower than that of water (presumably due to the large molecular radius of glycerol). Thus addition of glycerol to cell suspensions causes a transient water exit from the cells and a decrease in volume, followed by glycerol plus water entry and a re-swelling of the cells. Conversely, cells already equilibrated in glycerol, when shifted to an environment of lower glycerol concentration, should exhibit a transient swelling due to water uptake in the period prior to glycerol exit. If the cell is insufficiently mechanically strong, the glycerol gradient large and applied rapidly enough, a massive uptake of water should occur which could be great enough to cause cell rupture. Such a technique could therefore provide the basis of a simple method of causing bacterial disruption. Furthermore, it is possible that the resulting fragments could be easily fractionated to give membrane vesicles and isolated walls.

Preliminary experiments showed that rupture of *E. coli* by this method is unreliable. However, the literature showed that this method had previously been successfully used for lysis of *A. agilis*[78,79]. Accordingly cell fractionation by "glycerol shock" has been further studied using *A. vinelandii*.

Curves (Fig. 40) obtained for the effect of equilibration of *A. vinelandii* cells with various concentrations of glycerol followed by rapid dilution into weak buffer plus $MgSO_4$ indicates that for cells initially suspended in glycerol at concentrations

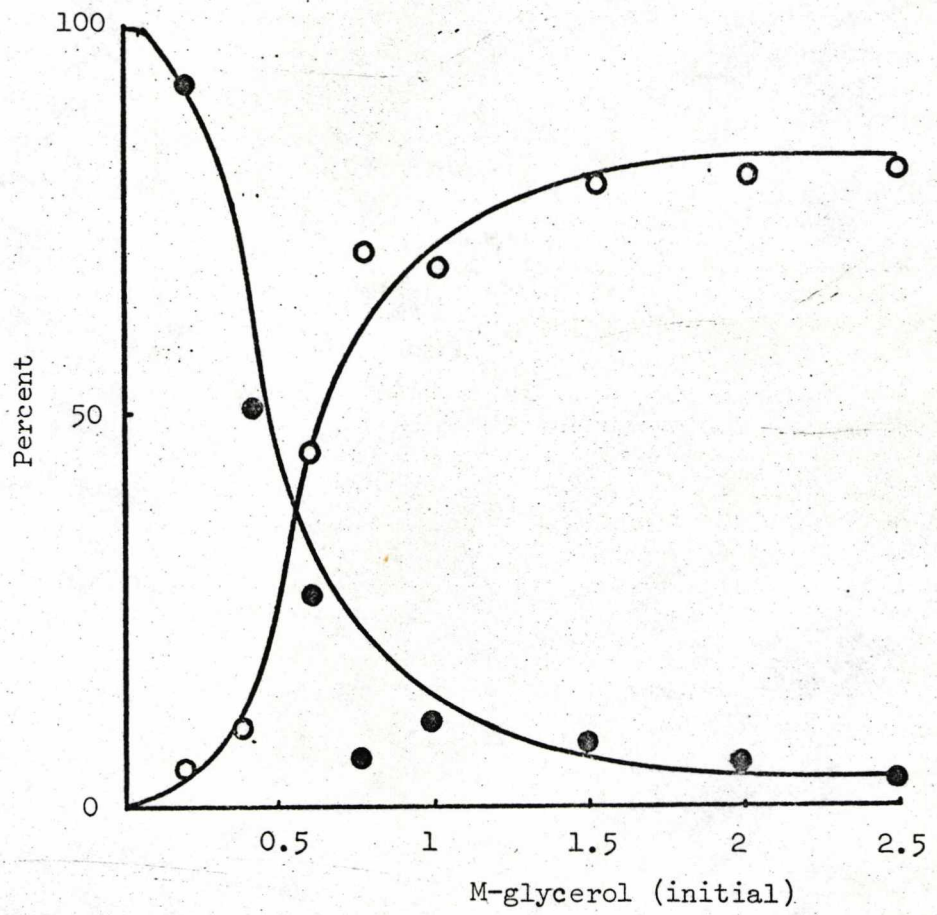


Fig. 40. Effect of glycerol concentration on cell lysis. Washed cells of *A. vinelandii* were mixed with an equal volume of glycerol in 50 mM Tris-HCl (pH 7.5) and after 5 min were ejected into 10 volumes of rapidly stirred cold buffer plus $MgSO_4$. Control experiments were done by the same method but cells were initially mixed with an equal volume of buffer alone. (O)% decrease in absorbance at 600nm (●) % intact cells estimated by counting the number of intact cells in suitably diluted bacterial suspension under phase contrast microscope.

of greater than 1.5 M less than 8% of cells remain intact after dilution into buffer, and the decrease in optical density is 80%. Fig. 41 shows the effect of initial bacterial concentration on lysis. Maximal lysis of cells take place when the initial concentration of the cell suspension is lower than 20 absorbance units at 600 nm (i.e. 10 absorbance units when mixed with an equal volume of glycerol), which is 10 mg dry weight per ml.

7 - Isolation of crude envelopes of *A. vinelandii*

The broken cell suspension prepared by osmotic shock was centrifuged at a low speed (1500 x *g* for 10 min) to remove any remaining intact cells. The supernatant containing crude envelopes and released soluble enzymes, etc. was centrifuged at 23,000 x *g* for 30 min. Even at this low force (compared with force required to sediment membrane particles from French press lysed cells, i.e. 150,000 x *g* 45 min or more[18,33]) crude envelope fractions were separated from the rest of the cellular material.

The distribution of cell components in the resulting supernatant and pellet was examined by various biochemical markers: KDO was chosen as a marker for the LPS layer of the cell wall, DAP for the peptidoglycan layer, cytochromes and oxidase activities for the cytoplasmic membrane, isocitrate dehydrogenase activity for the cytoplasmic enzymes[157] and RNAase I (namely the "latent" ribosomal RNAase [152,158], for the periplasmic space).

Fig. 42 shows cytochrome difference spectra and Table 6 shows the distribution of cell material in the supernatant

Fig. 41. Effect of cell concentration on lysis. Different concentrations of cells (*A. vinelandii*) suspensions were mixed with an equal volume of 3 M glycerol in 50 mM Tris-HCl (pH 7.5) and, after 5 min, samples were ejected into 10 volumes of rapidly stirred cold buffer. Control experiments were done by the same method but cells were mixed with an equal volume of buffer alone. (●)% intact cells, (O) % decrease in absorbance at 600 nm.

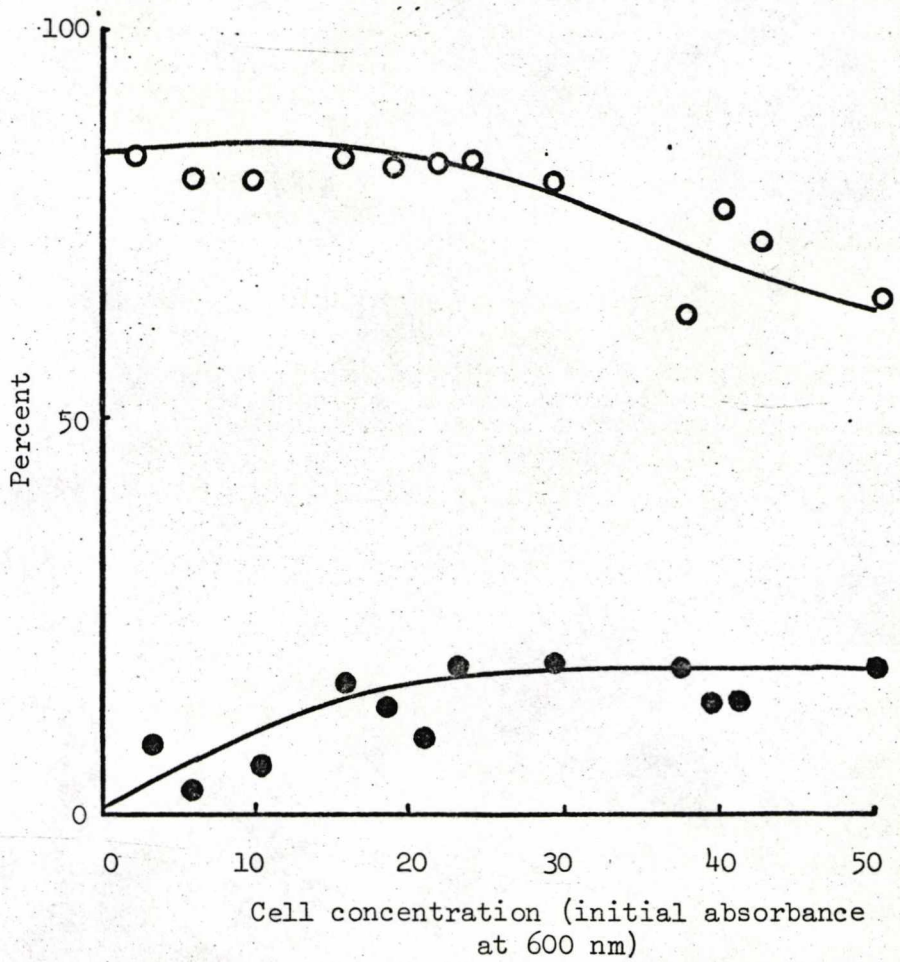

100
50
0
Percent
0
10
20
30
40
50
Cell concentration (initial absorbance at 600 nm)

Fig. 41.

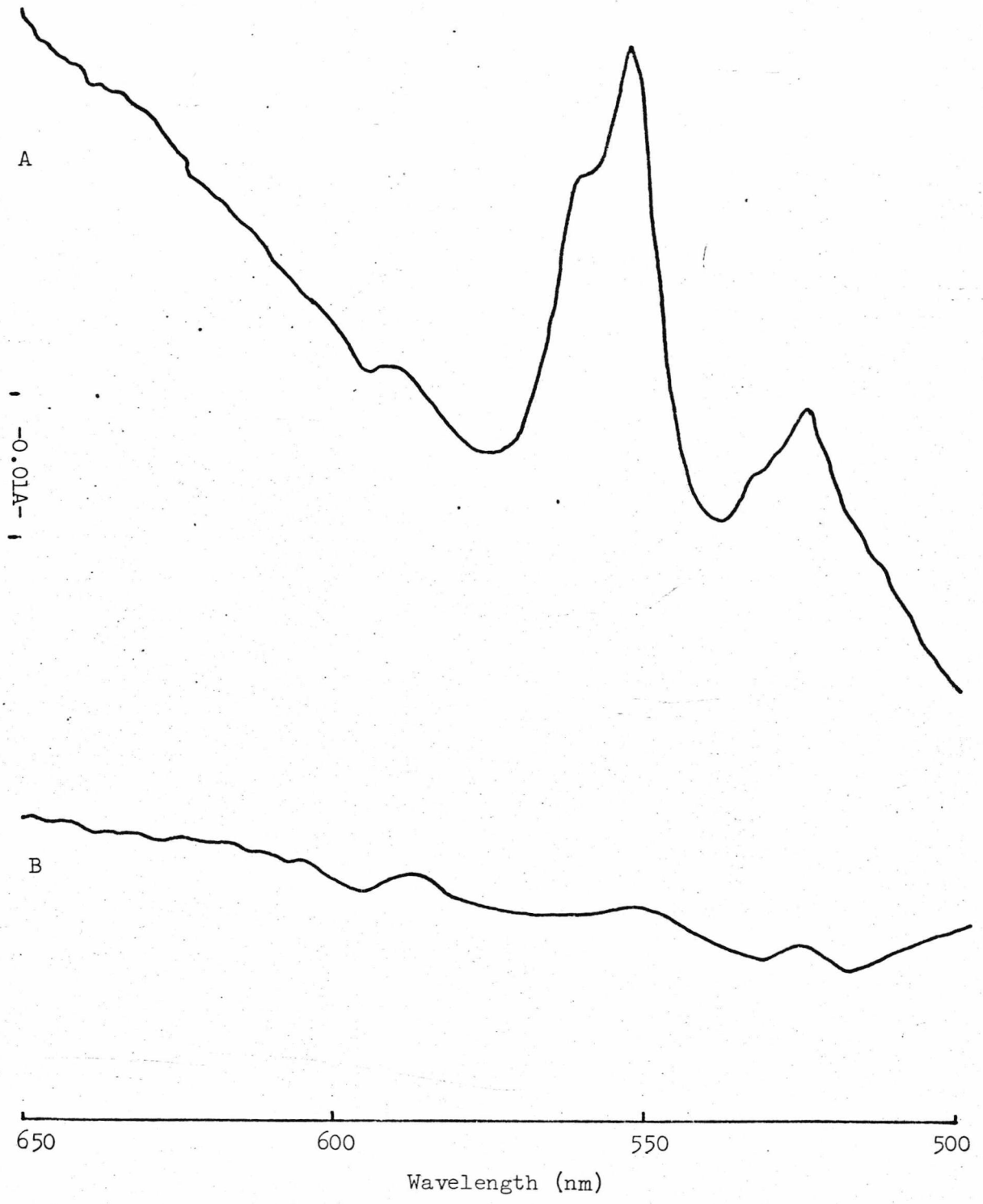


Fig. 42. Cytochrome spectra of *A. vinelandii* (A) pellet (crude envelope), (B) supernatant. In each case the final protein concentration was 1.05 mg/ml.

and pellet. Electron micrographs of crude envelope fragments are shown in Figs. 43 and 44.

TABLE 6. DISTRIBUTION OF CELL COMPONENTS IN SUPERNATANT AND CRUDE ENVELOPES OF *Azotobacter vinelandii* DISRUPTED BY GLYCEROL SHOCK

	Crude Envelopes	Supernatant
Protein (%) *	17.5	75
Specific protein concentration (mg/ml)	0.35	1.5
Cytochrome b (nmole/mg)±	1.1	0
Cytochrome c (nmole/mg)±	1.5	0
NADH oxidase (µg atoms oxygen/min/mg)	1.08	0
Succinate oxidase (µg atoms oxygen/min/mg)	0.15	0
Isocitrate dehydrogenase (µmole/mg protein/min)	0.005	0.2
RNAase (ΔA260/min)	0.2	0.8
LPS (relative %)	100	0
DAP(%)	100	0
Ratio of nucleic acid/ protein ($\Delta A260/_{\Delta A280}$)	1.17	1.71

* 7.5 % of the total protein was lost during treatment

± Refer to Fig. 42.

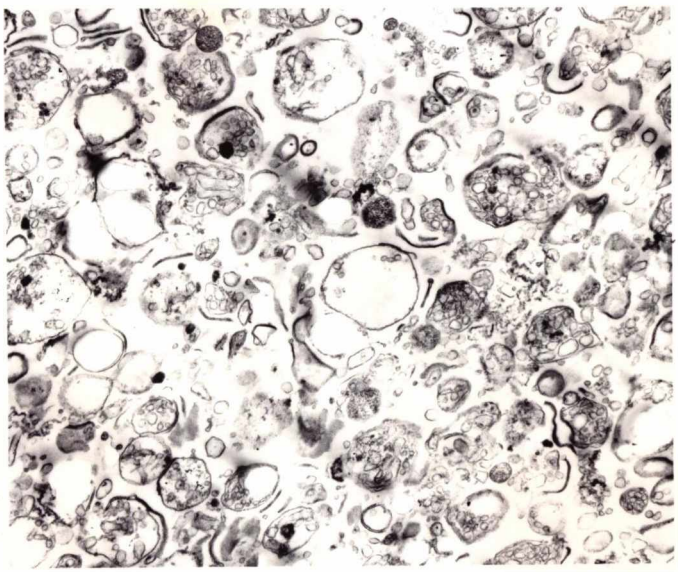


Fig. 43. Electron micrograph of *A. vinelandii* crude envelopes prepared by osmotic shock : x 7497.

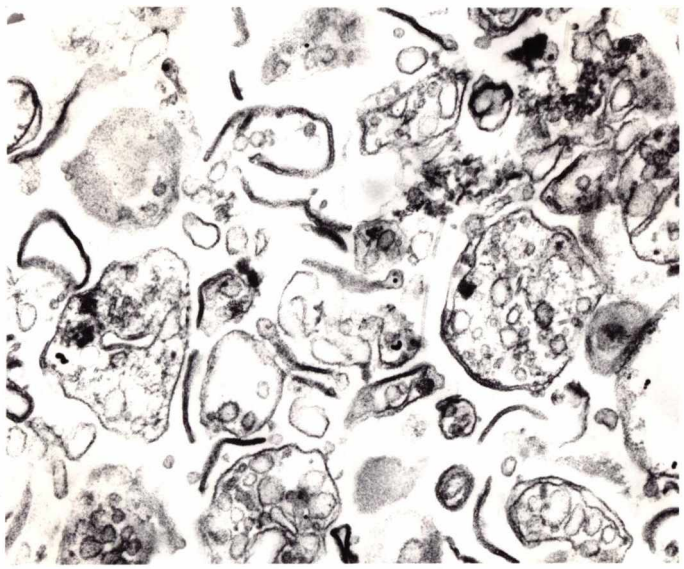


Fig. 44. Electron micrograph of *A. vinelandii* crude envelopes prepared by osmotic shock : x 29750.

8 - Orientation of *A. vinelandii* membrane vesicles

As already noted crude envelopes of *A. vinelandii* prepared by osmotic shock could be sedimented at relatively low centrifgual forces, indicating that the envelope was only minimally damaged. Biochemical tests were carried out to study the sidedness of the membrane vesicles obtained by this method.

A number of methods have been reported which can be used to determine the sidedness of membrane vesicles e.g. freeze-etching electon microscopy[74], uncoupling by ionophores[117], measuring ATPase and NADH dehydrogenase activities[66], studies of transport systems[66,159,160]. In this study the following procedures were used to determine orientation of membrane vesicles prepared by osmotic shock: (1) active transport of glucose; (2) effect of ion-translocating antibiotics on respiration; (3) accessibility of impermeable ferricyanide ion to the respiratory chain.

(1) Active transport of glucose into the crude envelope vesicles derived by osmotic shock treatment was studied using the method described by Barnes[114,115]. Glucose transport was also studied using vesicles of *A. vinelandii* prepared by the lysozyme-EDTA method, which are reported to be right-side out[73,74,160]. However, a number of workers have argued that during the preparation of membrane vesicles by lysozyme-EDTA a significant number of membrane vesicles become inverted[66,119,161].

As shown in Table 7, in spite of higher rates of oxygen uptake with all electron donors tested, membrane vesicles of

TABLE 7. EFFECT OF ELECTRON DONORS ON GLUCOSE UPTAKE AND OXYGEN UPTAKE BY MEMBRANE VESICLES FROM *A. vinelandii* PREPARED BY DIFFERENT PROCEDURES.

	Electron donor *	Glucose uptake Initial rate (nmole/mg/min)	Steady state (5 min) (nmole/mg)	Oxygen uptake rate (µg atoms mg/min)
Crude envelopes prepared by osmotic shock (sucrose-grown cells)	None	0.32	0.57	n.d.
	L-Malate	1.53	1.55	0.11
	NADH	0.68	0.98	1.10
	DL-Lactate	1.84	2.36	0.25
Crude envelopes prepared by osmotic shock (glucose-grown cells)	None	0.33	0.60	n.d.
	L-Malate	1.32	2.28	0.08
	NADH	0.82	1.03	0.55
	DL-Lactate	1.31	3.00	0.16
Membrane vesicles prepared by lysozyme-EDTA method (glucose-grown cells)	None	2.06	2.30	n.d.
	L-Malate	5.79	5.60	0.03
	NADH	1.02	1.28	0.59
	DL-Lactate	7.24	7.88	0.07
Intact cells (sucrose-grown cells)	None	1.96	2.19	n.d.
	L-Malate	2.44	3.04	0.06

n. d. not determined

* L-Malate, DL-Lactate 20 mM final concentration and NADH 10 mM.

A. vinelandii prepared by osmotic shock take up glucose at a lower rate than vesicles prepared by the lysozyme-EDTA, indicating the possibility that a significant number of vesicles are inverted.

(2) Effect of ionophores. Effect of ion-translocating antibiotics on the rate of respiration was followed by method described by John and Hamilton[117]. From Table 8, it can be seen that addition of either valinomycin or nigericin alone only slightly affects the rate of respiration, either in the presence or in the absence of K^+. However, addition of valinomycin, nigericin and K^+ caused a significant increase in the respiratory rate. Addition of NH_4^+ also resulted in an increase in the respiratory rate only in the presence of valinomycin.

TABLE 8. EFFECT OF VALINOMYCIN ON THE RATE OF RESPIRATION IN THE PRESENCE OF NIGERICIN, K^+ and NH_4^+

Additions	Rate of oxygen uptake (natoms/mg/min)	
	Control	+ Valinomycin
None	347	368
K^+ (10 mM)	362	375
Nigericin	362	362
K^+ (10 mM) + Nigericin	362	445
NH_4^+ (10 mM)	347	459

If vesicles are inverted (H^+ are pumped in) addition of nigericin in the presence of K^+ enables exchange of K^+ for H^+ and the further addition of valinomycin causes stimulation of respiratory rate because valinomycin facilitates the exit of K^+ ions which have been exchanged for H^+ by nigericin. Because there is a significant stimulation in the rate of respiration after addition of nigericin and K^+ it can be concluded that some vesicles are inverted.

(3) NADH-ferricyanide reductase activity.

The NADH dehydrogenase is located on the inside of correctly oriented membranes[66,120]. The site where ferricyanide obtains electron from the respiratory chain (when NADH is used as substrate) is on the internal membrane surface[66]. Therefore ferricyanide reductase activity can serve as a marker of sidedness of membrane vesicles[66] because addition of substances e.g. toluene or butanol (which cause the vesicles to become porous) to vesicles which are right-side out should cause an increase of enzyme activity.

As shown in Fig. 45 addition of toluene or butanol to membrane vesicles had only a slight effect on the rate of reduction of ferricyanide, indicating that most vesicles are inverted.

When examining the reductase of ferricyanide the respiration was inhibited by addition of 1 mM KCN.

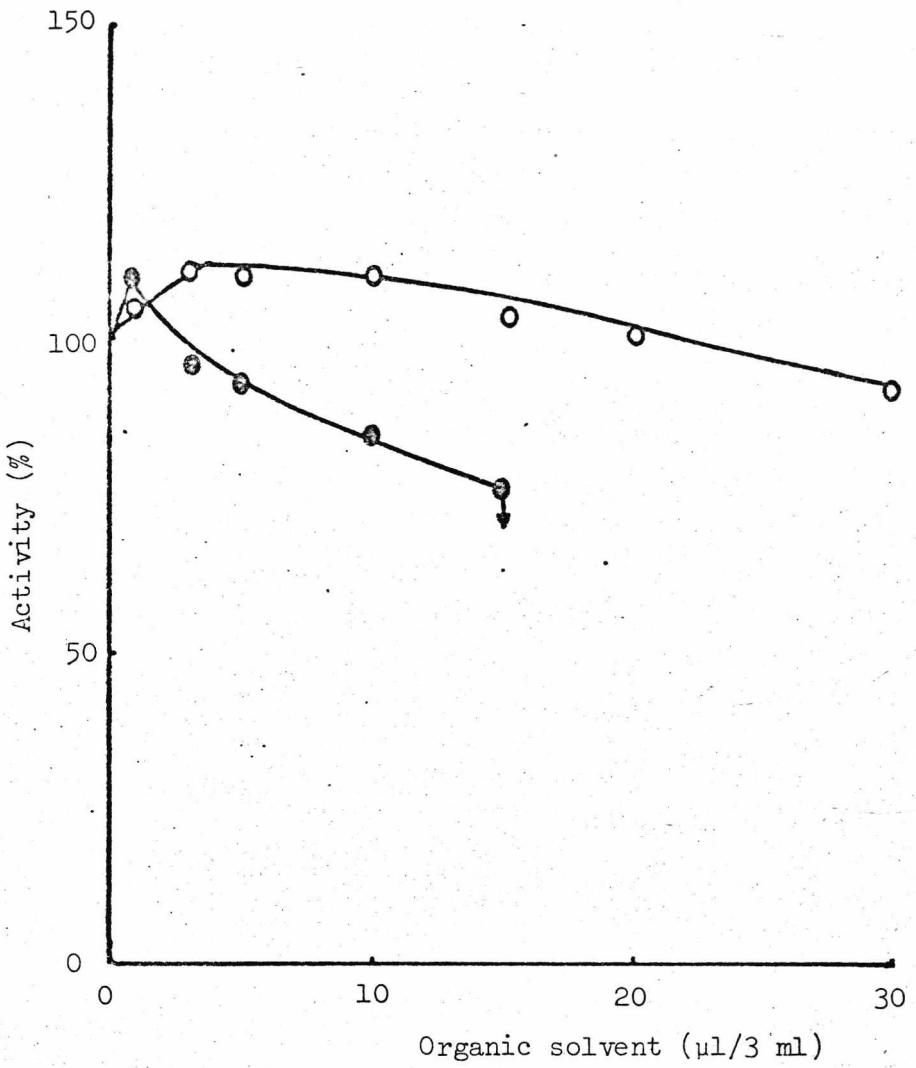


Fig. 45. NADH-ferricyanide reductase activity after addition of toluene (●) and butanol (O)

Orientation of vesicles was also examined by measuring the NADH oxidase activity, as shown in Fig. 46.

9 - Isolation of *A. vinelandii* wall and membrane fractions by sucrose density gradients

Envelope fractions (wall plus membrane) of *A. vinelandii* obtained by osmotic shock were separated by sucrose density gradients as noted in Chapter 2. The higher density fraction (coloured white) was the cell wall whilst the less dense fraction (reddish in colour) was the cytoplasmic membrane fraction. The purity of each band was examined by measuring the composition of the two layers.

Fig. 47 illustrates the distribution of nucleic acid and protein on the gradient and Fig. 48 shows the cytochrome distribution. Table 9 presents the LPS content, DAP distribution, NADH oxidase activity and cytochrome concentration of the bands. The morphology of the fragments in the fractions are illustrated in Fig. 49 and 50. The morphology of the bottom band (Fig. 49) was similar to that of intact cell walls, but the cytoplasmic membrane fraction consists of small vesicles with a single unit membrane (Fig. 50).

Efforts to separate the fractions derived from osmotically shocked cells without sonication and separation of fractions of sonicated cells was not successful (data not presented).

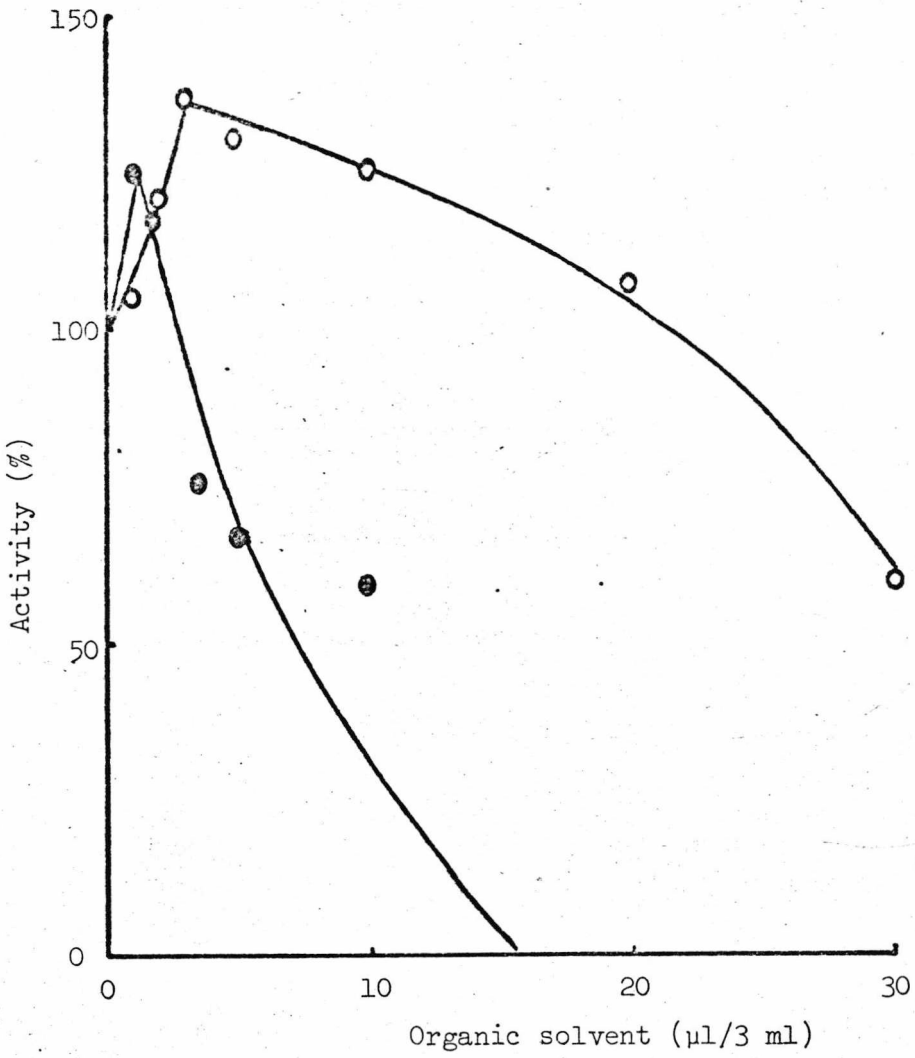


Fig. 46. Effect of toluene (●) and butanol (○) on stimulation of NADH oxidase activity.

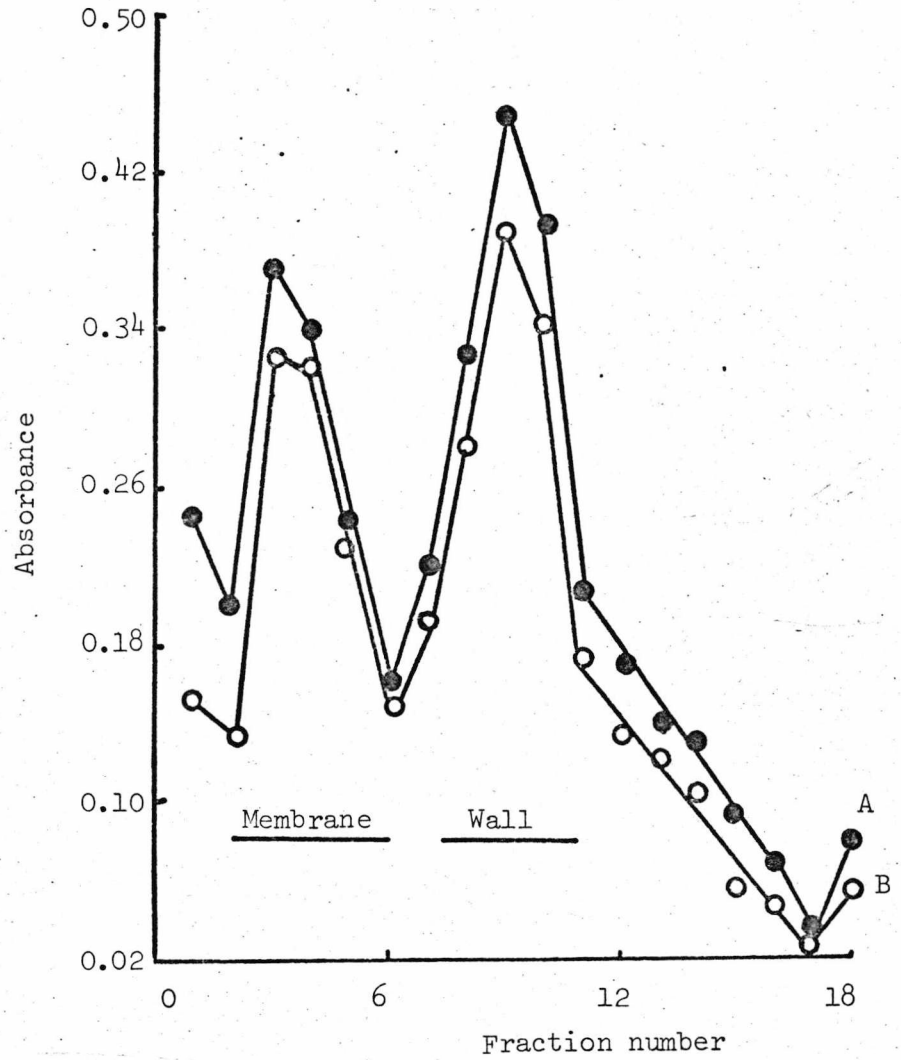


Fig. 47. Distribution of nucleic acid (A) and protein (B) in gradients. *A. vinelandii* envelope fractions were layered on sucrose density gradients and gradients were centrifuged at 20,000 x *g* for 16 h. The gradients were fractionated by pumping and optical densities of each fraction was read at 260nm and 280nm.

Fig. 48. Distribution of cytochromes, in the two fractions of *A. vinelandii*. (A) cytoplasmic membrane, (B) cell wall. In each case the final protein concentration was 1 mg/ml.

TABLE 9. DISTRIBUTION OF NADH OXIDASE, LIPOPOLYSACCHARIDE, DIAMINOPIMELIC ACID AND CYTOCHROMES IN CELL WALL AND MEMBRANE FRACTIONS OF *A. vinelandii* PREPARED BY OSMOTIC SHOCK TREATMENT AND SUCROSE DENSITY GRADIENT CENTRIFUGATION

	Membrane	Wall
LPS (%)	28.9	71.1
DAP (%)	28.88	71.12
NADH activity (µg atoms oxygen/ mg/min)	2.000	0.075
Cytochrome b*	2.2	0.4
Cytochrome c*	2.5	0.4

* nmole/mg protein (see also Fig. 48)

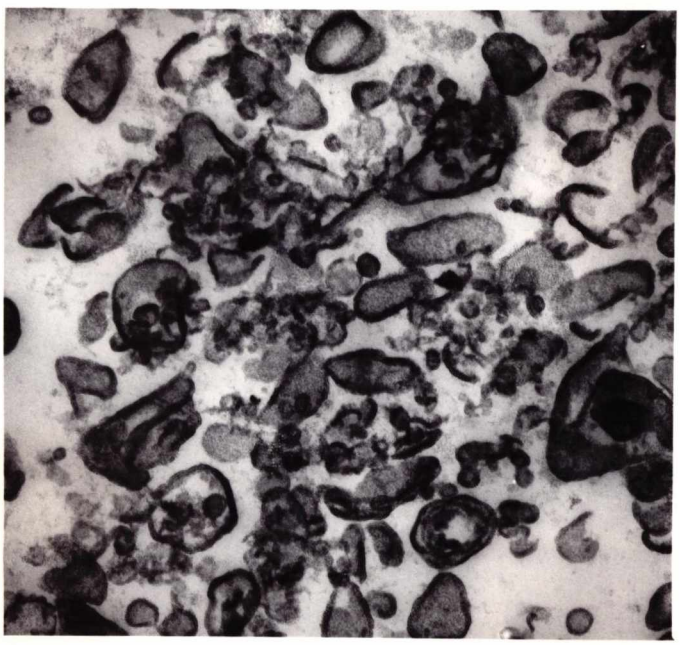


Fig. 49. Electron micrograph of *A. vinelandii* cell walls prepared by osmotic shock and sucrose density gradient. Pellets were suspended in 1% OsO_4 in Kellenberger buffer and were dehydrated and polymerized after washing in Kellenberger buffer and 0.5% uranyl acetate in room temperature (cf. Chapter 2 part 7) : x 47600.

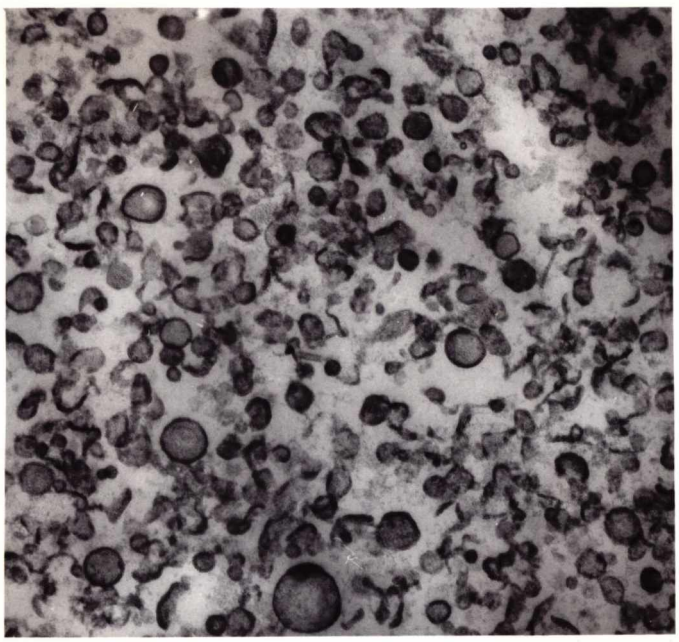


Fig. 50. Electron micrograph of *A. vinelandii* cell membranes prepared by osmotic shock and sucrose density gradient. Fractions were fixed dehydrated and polymerized as noted in Fig. 49 : x 47600.

CHAPTER 4

D I S C U S S I O N

Section 1

Turbidity and Volume Changes of *E. coli*

Use of the exclusion (sucrose and dextran) space technique, shows that increasing the medium concentration of non-penetrant solutes, in addition to causing a decrease in volume of the 'protoplast' caused the cell wall to contract, though to a lesser degree.

Since salts (NaCl and $MgCl_2$) cause a much greater contraction of the wall than does sucrose, it is presumed that this contraction is mainly due to ionic interaction rather than an osmotic effect. These results are qualitatively similar to earlier observations with *E. coli*[86]. Quantitative differences are due to the different growth conditions and phase of harvesting, as well as the more accurate technique used in the present studies.

Direct experiments on isolated cell walls of *E. coli* shows that addition of salts to Gram-negative bacterial cell walls cause proton release (Fig. 37) and contraction of the walls (Fig. 38) confirming that the contraction of the walls is due to ionic interaction. It is not known whether it is the murein layer or the outer layer of the wall which intracts with ions, causing the decrease in volume. Isolated walls of Gram-positive cells, which are principally murein and have no outer layer, exhibit ionic contraction[133,134], suggesting that it is the murein rather than the outer layer of isolated *E. coli* cell walls that intracts with ions.

The mechanically strong layer of the cell envelope of *E. coli*,

the wall, is therefore not completely rigid and exhibits some elasticity. The observations on contraction of cell walls confirm previous observations[162,163], and present evidence that the peptidoglycan layer is a structure that acts as an envelope for the cell and is an elastic layer rather than a rigid shell.

Electron micrographs of plasmolysed cells of *E. coli* (Figs. 23A, 24A and 26A) show that there are many points of adhesion between the cell wall and cytoplasmic membrane in cells plasmolysed by salts, but not in cells plasmolysed by sucrose. This contrasts with previous studies on *E. coli* [164-166]. However, in these studies cells were plasmolysed in media of much higher ionic strength (due to cells and added sucrose being suspended in nutrient medium or salts plus buffer). It is possible, therefore, that maintenance of the adherence sites between the wall and the membrane depends on the ionic strength of the plasmolysing agent. So far, no experiments have been undertaken specifically to vary the ionic strength of the medium at fixed osmolalities and also measure the number of adhesion sites present.

The adherence points have been implicated as phage receptor sites[165] or as the site of export of newly synthesized lipopolysaccharide from the inside of the cytoplasmic membrane[167]. It has been shown that the rate of bacteriophage attachment to *E. coli* depends on the concentration of ions in the medium.[168] It will be of interest to see whether these processes are affected by the ionic strength of the medium in plasmolysed cells and whether this can be related to the number of adherence points present.

In addition to the probable interaction of ions with the murein[133], the decrease in whole cell volume on addition of

salts could be due to the inward pull caused by the adherence points between the wall and the cytoplasmic membrane as the 'protoplast' decreases in size on osmotically induced, outwards passage of water across the cytoplasmic membrane. The difference in the decrease of cell volume caused by salts and sucrose could thus be explained by the lack of or few adherence points found in sucrose (low ionic strength) - plasmolysed cells. Further work is needed to confirm or disprove this possibility.

In *A. vinelandii*, the cell wall is much more flexible than in *E. coli* and only a little plasmolysis occurs[87,169], with distinct decrease in whole cell volume induced by both sucrose and electrolytes. On the other hand Gram-positive bacteria do not show volume changes except at very high medium osmotic pressures. This is probably due to much higher internal osmotic pressures[85,133,134].

Despite the differences in whole cell concentration caused by salts and sucrose , the turbidity changes are strictly dependent on the applied osmotic pressure and the volume of 'protoplast'. The turbidity changes are thus due entirely to the water movement across the cytoplasmic membrane, and are not measurably modified by the whole cell volume changes.

Section 2

Glycerol penetration

Since turbidity changes of *E. coli* are an effective measure of the volume of the 'protoplast', they can be used to measure penetration of solutes across the cytoplasmic membrane by simple diffusion, facilitated diffusion, or active transport.

Substrate entry into bacteria has been studied mainly by radioactivity measurements: the cell (or derived membrane vesicles) are exposed to labelled substrate, following by filtration and assay of the radioactivity remaining associated with the cell. Radioactivity studies are more useful for investigating active transport than for facilitated or simple diffusion, since in the latter case the quantity of radioactivity in the cell is not greatly different to that of the medium. Thus, except for active transport studies, this method lacks sensitivity and is of limited usefulness. Assay of simple and facilitated diffusion by turbidity changes of bacteria offers a simple and rapid alternative method of study.

Accordingly this technique was used for some preliminary studies of glycerol penetration into *E. coli*, in order to see if it is a useful technique. Glycerol penetration was selected for study because (1) entry occurs by simple diffusion and in induced cells by facilitated diffusion (2) mutants deficient in various expressions of glp operon* are available[135],

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*Wild-type of *E. coli* possess an inducible permeation system which catalyses facilitated diffusion of glycerol into the cell. The structural genes are glpF, glpK, glpD and glpR which are facilitator, glycerol kinase, glycerol 3-phosphate dehydrogenase and the repressor, respectively [135].

and (3) the rate of glycerol entry is too rapid to measure by other techniques.

Growth on glucose containing media results in a non-saturating, pH independent penetration of glycerol by simple diffusion, which can be followed easily and conveniently in a stopped-flow spectrophotometer. Growth on glycerol containing media causes induction of a saturable facilitated diffusion system (Fig. 30), and the availability of facilitator negative (F^-) mutants shows that this induction is under genetic control. The inducible penetration system is much more likely to be by facilitated diffusion than by active transport because (1) appreciable accumulation of glycerol by active transport would require expenditure of enormous quantities of energy owing to the rapid loss of glycerol back to the environment by simple diffusion out of the cells (2), there is no evidence in induced cell , for accumulation of glycerol to concentrations greater than that of the medium.

These results on glycerol penetration into *E. coli* confirm that measurement of turbidity changes of Gram-negative bacteria for simple and facilitated diffusion is a useful, sensitive method of assay. This technique should therefore be applicable to a wide range of similar studies.

Thermotropic lipid phase transition of cytoplasmic membranes of bacteria are well documented[170]. The phase changes occur at temperatures somewhat below the growth temperature[170], and the exact transition temperature depends on the lipid composition of membrane[136]. Such phase changes affect the

function of membrane-bound enzymes. It is interesting, therefore that in *E. coli* the rate of simple diffusion of water and glycerol across the lipid phase also exhibit transition temperatures, corresponding to lipid phase transitions (Fig. 32).

The fatty acid used to support growth of *E. coli* greatly affects the transition temperature[170]. Furthermore the temperature at which the transition occurs is lowered by increasing the degree of fatty acid unsaturation of the membrane lipids[136].

Studies on glycerol entry into an unsaturated fatty acid auxotroph of *E. coli* provided evidence and confirm previous work (ref. 136). As predicted, growth of the mutants on elaidic acid containing media resulted in cells with membranes having a higher transition temperature for glycerol entry (28-30°C) than oleic acid grown cells (13-15°C).

It has been shown[171], that there are differences in the properties of parts of the lipid phase of the *E. coli* cytoplasmic membrane. Changes in activation energy of membrane-bound enzymes above and below the transition temperature involve associated non-bulk phase, possibly non-bilayer and non-mobile lipid bound to the enzymes[13], and measurement of such activation energies may not be typical of the bulk-phase mobile lipid of the membrane.

The data in this thesis on the activation energy for the penetration of water and glycerol across, presumably, the mobile, non-protein associated fractions of the membrane lipids show that the lipid phase transitions are therefore important with

respect to functions (simple diffusion of water and solutes) of the membrane associated directly with the lipid bi-layer, as well as with enzymic activity of the integral protein.

The activation energy for glycerol penetration into *E. coli* is higher above the transition temperature than below it, yet the converse is true for water movement (Fig. 32). It is possible that this reflects the fact that in one case the water flow is out of the cell, whilst in the other the glycerol plus water movement is into the cell, i.e. the membrane exhibits sidedness. It is more likely, however, that the difference of effect is due to the more lipophilic properties of glycerol than water, or to their considerable difference of size and the difference in available "holes" for their permeation[172, 173].

Section 3

Orientation of Cytoplasmic Membrane Vesicles of *A. vinelandii*.

A. vinelandii, which apparently has a more flexible cell wall than *E. coli*, was lysed by glycerol osmotic shock treatment (internal pressure disruption) and crude envelopes were separated from the rest of the cell components at a relatively low centrifugal force. The ability to separate the crude envelopes from the soluble components of the cell at a lower centrifugal force (23,000 x *g* for 30 min) than the force typically required to separate the envelope fractions prepared by French pressure cell e.g. 150,000 x *g* for 45 min[18,33] and sonication indicates that the envelope fractions were only minimally damaged and presumably relatively large membrane vesicles are formed.

Membrane vesicles prepared by lysozyme-EDTA method have been extensively used to study active transport [72,73,115] because they have great advantages over intact cells[160]. Moreover, it has been reported that these vesicles are topologically closed and are relatively large [72].

Separation at a low centrifugal force of the envelope fraction from the soluble fraction of *A. vinelandii* (lysed by osmotic shock) and electron microscopic examination of the membrane fraction confirmed that membrane fractions are vesicular and also of large size.

So far studies in several laboratories on orientation of membrane vesicles prepared by different procedures have

indicated that vesicles prepared by French pressure cell and sonication are inside out [66,68] whereas the majority of vesicles prepared by lysozyme-EDTA are right side out [66,119,161]*

Studies on sidedness of membrane vesicles obtained by glycerol osmotic shock show that active transport of glucose takes place at a very considerably lower rate than the rate of uptake into vesicles obtained from lysozyme-EDTA treated cells. This suggests that the majority of vesicles are inside out or that not all the vesicles are completely enclosed.

The site whereby ferricyanide obtains electrons from the respiratory chain is on the internal cytoplasmic side of the plasma membrane [66], as is the NADH-dehydrogenase in correctly oriented membrane vesicles. Therefore, NADH-ferricyanide reductase or NADH oxidase activities can be used as markers for orientation of membrane vesicles. Furthermore addition of organic solvents (e.g. toluene) to permeabilise the membrane should increase the observed rate of ferricyanide reductase activity of right side out vesicles but have no effect on inside out vesicles (which will already have NADH-ferricyanide reductase and NADH oxidase activities since the enzyme is already exposed to its substrate). Results of the studies on the effect of toluene and butanol on the NADH-ferricyanide reductase and NADH oxidase activities shows that at low concentrations they have only a slight stimulatory effect on both activities, indicating that the site of reduction of

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* However, as discussed in the results, there is some controversy whether or not the lysozyme-EDTA vesicles are mono-oriented [66,119,161].

ferricyanide and the NADH dehydrogenase are both located principally on the external surface of vesicles prepared by glycerol osmotic shock. Therefore these results also suggest that vesicles are principally inverted but contain a small proportion of right side out vesicles.

Ion-translocating antibiotics (ionophores) are able to conduct ions across the membrane. Therefore, addition of ion-translocating antibiotics to suspension of inverted vesicles (protons are pumped into the vesicles by the respiratory system) would increase the rate of respiration because protons will be exchanged for cations.

Studies on the orientation of *A. vinelandii* vesicles, prepared by glycerol osmotic shock, using ion-translocating antibiotics shows that addition of nigericin in the presence of K^+ and valinomycin causes stimulation of respiratory rate (Table 8), indicating that some or all of the vesicles are inverted. Valinomycin facilitates the exit of K^+ which has been exchanged for H^+ by nigericin, thus facilitating the re-entry of H^+.

In total, the data on ion-translocating antibiotics, NADH-ferricyanide reductase and NADH oxidase activities and glucose transport indicate that formation of vesicles prepared by glycerol osmotic shock might be (d) or (e) in Fig. 15 but that the great majority of the vesicles are inverted. A small, but significant, fraction of membrane fragments are not enclosed to form vesicles and are either (1) separated from wall fractions but are not sealed

(Fig. 15 (a)) or (2) might be still associated with the wall. The latter suggestion is more likely because of the apparent difficulties in preparing relatively "pure" wall and "pure" membrane fractions by sucrose density gradient centrifugation of the crude osmotic shocked envelopes and the necessity for further treatment to separate the layers of envelope. It can also be concluded that in *A. vinelandii* the number of points of adhesion between the wall and the membrane is likely to be more than in *E. coli* (cf. Bayer [166]) in good agreement with lower degree of plasmolysis in this microorganism [87,169].

Section 4

Separation of Wall and Cytoplasmic Membrane of *A. vinelandii* after Glycerol Osmotic Shock

Several procedures have been developed for isolation and fractionation of cell envelope preparations of Gram-negative bacteria. These methods include gentle lysis by lysozyme-EDTA [30,68,70,71] and the much more drastic techniques of French pressure cell [18,33] or sonication [65]. During spheroplast formation by lysozyme-EDTA, the LPS layer of cell wall is broken by EDTA and the peptidoglycan layer split by the action of lysozyme. Therefore, this method is essentially useful for isolation of the cytoplasmic membrane. Since I was interested in the possibility of obtaining large quantities of cell walls of Gram-negative bacteria, I based my procedures on the glycerol osmotic shock (internal osmotic pressure disruption) technique.

Efforts to separate the envelope fractions of *A. vinelandii* directly after osmotic shock and by sucrose density gradient centrifugation were not successful. However, after further treatment (refer to results) the crude envelopes of *A. vinelandii* prepared by glycerol osmotic shock were separated into two distinctive fractions (wall and membrane). Biochemical and morphological studies of the two fractions indicated that the wall and membrane are extremely well separated, in comparison to published data on envelope fractionation of Gram-negative bacteria [18,33]. I therefore conclude that my procedure for isolation and fractionation

of *A. vinelandii* cell envelope fractions by the glycerol osmotic shock technique followed by sucrose density gradient centrifugation is a useful new method of cell envelope fractionation which can be extended to other Gram-negative bacteria, when isolation of both the cell wall and the cytoplasmic membrane is required. This method should also be used when large quantities of walls and cytoplasmic membrane are needed.

REFERENCES

1 - Glauert, A. M. and Thornley, M. J. (1969)
Ann. Rev. Microbiol. *23*, 159-198

2 - Kellenberger, E. and Ryter, A. (1958)
J. Biophys. Biochem. Cytol. *4*, 323

3 - Forsberg, C. W., Costerton, J. W. and McLeod, R. A. (1970)
J. Bacteriol. *104*, 1354-1368

4 - Schnaitman, C. A. (1971)
J. Bacteriol. *108*, 553-563

5 - Salton, M. R. J. (1961)
Bacteriol. Rev. *25*, 77-99

6 - Rogers, H. J. and Perkins, H. R. (1968)
In Cell Walls and Membranes
Edited by Cyril Long
E. and F. N. Spon Ltd.

7 - Schleifer, K. H. and Kandler, O. (1972)
Bacteriol. Rev. *36*, 407-477

8 - Costerton, J. W., Ingram, J. M. and Cheng, K. J. (1974)
Bacteriol. Rev. *38*, 87-110

9 - Reavely, D. A. and Burge, R. E. (1972)
Adv. Microbiol. Physiol. *7*, 1-71

10 - Rothfield, L. I. (1971) In *Structure and Function of Bacterial Membrane*, Academic Press, New York, London.

11 - Salton, M. R. J. (1967)
Ann. Rev. Bacteriol. *21*, 417-442

12 - Gross, W. (1971)
Angew. Chem. Internat. Edit. *10*, 388-395

13 - Singer, S. J. and Nicolson, G. L. (1972)
Science *175*, 720-731

14 - Danielli, J. F. and Davson, H. (1935)
J. Cell Comp. Physiol. *5*, 495

15 - Robertson, J. D. (1960) In *Progress in Biophysics and Molecular Biology*, Pergamon Press, Oxford.
vol. 10

16 - Lenard, J. and Singer, S. J. (1966)
Proc. Natl. Acad. Sci. U.S. *56*, 1828-1835

17 - Singer, S. J. (1973)
Science 180, 982-983

18 - Schnaitman, C. A. (1970)
J. Bacteriol. 104, 890-901

19 - Martin, E. L. and MacLeod, R. A. (1971)
J. Bacteriol. 105, 1160-1167

20 - Dreher, K. D., Schulman, J. H., Anderson, O.R. and Roels, D. A. (1967)
J. Ultrastruct. Res. 19, 586-599

21 - Cheng, K. J., Ingram, J. M. and Costerton, J. W. (1971)
J. Bacteriol. 107, 325-336

22 - Mitchell, P. (1961) Approaches to the Analysis of Specific Membrane Transport,
In T. W. Goodwin and O. Lindberg (ed.)
Biological Structure and Function
vol. 2 pp 581-603
Academic Press Inc., New York.

23 - Birdsell, D. C. and Cota-Robles, E. H. (1967)
J. Bacteriol. 93, 427-437

24 - Payne, J. W. and Gilvarg, C. (1968)
J. Biol. Chem. 243, 6291-6294

25 - Singh, A. P., Cheng, K. J., Costerton, J. W., Idziak, E. S. and Ingram, J. M. (1972)
Can. J. Microbiol. 18, 909-915

26 - Osborn, M. J. (1963)
Proc. Natl. Acad. Sci. U.S. 50, 499-506

27 - Rothfield, L. I. and Horne, R. W. (1967)
J. Bacteriol. 93, 1705-1721

28 - Osborn, M. J. (1969)
Ann. Rev. Biochem. 38, 501-558

29 - Lopes, J. and Inniss, W. E. (1970)
J. Bacteriol. 103, 238-243

30 - Osborn, M. J., Cander, J. E., Parisi, E. and Carson, J. (1972)
J. Biol. Chem. 247, 3962-3972

31 - Braun, V. and Boch, V. (1973) *FEBS Letts. 34,* 302-306.

32 - Braun, V. and Rehn, K. (1969)
Eur. J. Biochem. *10*, 426-437

33 - Schnaitman, C. A. (1970)
J. Bacteriol. *104*, 882-889

34 - Lenhninger, A. L. (1970)
In *Biochemistry*
Worth Publishers Inc. New York.

35 - Braun, V., Gnirke, H., Henning, U. and Rehn, K. (1973)
J. Bacteriol. *114*, 1264-1270

36 - Ghuysen, J. M. (1968)
Bacteriol. Rev. *32*, 425-464

37 - Weidel, W. and Pelzer, H. (1964)
Adv. Enzymol. *26*, 193-232

38 - Braun V. and Bosch, V. (1972)
Proc. Natl. Acad. Sci. U.S. *69*, 970-974

39 - Hantke, K. and Braun, V. (1973)
Eur. J. Biochem. *34*, 284-296

40 - Inouye, M., Shaw, J. and Shen, C. (1972)
J. Biol. Chem. *247*, 8154-8159

41 - Bosch, V. and Braun, V. (1973)
FEBS Letts *34*, 307-310

42 - Burman, L. G., Nordstrom, K. and Bloom, G. D. (1972)
J. Bacteriol. *112*, 1364-1374

43 - Forsberg, C. W., Costerton, J. W. and MacLeod, R. A. (1970).
J. Bacteriol. *104*, 1338-1353

44 - Salton, M. R. J. (1964)
In *Bacterial Cell Wall*
Elsevier Publishing Company, Amsterdam, London, New York.

45 - Lilly, M. D. (1962)
J. Gen. Microbiol. *28*, ii-iii

46 - Cutinelli, C. and Galdiero, F. (1967)
J. Bacteriol. *93*, 2022-2023

47 - Ellwood, D. C. and Tempest, D. W. (1972)
Adv. Microbiol. Physiol. *7*, 83-117

48 - Tempest, D. W., Dicks, J. W. and Meers, J. L. (1967)
J. Gen. Microbiol. *49*, 139-147

49 - Eagon, R. G. (1969)
Can. J. Microbiol. 15, 235-236

50 - Rogers, H. J. (1970)
Bacterial Rev. 34, 194-214

51 - Park, J. T. and Strominger, J. L. (1957)
Science 125, 99

52 - Costerton, J. W. (1970)
Rev. Can. Biol. 29, 229-316

53 - Anraku, Y. (1968)
J. Biol. Chem. 243, 3116-3122

54 - Heppel, L. A. (1971)
The Concept of Periplasmic Enzymes, pp 223-246,
In Rothfield, L. I. (ed.)
Structure and Function of Biological Membrane,
Academic Press, New York, London.

55 - Dvorak, H. F., Wetzel, B. K. and Heppel, L. A. (1970)
J. Bacteriol. 104, 542-548

56 - MacAlister, T. J., Costerton, J. W., Thompson, L.,
Thompson, J. and Ingram, J. M. (1972)
J. Bacteriol. 111, 827-832

57 - Wetzel, B. K., Spicer, S. S., Dvorak, H. F. and
Heppel, L. A. (1970)
J. Bacteriol. 104, 529-542

58 - Neu, H. C. and Heppel, L. A. (1965)
J. Biol. Chem. 240, 3685-3692

59 - Heppel, L. A. (1967)
Science 156, 1451-1455

60 - Heppel, L. A. (1969)
J. Gen. Physiol. 54, 95-109

61 - Stanier, R. Y., Doudoroff, M. and Adelberg, E. A.
In *General Microbiology*, Third edition (1970)
Original American edition by Prentic Hall Inc.,New
Jersey, U.S.A.

62 - De Pamphilis, M. L. and Alder, J. (1971)
J. Bacteriol. 105, 376-383

63 - De Pamphilis, M. L. and Alder, J. (1971)
J. Bacteriol. 105, 384-395

64 - De Pamphilis, M. L. and Alder, J. (1971)
J. Bacteriol. 105, 396-407

65 - Kaback, H. R. and Deuel, T. F. (1969)
Arch. Biochem. Biophys. 132, 118-129

66 - Futai, M. (1974)
J. Membrane Biol. 15, 15-28

67 - Hertzberg, E. L. and Hinkle, P. C. (1974)
Biochim. Biophys. Res. Commun. 58, 178-184

68 - Mevel-Nino, M. and Yamanota, T. (1974)
Biochim. Biophys. Acta 357, 63-66

69 - Milner, H. W., Lawrence, N. S. and French, C. S. (1950)
Science 111, 633

70 - Miura, T. and Mizushima, S. (1968)
Biochim. Biophys. Acta 150, 159-161

71 - Miura, T. and Mizushima, S. (1969)
Biochim. Biophys. Acta 193, 268-276

72 - Kaback, H. R. (1971)
Bacterial Membrane pp 99-120
In *Method in Enzymology* Vol. 22
B. Jacoby Editor, Academic Press Inc., New York.

73 - Kaback, H. R. (1972)
Biochim. Biophys. Acta 265, 367-416

74 - Altendrof, K. H. and Staehelin, L. A. (1974)
J. Bacteriol. 117, 888-899

75 - Leive, L. (1965)
Biochim. Biophys. Res. Commun. 21, 290-296

76 - Reposke, R. (1958)
Biochim. Biophys. Acta 30, 225-232

77 - Kaback, H. R. and Stadtman, E. R. (1966)
Proc. Natl. Acad. Sci. U. S. 55, 920-927

78 - Robrish, S. A. and Marr, A. G. (1961)
J. Bacteriol. 83, 158-168

79 - Pangborn, J., Marr, A. G. and Robrish, S. A. (1962)
J. Bacteriol. 84, 669-678

80 - Oltmann, L. F. and Stouthamer, A. H. (1973)
Arch. Mikrobiol. 93, 311-325

81 - Martin, H. H. (1963)
J. Theoret. Biol. 5, 1-35

82 - Scherrer, R. and Gerhardt, P. (1971)
J. Bacteriol. 107, 718-735

83 - Dick, A. D. T.
Water Movement in Cells
In *Membrane and Ion Transport*
Vol. 3 pp 211-250 By E. E. Bitter
First Published (1971) John Wiley & Son Ltd.
London, New York, Sydney, Toronto.

84 - Davson, H.
A Text Book of General Physiology;
vol. 1 pp 369-394
Fourth edition (1970) J. and A. Churchill, London.

85 - Mitchell, P. and Moyle, J. (1956)
Osmotic Function and Structure in Bacteria,
In *Bacterial Anatomy*, pp 150-180
Symposia of the Society for General Microbiology

86 - Knowles, C. J. (1971)
Nature New Biology 229, 154-155

87 - Knowles, C. J. and Smith, L. (1971)
Biochim. Biophys. Acta 234, 144-152

88 - Matts, T. C. and Knowles, C. J. (1971)
Biochim. Biophys. Acta 249, 583-587

89 - Alemohammad, M. M. and Knowles, C. J. (1974)
J. Gen. Microbiol. 82, 125-142

90 - Mager, I., Kuczynski, M. Schatzberg, G. and
Avi-Dor, Y. (1956)
J. Gen. Microbiol. 14, 69-75

91 - Avi-Dor, Y., Kuczynski, M., Schatzberg, G. and
Mager, I. (1956)
J. Gen. Microbiol. 14, 76-83

92 - Bovel, C. R., Packer, L. and Helgerson, R. (1963)
Biochim. Biophys. Acta 75, 257-266

93 - Stein, W. D. (1967)
The Movement of Molecules Across Cell Membranes,
Academic Press, New York.

94 - Rothstein, A. (1968)
The Relationship of the Cell Wall to Electrolyte and Volume Regulation in Microorganisms, In *Microbial Protoplasts, Spheroplasts and L-forms* pp 174-185
Guze, L. B. (ed)
The Williams and Wilkins Co., Baltimore.

95 - Epstein, W. and Schultz, S. G. (1965)
J. Gen. Physiol. 49, 211-234

96 - Knowles, C. J. (1973)
Pestic Sci. 4, 861-862

97 - Greville, G. D. (1969)
In *"Current Topics in Bioenergetics"*
(Sanadi, D. R. ed) vol. 3 pp 1-78
Academic Press, New York, London

98 - Williams, R. J. P. (1969)
In *"Current Topics in Bioenergetics"*
(Sanadi, D. R. , ed) vol. 3 pp 80-156

99 - Slater, E. C. (1971)
Quart. Rev. Biophys. 4, 35-72

100 - Pullman, M. E. and Schatz, G. (1967)
Annu. Rev. Biochem. 36, 652

101 - Wanino, W. W. (1970)
The Mammalian Mitochondria Respiratory Chain
Academic Press, New York

102 - Harold, F. M. (1972)
Bacterial Rev. 36, 172-230

103 - Skulachev, V. P. (1971)
In *"Current Topics in Bioenergetics"*
(Sanadi, D. R., ed) vol 4. pp 127-190
Academic Press, New York, London

104 - Wilson, D. F., Dutton, P. L. and Wagner, M. (1973)
In *"Current Topics in Bioenergetics"*
(Sanadi, D. R. and Packer, L., ed)
Vol. 5 pp 234-265
Academic Press, New York, London

105 - Mitchell , P. (1961)
Nature 191, 144-148

106 - Mitchell, P. (1966)
Biol. Rev. (Cambridge) *41*, 445-502

107 - Gel'Man, N. S.
The Respiration and Phosphorylation of Bacteria
Plenum Press, New York (1967)

108 - Kiesow, L. (1964)
Proc. Natl. Acad. Sci. U.S. *52*, 980-988

109 - Aleem, M. and Nason, A. (1960)
Proc. Natl. Acad. Sci. U.S. *46*, 763-769

110 - White, D. C. and Sinclair, P. R. (1971)
Adv. Microbial Physiol. *5*, 173-211

111 - Jones, C. W. and Redfearn, E. R. (1967)
Biochim. Biophys. Acta *143*, 340-353

112 - Ackrell, B. A. C. and Jones, C. W. (1971)
Eur. J. Biochem. *20*, 22-28

113 - Ackrell, B. A. C. and Jones, C. W. (1971)
Eur. J. Biochem. *20*, 29-35

114 - Barnes, Jr. E. M. (1972)
Arch. Biochem. Biophys. *152*, 795-799

115 - Barnes, Jr, E. M. (1973)
J. Biol. Chem. *248*, 8120-8124

116 - Tsukagoski, N. and Fox, C. F. (1971)
Biochemistry *10*, 3309-3313

117 - John, P. and Hamilton, W. A. (1971)
Eur. J. Biochem. *23*, 528-532

118 - Hampton, M. L. and Freese, E. (1974)
J. Bacteriol. *118*, 497-504

119 - Gorneva, G. A. and Ryabova, I. D. (1974)
FEBS Letts. *42*, 271-274

120 - Weiner, J. H. (1974)
J. Membrane Biol. *15*, 1-14

121 - Harold, F. M. (1970)
Adv. Microbial Physiol. *4*, 45-104

122 - Jackson, R. W. and De Moss, J. A. (1965)
J. Bacteriol. *90*, 1420-1425

123 - Boos, W. (1974)
Annu. Rev. Biochem. *43*, 123-146

124 - Kaback, H. R. (1970)
Current Topics in Membrane Transport
1, 35-99

125 - Kunding, W., Ghosh, S. and Roseman, S. (1964)
Proc. Natl. Acad. Sci. U.S. *52*, 1067-1074

126 - Kaback, H. R. (1968)
J. Biol. Chem. *243*, 3711-3724

127 - Barnes, E. M. and Kaback, H. R. (1971)
J. Biol. Chem. *246*, 5518-5522

128 - Walsh, C. T., Abeles, R. H. and Kaback, H. R. (1972)
J. Biol. Chem. *247*, 7858-7863

129 - Reeves, J. P., Lambardi, F. J. and Kaback, H. R. (1972)
J. Biol. Chem. *247*, 6204-6211

130 - Mitchell, P. (1970)
Symp. Soc. Gen. Microbiol. 20th, pp 121-166

131 - Hamilton, W. A. (1975)
Adv. Microbial Physiol. *12*, 1-53

132 - Niven, D. F. (1973)
Mechanisms of Energy Coupling to the Transport of Amino Acids by S. aureus
Ph.D. thesis, Department of Biochemistry, Marischal College, Aberdeen

133 - Marquis, R. E. (1968)
J. Bacteriol. *95*, 775-781

134 - Ou, Li-Tse and Marquis, R. E. (1970)
J. Bacteriol. *101*, 92-101

135 - Richey, D. P. and Lin, E. C. C. (1972)
J. Bacteriol. *112*, 784-790

136 - Wilson, G. and Fox, C. F. (1971)
J. Mol. Biol. *55*, 49-60

137 - Miller, J. H. (1972)
Experiments in Molecular Genetics
P.431 Cold Spring Harbor, Cold Spring Harbor Laboratory

138 - Gibson, Q. H. and Milnes, L. (1964)
Biochem. J. *91*, 161-171

139 - Gibson, Q. H. (1969)
Rapid Mixing (stopped-flow)
In *Method in enzymology* vol 16 pp 187-228
Fast Reaction Kustined, K., ed.
Academic Press, New York, London

140 - Finlayson, J. S. (1969)
In *Basic Biochemical Calculations*
Addison-Wesley Publoshing Company Inc.
Massachusetts, California, London, Ontario

141 - Bray, G. A. (1960)
Analyt. Biochem. 1, 279-285

142 - Black, S. H. and Gerhardt, P. (1962)
J. Bacteriol. 83, 960-967

143 - Spurr, A. R. (1969)
Ultrastructure Res. 26, 31-43

144 - Reynolds, E. C. (1963)
J. Cell Biol. 17, 208-216

145 - Glauert, A. M. (1965)
In *Techniques for Electron Microscopy*
pp 165-212, Kay, D. ed.,
Blackwell Scientific Publication Ltd.

146 - Gornall, A. G., Bardawill, C. S. and David, M.M. (1949)
J. Biol. Chem. 177, 751-763

147 - Weston, J. A. (1974)
The Respiratory system for Beneckea natriegens
Ph.D. thesis, University of Kent, Canterbury

148 - Jones, C. W. and Redfearn, E. R. (1966)
Biochim. Biophys. Acta 113, 467-481

149 - Arrigoni, D. and Singer, T. D. (1962)
Nature 193, 1256-1258

150 - Chapell, J. B. (1964)
Biochem. J. 90, 225-237

151 - Günther Srebert (1965)
In *Methods of Enzymatic Analysis*
pp 318-323, Bergmeyer, H. U. ed.,
Varley Chemie, Academic Press, New York, London

152 - Neu, H. C. and Heppel, L. A. (1964)
Biochim. Biophys. Res. Commun. 14, 109-112

153 - Aminoff, D. (1961)
Biochem. J. 81, 384-392

154 - Becker, B., Lechevalier, M. P., Gordon, R. E. Lechevalier, H. A. (1964)
Appl. Microbiol. 12, 421-423

155 - Perkins, H. R. (1965)
Biochem. J. 95, 876-882

156 - Scheie, P. O. (1969)
J. Bacteriol. 98, 335-340

157 - Alexander, M. and Wilson, P. W. (1955)
Notes 71, 252-253

158 - Neu, H. C. and Heppel, L. A. (1964)
J. Biol. Chem. 239, 3893-3900

159 - Short, S. A., Kaback, H. R., Kaczorowski, G., Fisher, J., Walsh, C. T. and Silverstein, S. C. (1974)
Proc. Natl. Acad. Sci. U.S.*71*, 5032-5036

160 - Kaback, H. R. (1974)
Science 186, 882-892

161 - Hare, J. F., Olden, K. and Kennedy, E. P. (1974)
Proc. Natl. Acad. Sci. U.S.*71*, 4843-4846

162 - Marquis, R. E. (1973)
J. Bacteriol. 116, 1273-1279

163 - Isaac, L. and Warem G. C. (1974)
J. Appl. Bacteriol. 37, 335-339

164 - Cota-Robles, E. H. (1963)
J. Bacteriol. 85, 499-503

165 - Bayer, M. E. (1968)
J. Virol. 2, 346-456

166 - Bayer, M. E. (1968)
J. Gen. Microbiol. 53, 395-404

167 - Mühlradt, P. F., Menzel, J., Golecki, J. R. and Speth, V. (1973)
Eur. J. Biochem. 35, 471-481

168 - Puck, T. T., Garen, A. and Cline, J. (1951)
J. Experimental Medicine 93, 65-88

169 - Knowles, C. J. and Smith, L. (1971)
Biochem. Biophys. Acta 234, 153-161

170 - Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T. and Wakil, S. J. (1971)
Proc. Natl. Acad. Sci. U.S. 68, 3180-3184

171 - Trauble, H. and Overath, P. (1973)
Biochim. Biophys. Acta 307, 491-512

172 - Stein, W. D. (1967)
The Movement of Molecules across Cell Membrane
pp 122-124 New York, Academic Press

173 - Ling, G. N. (1973)
Biophys. J. 13, 807-816