ASPECTS OF OUTER MEMBRANE PROTEIN SYNTHESIS IN ESCHERICHIA COLI B/r

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by

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Submitted by A. Boyd.

ABSTRACT

The growth and biogenesis of the outer membrane of E.coli B/r has been studied by investigating both the pattern of synthesis of outer membrane protein in the cell cycle, and the kinetics of synthesis and assembly of the major outer membrane protein species, the 36.5K porin.

To facilitate this study the detergent sarkosyl was used to separate the outer membrane from the cytoplasmic membrane of E.coli; comparison of these fractions with those obtained by centrifugation revealed that they were qualitatively identical.

Analysis of the cell cycle revealed that the rate of synthesis of outer membrane protein was constant through the cycle, with an abrupt doubling in rate occurring late in the cycle. Further experiments revealed that the rate of synthesis of outer membrane protein is not differentially affected by treatments which affect DNA synthesis, and it is therefore concluded that the cell cycle doubling in rate of synthesis is unrelated to the DNA replication cycle.

In other experiments the time was measured for the 36.5K porin to be translated, enter the cell envelope, and become associated with the sarkosyl-insoluble outer membrane. This was achieved by a pulse-chase method. The porin associated with the envelope as soon as it was completed; however, it took significantly longer to reach the outer membrane, indicating the existence of a sarkosyl-soluble intermediate form of the mature porin. It is concluded that this may be either a true cytoplasmic membrane intermediate, or a nascent form present in atypical regions of the outer membrane.

Finally, it was shown that an outer membrane protein whose synthesis has been reported to occur periodically in the cell cycle is, in fact, a protein involved in ferric-enterochelin uptake, and it is concluded that its apparent periodic synthesis is an artefact caused by the methods used to generate synchronous cultures.

CONTENTS

CHAPTER 1

INTRODUCTION

This thesis is concerned primarily with aspects of outer membrane protein synthesis in *E.coli* B/r. The pattern of synthesis of these proteins in the cell cycle will be discussed, together with the implications this may have for models of overall surface growth. There will also be discussion of outer membrane biogenesis.

In order to understand the unique nature of the outer membrane and the special problems of its biogenesis it is first necessary to be familiar with current knowledge of more typical biological membranes and how they are assembled. This then is the approach I will use in this Introduction. The first part will be a broad survey of membrane structure and of the structure and biosynthesis of membrane proteins. Building upon this foundation I will describe the structure of the *E.coli* cell envelope, and so arrive at a consideration of the structure and function of the outer membrane. Finally, I will discuss envelope biogenesis, and envelope growth in relation to the cell cycle.

I. THE STRUCTURE OF MEMBRANES AND MEMBRANE PROTEINS

I.(a) Membrane Structure

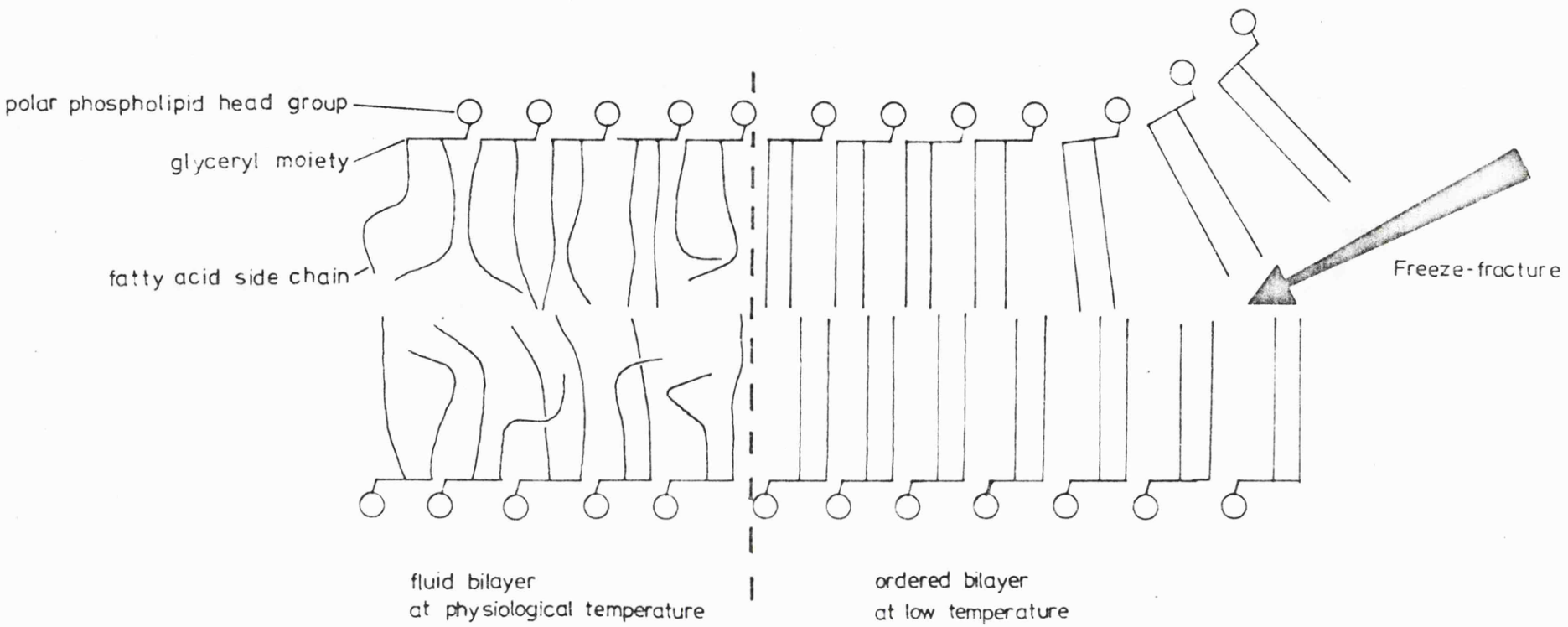
(i) The Fluid-Mosaic Model

The major molecular components of biological membranes are phospholipids and proteins. The fluid-mosaic model of membrane structure (Singer and Nicolson, 1972) is commonly accepted as the best description of the way in which these components combine to form a membrane. In this view the matrix of the membrane is a phospholipid bilayer, a structure which is formed spontaneously by aqueous dispersions of phospholipids. The stability of the bilayer arises from the low free energy state resulting from the segregation of the hydrophobic hydrocarbon chains away from the aqueous surroundings (see Fig. 1.1). The large body of evidence for the existence of the bilayer structure in biological membranes derives from the similarity between the physical properties of intact biological membranes and the corresponding properties of bilayers prepared from purified phospholipids (reviewed by Bangham, 1972). Two such properties which will be referred to below are, firstly, the formation of a "double-track" image in thin section electron micrographs; and secondly, the existence of a freeze-fracture cleavage plane through the hydrophobic phase of the bilayer (see Fig. 1.1).

The proteins of biological membranes have been divided into two classes according to their structural relationship with the membrane bilayer (Singer and Nicolson, 1972). Integral membrane proteins are only removed from the membrane by such drastic procedures as treatment with detergents, (e.g. sodium dodecylsulphate), bile salts, or chaotropic agents (e.g. guanidinium ions) (see Maddy and Dunn, 1976 for a review of methodology). The isolated integral membrane proteins

Figure 1.1: THE BILAYER STRUCTURE OF MEMBRANES

The diagram depicts the phospholipid bilayer which forms the matrix of biological membranes. Also represented are two properties of such bilayers which are referred to in the text : firstly, the order-disorder transition in the hydrophobic phase, which occurs with increasing temperature; and secondly, the existence of a freeze-fracture plane through the membrane bilayer.


polar phospholipid head group
glyceryl moiety
fatty acid side chain
Freeze-fracture
fluid bilayer
at physiological temperature
ordered bilayer
at low temperature

often have tightly bound lipid molecules and are insoluble in aqueous buffers at neutral pH in the absence of detergents. In the fluid-mosaic model it is proposed that integral membrane proteins, such as erythrocyte glycophorin (discussed in detail below), interact directly with the hydrophobic phase of the phospholipid bilayer, as well as having part of their structure exposed at one or both faces of the membrane. In contrast, peripheral membrane proteins (e.g. cytochrome c) are removed from the membrane by relatively mild procedures like sonication (mitochondrial coupling factor F1; Horstmann and Racker, 1970); treatment with chelating agents; or washing with buffers of high or low ionic strength (cytochrome c; Jacobs and Sanadi, 1960). Peripheral membrane proteins are usually recovered in a soluble, lipid-free form. They are thought to interact with the membrane surface, binding either to the polar head groups of the phospholipids, or to integral membrane proteins. The structures of integral and peripheral proteins are shown diagrammatically in Figure 1.2.

(ii) Membrane Fluidity

The matrix of the membrane, the phospholipid bilayer, is essentially an ordered fluid. The bilayers formed in aqueous dispersions of phospholipids exhibit a melting transition at a temperature characteristic of the phospholipids used (Chapman and Wallach, 1968). Below this temperature the hydrocarbon chains are rigid; above it they are free to move, with the degree of freedom increasing with distance from the polar head group. This property of membranes can be measured spectroscopically by electron spin resonance studies of the mobility of spin-labelled probe molecules (Hubbell and McConnell, 1969). Above the melting temperature of the bilayer lateral motion of phospholipid molecules can also occur, as shown by Kornberg and McConnell (1971) who used a spin-labelled phosphatidylcholine probe

incorporated into phosphatidylcholine bilayers, and found that the probe could diffuse at a rate of more than 0.05 μm/sec at 35°C. This lateral mobility of membrane phospholipids has been confirmed by Schlessinger et al. (1976) who measured the diffusion coefficient of a fluorescent lipid probe molecule in a biological membrane; the value of this diffusion coefficient, $D = 8 \times 10^{-9}$ cm^2/sec indicates rapid lateral diffusion in the phospholipid matrix. The other type of movement possible for a phospholipid molecule, flip-flop, or movement from one side of the bilayer to the other, has been found to be extremely rare (Kornberg and McConnell, 1971), or even undetectable (Rothman and Dawidowicz, 1975), in model systems. This is thought to be due to the high activation energy of this process, which would require a polar or charged phospholipid head group to cross the bilayer.

The proteins of biological membranes are also free to undergo lateral diffusion in the plane of the fluid bilayer, as has been shown by: (1) intermixing of surface antigens after formation of mouse-human heterokaryons (Frye and Edidin, 1970); (2) diffusive decay of a patch of fluorescent-antibody-labelled surface receptors on the membrane of cultured muscle fibres (Edidin and Farnborough, 1973); and (3) recovery of fluorescence intensity in a laser-bleached area on the surface of fluorescent-labelled myoblasts (Schlessinger et al., 1976). Edidin and Farnborough (1973) estimated a membrane protein diffusion coefficient, D, of about 10^{-9} cm^2/sec, which is comparable to that measured for membrane phospholipids (see above). However, the data of Schlessinger et al. (1976) indicate a value of D about two orders of magnitude less, which they interpreted as showing a degree of restriction upon mobility, In general, this could arise from the formation of long-range order in the membrane or from association of membrane proteins with the underlying cytoskeletal framework.

There is considerable evidence to show that the fluidity of the phospholipid bilayer of biological membranes is essential for cellular function (reviewed by Melchior and Stein, 1976). Thus the membranes of eukaryotic cells contain cholesterol, which increases the fluidity of the bilayer, whereas the membrane phospholipids of prokaryotic cells contain unsaturated, branched chain, or cyclopropyl fatty acid side chains for the same reason (Raetz, 1978). The disorder-order phase transitions produced in membranes by cooling are associated with breaks in the Arrhenius plots of the activities of membrane proteins such as the lactose permease (Overath and Träuble, 1973) and the NADH oxidase (Morrisett et al., 1975) of E.coli. In both of these studies the correlation between the phase transition in the bilayer and the effect upon enzyme activity was strengthened by using auxotrophic strains of E.coli unable to synthesise unsaturated fatty acids. The unsaturated fatty acid content of the membrane phospholipids of such a strain depends entirely upon the exogenous supply, and so cultures which have different membrane fluidities and, consequently, different transition temperatures, can be produced. The effect upon enzyme activity is always found to occur at the transition temperature. In wild-type strains of E.coli, membrane fluidity appears to be controlled by variation of the content of unsaturated fatty acids. Thus, cultures of the same bacterial strain growing at decreasing temperatures contain higher levels of unsaturated fatty acids (Sinensky, 1971), and seem to maintain a membrane fluidity sufficient to keep the transition temperature about 10°C below the growth temperature (Melchior and Stein, 1976). Sinensky (1971) has demonstrated that the membrane-associated enzyme responsible for adding fatty acids to the phospholipids in E.coli, acyl CoA : glyceryl-3-phosphate transacylase, exhibits a temperature-dependent discrimination between unsaturated and saturated fatty acids. It seems possible, therefore,

that the fluidity of the bilayer modulates the activity of the enzyme in a homeostatic control loop.

(iii) The Asymmetry of Biological Membranes

It is clear that a bilayer membrane has two distinct faces. In the case of the plasma membrane of, for example, the erythrocyte, one face is in contact with the cytoplasm while the other is exposed to the cell's surroundings. In general, it has been found that the two faces of a biological membranes differ in their macromolecular composition (Rothman and Lenard, 1977). This asymmetry of membranes has become apparent from experiments which define the topography of membrane components. These experiments exploit the impermeability of membranes to many low molecular weight reagents, and to most macromolecules. If intact cells are treated with a reagent or enzyme directed against a membrane component, and if this reagent cannot cross the membrane, then only those molecules, or regions of molecules, exposed at the cell surface will be accessible for reaction. If, however, the membrane barrier is disrupted, as for example in erythrocyte ghost membranes prepared by osmotic lysis of erythrocytes, then both faces of the membrane are made accessible to the reagent.

This general strategy has been extensively used to investigate the asymmetry of the erythrocyte membrane (reviewed by Marchesi et al., 1976). Proteins exposed at the cell surface can be identified by their accessibility in intact cells to proteases (Philips and Morrison, 1971; Cabantchik and Rothstein, 1974); lactoperoxidase-catalyzed radio-iodination (Reichstein and Blostein, 1973); or reaction with non-penetrating reagents such as formylmethionyl(sulphone)methylphosphate (FMMP) which reacts with primary amino groups of both proteins and phospholipid head groups (Bretscher, 1971). By these criteria only two

of the major proteins of the erythrocyte are exposed at the outer surface (glycophorin and band 3 protein); and the phospholipids are distributed asymmetrically with phosphatidylethanolamine and phosphatidylserine located mostly in the cytoplasmic leaflet of the bilayer (Bretscher, 1972). An asymmetrical distribution of phospholipids has also been demonstrated in the cytoplasmic membrane of Bacillus megaterium (Rothman and Kennedy, 1977a). In this case the amino-group-directed reagent used, trinitrobenzenesulphonate (TNBS), does not penetrate membranes at 3°C but penetrates slowly at 15°C. Since at the lower temperature only one-third of the phosphatidylethanolamine of the membrane of intact cells was accessible to TNBS, whereas at the higher temperature one third reacted rapidly and the remainder reacted slowly, it was concluded that two-thirds of the membrane phosphatidylethanolamine is located at the cytoplasmic face of the bilayer. The role of the phospholipid asymmetry in biological membranes is completely obscure. It seems unlikely that it is due to specific lipid-binding requirements of membrane proteins since these would be expected to be satisfied by a relatively small degree of asymmetry. Other possible roles include a need for different fluidities in the two leaflets of the bilayer; and a need for different surface tensions in the two leaflets, to produce membrane curvature (discussed in Rothman and Lenard, 1977).

I.(b) Membrane Protein Structure

The structures of many soluble proteins, such as cytochrome c and haemoglobin, have been elucidated at the tertiary, and even quaternary, level. Knowledge of these structures has made it possible to make predictions of secondary and tertiary structures from the primary sequence of amino acids (reviewed by Chou and Fasman, 1978). In

Figure 1.2: THE STRUCTURE OF MEMBRANE PROTEINS

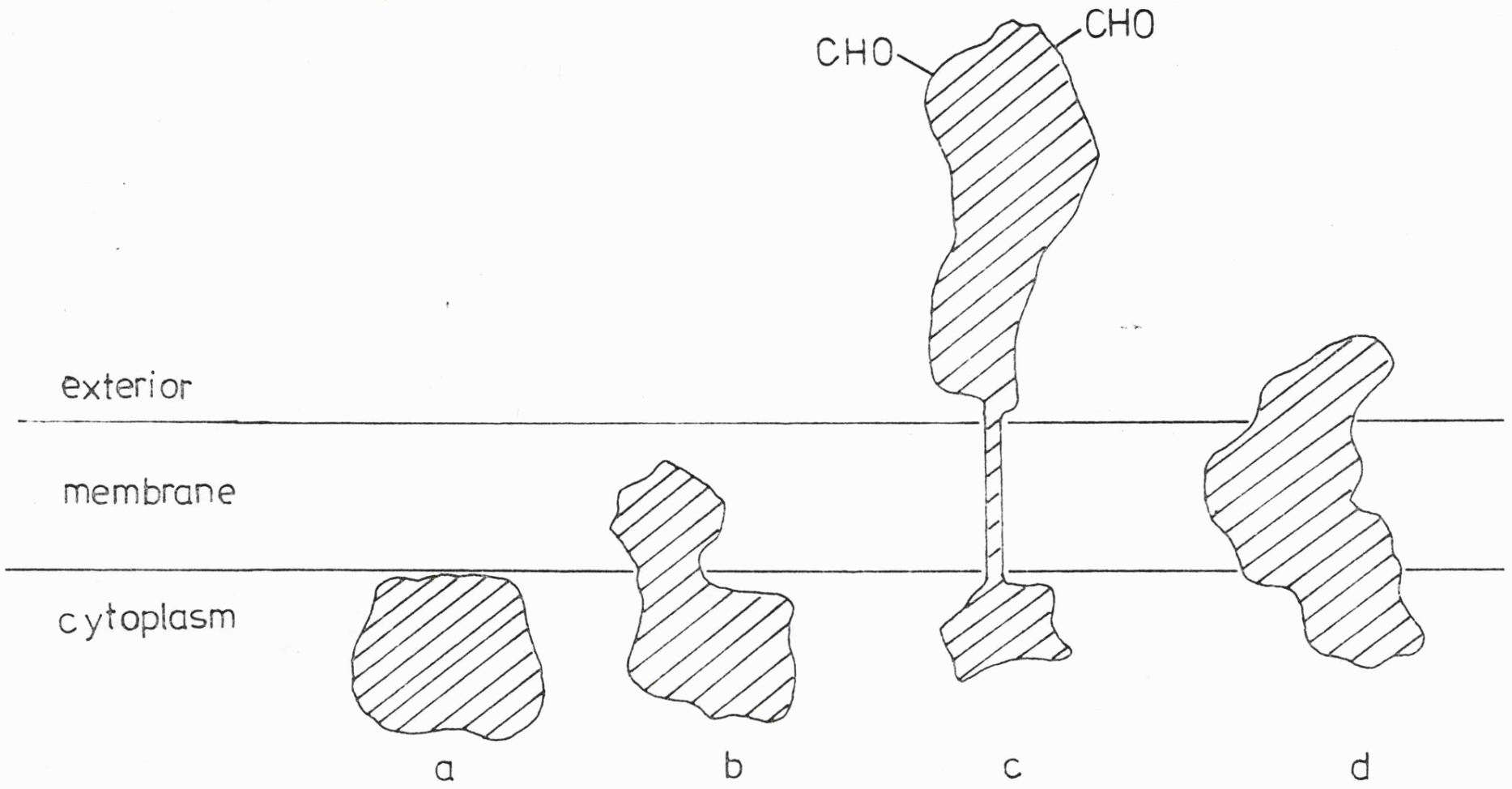
(a) A peripheral membrane protein is depicted interacting with the polar surface groups of the phospholipid bilayer. Peripheral proteins may also interact directly with integral membrane proteins.

(b) An integral endoprotein, i.e. a protein which interacts with the hydrophobic phase of the membrane, but which does not completely penetrate the bilayer.

(c) An integral ectoprotein, i.e. a protein which interacts with the hydrophobic phase of the bilayer, and which has a polar domain at the external surface of the membrane. The intramembrane domain of the protein is a single section of the polypeptide chain.

(d) An integral protein which has a globular structure embedded within the hydrophobic phase.

Examples of each class are discussed in the text.


CHO
CHO
exterior
membrane
cytoplasm
a
b
c
d

contrast, our knowledge of the structures of integral membrane proteins is very limited, since these proteins are typically insoluble, and do not therefore lend themselves readily to standard analytical techniques.

As pointed out by Singer (1971), the same thermodynamic laws must govern the folding of both soluble and membrane proteins. In aqueous solution the folding of a polypeptide maximizes the capacity for hydrogen bonding between groups exposed on the surface of the folded molecule and the surrounding solvent molecules. Other hydrogen bonds between the carbonyl and amino groups of the peptide backbone, are formed internally in α and β structures; hydrophobic groups are, in general, forced to the centre of the folded structure where they can form van der Waals contacts. It is the unfavourable decrease in solvent entropy arising if a hydrophobic group is in contact with the surrounding water which drives this folding process. The essential point is that folding of the protein molecule is determined by the interactions between the polypeptide and its surroundings, not by the polypeptide sequence alone. Clearly the forces operating in the hydrophobic phase of a phospholipid bilayer, or at the interface between the hydrophobic and hydrophilic phases at the membrane surfaces, will be very different from those in an entirely aqueous environment. It might be expected that membrane-associated domains of membrane proteins will have hydrophobic groups lying at the molecular surface where they can interact with the surrounding hydrocarbon phase, whilst charged and hydrophilic groups will be forced inwards.

Comparison of the amino acid composition of soluble and membrane proteins has revealed only a slight increase in overall hydrophobicity in the latter class (Capaldi and Vanderkooi, 1972). This is not too

surprising since many membrane proteins are amphiphilic molecules, interacting with both hydrophobic and hydrophilic environments. The best characterized integral membrane protein provides an example of amphiphilic structure : this is glycophorin, a major integral glycoprotein of the erythrocyte membrane. Glycophorin is accessible to various treatments in intact cells, including labelling by FMMP. When the tryptic peptides of FMMP-labelled protein from intact cells and from ghost membranes are compared it is found that two additional peptides are labelled in the latter case (Bretscher, 1971), indicating that the glycophorin molecule has regions exposed at both membrane surfaces, and is, therefore, a transmembrane protein. The entire primary sequence of the 131 amino acid residues of the protein has been determined (Tomita and Marchesi, 1976), and it is clear that the externally exposed, glycosylated, region of the protein is the amino-terminal part of the polypeptide chain. An internal sequence of the protein consists of 23 nonpolar residues, many of which are hydrophobic; this segment of the protein was originally isolated as an insoluble tryptic peptide (Segrest et al., 1973) and shown to have a hydrophobicity comparable to similar internal sequences in membrane-associated coliphage proteins, and to the antibiotic ionophore gramicidin A (Segrest and Feldmann, 1974). These workers have also pointed out that this length of polypeptide chain is sufficient to span a phospholipid bilayer if the chain is folded in α-helical conformation, although there is no evidence that it does exist in this form in the membrane. On the basis of all the above evidence the structure proposed for glycophorin is of two hydrophilic domains separated by a hydrophobic domain which anchors the molecule in the membrane (Marchesi et al., 1976; see Fig. 1.2).

A membrane protein which occurs under certain conditions in the cytoplasmic membrane of E.coli is thought to have a structure basically similar to that of erythrocyte glycophorin. This is the major coat protein of a group of closely-related filamentous phages M13, f1 and fd. The protein, which is present in abundance in the cytoplasmic membrane of phage-infected cells, has a molecular weight of about 5000 daltons and consists of a known sequence of 50 amino acids (Ashbeck et al., 1969; Nakashima and Konigsberg, 1974). Physical studies of the virus have led to a model of virus structure (Marvin and Wachtel, 1975) in which the DNA at the centre of the viral filament is surrounded by mainly α-helical coat proteins arranged in overlapping layers of molecules, like roof tiles. In this structure the basic carboxy-terminal region of the protein interacts with the DNA while the amino-terminal region is exposed at the surface of the virion. Antibodies raised against the virion are found to be directed against the amino-terminal region of the coat protein and have been used to demonstrate that this part of the polypeptide is exposed at the exterior of the cytoplasmic membrane of infected cells (Wickner, 1975; Wickner, 1976). The coat protein contains a sequence of 19 non-polar, and predominantly hydrophobic, residues (Segrest and Feldmann, 1974). This segment of the protein is thought to span the membrane, with the basic carboxy-terminal region located at the cytoplasmic face of the membrane where it can interact with viral DNA during virion formation (Smilowitz, 1974). Taken altogether this evidence strongly suggests that the viral coat protein is a transmembrane molecule with the same type of linear domain structure as glycophorin (see Fig. 1.2).

Another type of amphiphilic protein structure is exemplified by cytochrome b5, a component of the liver microsomal electron transport

chain (Strittmatter et al., 1974). The cytochrome, assayed spectroscopically, may be isolated from microsomal membranes in two ways : treatment with trypsin releases a globular haemoprotein of molecular weight 11.7K (Omura and Takesue, 1970), whereas extraction with detergent releases a form of the protein of molecular weight 16.7K carrying an additional carboxy-terminal sequence of 40, predominantly hydrophobic, amino acids (Spatz and Strittmatter, 1971). The detergent-solubilized form is thought to be the complete cytochrome b5 molecule. The structure proposed by Spatz and Strittmatter for the membrane-associated molecule is of a hydrophobic carboxy-terminal domain inserted into the membrane bilayer, and a globular hydrophilic domain which protrudes into the surroundings (see Fig. 1.2). When the detergent-solubilized form is freed of detergent it aggregates to form soluble octamers, presumably by interaction of the hydrophobic domains. This octameric protein can be added back to membranes to reconstitute functional cytochrome activity in a short electron transport chain (Spatz and Strittmatter, 1971), and has also been used to study interactions of the protein with detergent micelles (Robinson and Tanford, 1975).

It has been proposed by Rothman and Lenard (1977), that membrane proteins like glycophorin and M13 coat protein which have extracytoplasmic domains should be termed ectoproteins, whereas proteins with no such domain should be termed endoproteins (summarized in Fig. 1.2). Cytochrome b5 may be tentatively assigned to the latter class although there is no evidence that part of the hydrophobic domain does not completely penetrate the membrane. The basic difference between the structures of endoproteins and ectoproteins has been suggested by Rothman and Lenard to reflect two different mechanisms of

biosynthesis and membrane insertion of integral membrane proteins, a point which will be discussed in detail in Section II.(c).

One other integral membrane protein whose structure is quite well characterised is the bacteriorhodopsin of the purple membrane of the bacterium Halobacterium halobium (reviewed by Henderson, 1977). This protein occurs in quasicrystalline patches in the bacterial membrane and has, therefore, been found to generate informative electron diffraction patterns (Henderson and Unwin, 1975). The protein, of molecular weight 26K, seems to consist of seven rod-like domains, all about 40 nm in length, which are within the membrane and approximately perpendicular to the plane of the bilayer. These domains are thought to be α-helices since it is known from spectroscopic measurements that the purple membrane is rich in α-structure. The protein has not been sequenced, but eventually the comparison of the sequence with the physical structure could reveal important general rules about membrane protein structure. It is, however, already clear from the physical studies that this protein, which forms a transmembrane light-driven proton pump, has a much more complex relationship with the phospholipid bilayer than the simple linear domain structure exemplified by glycophorin. This difference in relationship with the membrane bilayer probably arises from the fact that proteins such as bacteriorhodopsin and other permeases have a transmembrane function whereas for proteins like glycophorin the bilayer may serve merely as anchorage.

II. MOLECULAR ASPECTS OF MEMBRANE PROTEIN SYNTHESIS

How do membrane proteins acquire their final conformation in the membrane bilayer? This question cannot be answered merely by elucidating the structures of membrane proteins : a full answer will require an understanding of the various factors which determine how and why certain proteins become associated with membranes. In particular we might ask do these polypeptides, which are often insoluble when isolated from membranes, ever exist in a membrane-free form; how do the proteins fold in the phospholipid bilayer; how do the polar extracytoplasmic domains of ectoproteins cross the hydrophobic bilayer; and, in cells possessing several intracellular membrane systems, what determines which membrane is entered by a particular protein? Some aspects of these problems will be described in this Section.

II.(a) Secretory Proteins of Eukaryotes : The Signal Hypothesis

Before discussing in detail the problem of biosynthesis of membrane proteins, I will describe recent advances in our understanding of the biosynthesis of secreted proteins. This is relevant here since these proteins have a biosynthetic feature in common with membrane ectoproteins. Ectoproteins have a hydrophilic domain at the extracytoplasmic face of the membrane in which they are found; this domain must cross the hydrophobic bilayer from its site of synthesis in the cytoplasm. Secretory proteins are soluble proteins which are exported from a cell, and which must, therefore, cross a membrane : examples include pancreatic zymogens such as chymotrypsinogen; immunoglobulins; and peptide hormones such as insulin.

Eukaryotic cells which secrete proteins are seen in electron

micrographs to be rich in rough endoplasmic reticulum : an example of such a cell type is the pancreatic exocrine cell, active in the synthesis and export of pancreatic zymogens (reviewed by Palade, 1975). The endoplasmic reticulum (ER) of eukaryotic cells is an intracellular system of membrane-bounded tubules. Areas of the ER are found to be studded with ribosomes bound to the cytoplasmic face of the membrane - this is the rough ER; smooth regions of the ER are the sites of processes such as phosphatide and triacylglycerol synthesis, mixed-function oxygenation, and fatty acid desaturation. In a subcellular fractionation the ER is recovered in the form of rough and smooth membrane vesicles, or microsomes. Whilst most eukaryotic cell types possess some rough ER, specialised secretory cells like the pancreatic exocrine cell are literally full of it. This correlation between secretory activity and the presence of rough ER gave rise to the idea that the ribosomes of the rough ER are involved in synthesis of secreted proteins, and this received experimental support when Siekevitz and Palade (1960) showed that rough microsomes isolated from pancreatic exocrine cells were active in synthesis of chymotrypsinogen. Confirmation that this occurs *in vivo* came from an autoradiographic study by Caro and Palade (1964), which demonstrated that pulse-labelled proteins are synthesised in the rough ER of the exocrine cell. This study also demonstrated the pathway by which these proteins reach the cell exterior, and this is summarised in Figure 1.3(a). Thus, newly synthesised proteins were seen to enter cytoplasmic, membrane-bounded vesicles formed by budding of the ER membrane in a structure called the Golgi complex; these vesicles represent a storage form of the secreted protein, which eventually leaves the cell when the vesicle membrane fuses with the plasma membrane.

Figure 1.3: THE ROLE OF MEMBRANE-BOUND POLYSOMES IN EUKARYOTES AND PROKARYOTES

N.B. The existence of amino-terminal signal sequences is not represented in this figure.

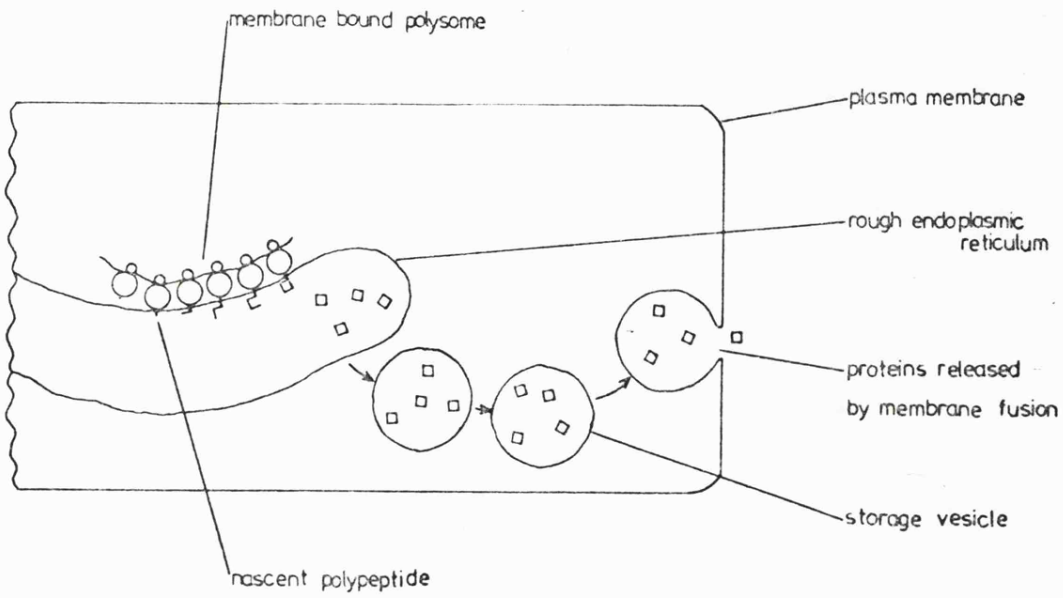
(a) Protein secretion in eukaryotes

Note that once the protein is extruded into the cisterna of the endoplasmic reticulum it is topologically extra-cytoplasmic, and is thought to leave the cell without crossing any other membrane.

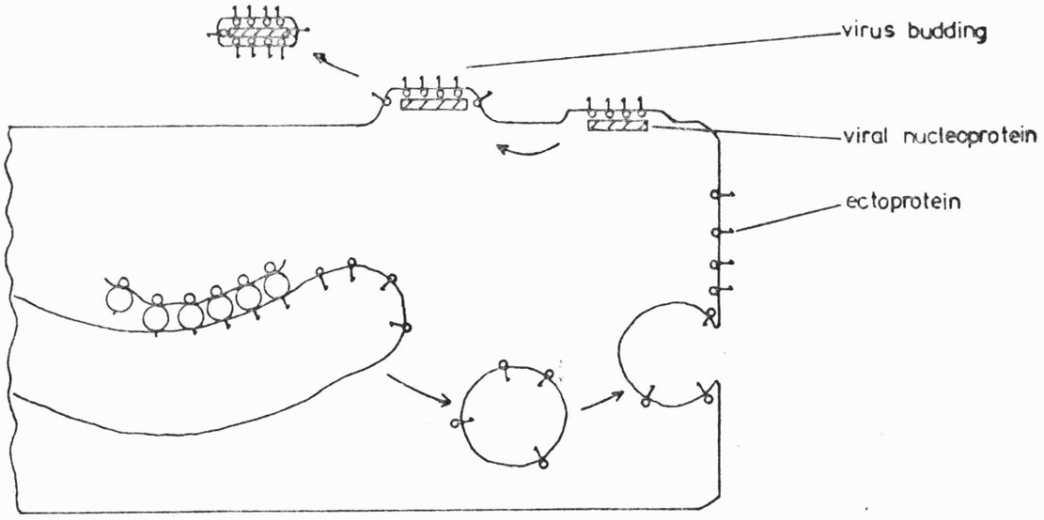
(b) Ectoprotein synthesis in eukaryotes

The diagram depicts the synthesis of integral ectoproteins in the rough endoplasmic reticulum. Note that the domain of the protein which is extruded into the cisterna is extra-cytoplasmic, and that it can reach the cell surface without crossing any other membrane. Note also that this topology is maintained during the budding of a virus such as vesicular stomatitis virus.

a

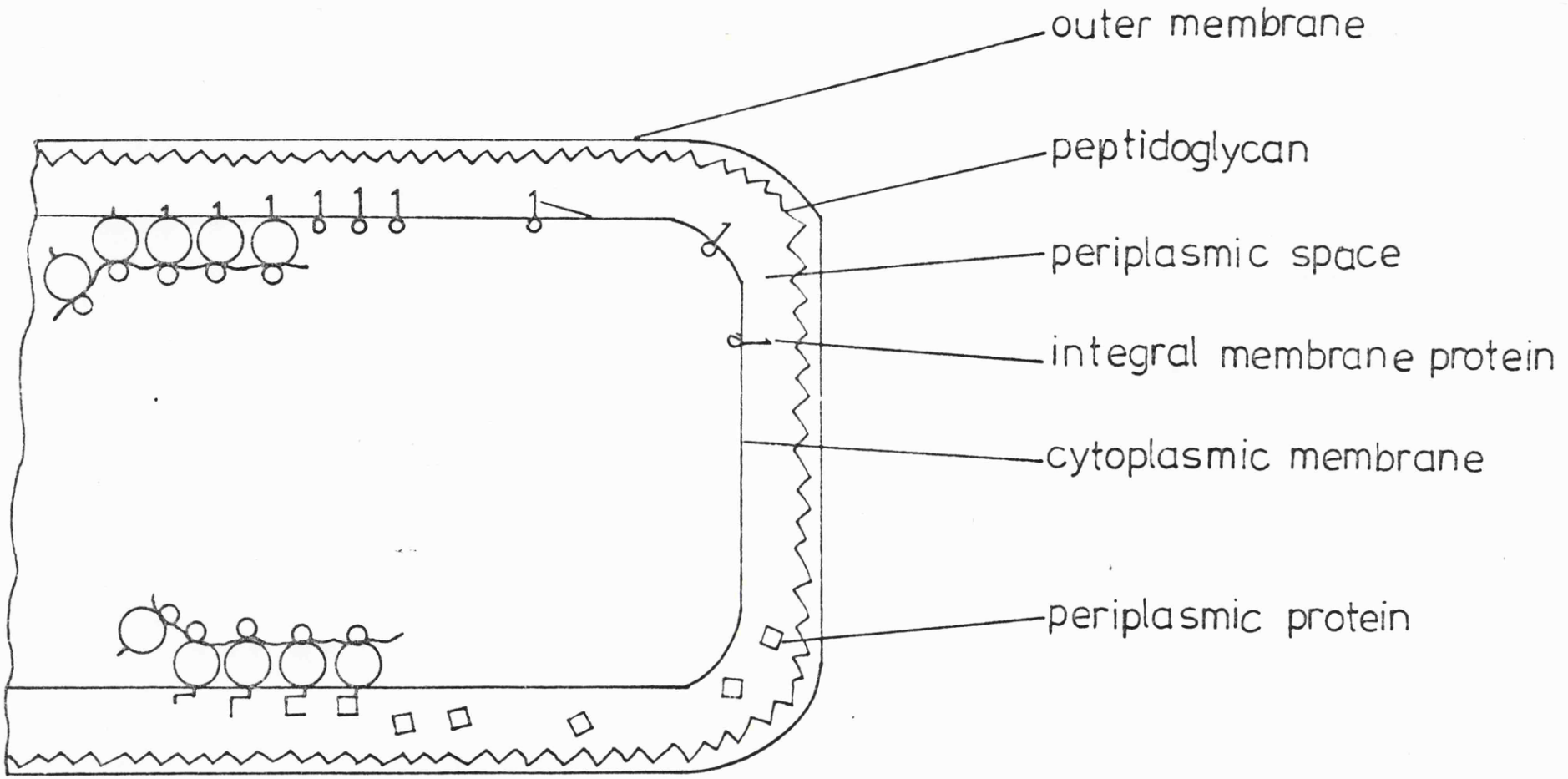

membrane bound polysome
plasma membrane
rough endoplasmic reticulum
proteins released by membrane fusion
storage vesicle
nascent polypeptide

b


virus budding
viral nucleoprotein
ectoprotein

(c) Synthesis of periplasmic and membrane proteins in E.coli

No attempt has been made to represent the synthesis of outer membrane proteins.


outer membrane
peptidoglycan
periplasmic space
integral membrane protein
cytoplasmic membrane
periplasmic protein

The nature of the membrane-ribosome association in the rough ER was probed by Sabatini *et al.* (1966) who found that dissociation of the ribosomes of rough microsomes by EDTA left the large ribosomal subunit attached to the membrane, and concluded that it is this subunit which interacts with the membrane *in vivo*. The demonstration by Sabatini and Blobel (1970) that growing polypeptide chains of membrane-bound ribosomes are completely protected from proteolytic attack in microsomal preparations led to the idea that the growing polypeptide chain is extruded directly across the membrane into the cisternal space of the rough ER. As shown in Figure 1.3(a), a protein in the cisternal space is already, topologically, outside the cell.

Two general hypotheses can be formulated to explain the existence of membrane-bound ribosomes : either the membrane-bound ribosome is a permanent structure which interacts only with those mRNA molecules encoding secreted proteins; or free ribosomes already translating such messengers are then directed to the membrane in some way. The work of Blobel and Dobberstein (1975) suggested that it is a special property of the nascent amino-terminal section of a secreted protein which causes membrane association of polysomes. These workers purified mRNA from a murine myeloma, the cells of which secrete immunoglobulins, and found that this messenger directed the synthesis *in vitro* of a polypeptide larger than authentic immunoglobulin light chain by 2-3K. Membrane-bound polysomes from the same cells produced authentic light chains only, but when such polysomes were detached from the membrane using a detergent and then allowed to complete translation, the products included both normal and heavy forms of the immunoglobulin light chain. The authors concluded that the immunoglobulin is synthesised as a precursor or preprotein, which is proteolytically processed during

translation and extrusion of the polypeptide chain, and proposed the signal hypothesis to explain the various phenomena associated with protein secretion. Briefly, this hypothesis states that mRNAs encoding secreted proteins contain, immediately after the initiation signal, a sequence which encodes a 'signal' sequence of amino acids. The signal sequence causes attachment of the nascent polypeptide-ribosome complex to the membrane and promotes the formation of a transmembrane tunnel by pre-existing membrane proteins; this tunnel provides a polar channel through which the nascent secreted protein is extruded. Since mature secreted proteins do not have a common amino-terminal sequence, and in the light of the existence of a preprotein form of immunoglobulin light chain, it was further proposed that the amino-terminal signal sequence must be removed during translation and extrusion across the membrane.

Since it was originally proposed the signal hypothesis has received considerable experimental support, in that it has been shown that many secreted proteins are synthesised as preproteins *in vitro* in the absence of membranes. Some examples are: immunoglobulin chains (Schechter and Burstein, 1976); pancreatic zymogens (Devillers-Thiery *et al.*, 1975); egg white lysozyme (Palmiter *et al.*, 1977); prolactin (Maurer *et al.*, 1977); serum albumin (Strauss *et al.*, 1977); growth hormone (Sussman *et al.*, 1976; Lingappa *et al.*, 1977); and insulin (Chan *et al.*, 1976). These studies have shown, in most cases, that the extra sequence is present as an amino-terminal extension to the authentic form of the protein. These extensions or signal sequences are all extremely hydrophobic in nature, with homologous, but not identical, signal sequences being present in preproteins synthesized by the same cell type (Devillers-Thiery *et al.*, 1975). This hydrophobicity of the signal sequences suggests that the nascent preprotein may interact directly

with the phospholipid bilayer rather than with the membrane receptor proteins envisaged in Blobel and Dobberstein's original hypothesis. The hydrophobicity also provides a reasonable explanation for the need for proteolytic processing, since a hydrophobic sequence might interfere with protein folding or might even lead to stable membrane association.

Blobel and co-workers have also demonstrated *in vitro* translocation and processing of secreted proteins. In an *in vitro* system driven by bovine pituitary RNA, the major products in the absence of membranes were preprotein forms of prolactin and growth hormone (Lingappa *et al.*, 1977). Synthesis of these proteins after the addition of microsomes prepared from canine pancreas led to the appearance of processed prolactin and growth hormone; these processed products were shown to be inaccessible to proteases unless detergent was added, indicating that they had been translocated into the microsomal vesicular spaces. No translocation or processing occurred if microsomes were added after completion of translation, suggesting a requirement for concurrent translation and translocation. In another study, by Shields and Blobel (1977), it was found that pre-proinsulin, encoded *in vitro* by mRNA from fish pancreatic β cells, was translocated and faithfully processed by microsomes from canine pancreas, demonstrating evolutionary conservation of the mechanisms involved.

The picture of protein secretion which has emerged from these studies is as follows: initiation of translation of mRNAs encoding secreted proteins occurs upon free ribosomes; the amino-terminal sequence of the primary product of translation protrudes from the large ribosomal subunit and, due at least partly to its hydrophobic nature, causes attachment of the translation complex to the membrane of the ER; and finally this primary association in some way leads to extrusion of the

growing polypeptide chain across the membrane and into the cisternal space of the ER. Proteolytic removal of the signal peptide is thought to occur duing translation and extrusion, presumably at the extra-cytoplasmic face of the membrane.

The other major postulate of the signal hypothesis is that specific membrane proteins of the ER are involved in the formation of a polar tunnel across the hydrophobic bilayer to facilitate the passage of polar polypeptide chains. Two sets of membrane proteins which seem to participate in protein secretion have been identified. Warren and Dobberstein (1978) have demonstrated that washing rough microsomes from dog pancreas with 0.5M KCl abolishes their activity in _in vitro_ translocation and processing of immunoglobulin light chain preprotein. This salt-extractable protein fraction can be added back to washed rough microsomes to reconstitute activity; however these proteins are not solely responsible for protein transfer since they do not confer such activity upon smooth microsomal membranes, phospholipid vesicles, or trypsin-treated rough microsomal membranes. Presumably these proteins are peripherally attached to the cytoplasmic face of the rough ER membrane, and Warren and Dobberstein have proposed that they are active in ribosome binding rather than protein transfer.

Kreibick _et al_. (1978a;b) have detected two proteins of molecular weights 65K and 63K in the rough ER of rat liver which are absent from the smooth ER. These proteins are not removed from the membrane by extremes of pH or ionic strength, and are, by these criteria, integral membrane proteins. Regions of both proteins are accessible to lacto-peroxidase-catalyzed iodination in intact microsomes, and further sites of iodination are revealed by detergent treatment, suggesting that the proteins have a transmembrane conformation. When rough microsomes

are solubilized by treatment with neutral detergents the two proteins are recovered in association with the ribosomes, tempting speculation that the proteins may be the postulated 'tunnel' proteins.

Although the signal hypothesis is rapidly attaining the status of a dogma it must be emphasised that the details of protein secretion are by no means clear. For example, the exact behaviour of the signal sequence itself is difficult to imagine - is it energetically reasonable that once the hydrophobic peptide is inserted into the bilayer it will traverse it and be discharged; and if it is not extruded how does the site of proteolytic cleavage encounter the signal peptidase which, according to the available evidence, lies at the extracytoplasmic face of the membrane? Another aspect of the process of translocation which is not understood is the source of the energy which drives extrusion. Two suggestions have been made : if the ribosome is firmly anchored to the membrane then energy available from peptide bond formation itself may be utilised; alternatively, folding of the protein at the extracytoplasmic face of the membrane may act to pull the chain through the membrane (Smith et al., 1977).

Finally, mention must be made of a glaring exception to the rule that all secreted proteins are synthesised as preproteins. McReynolds et al. (1978) have determined the sequence of the mRNA encoding the secreted protein, chicken ovalbumin, and have found that the initiation codon immediately precedes the first amino acid residue of authentic ovalbumin; furthermore, the amino-terminal sequence of the protein does not resemble the signal sequences of other secreted proteins, ruling out the possibility that ovalbumin is, in fact, an unprocessed pre-protein. The significance of this result is not clear; it is possible that ovalbumin secretion occurs by very special mechanisms, making it

the exception that proves the rule.

II.(b) Secretory Proteins of Prokaryotes

Bacteria produce proteins which are analogous to the secretory proteins of eukaryotes. In gram positive species these are extracellular enzymes such as the exopenicillinase of Bacillus licheniformis (reviewed by Lampen, 1978), whereas in gram negative species they are most commonly found in the periplasmic space between the cytoplasmic and outer membranes (reviewed by Heppel, 1971). The periplasmic proteins of E.coli include: β lactamases (Richmond and Sykes, 1973); binding proteins involved in sugar transport and chemoreception (reviewed by Parkinson, 1977); and various degradative enzymes such as alkaline phosphatase and several nucleotidases (Beacham et al., 1977).

Extracellular enzymes and periplasmic proteins must cross the cytoplasmic membrane of the cell to reach their final location. There is increasing evidence that the process of secretion of these bacterial proteins is similar in many respects to the process of extrusion of proteins across the membrane of the rough endoplasmic reticulum in eukaryotic cells. However, since bacteria such as E.coli possess no detectable intracellular membranes analogous to the endoplasmic reticulum, exported proteins are presumably extruded directly across the cytoplasmic membrane into the periplasm or extracellular space, as shown in Figure 1.3(c). In support of this hypothesis, membrane-bound polysomes have been identified in electron micrographs of membrane fractions from E.coli (Cancedda and Schlessinger, 1974), and these preparations of polysomes were shown to contain 80% of the nascent chains of alkaline phosphatase present in the cells. In a more detailed study Randall and Hardy (1977) have isolated and compared the properties of membrane-bound and free polysome fractions of E.coli.

After separation on sucrose equilibrium gradients the two polysome fractions were allowed to complete translation *in vitro*, in the absence of further initiations (read-out translation). The products of translation by the free polysome fraction were soluble, and included elongation factor Tu, which was identified immunologically. In contrast the membrane-bound polysomes produced both soluble proteins and membrane-bound proteins; this latter class of product probably represents integral membrane proteins since they were not removed from the membrane fraction by salt treatment or by sonication. The major soluble product of translation by the membrane-bound polysomes, identified by precipitation by specific antibody, was the maltose-binding protein, a periplasmic protein. Thus it seems possible that both exported proteins and integral membrane proteins of *E.coli* are synthesised by membrane-bound polysomes. There is also some more direct evidence that nascent chains of alkaline phosphatase are extruded across the membrane. Spheroplasts of *E.coli* (lysozyme-treated cells which lack the peptidoglycan layer and most of the outer membrane) were labelled with a membrane-impermeant reagent directed against primary amino groups (Smith *et al.*, 1977). A membrane-bound polysome fraction prepared from these spheroplasts was then allowed to read out existing rounds of translation, and the labelled products were found to include alkaline phosphatase. This finding is consistent with the idea that this periplasmic protein is synthesised on membrane-bound polysomes, with nascent chains spanning the cytoplasmic membrane with their amino termini exposed at the extracytoplasmic face of the membrane.

There are also several lines of evidence to suggest the existence of precursor forms of prokaryotic secreted proteins analogous to the preprotein forms of secretory proteins of eukaryotic cells. Inouye and

Beckwith (1977) isolated DNA from a ø80 transducing phage carrying the phoA gene, which encodes alkaline phosphatase, and used it to direct protein synthesis in an in vitro transcription-translation system. The phoA gene product was identified immunologically and found to have a molecular weight several thousand daltons greater than that of the alkaline phosphatase subunit found in vivo. This putative preprotein produced alkaline phosphatase activity upon prolonged incubation and was also found to be processed to the normal size by an E.coli outer membrane fraction. The finding that the processing activity is located in the outer membrane is somewhat surprising, since it indicates a requirement for both membranes of the E.coli cell envelope in the secretion process. The unprocessed preprotein adhered to a hydrophobic affinity column (decylagarose) suggesting a hydrophobic nature for the putative precursor. Although there is no evidence that an amino-terminal signal sequence is responsible for the various properties of the phoA gene product, all the data are consistent with the existence of such a sequence. The additional finding that the unprocessed polypeptide can give rise to active enzyme indicates that in this case at least, the presence of a signal peptide does not interfere with protein folding. Additional evidence for the existence of signal sequences in secreted proteins of prokaryotes has come from further analysis of the products of read-out translation by membrane-bound polysomes from E.coli. Randall et al. (1978) have reported that immunoprecipitated translation products corresponding to the periplasmic binding proteins for maltose and arabinose have molecular weights greater than those of the mature proteins by about 2K; it seems likely that these are preproteins, analagous to the preprotein forms of eukaryotic secreted proteins. The putative preprotein form of the arabinose binding

protein was also detected *in vivo*, in pulse-labelled cells. Taken at face value, this result suggests that the preprotein may exist transiently, in contrast to eukaryote preproteins which are presumed to be processed during translation. However, some form of read-out translation during isolation of membrane-bound polysomes, occurring in the absence of normal processing, could lead to such a detection of *in vivo* preprotein.

There is one other *E.coli* protein which should, in my opinion, be classed as a periplasmic protein, and whose biosynthesis has been investigated in detail. This is the peptidoglycan lipoprotein, whose structure and cellular location will be discussed in Section III. Inouye *et al.* (1977) have found that in a cell-free protein synthesising system the mRNA for the lipoprotein directs the synthesis of a pre-protein form of the lipoprotein. This preprotein has an amino-terminal extension consisting of a known sequence of 20 amino acids, of which some 60% are hydrophobic in nature. This is clearly very similar to the signal sequences found in eukaryote preproteins. A single amino acid change in the sequence of the lipoprotein in a mutant strain of *E.coli* has been shown to lead to a failure to process the preprotein (Lin *et al.*, 1978). Unlike the normal lipoprotein, which is processed, covalently modified by addition of lipid groups, and covalently attached to the peptidoglycan sacculus, the unprocessed mutant protein has no attached lipid and is not linked to the peptidoglycan (Wu *et al.*, 1977) but is found in association with both the cytoplasmic and outer membranes of the cell envelope. This membrane association may reflect a relatively non-specific association with the membrane bilayers, via the uncleaved signal region.

Turning to the extracellular enzymes of gram-positive bacteria, the exopenicillinase of Bacillus licheniformis is the best characterised example (reviewed by Lampen, 1978). This soluble protein is found in the culture supernatent; however a substantial part of the penicillinase activity remains attached to the cell membrane. This membrane enzyme has been found to possess an additional sequence of 25 amino acid residues at its amino terminus when compared to the soluble exoenzyme, and also has a phosphatidic acid residue covalently attached to its amino-terminal serine residue, making the membrane enzyme a phospholipoprotein. This phospholipoprotein is attached to the outer surface of the cell membrane, and is converted to the free exoenzyme by the action of a specific protease found in the cell envelope. The phospholipopeptide which is removed by proteolysis is not a signal sequence since it consists of mainly polar residues and also does not have an amino-terminal methionine residue, indicating that it is not the primary product of translation. Sarvas et al. (1978) have used DNA from a transducing phage carrying the penicillinase gene from B. licheniformis to direct the synthesis of this protein in vitro, and have found that the penicillinase product has a higher molecular weight than the phospholipoprotein form of the enzyme, caused by the presence of extra material at the amino terminus.

It seems likely, therefore, that the molecular details of protein secretion in gram positive bacteria are similar to those of secretion in gram negative bacteria. Furthermore, these studies of protein secretion in prokaryotes are revealing many similarities to the corresponding process in eukaryotic cells (i.e. extrusion of proteins into the cisterna of the endoplasmic reticulum).

II.(c) Biosynthesis of Integral Membrane Proteins

As described in Section I, membrane proteins may be divided into three general classes : peripheral membrane proteins; integral endoproteins; and integral ectoproteins. Because of their different structural relationships with the membrane bilayer, each of these three classes of membrane protein presents different problems for biosynthesis.

Peripheral membrane proteins are soluble proteins which are superficially associated with the membrane. There is no obvious reason why these proteins should not be synthesised upon free, cytoplasmic ribosomes; the completed and folded polypeptide could subsequently associate with specific membrane components. In the case of cytochrome c this role could be fulfilled by cytochrome oxidase, since cytochrome c can be chemically cross-linked to this integral membrane complex (Erecinska et al., 1975). Another example of a peripheral membrane protein interacting with integral membrane components is afforded by the nitrate reductase complex of E.coli. This is a respiratory enzyme complex found in the cytoplasmic membrane of cells growing anaerobically in the presence of nitrate. The complex, which can be solubilized from the membrane by Triton X-100, consists of several copies of each of two components A and B, and of cytochrome b. Components A and B are removed from the membrane by mild heat treatment at alkaline pH, indicating that they are peripherally associated with the membrane, whilst the cytochrome b component appears to be an integral membrane protein (Garland et al., 1975). Macgregor (1976) has shown that a hemA mutant of E.coli, which cannot synthesise haem groups unless supplied with δ-aminolaevulinic acid, fails to synthesise normal cytochrome b. Under these conditions the A and B subunits of nitrate reductase accumulate in the cytoplasm. When the cells are supplied with δ -aminolaevulinic

acid they begin to synthesise cytochrome b, whereupon pre-existing A and B subunits associate with the membrane to form normal, membrane-bound nitrate reductase. In this system completed soluble proteins are clearly capable of functional association with their membrane receptor, supporting the idea that such peripheral proteins are synthesised on cytoplasmic ribosomes.

Endoproteins have been defined as those integral membrane proteins which do not have an extracytoplasmic domain. In the case of cytochrome b_5 (an endoprotein discussed in Section I), we might imagine that the hydrophobic carboxy-terminal domain of the protein inserts into the hydrophobic membrane bilayer after the completion of translation, by a mechanism analogous to the <u>in vitro</u> association of the cytochrome with membranes (discussed in Section I). It seems, therefore, that endoproteins like peripheral membrane proteins, could be synthesised upon cytoplasmic polysomes. In support of this is the finding that two integral endoproteins of rabbit reticulocytes are synthesised <u>in vivo</u> upon cytoplasmic polysomes (Lodish, 1973; Lodish and Small, 1975). The problem of a membrane-specific insertion of endoproteins (for example, cytochrome b_5 is found only in the membrane of the smooth endoplasmic reticulum) could be solved in several ways : perhaps the endoprotein molecule is synthesised as a precursor which does not have affinity for membranes, but which is proteolytically activated by an enzyme in the target membrane.

In contrast, special mechanisms involving membrane-bound ribosomes have been proposed to explain the transmembrane insertion of the third class of membrane proteins, ectoproteins. Ectoproteins such as erythrocyte glycophorin have a hydrophilic amino-terminal domain at the extracytoplasmic face of the membrane bilayer. Since the problem of

transferring this hydrophilic domain across the hydrophobic bilayer is analogous to the problem of transferring a secreted protein across the membrane of the rough endoplasmic reticulum, Rothman and Lenard (1977) have proposed that the solution to the problem is also the same. The signal hypothesis has been extended by these authors to include transmembrane insertion of ectoproteins : it is proposed that these proteins are synthesised as preproteins with an amino-terminal signal sequence identical in nature and function to the signal sequences of the presecretory proteins discussed earlier. The hydrophilic amino-terminal domain is seen as being extruded through a proteinaceous tunnel; however, when the internal hydrophobic domain of the protein enters, it disrupts the tunnel, allowing stable association of the ectoprotein with the bilayer. In this strictly defined form the hypothesis can only explain the transmembrane insertion of proteins like glycophorin which have a simple linear domain structure of the type depicted in Figure 1.2.

The only eukaryote ectoprotein whose biosynthesis and transmembrane insertion have been investigated is the envelope glycoprotein of vesicular stomatitis virus (VSV). VSV is an RNA rhabdovirus which consists of an RNA-protein nucleocapsid enclosed by a bilayer membrane. This viral membrane arises from the host cell plasma membrane by a budding process, and contains, as its only protein species, many copies of a single virus-encoded glycoprotein which protrudes from the viral surface as observable spikes (reviewed by Lenard and Compans, 1974). VSV-infected Chinese hamster ovary cells synthesise two membrane proteins (one of which is the spike glycoprotein), and three soluble proteins. Morrison and Lodish (1975) have shown that in these infected cells glycoprotein-specific mRNA is found only in association with membrane-bound polysomes, and have suggested that the membrane glycoprotein is

synthesised upon membrane-bound ribosomes of the rough endoplasmic reticulum.

The topography of insertion of the glycoprotein into the membrane of the endoplasmic reticulum, its movement to the plasma membrane, and its assembly into virus is shown in Figure 1.3(b). It can be seen that the glycosylated spike of the protein corresponds to the domain which must cross the endoplasmic reticular membrane during transmembrane insertion. In support of this model is the finding that the glycoprotein is present as an asymmetric transmembrane protein in the rough endoplasmic reticulum of infected cells, with most of the polypeptide in the bilayer or cisterna (unpublished data referred to in Katz et al., 1977). However, a small carboxy-terminal region of the protein is apparently exposed at the cytoplasmic face of the endoplasmic reticular membrane (presumably this was deduced from accessibility to protease action in rough microsomes). These data indicate that VSV glycoprotein has the same sort of linear domain structure as erythrocyte glycophorin.

Purified VSV glycoprotein mRNA has been used to direct the in vitro synthesis of the glycoprotein (Katz et al., 1977), which, in the absence of membranes, leads to the production of an unglycosylated form of the protein which has a greater electrophoretic mobility than the glycoprotein. In contrast, translation in the presence of canine pancreatic microsomes (stripped of endogenous polysomes) leads to the appearance of a partially glycosylated form of the protein which has also been detected in the endoplasmic reticulum in vivo. In addition, it was found that whilst the unglycosylated protein synthesised in the absence of membranes is completely degraded by trypsin, the glycosylated form synthesised in the presence of membranes is almost completely resistant to protease action. This resistance to proteolysis was lost

in the presence of detergents. Katz and co-workers concluded that the protein is inserted into the membrane *in vitro* in a configuration identical to that found *in vivo*; glycosylation in the presence of membranes is thought to occur as a result of this transmembrane insertion. A refined version of this cell-free system has been used to define the timing of insertion. Rothman and Lodish (1977) achieved a synchronous burst of synthesis of the protein by adding an inhibitor of initiation of translation a short time after the start of the experiment. Insertion of the protein into the membrane (assayed as appearance of the partially glycosylated form) was found to occur only if membranes were present before an early point in translation, approximately corresponding to the emergence of the 40th amino acid residue from the large ribosomal subunit. If membranes were added after this point, glycosylation (and, by inference, transmembrane insertion) did not occur. There seems to be a requirement for an interaction between the membrane and the nascent amino-terminal region of the protein, which cannot occur after a certain degree of folding of the nascent polypeptide has occurred. Because of the variations in electrophoretic mobility between the unglycosylated and glycosylated forms of the protein it was not possible to detect a preprotein form on the basis of molecular size. However, there is now evidence that such a preprotein does exist, with an amino-terminal signal sequence which is removed during formation of the mature glycoprotein (Lodish, personal communication). The details of the biosynthesis of the VSV glycoprotein seem to fit very closely to the predictions of the signal hypothesis for ectoprotein synthesis.

The importance of the amino-terminal sequence of a membrane protein of *E.coli* has been demonstrated by a very different approach. Silhavy *et al.*, (1976) have constructed strains of *E.coli* in which the amino-

terminal sequence of the malF protein is fused with the carboxy-terminal part of the lacZ protein, β-galactosidase. The product of the malF gene has not yet been identified, but it is probably a component of the maltose transport system in the cytoplasmic membrane. The product of the fused genes enabled the cells to utilise lactose as a carbon source, and was found to be a β-galactosidase associated with the cytoplasmic membrane. Mutation of the lacY gene, which encodes the lactose permease, prevented the fusion strain from growing on lactose. This evidence led the authors to conclude that the fusion protein is a β-galactosidase which is associated with the inner face of the cytoplasmic membrane, through the amino-terminal segment of the malF gene product. It is not known, however, how much of the fusion protein is specified by the malF gene nor whether the malF gene product is an endo- or ectoprotein.

Apart from the VSV glycoprotein, the only membrane ectoproteins whose synthesis and transmembrane insertion have been studied, occur in the cytoplasmic membrane of E.coli under certain conditions. These are the coat proteins of the closely-related filamentous phages M13, fd, and f1, whose structure was discussed in Section I. The mRNA encoding the fd coat protein was sequenced by Sugimoto et al. (1977), and from this sequence it was deduced that the primary product of translation should have an amino-terminal extension of 23 predominantly non-polar amino acids. This was confirmed for the coat protein of f1 when Chang et al. (1978) showed that it is synthesised in a preprotein form in a cell-free transcription-translation system programmed by viral DNA. The positions of several amino acid residues in the amino-terminal sequence of pre-coat protein were found to be consistent with the mRNA sequence. Chang et al. also found that the preprotein was

processed to mature coat protein when synthesis occurred in the presence of membrane vesicles (cytoplasmic face out) from E.coli; they inferred from this result that transmembrane insertion had occurred in the cell-free system, although no direct evidence for this was presented.

One major tenet of the signal hypothesis which has been upheld by experimental findings is that transmembrane insertion must occur during translation, and that it is this which leads to an asymmetric configuration for ectoproteins. An interesting exception to this has emerged from studies of the M13 coat protein. By diluting a cholate-solubilized solution of purified coat protein and phospholipids, Wickner (1976), prepared vesicles containing membrane-bound protein. The conformation of the coat protein in the membranes of these vesicles was probed using antibody directed against the amino terminus of the protein. It was found that when the vesicles were formed near the transition temperature of the phospholipids, the amino-terminal domain of the protein was located exclusively at the outer surface of the vesicles. It is not clear what significance this property of asymmetric self-assembly might have for in vivo membrane insertion. More recently Wickner et al. (1978) used M13 DNA to direct coat protein synthesis in a cell-free system in the absence of membranes, and the product was a form of the protein which contained an extra chymotryptic fragment, as would be expected for a preprotein. This preprotein was initially present in a soluble form which was slowly converted into a rapidly sedimenting form. The molecular size of the soluble preprotein indicated that it was actually present as a soluble multimeric aggregate, possibly complexed with DNA. The rapidly sedimenting product was presumed to arise from the association of the preprotein with residual

membranes present in the cell-free system. Membrane-association was greatly enhanced by the addition of E.coli membrane vesicles, and with equal efficiency by unilamellar phospholipid bilayer vesicles. These membranes were added 30 min after initiation of synthesis, when more than 50% of total preprotein synthesis had been completed : there was no requirement for concurrent translation and membrane association. This membrane-associated protein was only slightly accessible to added chymotrypsin, but this accessibility was greatly increased by addition of the detergent sarkosyl, presumably as a result of the disruption of the membrane permeability barrier. In agreement with this conclusion it was found that when the preprotein became associated with phospholipid vesicles preloaded with chymotrypsin, the preprotein was extensively degraded. It was inferred from these data that large regions of the preprotein of the phage coat protein had penetrated to the inner surface of the vesicle membrane. Wickner et al suggest that the soluble pre-coat protein has the property of asymmetric self-assembly into membrane bilayers, with no requirement for other membrane proteins. If it can be shown more conclusively that the pre-coat protein is capable of acquiring a transmembrane conformation in phospholipid bilayers, with polar residues crossing the bilayer, then the thermodynamic arguments for transmembrane tunnels will be considerably weakened. It is possible that once the signal sequence has caused an association between the nascent protein and the membrane, spontaneous folding processes occurring at the hydrophobic-hydrophilic interfaces of the membrane lead to the formation of a transmembrane conformation; after all, hydrophobic amino acid residues are"extruded" from the ribosome into the aqueous cytoplasm without a requirement for hydrophobic "tunnels". However, the evidence relating to M13 coat protein synthesis should not be pushed too far :

this protein is clearly not a typical membrane protein since it ultimately leaves the membrane during virus formation. Conversely the 'typical' transmembrane structure of ectoproteins like VSV glycoprotein cannot be assumed to be typical of all integral membrane proteins. As I pointed out in Section I, the truly typical transmembrane proteins, such as permeases, may be better exemplified by the bacteriorhodopsin of H.halobium, a protein which has a folded globular structure within the bilayer. Hypotheses which can satisfactorily explain the acquisition of membrane insertion of these proteins have yet to be formulated.

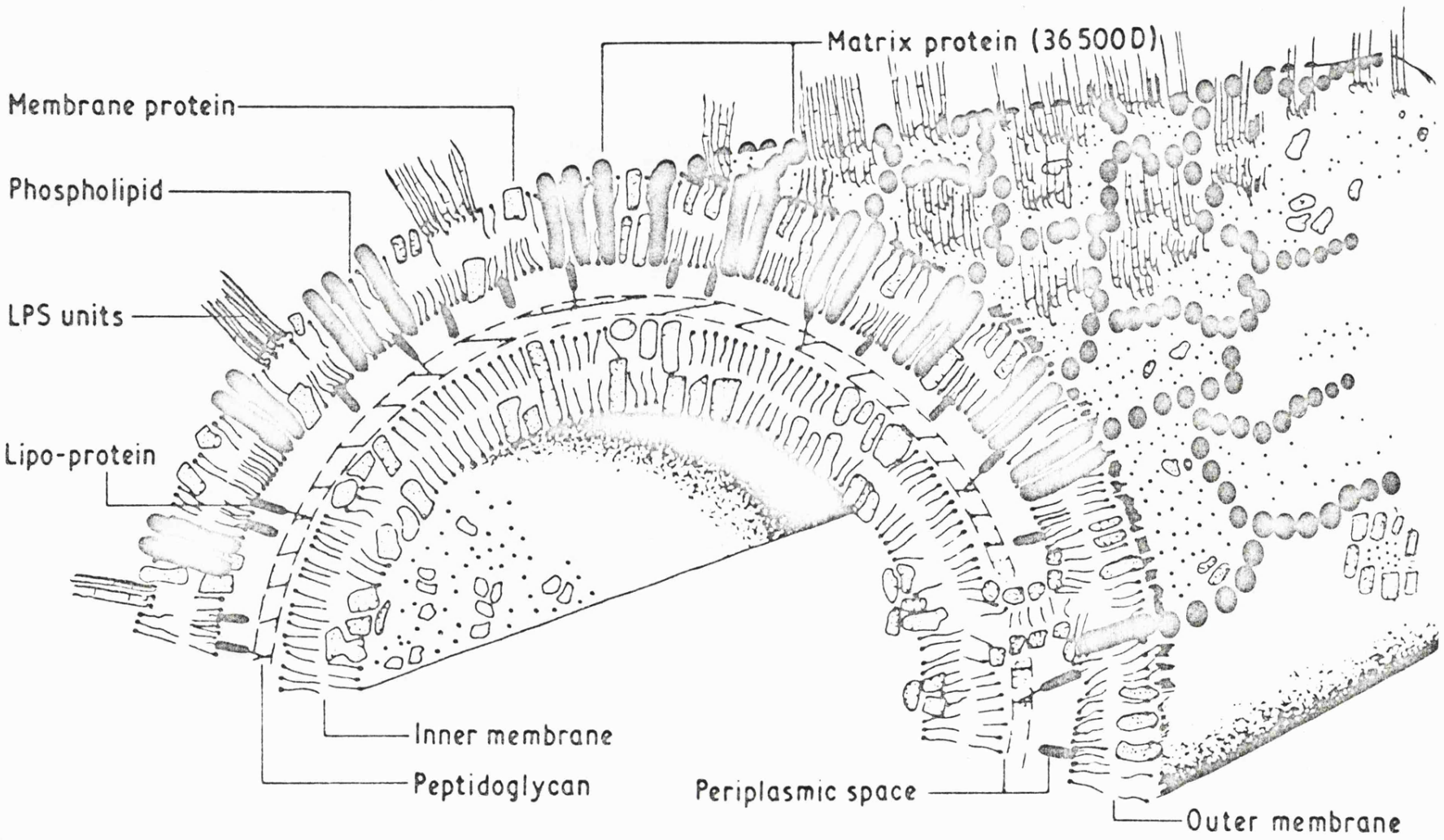
III. THE CELL ENVELOPE OF E.COLI

The cell envelope of gram negative bacteria such as E.coli is a complex, three-layered structure (Murray et al., 1965; de Petris, 1967) as depicted diagrammatically in Figure 1.4. A molecule leaving the cell would encounter these layers in the following order : firstly the inner or cytoplasmic membrane; next the peptidoglycan or murein layer; and lastly the outer membrane. In this Section I will briefly describe the structure of the cytoplasmic membrane and then consider the structures and interrelationships of the two outer layers in greater detail.

III.(a) The Inner or Cytoplasmic Membrane

The cytoplasmic membrane is composed of phospholipid and protein (Osborn et al., 1972a) and displays a trilaminar image in thin-section electron micrographs of whole cells (de Petris, 1967). It possesses an extensive freeze-fracture plane (Bayer, 1975; Iyer and Adshead, 1977) and is therefore, by these criteria, a typical bilayer membrane. SDS-PAGE analysis of the membrane reveals the presence of many polypeptide species; Ames and Nikaido (1976) have estimated that the majority of the 150 polypeptides resolved in whole envelopes by two-dimensional electrophoresis are from the cytoplasmic membrane. This multitude of polypeptides might be expected in view of the many functions which this membrane performs. Thus the cytoplasmic membrane contains the enzyme systems of oxidative phosphorylation (reviewed by Harold, 1977); of active transport (e.g. the lactose permease); and also those responsible for the synthesis and translocation of components of the outer layers of the envelope.

Figure 1.4: STRUCTURE OF THE E.COLI CELL ENVELOPE (DRAWN BY I.B. HOLLAND) SHOWING THE RELATIONSHIP BETWEEN THE THREE MAIN LAYERS


Matrix protein (36500D)
Membrane protein
Phospholipid
LPS units
Lipo-protein
Inner membrane
Peptidoglycan
Periplasmic space
Outer membrane

Because the cytoplasmic membrane is, by all criteria, a typical biological membrane it constitutes an almost ideal system for the study of structure and biosynthesis of membrane proteins. Its potential has not been exploited, however, because of the problem of separating it from the outer membrane, and because of its relative complexity. However, the techniques of biochemical genetics are beginning to provide insights into the nature of membrane proteins such as the ATPase (see, for example, Rosen et al., 1978), and future studies of the cytoplasmic membrane of E.coli will, no doubt, reveal basic principles governing biological membranes in general.

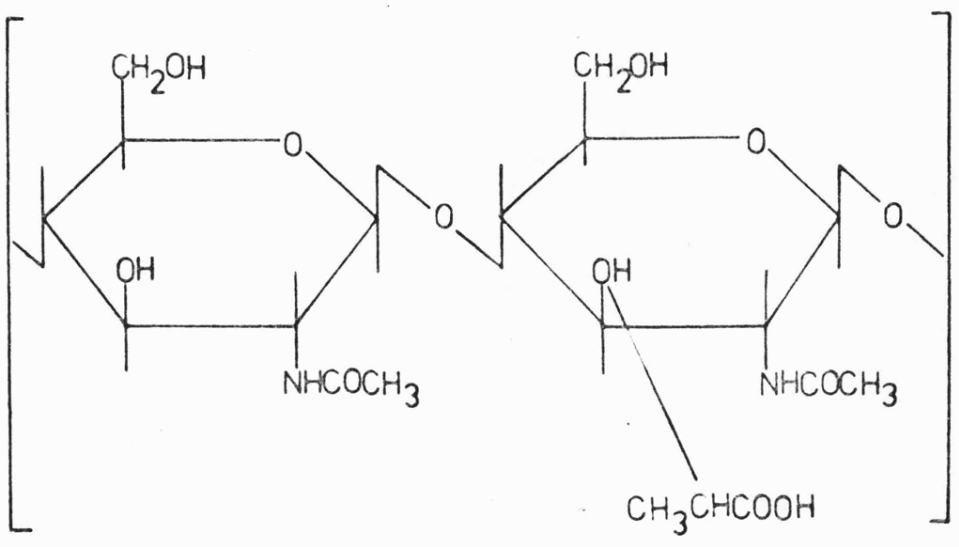
III.(b) The Peptidoglycan Layer

In E.coli the murein or peptidoglycan layer consists of a backbone of glycan chains composed of alternating residues of the amino sugars N-acetylglucosamine and N-acetylmuramic acid (Fig. 1.5). These amino sugars are joined in β(1-4) linkage like the glucose units in cellulose. Projecting from the N-acetylmuramic acid residues are tetrapeptides of sequence : L-alanine-D-glutamate-meso-diaminopimelate-D-alanine. These peptides are attached to the glycan chains by amide bonds between the carboxyl groups of the muramate residues and the amino-terminal amino group of the tetrapeptide. Adjacent glycan chains are cross-linked via the tetrapeptides by peptide bonds between the carboxyl group of the D-alanine residues and the free amino group of the meso-diaminopimelate residues; about half of the peptides participate in this cross-linking. The resultant network forms a layer which encloses the cell, conferring rigidity upon it and protecting it from hypotonic shock. The way in which the peptidoglycan is modelled to form a rod-shaped sacculus is not understood, although it is clear that the peptidoglycan does not have inherent shape-

Figure 1.5: THE STRUCTURE OF PEPTIDOGLYCAN

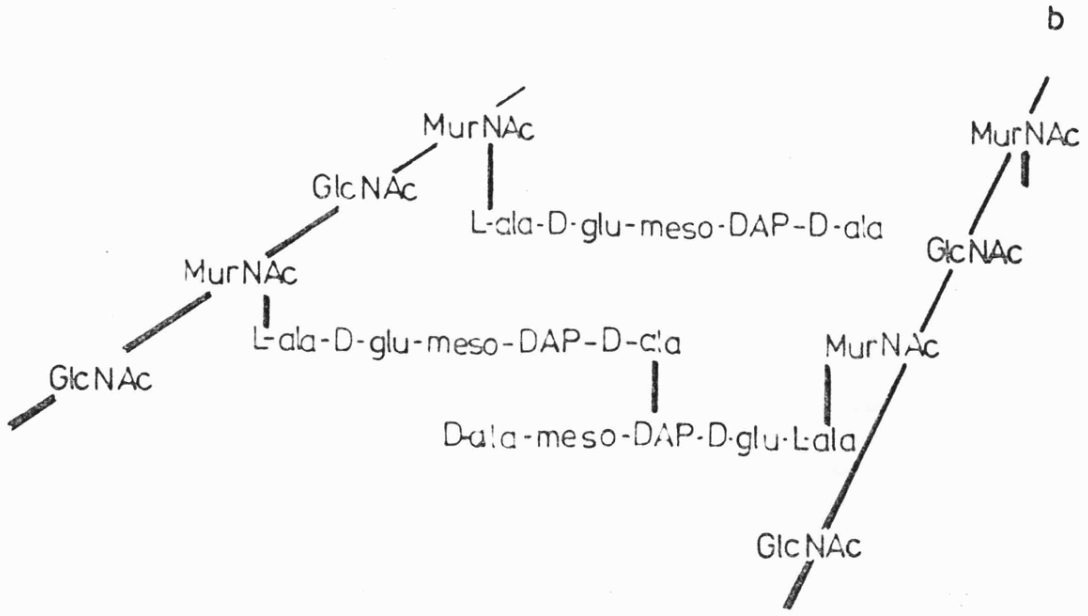
(a) The disaccharide which forms the repeating unit of the glycan chains of peptidoglycan.

(b) Peptidoglycan crosslinking : two parallel glycan chains are linked via peptide chains.



N-acetyl glucosamine (GlcNAc)

N-acetyl muramic acid (MurNAc)



determining properties. The possible role of certain cytoplasmic membrane proteins, the penicillin-binding proteins, in determining the growth and shape of the peptidoglycan sacculus will be discussed in Section IV.

The Peptidoglycan Lipoprotein

The structure of the peptidoglycan-associated lipoprotein has been reviewed by Braun (1975). The lipoprotein has a molecular weight of about 7K and is present in great abundance in the cell envelope of E.coli, S.typhimurium and other gram negative bacteria. The E.coli lipoprotein consists of a known sequence of 58 amino acids with a carboxy-terminal lysine and an amino-terminal cysteine, the latter being modified by attachment of a diacyl glyceryl residue and also of an acyl group joined in amide linkage to the α-amino group of the cysteine. Approximately one third of the lipoprotein molecules of the cell are bound to the peptidoglycan by covalent linkage between the ϵ-amino group of the carboxy-terminal lysine of the protein and the carboxyl group of the diaminopimelate residues of the peptidoglycan. About one tenth of the diaminopimelate residues participate in this binding. Both the peptidoglycan-bound and the peptidoglycan-free forms of the lipoprotein are thought to be associated with the outer membrane due to hydrophobic interactions discussed below.

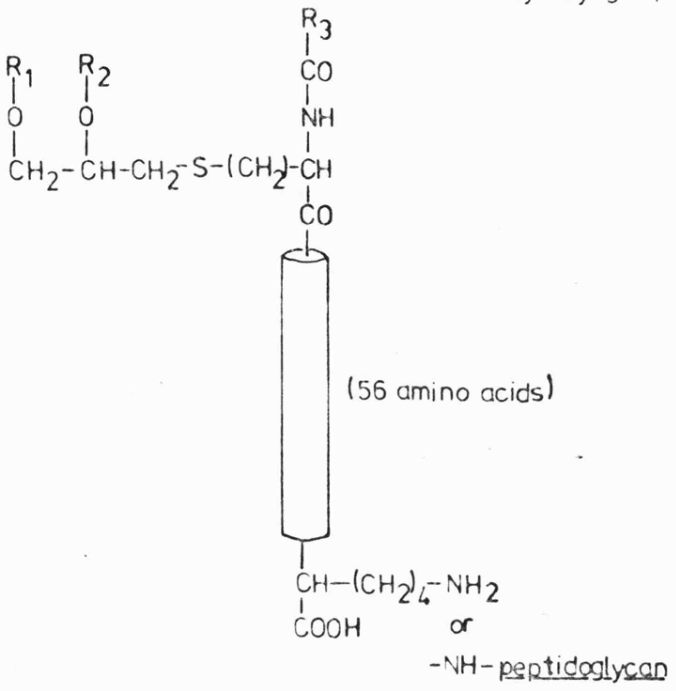
The sequence of the lipoprotein displays a regular pattern of hydrophobic residues such that if it is folded into an α-helix the resulting rod-shaped molecule would have a strip of hydrophobic residues running down one side. Since physical studies of purified lipoprotein have revealed that it does indeed have a very high content of α-helical structure it seems likely that this arrangement of hydrophobic residues is important to the structure and function of

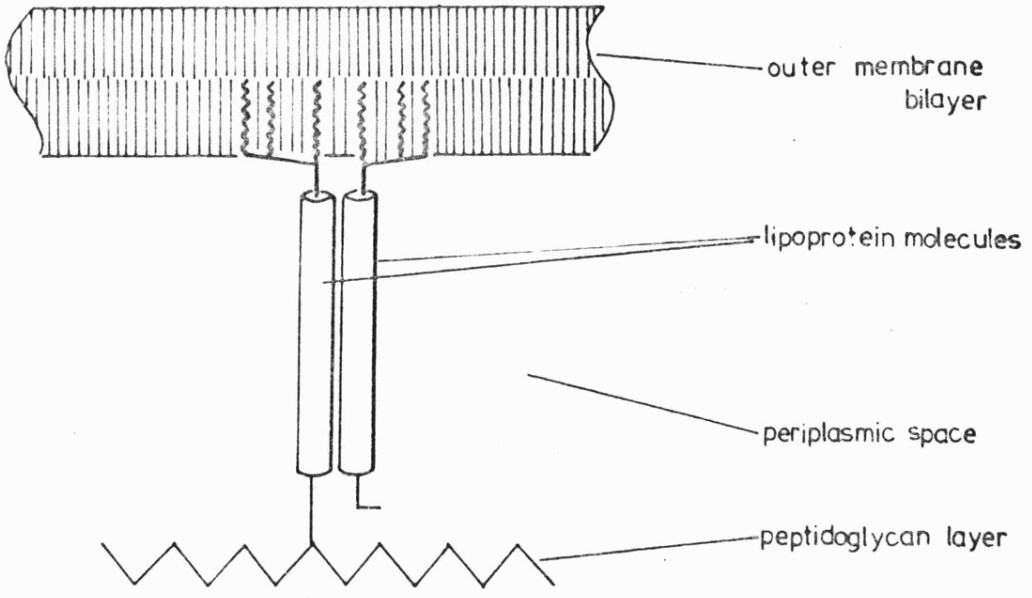
Figure 1.6: THE PEPTIDOGLYCAN LIPOPROTEIN

(a) The basic molecular structure of the lipoprotein. The bulk of the peptide chain is thought to form a single α-helix.

(b) Interactions of the lipoprotein with the cell envelope. The diagram depicts a dimer of the lipoprotein; no attempt has been made to represent the coiled-coil structure of this dimer, discussed in the text. One of the lipoprotein molecules is covalently associated with the peptidoglycan layer; both molecules undergo hydrophobic interactions with the outer membrane bilayer.

R_1, R_2, R_3 are
fatty acyl groups.





the protein. Based upon this structural evidence, and upon the association of the lipoprotein with the outer membrane, Inouye (1975) has proposed that six α-helical lipoprotein molecules come together in a hexagonal array to form a transmembrane pore through the outer membrane : the hydrophobic strips of the individual molecules are presumed to form an outer hydrophobic surface which interacts with the membrane bilayer while hydrophilic residues line the water-filled pore. There is no direct evidence, however, that the lipoprotein spans the bilayer in this way, and since the pore function in the outer membrane has now been ascribed to other outer membrane proteins it seems unlikely that the pore structure is the correct configuration for the lipoprotein.

Another configuration of the lipoprotein in the cell envelope has been proposed by Braun (1975) and championed by Maclachlan (1978). In this model the α-helical lipoprotein acts as a 'linker' between the outer membrane and the peptidoglycan, with rod-like molecules spanning the periplasm (Fig. 1.6). Maclachlan has pointed out the similarity between the arrangement of hydrophobic residues in the lipoprotein and that found in the muscle protein tropomyosin, which forms a two-stranded coiled-coil of symmetrically paired helices (Hodges *et al.*, 1972) stabilised by interactions between hydrophobic residues. It seems possible that two α-helical lipoprotein molecules also interact by means of their hydrophobic edges to form a compact coiled-coil structure, similar to that originally envisaged by Braun. Such a rod-like lipoprotein structure would have a completely hydrophilic surface and would therefore be perfectly suited to the periplasmic 'linker' function. Direct experimental evidence for the linking of the peptidoglycan to the outer membrane by the lipoprotein

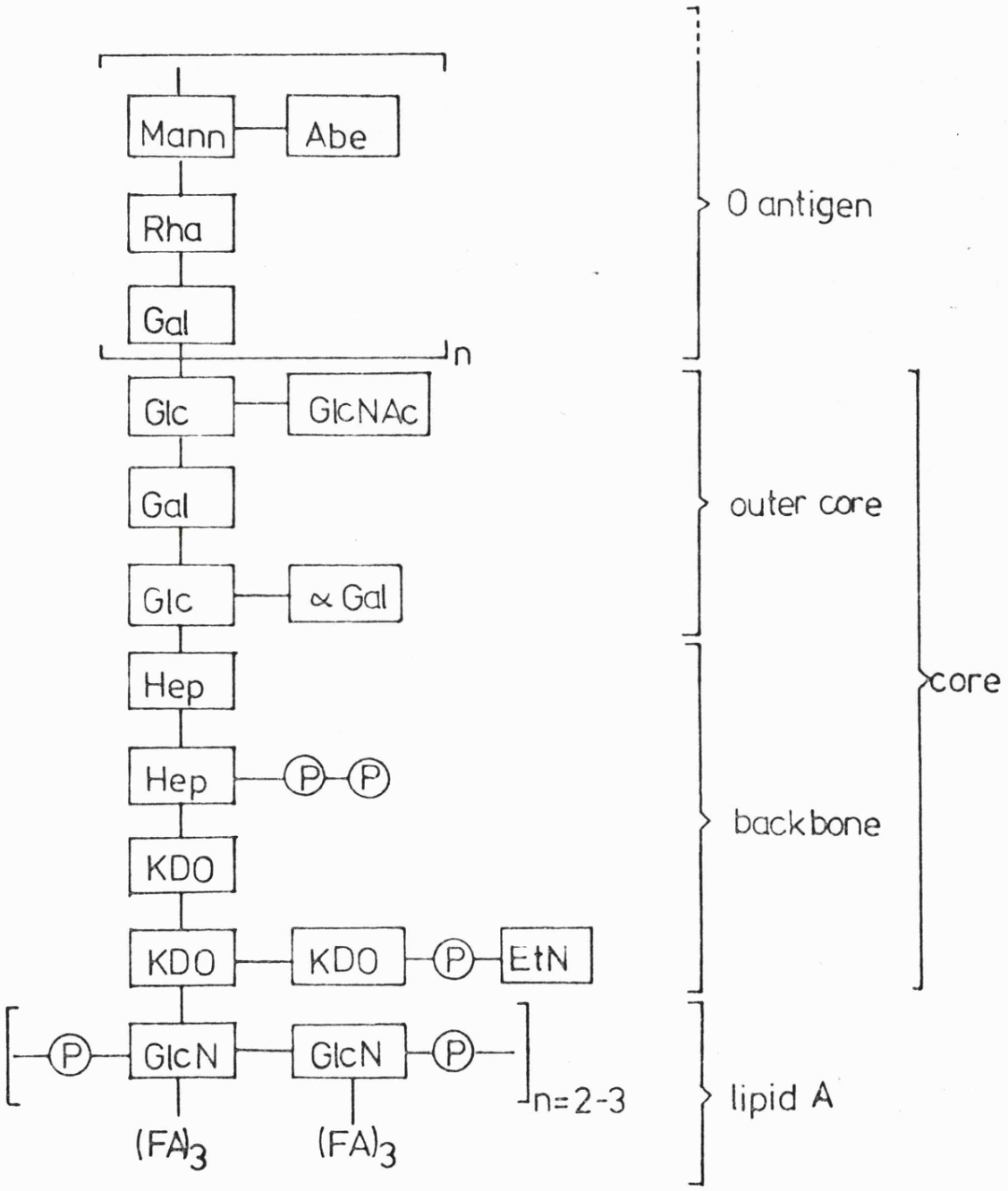
has come from careful characterisation of mutant strains of E.coli and S.typhimurium which have an altered lipoprotein. Yem and Wu (1978) have studied a mutant of E.coli producing an altered form of the lipoprotein which fails to bind to the peptidoglycan in vivo. This defect causes several phenotypic changes in the bacterium including the appearance of outer membrane blebs, a finding consistent with a role for the protein as a linker. A complementary study has been carried out by Fung et al. (1978) who found the same outer membrane blebs in the lkyD mutant of S.typhimurium which lacks the peptidoglycan-bound form of the lipoprotein, and in the lpo mutant of E.coli which produces no detectable lipoprotein at all. Sonntag et al. (1978) have found that the formation of outer membrane blebs is much more pronounced in mutant strains lacking both the lipoprotein and the major outer membrane protein d (see Section III.(d)); this suggests the possibility of some cooperation between these two proteins in linking the outer membrane to the peptidoglycan.

In summary, the weight of evidence favours the hypothesis that the many copies of the lipoprotein form a structural link between the two outer layers of the cell envelope. In this view the lipid chains of the lipoprotein are intercalated into the inner leaflet of the outer membrane while the remainder of the molecule spans the gap between the outer membrane and the peptidoglycan. A potential role of the lipoprotein linker in septation will be discussed in Section IV.

Figure 1.7: THE STRUCTURE OF LIPOPOLYSACCHARIDE

The diagram represents the LPS molecule of smooth strains of Salmonella typhimurium (Nikaido et al., 1977). The LPS molecule of E.coli K12 is thought to resemble this structure, except that the O antigen is absent.

Abe	:	abequose	Glc NAc	:	N-acetylglucosamine
Mann	:	mannose	KDO	:	3-deoxy-D-manno-octulosonic acid
Rha	:	rhamnose	EtN	:	ethanolamine
Gal	:	galactose	GlcN	:	glucosamine
Glc	:	glucose	P	:	phosphate
Hep	:	heptose	FA	:	fatty acid side chain


Mann
Abe
Rha
Gal
n
Glc
GlcNAc
Gal
Glc
∝ Gal
Hep
Hep
P
P
KDO
KDO
KDO
P
EtN
P
GlcN
GlcN
P
n=2-3
(FA)3
(FA)3
O antigen
outer core
backbone
core
lipid A

III.(d) Outer Membrane Structure and Function

In thin-section electron micrographs, both of whole cells (Murray et al., 1965; de Petris, 1967) and of membrane preparations (Osborn et al., 1972a), the outer membrane of gram negative bacteria displays the trilaminar image typical of biological membranes. The outer membrane also displays a fracture plane in freeze-fracture electron micrographs (Verkleij et al., 1977), although this seems to form less readily than the fracture plane in the cytoplasmic membrane (Bayer, 1975; Iyer and Adshead, 1977). By these criteria the outer membrane is a bilayer structure.

(i) Asymmetry in the Outer Membrane

The outer membrane may be separated from the underlying layers of the cell envelope by treating cells with EDTA and lysozyme to degrade the peptidoglycan layer, and then subjecting the residual membranes to ultracentrifugation in a sucrose density gradient. Under these conditions the outer membrane is separated from the cytoplasmic membrane by virtue of its greater density (Osborn et al., 1972a). The isolated outer membrane contains protein, phospholipid, and also all of the lipopolysaccharide (LPS) of the cell envelope. The LPS is an amphiphilic molecule consisting of a lipophilic moiety - lipid A - and a hydrophilic oligosaccharide chain. In Figure 1.7 the structures of the LPS of Salmonella typhimurium and of E.coli K12 are shown. The loss of the distal region, or O-antigen, of the LPS of S.typhimurium due to mutation gives rise to so-called rough strains. By comparing the structure of the E.coli K12 LPS with that of S.typhimurium it can be seen that E.coli K12 is in fact a rough strain, presumably a variant of some wild smooth strain of E.coli. The oligosaccharide of the LPS forms the major antigenic determinant

of the intact cell, a fact which points to the exposure of the LPS at the cell surface. Muhlradt and Golecki (1975) have shown that ferritin-conjugated antibodies directed against the oligosaccharides of the LPS interact only with the outer face of carefully isolated sheets of outer membrane, evidence which indicates that in vivo the LPS is asymmetrically distributed in the outer membrane, with lipid A groups associated with the outer leaflet of the membrane bilayer and with oligosaccharides extending outwards from the cell surface (see Fig. 1.4).

The asymmetric distribution of the LPS is thought to be responsible for some of the unusual permeability properties of the outer membrane. Phospholipid bilayer membranes typically allow small nonpolar molecules to diffuse freely across whilst preventing the passage of polar molecules. In contrast, the outer membrane is freely permeable to small polar molecules (discussed below in Section III.(d)(iii)) but is impermeable to nonpolar molecules. It is this barrier property of the outer membrane, discussed by Nikaido (1976), which confers upon gram negative bacteria their insensitivity to certain antibiotics (e.g. novobiocin), dyes (e.g. crystal violet) and bile salts. The observation of this low permeability of the intact outer membrane to nonpolar compounds has led to the idea that there is no phospholipid bilayer in the membrane which is accessible from the cell exterior. There are however two opposing views as to how this inaccessibility arises. Nikaido has argued that in fact there is no phospholipid bilayer at all in the outer membrane, but that the outer leaflet of the bilayer is formed exclusively by the lipid groups of the LPS, whose number approximately equals that of the lipid chains of the outer membrane phospholipids. In support of this proposal Kamio and

Nikaido (1976) have shown that in wild-type strains of S.typhimurium the polar head groups of the outer membrane phospholipids are inaccessible in intact cells to reaction with external phospholipase C or with cyanogen-bromide-activated-dextran. The same result is obtained using rough mutant strains which synthesise a truncated LPS with only 6 saccharide units, as compared to the 40 units of wild-type strains. In a deep rough mutant however, in which the LPS oligosaccharide is further reduced by one unit, Kamio and Nikaido detected two changes. Firstly the amount of protein in the outer membrane was markedly reduced and was replaced by phospholipid; and secondly the phospholipid head groups in the outer membrane were accessible to external phospholipase C. On the basis of this evidence Nikaido has concluded that in wild-type and rough strains there is virtually no phospholipid in the outer leaflet of the outer membrane, but that in deep rough strains the synthesis of outer membrane proteins is somehow reduced, with the missing protein being replaced by regions of phospholipid bilayer. This results in the appearance of accessible phospholipids in intact cells and also to an increased permeability of the outer membrane to nonpolar substances (Nikaido, 1976). Nikaido argues that the alternative explanation of these phenomena, that the oligosaccharides of the LPS form a steric barrier to phospholipase C and a polar barrier to nonpolar substances, is unlikely in view of the marked differences with respect to these properties of the outer membranes of rough and deep rough strains which occur with the removal of just one saccharide unit from the LPS.

The opposing viewpoint, that steric factors are responsible for these phenomena is argued by van Alphen, L., et al. (1977). These workers have shown that phospholipid head groups are also inaccessible

to phospholipases in intact cells of E.coli K12, but have obtained evidence that this is due to a masking effect of proteins and LPS. Thus van Alphen et al. used phospholipases to probe the surface of a wide range of mutant strains which either have shortened LPS oligosaccharides or lack specific major outer membrane proteins. Certain mutants which lack such proteins, but which have normal LPS were found to produce compensatory amounts of other proteins, and do not therefore have increased levels of outer membrane phospholipids. Nevertheless these same mutants have outer membrane phospholipids which are accessible to external phospholipases, consistent with the idea that in wild-type strains the phospholipids in the outer leaflet are indeed masked by proteins.

At present it is not possible to choose between this view of outer membrane structure and that proposed by Nikaido. It does seem fairly certain however that the outer membrane is a bilayer with much of its outer leaflet contributed by LPS lipid A groups, and that this asymmetric distribution of the LPS in some way accounts for the unusual impermeability of the membrane to nonpolar compounds.

(ii) The Outer Membrane is not a Fluid-Mosaic Structure

Reference was made in Section I of this Introduction to the fluid nature of biological membranes. Both the phospholipids and the protein of membranes are, in general, found to undergo rapid lateral diffusion. There is much evidence which suggests that in contrast, the lateral diffusion of the molecular components of the bacterial outer membrane is relatively restricted.

The properties of the phospholipids in the outer membrane have been probed using physical methods, with the cytoplasmic membrane serving for comparison. Overath et al. (1975) examined the order-

disorder transition of the phospholipids of isolated outer and cytoplasmic membranes of E.coli and concluded that only a fraction of the phospholipids of the outer membrane undergo the phase transition, indicating that much of the phospholipid already exists in an ordered state at physiological temperatures. This ordered state of the outer membrane phospholipids is also indicated by the data of Cheng et al. (1974) who measured the properties of a fluorescent probe in isolated membranes and found that the microviscosity of the outer membrane is twice that of the cytoplasmic membrane. It thus seems likely that the phospholipids do not form a truly fluid matrix in the outer membrane.

The mobility of the LPS of the outer membrane has been investigated by Muhlradt et al. (1974) who used a strain of S.typhimurium which lacks the enzyme galactose epimerase as a result of a mutation in the galE gene. This strain produces a truncated LPS oligosaccharide with distinctive antigenic properties. Addition of galactose to a growing culture of the mutant causes immediate production of new LPS molecules with a complete oligosaccharide, which have wild-type antigenic properties. In such an experiment addition of ferritin-labelled antibody directed against complete LPS allowed visualisation of clusters of newly-synthesised LPS inserted into the outer membrane at many discrete sites in the cell surface. By following the movement of the newly-synthesised LPS away from these insertion points it was possible for Muhlradt et al. to estimate a diffusion coefficient for the LPS in the outer membrane. This coefficient was several orders of magnitude lower than that expected for a constituent of a typical membrane, suggesting that the movement of LPS molecules is somehow restricted.

The mobility of the proteins of the outer membrane also seems to be restricted. Direct visualisation of this low mobility has come from a study of an E.coli mutant which produces a functional outer membrane receptor protein for phage T6 at low but not at high temperature. Begg and Donachie (1977) found that when the synthesis of the T6 receptor was turned off by a temperature shift the pre-existing receptors, detected by phage attachment in electron micrographs, tended to remain in the same region of the cell surface. During subsequent cell growth new regions of receptor-free outer membrane were formed at the cell poles; pre-existing receptors did not diffuse into these regions, again indicating a restriction upon mobility of molecules in the outer membrane.

Taken all together the evidence relating to the mobility of the molecular components of the outer membrane strongly suggests a degree of order much greater than that expected for a biological membrane. Several possible determinants of this order will now be considered. Since it is the LPS which is unique to the outer membrane, and which determines some of its unusual permeability properties, it seems reasonable that the LPS may also determine the lack of fluidity in the outer membrane. There are several lines of evidence which indicate that adjacent LPS molecules may indeed interact to form extended structures in the plane of the membrane. As shown in Figure 1.7 the core region of the LPS molecule contains several phosphate residues and it is thought that some or all lipid A moieties may be cross-linked by the formation of phosphodiester bonds. There are also a number of ionisable groups in the inner core region which may participate in the formation of salt bridges with divalent cations such as Ca^{2+} and Mg^{2+}; this would explain the finding that EDTA, which

chelates divalent cations, disrupts the outer membrane causing release of LPS and outer membrane proteins into the medium (Leive, 1967; Bayer and Leive, 1977). The structured nature of a layer of LPS has been demonstrated by the finding by Nikaido *et al.* (1977a) that the parts of the LPS lipid chains near the surface of an artificial, pure LPS bilayer do not undergo an order-disorder phase transition even at temperatures as high as 70°C. Furthermore, Rottem (1978) has incorporated lipid A molecules into artificial phospholipid bilayer membranes and demonstrated that this reduces the mobility of spin-labelled fatty acids incorporated into these membranes. This evidence suggests that since the outer leaflet of the outer membrane bilayer is composed predominantly of lipid A moieties of the LPS then it must possess a high degree of rigidity at physiological temperatures. Evidence for the involvement of the LPS oligosaccharide in LPS interactions has come from work by Rottem and Leive (1977) who incorporated a spin-labelled fatty acid probe into isolated membranes from *E.coli* J5, a *galE* mutant of *E.coli* 0111:B4 which is a smooth strain of *E.coli*. The mobility of the probe was lower in the outer membrane than in the cytoplasmic membrane. It will be recalled that *galE* mutants synthesise a truncated LPS oligosaccharide unless supplied with galactose. Thus Rottem and Leive were able to demonstrate that the mobility of the probe was reduced even further when complete LPS was present in the outer membrane. This finding suggests that the oligosaccharide chains of adjacent LPS molecules may interact to form a matrix of LPS possessing long range order. It seems unlikely however that this oligosaccharide interaction is of great importance in most laboratory strains of *E.coli* since, as explained above, these are rough mutants which lack the O-antigen, unlike *E.coli* 0111:B4. Thus *E.coli*

J5 grown in the absence of galactose produces LPS which resembles E.coli K12 LPS more closely than does that produced in the presence of galactose. In summary, the evidence presented here suggests that LPS interactions of several types may lead to the formation of a rather ordered LPS matrix in the outer leaflet of the outer membrane.

Although the lateral mobility of outer membrane proteins may be reduced by interactions with the LPS matrix there is also good evidence for some specific protein-protein interactions which may lead to the formation of another ordered matrix in the outer membrane. The major proteins of the outer membrane of E.coli are present in many copies : for example in E.coli B there are 10^5 molecules/cell of the major protein of molecular weight 36.5K (Rosenbusch, 1974). This protein is unusually resistant to SDS solubilisation below 70°C, so that when whole cells are lysed in a warm SDS solution a non-covalent, rod-shaped complex of the protein covering the peptidoglycan sacculus is recovered (Rosenbusch, 1974). When these structures are examined by electron diffraction it is found that the protein molecules form a regular hexagonal lattice. The association with peptidoglycan in sacculi is also characteristic of the major protein species of E.coli K12 and of other gram negative species (Lugtenberg et al., 1977), and indeed Lugtenberg has proposed that it reflects a strong non-covalent interaction in vivo between these proteins of the outer membrane and the underlying peptidoglycan layer. However, the findings that the same ordered protein structure can be isolated from cells in which the peptidoglycan layer has been extensively degraded by lysozyme (Steven et al., 1977), and that the protein forms ordered structures when reconstituted with LPS to form vesicles (Yamada and Mizushima, 1978), indicate that the formation of structure must be determined by protein-

protein interactions. The question remains as to whether the protein matrix reflects the *in vivo* organisation of the outer membrane or whether it arises by a crystallisation process during delipidisation of the membrane. If the hexagonal lattice structure does exist *in vivo* then it will clearly contribute to the lack of fluidity in the outer membrane.

Finally, in considering possible determinants of order in the outer membrane, mention must be made of the possible contribution of the peptidoglycan lipoprotein to the structure of the outer membrane. The structure of this protein was discussed in Section III.(c). In the present context it should be recalled that the lipid moiety of this protein is thought to be inserted into the inner leaflet of the outer membrane bilayer. Since there are about 2×10^5 lipoprotein molecules/cell bound covalently to the peptidoglycan (Braun, 1975), then there are an equal number of lipid moieties in the inner leaflet of the outer membrane which are essentially immobilised. Furthermore, if as has been suggested (Maclachlan, 1978) one free lipoprotein molecule is associated with each bound molecule this number of immobilised lipids may be doubled. Braun (1978) has estimated that up to one quarter of the fatty acid chains of the inner leaflet may be contributed by the lipoprotein. The lipoprotein may also interact with the major protein matrix of the outer membrane as suggested by the finding by Yamada and Mizushima (1978) that protein-peptidoglycan complexes (which closely resemble the *in vivo* matrix) are formed *in vitro* only if the peptidoglycan sacculi used in the reconstitution possess bound lipoprotein.

There are then four potential sources of order in the outer membrane. Firstly, the LPS molecules of the outer leaflet of the

membrane may interact to form an ordered matrix which contributes many lipids to the hydrophobic phase of the membrane; secondly, a large proportion of the lipid of the inner leaflet is likely to be immobilised since it is contributed by the peptidoglycan-bound lipoprotein; thirdly, a major protein component of the outer membrane forms an ordered lattice in the plane of the membrane; and lastly, some outer membrane proteins may interact with the underlying peptidoglycan-lipoprotein layer. The outer membrane may thus consist of several matrices, perhaps interwoven with one another, to produce a membrane which might best be described as a rigid mosaic. The ordered nature of the outer membrane, and its structural relationship with the peptidoglycan layer may also have implications for its biogenesis, as discussed in Sections IV and V, and in the body of this thesis.

(iii) Pores in the Outer Membrane

The Porin Hypothesis and the Role of the Major Proteins

In contrast to typical membranes including the bacterial cytoplasmic membrane, the outer membrane of gram negative bacteria such as E.coli is highly permeable to small polar molecules including sugars and amino acids. The cell wall of E.coli (i.e. the peptidoglycan plus the outer membrane) was originally characterised as a 'molecular sieve' by Payne and Gilvarg (1968) who investigated the ability of families of homologous oligopeptides to relieve amino acid auxotrophy. They found, for example, that a lysine auxotroph will grow when supplied with tetralysine but not pentalysine, and they concluded that the larger molecules were excluded from passive pores in the cell wall, making them inaccessible to periplasmic exopeptidases. In later studies Nakae and Nikaido (1975) measured the movement of sugars into the

periplasmic space of plasmolysed cells of S.typhimurium and found that degradation of the peptidoglycan layer did not increase the permeability measured in this assay. From this they concluded that the sieve property of the cell wall resides in the outer membrane. In support of this conclusion is the finding that preparations of the major proteins of the outer membrane of S.typhimurium (Nakae, 1976a) and of E.coli B (Nakae, 1976b) can be reconstituted with phospholipids and lipopolysaccharides to form membrane vesicles whose passive permeability properties resemble those of the outer membrane in vivo. Nakae has proposed that these major proteins form water-filled pores which penetrate the outer membrane to allow free diffusion of polar molecules of molecular weight less than 700 daltons, and has suggested that these proteins be named porins.

Before going on to discuss the evidence in support of the porin hypothesis in greater detail I will describe the putative porins, the major proteins of the outer membrane. High resolution SDS-PAGE analysis of the outer membrane of E.coli has revealed the presence of several abundant proteins whose molecular weights fall in the range 35-45K. There are various nomenclatures used in the literature for these proteins; that due to Lugtenburg et al. (1975) will be adopted here. In this nomenclature the four major protein species of E.coli K12 are designated proteins a, b, c and d in order of decreasing molecular weight. The outer membrane of E.coli B and B/r contains only two major species, of electrophoretic mobility identical to proteins b and d. In all K12 strains protein a is normally absent at 37°C, appearing only at higher temperatures or in certain mutants; it will not be considered further.

It will be recalled from Section III.(d)(ii) that certain major outer membrane proteins are recovered in association with the peptidoglycan sacculus when cells are lysed in warm solutions of SDS. Proteins with this property have been termed matrix proteins, after Rosenbusch (1974). Proteins b and c in E.coli K12 and protein b in E.coli B are matrix proteins, and analogous proteins have been found in the outer membranes of other strains of E.coli, of S.typhimurium, and of several other species of gram negative bacteria (Lugtenberg et al., 1977). The formation of an association between purified matrix proteins and isolated peptidoglycan sacculi has suggested to many that matrix proteins interact with the peptidoglycan in vivo, (Hasegawa et al. (1976); Yu and Mizushima, 1977). There is, however, no direct evidence for this and indeed when Endermann et al. (1978) added a cross-linking reagent to cell envelopes they found that only protein d, not the matrix proteins b and c, could be linked to the peptidoglycan. It is these major proteins, the matrix proteins b and c which are thought to be the major pore-forming molecules in the outer membrane. Both proteins act as phage receptors (Henning et al., 1977) and protein b is accessible to reaction with cyanogen-bromide-activated-dextran in whole cells of E.coli B, evidence which indicates that both proteins are exposed at the cell surface. Proteins with a pore function would be expected to be transmembrane proteins; at present however the apparent association with the peptidoglycan constitutes the only evidence that the matrix proteins are exposed at the inner face of the membrane.

Physiological studies of a mutant strain of E.coli B have complemented the reconstitution experiments of Nakae referred to earlier, and have provided sound evidence in support of the porin

hypothesis by demonstrating the importance of the matrix protein b *in vivo*. The mutant in question was isolated on the basis of its very slow growth in media containing low concentrations of glucose as sole carbon source, but it was subsequently found to have a pleiotropic defect which increased the apparent Michaelis constant for the uptake of many sugars, amino acids and anions (von Meyenburg, 1971); further characterisation of the mutant revealed that it lacks protein b (Bavoil *et al.*, 1977). In wild-type strains it is the cytoplasmic membrane permease which determines the Michaelis constant for overall transport, since the outer membrane allows free diffusion. In the mutant lacking the porin however, the permeation of the outer membrane becomes the rate-limiting step. In normal laboratory culture conditions this defect does not manifest itself since growth substrates are always present in excess. This explains the finding that strains lacking all major proteins have normal growth rates in standard media (Henning and Haller, 1975).

Other studies of outer membrane permeability have provided general support for the porin hypothesis. Beacham *et al.* (1977) characterised mutants of *E.coli* K12 in which the expression by intact cells of periplasmic enzymes such as nucleotidases is reduced. These so-called 'cryptic' mutants were found to lack certain porins (see also van Alphen, W., *et al.* (1978a)). This result is explained in terms of the failure of enzyme substrates to penetrate the outer membrane of the mutants and thus reach the enzymes located in the periplasm. Using the same technique Nikaido *et al.* (1977b) assayed periplasmic β-lactamase activity in intact cells of mutant strains of *S.typhimurium* lacking two major outer membrane proteins, and in this way demonstrated a deficiency in the passage of β-lactam antibiotics across

the outer membrane of the mutants. Finally, the importance of the outer membrane pores in allowing passage of ions into the cell is indicated by the finding by Luktenhaus (1977) that strains of E.coli B/r, selected as being resistant to copper, specifically lack outer membrane protein b.

The evidence presented above provides strong support for the theory that the outer membrane matrix proteins form pores; other studies are beginning to give clues to the molecular details of pore structure and function. It will be recalled that protein b of E.coli B/r forms a hexagonal lattice in peptidoglycan-protein sacculi. Steven et al. (1977) have obtained high resolution electron diffraction images from protein b lattices in a form free of peptidoglycan, and have described the unit cell of the lattice as having three-fold symmetry and as containing triple indentations, tentatively identified as pores through the protein layer. The structure of the pores has been further characterised by Schindler and Rosenbusch (1978) who incorporated protein b into planar lipid membranes and detected the formation of aqueous channels which allowed passage of ions and of small uncharged molecules. The diameter of the channels formed in these experiments was estimated to be 1 nm, but the data also indicated that the smallest pore-forming entity contained three of these 1 nm channels. Similar data have been presented by Benz et al. (1978). These data, taken together with the ultrastructural analysis of Steven et al. discussed above, suggest that the lattice consists of trimeric porin aggregates with each trimer forming three transmembrane channels or pores.

The Two Porins of E.coli K12

The finding that E.coli K12 possesses two matrix protein species, b and c, in contrast to the single species in E.coli B/r, raises the question of the relationship between these proteins and also suggests that there may be classes of pores with different permeability properties. In fact, the relationship between proteins b and c of E.coli K12 is only just beginning to become clear. Mutants lacking either one, or both, of the proteins have been selected on the basis of their resistance to colicins or phages. Mutants lacking only protein c are resistant to phages PA-2 (Diedrich et al., 1977), TuIb (Henning et al., 1977), and Me1 (Verhoef et al., 1977); these mutations all appear to map at a single locus, par, at 48 min. on the E.coli K12 genetic map. Some mutants tolerant to colicins A, E_2, E_3, K and L are found to lack protein b, and these mutations map at 21 min., at the tolF locus (Chai and Foulds, 1977). Another class of colicin tolerant strains lack both proteins and map at the ompB locus at 74 min; the porin-less mutant of E.coli B/r described earlier is mutated at a locus designated kmt lying at 74 min on the genetic map of E.coli B/r, which is presumably identical to the ompB locus in E.coli K12. Although it was suggested by several authors that proteins b and c might be modified versions of a single gene product, perhaps encoded by the ompB gene, recent evidence has made it increasingly likely that proteins b and c are the products of two separate genes. Ichihara and Mizushima (1978) have determined the amino acid composition, the cyanogen bromide peptide pattern, and the sequence of the first 12 amino-terminal residues of the two proteins, and have concluded that they differ significantly in all three respects. These sequence data

are particularly interesting : protein b has valine at position 3 and leucine at position 11 whereas protein c has isoleucine and phenylalanine respectively at these positions; the remaining residues are identical in the two proteins. The overall similarities of the two sequences make it unlikely that c is derived from b by proteolytic cleavage of an amino-terminal fragment, whilst the amino acid differences argue very strongly against the single gene hypothesis. Both substitutions could arise, however, from single base changes, suggesting that the two proteins have arisen from a single ancestral sequence. Lugtenberg et al. (1978) have analysed cyanogen bromide peptides and proteolytically-generated peptides of proteins b and c and have also concluded that the two proteins are substantially different. Although it now seems extremely unlikely that covalent modifications of a single polypeptide chain are responsible for the existence of proteins b and c, post-translational modifications do occur. Thus Diedrich and Schnaitman (1978) have detected α-amino-adipic acid -semialdhyde residues in both proteins. These modified lysine residues could allow cross-linking of porin molecules to one another, or to the peptidoglycan.

Some evidence that proteins b and c may form subsets of pores with different specificities has come from a study by van Alphen, W., et al. (1978a) who concluded that protein b is more efficient in promoting the passage of certain nucleoside monophosphates, whilst both proteins seem to have equal efficiencies in allowing passage of β-lactam antibiotics across the outer membrane. It is difficult to reconcile these data with the hypothesis that the proteins form passive diffusion pores.

Other Outer Membrane Porins

The discovery of the pore function of the two matrix proteins has led to the demonstration by Manning et al. (1977) of a pore function for the major outer membrane protein d which occurs in both E.coli K12 and E.coli B/r. This protein acts as the receptor for phages K3 (van Alphen, L., et al., 1977) and TuII* (Datta et al., 1977), and is strongly implicated in the early stages of F-pilus-mediated conjugation (Manning and Reeves, 1977). These receptor functions, together with the susceptibility of the protein to cross-linking to the peptidoglycan (Endermann et al., 1978) make it likely that the protein has a transmembrane configuration. Manning et al. (1977) carefully characterised ompA mutants which specifically lack protein d and found that they have a specific defect in the uptake of proline and glutamine. Although such specificity is again difficult to reconcile with a passive pore function it seems likely that protein d forms some sort of transmembrane channel. It also seems strange that proline and glutamine should be excluded from the pores determined by proteins b and c.

Some other outer membrane proteins also seem to possess porin-type activity, although these are not well understood. The first of these is the protein of molecular weight 25K which acts as receptor for phage T6. Hantke (1976) has shown that mutants lacking this protein are deficient in uptake of a range of nucleosides; the low specificity of the defect suggests a semi-specific pore function for the protein rather than a specific permease function. The second example of a possible porin is the phage λ receptor protein, the product of the malB gene. This protein is recovered in non-covalent

association with peptidoglycan sacculi, making it another matrix protein. Phage λ resistant strains of E.coli lack the protein and are found to have a large increase in the apparent Michaelis constant for maltose uptake; furthermore they are quite unable to take up higher maltodextrins (Szmelcman et al., 1976). Work by von Meyenburg and Nikaido (1977) indicates that the presence of the malB gene product in the outer membrane of porin-deficient strains of E.coli enhances the uptake of sugars such as glucose and lactose, but not of histidine or 6-aminopenicillanic acid. Spontaneous revertants of the kmt mutant of E.coli B/r were found by these workers to include strains producing large amounts of the malB gene product. It seems probable, therefore, that the protein forms pores through the outer membrane which allow the passage of sugars including the large maltotriose molecule. Szmelcman et al. (1976) have suggested that this inducible protein may have evolved specifically to allow utilisation of the mixture of linear oligosaccharides originating from hydrolysis of starch and glycogen in the gut. I would suggest that this porin has become incorporated into the inducible maltose-utilising system because its constitutive presence in the outer membrane would lead to a deleterious increase in permeability to other, undesirable, molecules; it is only in the presence of maltose and maltodextrins that the benefits of having the protein in the outer membrane outweigh the disadvantages.

Finally, mention must be made of recent evidence for silent porin genes in E.coli K12. Several groups have reported the presence of electrophoretically novel proteins in the outer membrane of revertants of porin-less strains (Henning et al., 1977; Foulds and Chai, 1978; van Alphen, W., et al., 1978a), and Pugsley and Schnaitman (1978) have shown that mutations in at least three genetic

loci distinct from those already known to be associated with the porins (par, tolF, ompB) can give rise to the appearance of new, immunologically distinct porins. Lugtenberg et al. (1978) have analysed peptide patterns of one of these proteins and found it to be completely different from either protein b or c. This evidence suggests that the porin system of E.coli may be both more complex and more vital to the cell than has been assumed.

In conclusion, there is overwhelming evidence in support of a porin function for the major proteins of the outer membrane. It seems that these proteins form a highly ordered lattice in the membrane which is penetrated by transmembrane channels. The finding of some specificity of pores for substrates is somewhat puzzling since a central feature of the porin hypothesis is that the pores are passive diffusion channels : in addition, E.coli B/r seems to manage quite well with only one species of matrix protein. The porins are clearly proteins which are very important to the cell : for example, as shown in this thesis, protein b of E.coli B/r forms the single most prominent band in electrophoretic analyses of total cell protein.

(iv) Specific Transport Systems of the Outer Membrane

The apparent exclusion limit for free diffusion of polar compounds across the outer membrane is around 600 daltons (Nakae and Nikaido, 1975), and is presumably defined by the size of the porin-specified pores. There are two well-characterised examples of specific transport systems in the outer membrane facilitating the passage of metabolites which are excluded by the pores. These transport systems will now be discussed.

The transport of vitamin B_{12} by E.coli requires the presence of a specific outer membrane protein of molecular weight 60K (White et al.,

1973). This protein is the receptor for the E colicins and for phage BF23 (DiMasi *et al.*, 1973; Bradbeer *et al.*, 1976) and is the product of the *btu* (or *bfe*) gene. The need for this specific transport system presumably arises because the vitamin B_{12} molecule is excluded from the porin-specified pores because of its size (1350 daltons).

The acquisition of iron presents *E.coli* (and other organisms) with a difficult problem since, at physiological pH, ferric ions form insoluble hydroxy-polymers. To overcome this problem the bacterium relies upon the action of various compounds which chelate ferric ions; the cell transports iron in these various chelated forms. The iron chelate complexes are presumably excluded by the porin-specified pores since specific outer membrane transport systems which function in iron transport have been identified. The outer membrane proteins involved in two of these transport systems have also been identified as phage and colicin receptors. The product of the *tonA* gene is an outer membrane protein which acts as receptor for phages T1, T5 and ø80, and also for colicin M; *tonA* mutants are resistant to these agents and are specifically impaired in ferrichrome-mediated iron transport (Hantke and Braun, 1975). Ferrichrome is a complex molecule, of molecular weight 740, which is not produced by *E.coli* but is synthesised and secreted by certain fungi. In contrast, the chelator enterochelin, of molecular weight 746, is synthesised and secreted by *E.coli*; the enzymes responsible for enterochelin synthesis are the products of a cluster of *ent* genes mapping at 13 minutes, and their synthesis is coordinately repressed by the presence of iron in the medium. The colicin B receptor, an outer membrane protein of molecular weight 81K, is the product of the *feuB* locus which maps near the *ent* genes. Hancock *et al.* (1976) found that colicin B-resistant *feuB* mutants are defective

in enterochelin-mediated iron transport, indicating the role of this protein in transport. During growth under conditions of iron-limitation resulting, for example, from the presence of a nonutilisable chelator such as α, α' dipyridyl, the feuB and tonA gene products are produced in increased amounts. Two other induced outer membrane proteins are found under these conditions. These are the colicin I receptor of molecular weight 74K, and a protein of molecular weight 83K with no known receptor function; however, these proteins have no known role in iron transport.

In contrast to these transport systems which are induced by iron deficit, a third system is only induced by the presence of its specific chelator, citrate. When citrate is added to the medium a protein of molecular weight 81K appears in the outer membrane and is presumed to be responsible for citrate-mediated iron transport (Hancock et al., 1976; Frost and Rosenberg, 1973).

Apart from iron deficit there is another circumstance under which high levels of the inducible proteins are found in the outer membrane. This occurs in certain phage T1 resistant strains, mutant at the tonB locus. Such mutations are pleiotropic, conferring resistance to phage Ø80 and colicins B, I and V (Gratia, 1962; Davis and Reeves, 1975). In fact infection by a phage such as T1 is a two-stage process : the first stage, a reversible binding to the outer membrane receptor (the tonA protein in the case of phages T1 and Ø80), is followed by an energy-dependent transition to an irreversibly-bound state (Hancock and Braun, 1976). Similarly, killing by colicins is a two-stage process; mutants which bind colicins but are nevertheless insensitive are deficient in an energy-dependent translocation step and are said to be colicin-tolerant (Holland, 1975). The tonB mutant is thus both phage

- and colicin-tolerant. The tonB mutation also causes defects in the transport of vitamin B12 (Bassford et al., 1976) ferric enterochelin (Pugsley and Reeves, 1976) and ferrichrome (Braun et al., 1976), and there is some speculation that it is the energy-dependent step which is defective (Hancock et al., 1977). It seems possible, therefore, that the tonB gene product (as yet unidentified) may function in making energy from the proton gradient across the cytoplasmic membrane available for use by the outer membrane systems.

IV. MOLECULAR ASPECTS OF ENVELOPE BIOGENESIS IN E.COLI

In this Section I will discuss our knowledge of the biosynthesis and translocation of envelope components; in Section V I will go on to consider the coordination of the synthesis of the cell envelope with cell growth and division.

IV.(a) Biogenesis of the Cytoplasmic Membrane

(i) Phospholipids

Phospholipid biosynthesis in E.coli has been comprehensively reviewed by Raetz (1978). Most of the biosynthetic enzymes involved in the process are found in association with the cytoplasmic membrane (White et al., 1971; Bell et al., 1971). A clue to the way in which new phospholipid molecules are inserted into a pre-existing bilayer has come from work by Rothman and Kennedy (1977b), who found that newly-synthesised phosphatidyl ethanolamine in Bacillus megaterium is first detected in the inner, cytoplasmic leaflet of the cytoplasmic membrane (where it is inaccessible to reaction with trinitrobenzenesulphonate - see Section I.(a)iii); this newly inserted phospholipid rapidly equilibrates across the bilayer in accordance with the steady state asymmetric distribution of this particular phospholipid species in the membrane. The rate of this transmembrane movement was found by Rothman and Kennedy to be much greater than that measured in synthetic bilayers or in non-growing cells such as erythrocytes. Thus, in actively growing cells, newly-synthesised phospholipids may be inserted into the inner leaflet of the membrane bilayer by the membrane-bound enzymes of biosynthesis, and then subjected to a transmembrane movement by a facilitated process which enables phospholipids to reach the outer leaflet of the bilayer.

(ii) Proteins of the Cytoplasmic Membrane

The evidence relating to the biosynthesis of the coat proteins of phages M13, f1 and fd was discussed in Section II. These proteins, which are transmembrane proteins of the cytoplasmic membrane of infected E.coli, seem to be made in preprotein form with a hydrophobic amino-terminal signal sequence which presumably directs the protein into the membrane. There is no evidence relating to the biosynthesis of more typical membrane proteins such as the lactose permease. The finding of a signal sequence for such a protein would greatly strengthen the hypothesis that such sequences are necessary for transmembrane insertion of membrane proteins in general.

(iii) Requirement for Concomitant Synthesis of Proteins and Phospholipids

The simplest model of integration of proteins into the cytoplasmic membrane requires only that there is a pre-existing bilayer. An alternative to this, suggested by Hsu and Fox (1970), is that there is a requirement for concomitant synthesis of phospholipids and membrane proteins. Hsu and Fox reported that the induced synthesis of the lactose transport system in E.coli was reduced in mutant strains which are unable to synthesise phospholipids unless they are supplied with glycerol. Better designed experiments were subsequently carried out by Weisberg et al. (1975), who used similar, lipid-synthesis-defective mutants which were also defective in glycerol catabolism; this ensured that glycerol deprivation did not constitute removal of a carbon source. In this study the induced rates of synthesis of functional lactose permease and β-galactosidase were compared in the absence of phospholipid synthesis; no differential effect upon permease synthesis was seen. In accordance with this result McIntyre and Bell (1975) have reported that when similar mutants are deprived of glycerol they

nevertheless continue to synthesise bulk membrane protein until the protein/phospholipid ratio in the membrane has increased by about 60%; similar results have been obtained in experiments with lipid-synthesis mutants of Bacillus subtilis (Mindich, 1975). It seems clear that there is no need for concomitant synthesis of phospholipid with membrane proteins. One interesting exception to this rule is the finding that in phage f1-infected E.coli cells, in which phospholipid synthesis has been blocked (as described above), the accumulation of the membrane-associated coat protein is differentially reduced in comparison with soluble phage proteins (Cashman and Webster, 1977). This result once again indicates the potential pitfalls awaiting those who draw general conclusions about membrane protein synthesis from phage systems.

IV.(b) Biogenesis of the Outer Membrane

(i) Biosynthesis of the Lipopolysaccharide; Insertion Sites

The structures of the lipopolysaccharides of S.typhimurium and E.coli K12 are shown in Figure 1.7. The details of LPS biosynthesis have been reviewed by Nikaido (1973) and Meadow (1974). Very little is known about the synthesis of the lipid A group, or the transfer of the 3-deoxy-D-manno-octulosonic acid and heptose subunits to the growing molecule, although conditional mutants defective in these steps have now been isolated (Lehmann et al., 1977). It has been established however, that the outer core region is synthesised by sequential addition of UDP-linked sugars to the backbone-lipid A molecule. In S.typhimurium, (and, presumably, in smooth strains of E.coli), nucleotide-linked sugars are transferred sequentially to an isoprenoid carrier molecule in the cytoplasmic membrane, to form the basic repeating unit of the O-antigen; these units are, in turn, polymerised and transferred

finally to the terminal group of the core oligosaccharide. The work of Osborn et al. (1972a;b) established that in S.typhimurium the enzymes which synthesise the LPS molecule are located exclusively in the cytoplasmic membrane. Newly-synthesised LPS was accordingly found at first in the cytoplasmic membrane from where it rapidly moved to the outer membrane.

There is considerable evidence that the translocation of LPS to the outer membrane occurs at specific points in the cell envelope. It will be recalled from the discussion of outer membrane fluidity in Section III that Muhlradt et al. (1974) demonstrated that newly synthesised LPS appears at specific points in the outer membrane of S.typhimurium, rather than being randomly intercalated over the whole surface; the number of these insertion sites was estimated as about 250 per cell. In E.coli the existence of LPS insertion sites in the outer membrane has been demonstrated by Kulpa and Leive (1977). These workers used E.coli J5, a galE mutant strain which, in the absence of exogenous galactose, synthesises a truncated LPS molecule. This results in the production of an outer membrane with a decreased density as detected in equilibrium sucrose gradients. Kulpa and Leive exposed such cells to a brief pulse of galactose, whereupon a new dense peak of outer membrane was found upon sucrose gradient analysis. If, in this experiment, complete LPS molecules were intercalated randomly over the whole cell surface, the density of outer membrane fragments would be expected to shift gradually to that characteristic of galactose-grown cells. The detection of dense outer membrane fragments after a very short period of growth in galactose indicates that small homogeneous regions were formed which contained complete LPS molecules. When

galactose-pulsed cells were returned to galactose-free medium the dense peak of outer membrane quickly disappeared. This observation is crucial to the interpretation of these experiments since it rules out the possibility that complete LPS is, in fact, intercalated randomly but then forms homogeneous domains during membrane isolation. From the proportion of total outer membrane material found in the dense fraction after galactose pulsing, and knowing the mean surface area of the dense outer membrane fragments, Kulpa and Leive estimated that there are 10-22 insertion sites per cell. The discrepancy between this estimate and that of Mühlradt et al. (1974) is unexplained.

Such insertion sites in the outer membrane can be tentatively identified with the zones of adhesion observed in the cell envelopes of gram negative bacteria by Bayer (1968). These adhesion zones are regions of stable, intimate contact between the cytoplasmic and outer membranes, which are visible in thin section electron micrographs of plasmolysed cells. In extremely elegant experiments Bayer (1975) demonstrated that the adhesion zones are sites of LPS insertion by using the following experimental system. In Salmonella anatum infection by the phage ε15 leads to a change in the antigenic nature of the LPS being synthesised, a phenomenon known as phage conversion. Newly infected cells were exposed to ferritin-conjugated antibodies directed against the phage-specific LPS, plasmolysed to locate adhesion zones, and then examined in the electron microscope. Clusters of electron-dense ferritin molecules correlated well with the adhesion zones, providing strong evidence for the role of these structures in LPS translocation. The nature of the adhesion zones remains unclear, and recent attempts to isolate these specific regions of the cell envelope have failed (Crowlesmith et al., 1978).

As discussed in Section III, the LPS may exist in an ordered matrix in the outer membrane because of extensive interactions with itself and with other components such as the matrix proteins. The insertion sites discussed above may thus represent growing points for a process of surface growth somewhat similar to crystallisation.

(ii) Outer Membrane Phospholipids

The finding that all phospholipid synthesis occurs in the cytoplasmic membrane (Section IV(a)(i)) leads to the conclusion that phospholipids must also be translocated to the outer membrane, possibly at the LPS insertion sites. Movement of phospholipid between the two membranes has been demonstrated by Jones and Osborn (1977a;b) who added phospholipid bilayer vesicles to intact cells of S.typhimurium. This treatment produced significant transfer of vesicle lipid to the cell envelope by a process dependent upon the ionic conditions and the structure of the LPS of the recipient cells. Further analysis revealed that the transferred lipids appeared in both the outer and cytoplasmic membranes; Jones and Osborn concluded that this indicates a rapid translocation of phospholipid from the presumptive site of primary incorporation in the outer membrane, and have suggested that this translocation occurs by a reverse flow through the channels responsible for outer membrane bilayer synthesis. It was also found that when LPS was included in the vesicle membranes it entered the outer membrane but did not reach the cytoplasmic membrane, suggesting that LPS translocation may be irreversible.

(iii) Outer Membrane Protein Synthesis

The way in which specific proteins are assembled into the outer membrane is a major puzzle. What are the factors which determine that only these proteins reach the outer membrane? Recent experiments have suggested that amino-terminal signal sequences may be at least partly responsible for directing outer membrane proteins to their final cellular location. The first indication that this might be so came from a study carried out by Sekizawa et al. (1977), who found that E.coli, when briefly treated with toluene, produces new forms of the major outer membrane proteins in the cell envelope. These novel polypeptides had molecular weights some 2-3K greater than the normal outer membrane proteins, and it was concluded that they represent precursor forms analogous to the preproteins of secreted polypeptides discussed in Section II. The action of toluene in this system is not understood, but may be due to inhibition of proteolytic processing enzymes. Unfortunately Sekizawa et al. did not determine whether the putative preproteins reached the outer membrane.

Confirmatory evidence for the hypothesis that outer membrane proteins have signal sequences has been presented by Randall et al. (1978). This work was discussed in detail in Section II.(b). Briefly, membrane-bound polysomes were isolated from E.coli and allowed to complete existing rounds of translocation in the absence of reinitiation. Amongst the products of this system Randall et al. found a new form of the outer membrane phage λ receptor (the malB gene product) which was identified immunologically and had a molecular 2-3K greater than the normal protein. The appearance of this novel polypeptide was presumably due to the presence of an amino-terminal signal sequence. The importance of the amino-terminal sequence of the malB gene product, or

of its preprotein form, has also been elegantly demonstrated by Silhavy *et al.* (1977) who constructed strains of *E.coli* in which the region of the *malB* gene encoding the amino terminus of the *malB* protein is fused to the *lacZ* gene encoding β-galactosidase. Two such fusion strains were analysed : one of these possessed a wholly cytoplasmic β-galactosidase activity whereas the other strain possessed an envelope-associated enzyme. Further analysis revealed that this envelope-associated enzyme was equally distributed between the cytoplasmic and outer membranes, and that some of it was accessible to reaction with fluorescent antibodies at the surface of intact cells. Unfortunately, in the two fusion proteins analysed by Silhavy *et al.* the precise contribution of the amino-terminal region of the *malB* protein has not been established; however, from knowledge of the approximate extent of the amino-terminal deletiongenerated in the *lacZ* gene during strain construction, and the electrophoretic mobility of the fusion proteins, it may be tentatively concluded that the membrane-associated fusion protein contains a larger piece of *malB* specified polypeptide than does the cytoplasmic fusion protein. Possession of a part of the amino-terminal sequence of the *malB* protein, including any signal sequence, is clearly sufficient to direct a large soluble protein into the outer membrane.

The existence of these precursor forms of outer membrane proteins does not really answer the question posed above : how do specific proteins reach the outer membrane? This question will be discussed in more depth in Chapter 10. I will restrict myself to merely listing some possibilities here. Firstly, the presence of signal sequences may simply reflect the synthesis of outer membrane proteins as periplasmic or cytoplasmic membrane proteins which, under the control of other

factors, are subsequently translocated to the outer membrane. Thus, for example, nascent outer membrane proteins inserted into the cytoplasmic membrane might interact with nascent LPS and enter the outer membrane via the LPS insertion sites. Alternatively, nascent outer membrane proteins may possess a special class of signal sequence which produces extrusion across the cytoplasmic membrane but which escapes processing at this particular stage and so directs the nascent protein into the outer membrane. A final possibility which must be considered is that there are specific sites in the envelope where nascent outer membrane proteins may be inserted directly into the outer membrane. What evidence there is relating to this question will be discussed in Chapter 10.

IV.(c) The Biosynthesis of Peptidoglycan in Cell Growth and Division

(i) Peptidoglycan Biosynthesis : Growth Zones

The structure of the peptidoglycan was described in Section III. The early steps in biosynthesis occur in the cytoplasm, culminating in the formation of UDP-linked forms of N-acetylglucosamine and N-acetylmuramic acid - pentapeptide; the latter molecule contains the tetrapeptide present in peptidoglycan, together with an additional carboxy-terminal D-alanine residue. These nucleotide-linked units are sequentially transferred to a membrane-bound isoprenoid carrier molecule (the same carrier which is involved in LPS biosynthesis) to form covalently-linked N-acetylglucosamine-N-acetylmuramic acid - pentapeptide, which is the basic repeating unit of peptidoglycan. This unit is now translocated, in lipid-linked form, to the external face of the cytoplasmic membrane where it is attached to the end of a growing

glycan chain (composed of similar units) to form nascent peptidoglycan. Nascent peptidoglycan is incorporated into the peptidoglycan network by the formation of peptide crosslinks; this occurs by a transpeptidation reaction involving loss of the terminal D-alanine of the pentapeptide. Another enzymic activity, carboxypeptidase, is responsible for the removal of this terminal D-alanine from those peptides not participating in cross-linking.

In order to investigate the topology of insertion of nascent peptidoglycan into the sacculus, Ryter et al. (1973) labelled cells with a brief pulse of radioactive meso-diaminopimelate, and detected the appearance of label in the sacculus by high resolution autoradiographic electron microscopy. In this way Ryter et al. demonstrated the existence of a centrally-located 'growth zone' at which newly synthesised peptidoglycan first appeared; however, this newly inserted peptidoglycan was rapidly redistributed over the whole surface. Further analysis of similar experiments led Schwartz et al. (1975) to postulate the existence of a centrally-located growth zone and, in addition, two growth zones, situated midway between cell centre and cell poles, which were apparent in larger cells; it was suggested that the central growth zone may participate in cell elongation and, towards the end of the cell cycle, in cell division, whereas the peripheral zones may function only in cell elongation. Further evidence for the existence of growth zones in the sacculus has come from the study of autolytic sites. At fairly high concentrations ampicillin produces spheroplasts which arise at very localised sites in the envelope; this is thought to arise as a result of effects at sites of active peptidoglycan synthesis, or growth sites. Donachie and Begg (1970) have reported that these sites exist at a constant distance from one cell pole, and concluded

that sacculus growth is zonal and essentially asymmetric. In contrast Staugaard et al. (1976) found that the ampicillin-induced spheroplasts tended, except in very young cells, to arise at the cell centre. Thus, they concluded that the cell centre is 'marked' from early in the cycle and may be involved in elongation of the peptidoglycan sacculus. The existence of growth zones for peptidoglycan has also been quite clearly established in the gram-positive bacteria Streptococcus faecalis (Higgins and Shockman, 1970), and Bacillus subtilis (Burdett and Higgins, 1978), and it is a popular notion that localised growth is a general feature of bacterial peptidoglycan growth.

(ii) Septum Formation and Cell Division

A study of the morphology of septation and cell division in E.coli has been carried out by Burdett and Murray (1974). These workers generated synchronously growing cultures of E.coli B by the membrane elution technique (Helmstetter, 1967) and analysed electron micrographs of cells at various stages of the cell cycle. The first division event which they detected was segregation of the nucleoid, followed by the appearance of a bleb of outer membrane at the centre of the cell surface. This was followed by the formation of a centrally-located notch in the peptidoglycan and cytoplasmic membrane, which rapidly formed a septum from which the outer membrane was excluded. The final cell cleavage event was preceded by rapid ingrowth of the outer membrane to cover the nascent polar caps. Mutations at the envA locus seem to interfere specifically with a late stage in this process; such mutants form chains of cells which are separated by a peptidoglycan septum but which have not achieved the final act of cell separation (Normark et al., 1971).

The process of septation clearly comprises a switchover from synthesis of longitudinal peptidoglycan to synthesis of transverse, septal, peptidoglycan, and also involves a change in the association of the outer membrane with the peptidoglycan. Since the lipoprotein is thought to act as a linker between outer membrane and peptidoglycan (see Section III) it thus may well have a role in the septation process. In support of this hypothesis Fung et al. (1978) have reported that the lipoprotein mutations lpo in E.coli and lky in S.typhimurium cause defective septation, with the efficiency of outer membrane invagination being severely reduced. Nevertheless, an obligatory role for the lipoprotein in septation seems unlikely since both lpo and lky mutants are able to complete cell division.

(iii) Penicillin-Binding Proteins

Antibiotics of the β-lactam group, such as penicillins and cephalosporins, have various effects upon the morphology of bacteria, depending upon the concentration of the particular antibiotic being used (see Spratt, (1978), for a review of this and other aspects of the mechanism of action of penicillin). For most β-lactam antibiotics the lowest concentration which produces any morphological effect results in the formation of filamentous cells due to selective inhibition of cell division; these filaments eventually lyse. At higher concentrations cell lysis occurs more rapidly, and with some penicillins bulges develop at the centre of the elongating filament. At the highest concentrations virtually all β-lactam antibiotics rapidly inhibit cell elongation and cause cell lysis. A fourth effect of some antibiotics was discovered when it was shown that the compound mecillinam causes formation of large spherical cells which eventually lyse (Lund and

Tybring, 1972).

These various effects of β-lactam antibiotics are due to interference with various aspects of the process of peptidoglycan synthesis. Thus, in vitro transpeptidation and carboxypeptidation reactions are inhibited by penicillin (Araki et al., 1966; Izaki et al., 1966), and since penicillin binds irreversibly to bacterial cells, Tipper and Strominger (1965) suggested that the antibiotic forms covalent complexes with the penicillin-sensitive enzymes. This was confirmed by the identification of several penicillin-binding proteins (PBPs) in E.coli (Spratt and Pardee, 1975); these PBPs are found in the cytoplasmic membrane and are tacitly assumed to include most or all of the penicillin-sensitive enzymes of the cell.

The individual effects of various β-lactam antibiotics upon cell morphology correlate well with their relative affinities for the PBPs so that it is possible to deduce that particular PBPs are concerned with elongation of the sacculus, formation of the septum, or determination of cell shape (Spratt, 1975). Thus, for example, ampicillin has a higher affinity for PBP3 than for the other proteins; the specific effect of low concentrations of ampicillin upon cell division suggests that PBP3 is directly involved in septum formation. This conclusion is supported by the finding that benzylpenicillin, another antibiotic with high affinity for PBP3, also produces filaments at low concentrations, and also by the isolation of mutant strains which form filaments at 42°C and which have a thermolabile PBP3 (Spratt, 1978). It has been established by similar approaches that PBP2 is responsible for cell shape determination and PBP 1B is concerned with cell elongation. Two other PBPs, 5 and 6, are identical to the major D-alanine carboxy-

peptidase 1a of the cell (Spratt and Strominger, 1976), whose function *in vivo* is not yet known. Since the other PBPs have not yet been purified in active form their enzymic activities remain uncharacterised. It seems likely, however, that a major part of our understanding of cell growth and division must come from the study of these proteins.

V. ENVELOPE GROWTH IN RELATION TO THE CELL CYCLE OF E.COLI

(a) Salient Features of the Cycle : DNA Replication and Cell Division

In this section I will consider our present understanding of the cell cycle of E.coli, with particular reference to the way in which the cell surface grows. The commonly accepted explanation of the way in which DNA replication is controlled in the cycle provides a useful starting point for a discussion of this type.

The E.coli chromosome is a circular duplex DNA molecule which is replicated at a single pair of replication forks. These forks arise at a specific point on the chromosome, the origin of replication (ori C at 83 minutes; von Meyenburg et al., 1977), and move in opposite directions until they meet halfway around the chromosome at the terminus of replication. The time taken for this process to occur has been designated C, and seems to be constant and approximately equal to 40 minutes for E.coli B/r growing with generation times less than about 70 minutes (Helmstetter, 1967; Helmstetter and Cooper, 1968; Cooper and Helmstetter, 1968; Helmstetter and Pierucci, 1976).

It was proposed by Maaløe and Kjeldgaard (1966) that whilst the rate of elongation of a macromolecule may be constant at various growth rates, the rate of synthesis of the macromolecule may be regulated by controlling the frequency of initiation of its synthesis. In the case of DNA synthesis, a new round of chromosome replication is thought to be initiated when the quotient, cell mass/number of chromosome origins, reaches a critical value, termed the initiation mass (Donachie, 1968). The process of initiation halves this quotient by duplicating the origin; subsequent cell growth then increases the value of the quotient until it again reaches the critical value after one mass doubling time,

τ minutes later. In this way the rate of initiation of replication is coupled to the growth rate of the cell. If $\tau > C$ then there will be gaps between successive rounds of replication, whereas if $\tau < C$ then the initiation mass will be attained whilst the previous round of replication is still in progress. Thus, gaps between rounds of replication are detected only in slowly-growing cells (Helmstetter, 1967).

Several authors have proposed models to explain how the rate of initiation of DNA replication might be matched to the rate of growth (Helmstetter et al., 1968; Pritchard et al., 1969; Sompayrac and Maaløe, 1973). In these models the period of growth between initiations is regarded as the time taken either to accumulate a critical amount of some positive initiation factor, or to dilute out an inhibitor of initiation. Recent evidence relating to this point has been reviewed by Pritchard (1978).

Turning now to the control of the timing of cell division, it is clear that a tight coupling between division and chromosome replication might be predicted a priori since the former process cannot be efficiently accomplished unless replication provides daughter genomes for distribution among daughter cells. The finding by Cooper and Helmstetter (1968) of a fixed period, D, (found, over a range of growth rates to be approximately equal to 20 minutes), which elapses between termination of a round of replication and the subsequent cell division event, provided circumstantial evidence for such a coupling. This observation has been widely interpreted as reflecting the timing of cell division from some stage of replication; thus, for example, initiation of replication could set in motion a division process lasting C+D minutes,

or alternatively, termination could trigger a division, D minutes later. The notion that it is termination of replication from which division is timed has received support from demonstrations that the inhibition of DNA synthesis in exponentially growing cultures by nalidixic acid or by thymine starvation produces a period of residual division lasting for about 20 minutes (Inouye, 1971; Helmstetter and Pierucci, 1968); in addition, the fractional increment in cell number in these experiments equals the fraction of cells in the population which are expected to be in that part of the cell cycle between termination and division. It is possible, however, that these observations may be due to the existence of a regulatory mechanism which acts specifically under conditions of inhibition of DNA synthesis to turn off cell division; thus the timing of division might normally be determined by factors unrelated to DNA synthesis, and might only be subject to a regulation by the replication cycle under rather special circumstances. In fact, treatments such as thymine starvation, nalidixic acid treatment or UV irradiation cause DNA breakdown and lead to the induction of a group of functions known collectively as the SOS system (Witkin, 1976). This group of functions includes transient inhibition of cell division and also induced synthesis of the recA gene product. Inouye (1971) has demonstrated that in a recA mutant, after thymine starvation or nalidixic acid treatment, extensive cell division continues to produce DNA-less cells. Recent evidence has indicated, however, that induced synthesis of the recA protein (as opposed to the uninduced cellular level) is not strongly correlated with the onset of division inhibition (Darby and Holland, 1979).

An approach to understanding the nature of the D period which may avoid the complications discussed above is based upon the

observation by Pritchard and Zaritsky (1970), that in certain thymine-requiring mutants of E.coli the replication velocity may be decreased, without affecting growth, by lowering the concentration of exogenously-supplied thymine. Thus, at limiting thymine concentrations, a novel mode of balanced growth is established in which replication forks are moving slowly around the chromosome; addition of excess thymine relieves this limitation upon replication velocity, and the forks accelerate to produce an increase in the rate of DNA synthesis in the culture (Zaritsky and Pritchard, 1973). Analysis of the kinetics of cell division in such an experiment reveals an increase in the rate of division occurring 20 minutes after the addition of thymine (Meacock and Pritchard, 1975). This is clearly consistent with the hypothesis that the D period is an obligatory period elapsing between termination of replication and division; if division were timed from an earlier stage of replication then the increased rate of division would be expected to occur correspondingly later after the addition of thymine.

A somewhat different view of the nature of the D period was proposed by Jones and Donachie (1973), who suggested that cell division is timed to occur C+D minutes after initiation of replication. Thus the D period would merely reflect the difference in length of two parallel cycles initiated at the same time, namely a replication cycle lasting C minutes and a division cycle lasting C+D minutes. To explain the effects of inhibition of DNA synthesis described above it was further proposed that termination of replication provides a positive signal which is necessary if the division cycle is to be completed. Although this model cannot explain the effect of thymine limitation, it does predict that C and D should be related; if C is increased, then

D should be decreased by an equivalent amount. Indeed, Meacock and Pritchard (1975) found that under conditions of thymine limitation, as C was increased, so D decreased, although C+D did not remain constant. It has recently been demonstrated, however, that the cell shape changes which occur during thymine limitation (Zaritsky and Pritchard, 1973) are most likely due to a direct effect of the limitation upon surface synthesis (as discussed below). Since the D period may reflect, at least in part, the time taken for septal surface synthesis, the interpretation of experiments involving thymine limitation must be approached with caution.

Koch (1977) has questioned the basic assumption that cell division is 'timed' from any stage of the replication cycle. This dissenting view is based upon the observation that the variation in cell size at initiation of replication is greater than the variation in size at division. How, Koch argues, can an event which occurs in cells of a wide range of sizes control a process which occurs later in cells of a narrow size range? The small variation in cell size at division suggests an alternative, that it is the attainment of a critical cell mass which determines when cell division occurs. This sort of model could once more include a special 'veto' mechanism, enabling division to be turned off by any disruption of DNA replication. Circumstantial evidence which supports the idea that there is no obligatory requirement for DNA synthesis before cell division can occur has come from a study by Spratt and Rowbury (1971a,b) of a thermosensitive DNA initiation mutant of S.typhimurium. At the restrictive temperature this mutant completed existing rounds of replication but did not initiate new rounds. Residual cell division occurred for C+D minutes, as expected

from both models discussed earlier; however, division then continued, producing small DNA-less cells. Similar effects have also been observed in a mutant strain of E.coli which is thermosensitive for initiation of DNA synthesis (Hirota et al., 1968). Thus, under these conditions at least, DNA replication and cell division may be disassociated.

(ii) Growth of Cells and of the Cell Surface

Cells of E.coli are cylindrical rods with rounded ends. At any one growth rate cells have a fixed radius and grow solely by elongation (Marr et al., 1966). Cell mass is thought to be produced exponentially during growth, since the rates of synthesis of stable RNA and protein appear to increase continuously during the cycle. Thus, there is a direct proportionality between cell size and the rate of synthesis of RNA and protein (Ecker and Kokaisl, 1969), and measurements of the rates of synthesis of RNA and protein in cells of different age classes indicate that these rates increase exponentially as a function of cell age (Dennis, 1971; Meacock, 1975; Churchward and Holland, 1976a).

A possible alternative to exponentially increasing rates of production of components such as RNA and protein is accumulation by a linear mode; that is, production at a constant rate with an abrupt doubling in rate occurring at some point during the cell cycle. The difference between linear and exponential patterns of growth is very small - the theoretical curves differ by only 6% at the point of maximum deviation. It is thus the large difference in the cell age dependence of rates of synthesis of components produced linearly or exponentially which has provided the evidence for exponential growth of RNA and protein discussed above. In the case of cell parameters for which a rate of synthesis cannot be directly measured, the

determination of the pattern of production thus relies upon detection of the very small difference between linear and exponential accumulation curves. Nevertheless, it has been claimed that cell length and cell volume, in contrast to cell mass, increase linearly in synchronous cultures (Kubitschek, 1968; Ward and Glaser, 1971; Donachie et al., 1976). Another approach to determining the kinetics of cell elongation (and thus, volume increase) is exemplified by a study by Cullum and Vicente (1978), who have analysed length distributions in exponentially-growing populations, using mathematical methods developed by Collins and Richmond (1962); Cullum and Vicente concluded that elongation does indeed proceed linearly. This pattern of elongation may be a general feature of the growth of rod-shaped bacteria, since Sargent (1975) has concluded that the gram-positive rod Bacillus subtilis also elongates linearly.

Hypotheses which include propositions of an exponential increase in cell mass, coupled with linear cell volume increase, predict that the cell density will fluctuate during the cycle. This prediction has been confirmed by Poole (1977), who found that E.coli K12 displays cell age-dependent fluctuations in buoyant density. The cells, growing with a doubling time of 44 minutes, had a minimum density in mid-cycle and a maximum density (corresponding to the theoretical doubling in rate of volume increase) around the time of cell division. In contrast to Poole's findings, unpublished data referred to by Koch and Blumberg, (1976) indicate that cell density is constant during the cell cycle. This discrepancy can only be resolved by further experiments.

It is implicit in the foregoing discussion that any linear increase in cell length and cell volume must arise because the cell surface is laid down at a constant rate. Another prediction of the linear growth

hypothesis is, therefore, that at least some components of the cell envelope are synthesised linearly. I will now consider some direct evidence for linear envelope growth and then go on to discuss the theoretical framework into which this evidence may be placed.

Since it is the rigid peptidoglycan layer of the cell envelope which defines the cell surface area, it is this surface component which is the most likely candidate for linear accumulation. Hoffman et al. (1972) generated synchronous cultures by the membrane elution method and measured the rate of synthesis of peptidoglycan in cells of different ages by pulse-labelling with radioactive D-glutamate. They concluded that the rate of peptidoglycan synthesis was constant, with a doubling in rate occurring near the time of septation. Using a somewhat different approach Koppes et al. (1978) pulse-labelled slowly-growing E.coli with radioactive meso-diaminopimelate and used autoradiography to determine the relationship between cell length and the rate of peptidoglycan synthesis. The conclusion drawn from this study was again that a doubling in rate of synthesis occurred late in the cell cycle.

If two cell components accumulate, one linearly, the other exponentially, then the relative amounts of the two components will fluctuate during the cell cycle (as discussed above for cell density, which is a measure of the relative amounts of cell mass and cell volume). The amplitude of such fluctuations will, however, be very small, equalling the 6% maximum deviation between the linear and exponential accumulation curves. If we now assume that the peptidoglycan layer of the envelope is synthesised linearly, then what constraints does this impose upon the growth of the other surface layers, the cytoplasmic and outer membranes? If these membranes are synthesised exponentially

then we might predict that the resulting fluctuations in membrane surface/peptidoglycan surface could easily be accommodated either by appropriate changes in the mean intermolecular distances in the membrane (the 'packing' of the membrane components), or by the formation of bulges or invaginations of membrane. There would thus seem to be no compelling reason why these plastic surface layers should grow in step with the rigid peptidoglycan layer. Nevertheless, Churchward and Holland (1976a) have reported that bulk envelope protein (but not phospholipid) accumulates linearly; and Hakenbeck and Messer (1977) reported that both phospholipid and protein of separated cytoplasmic and outer membranes accumulate linearly. Churchward and Holland placed the doubling in rate in mid-cycle (for τ = 65 minutes) whereas Hakenbeck and Messer placed it towards the end of the cycle, about 15 minutes before division (for τ = 40 minutes).

My discussion of cell surface growth in relation to the cell cycle has, so far, been somewhat artificial in that I have not mentioned a line of research which has complemented, and in many cases inspired, the study of the cell cycle kinetics of surface synthesis. I did, however, briefly describe above how cells growing at a particular rate have a fixed radius of cross-section and grow solely by elongation. Growth at different rates, however, produces variations in cell radius; the faster the growth rate then the greater is cell radius (Schaechter _et al._, 1958; Grover _et al._, 1977). If, as a first approximation, we consider the cell as being cylindrical, then an expression for the mean surface/volume ratio of the cells may be written:

$$S/V = 2/R$$

where S is surface area

V is cell volume

R is cell radius

Thus, as cell radius increases, so the mean surface/volume of the cells decreases. If this expression is rewritten in terms of real cellular components by replacing surface with envelope, and volume with mass, then it may be concluded that envelope/mass should decrease with increasing cell radius. Since cell radius increases with growth rate, and if it is accepted that mean cell density is independent of growth rate (Kubitschek, 1974), then envelope/mass must, in contrast to cell mass, decrease as the growth rate increases. It has been the effort of several groups to explain this aspect of cell growth, i.e. the change of cell shape with growth rate, which has stimulated all of the research into patterns of cell surface growth.

In order to explain these shape changes, Pritchard (1974), pointed out that if the cell surface was synthesised linearly with the rate doubling being triggered in some way by replication of a specific gene, then this would lead directly to the observed changes in envelope/mass. Putting this another way, the envelope/mass of cultures with different growth rates would simply reflect the underlying changes in gene/mass, or gene concentration. This parameter, gene concentration, has been defined and discussed by Chandler and Pritchard (1975) and may be written, for any gene as:

mean gene concentration in a culture $= {}^{1}/_{k} \cdot 2^{-Cx/\tau}$

where k denotes initiation mass

C = replication time

τ = doubling time

x = fractional distance of gene from origin of replication

Note that any increase in growth rate (decrease in τ) will decrease gene concentrations; the magnitude of this effect is zero at the origin and maximal at the terminus. In fact, evidence has accumulated which suggests that surface growth is not linked to DNA replication. Thus, other manipulations of bacterial cultures which produce changes in gene concentration, namely growth of dnaA and repA mutants at various temperatures (Pritchard et al., 1978; Zaritsky and Woldringh, 1978), do not produce the shape changes predicted by the model of Pritchard (1974). It is still possible, however, that the doubling in rate of surface synthesis could occur at a fixed time before division, merely coinciding with replication of a particular gene, without being causally linked to replication. This type of model has been recently proposed by Rosenberger et al. (1978) who have suggested that the cell surface, or some component of the cell surface, is laid down at a constant rate at specific sites or zones, whose number doubles at a fixed point in the division cycle, d minutes before division. From analysis of the shape of E.coli B/r at different growth rates Rosenberger et al have estimated d to be approximately equal to 45 minutes. In this model the triggering is seen to result from the

fluctuations in cell density arising as a result of the imbalance between mass increase and surface increase; thus, at a critical cell density, the inauguration of new growth zones is triggered. The exact form of the model depends upon whether it is assumed that the rate of synthesis of surface per zone is constant, or varies with growth rate : if the rate per zone is assumed to be proportional to growth rate then d is a constant, whereas if the rate per zone is constant, then d will vary somewhat with growth rate. Once again the most likely candidate for the surface layer which is subject to the proposed regulatory mechanism is clearly the peptidoglycan; evidence for zonal synthesis of peptidoglycan was discussed in Section IV.

It has been suggested (Previc, 1970; Pritchard, 1974) that a doubling in rate of surface synthesis may be necessary to allow septation and cell division to occur. Pritchard (1974) has argued that peptidoglycan may be laid down at a nascent septum (as is the case in Streptococcus faecalis (Higgins and Shockman, 1970)), but that the increase in hydrostatic pressure within the cell, arising out of the linear-log imbalance between accumulation of mass and volume, causes this nascent wall to 'peel apart'. The sudden excess of capacity for peptidoglycan synthesis resulting from the rate doubling may thus allow septal wall synthesis to commence.

In summary, I hope that it is clear from the foregoing discussion that an understanding of the kinetics of cell surface growth may help to explain how the surface grows (i.e. whether growth is zonal); what constraints there are upon cell shape; and also may lead to a better understanding of the nature and timing of septation and cell division.

VI. AIMS OF THE PRESENT STUDY

When this study was commenced the idea that the rate of cell surface synthesis is linked to DNA replication was still popular. The observations made upon the kinetics of envelope protein synthesis made by Churchward and Holland (1976a) suggested that the rate of synthesis of this surface component might be a useful indicator of the overall rate of surface synthesis. I set out, therefore, to extend these observations and to investigate the relationship between envelope protein synthesis and DNA replication.

Churchward and Holland (1976a, b) had also described an envelope protein synthesised periodically in the cell cycle : I also set out, therefore, to investigate the significance of this phenomenon.

CHAPTER 2

MATERIALS AND METHODS

1. BACTERIAL STRAINS

The strains of *E.coli* used in most of the experiments were *E.coli* B/r (substrain F) LEB16 and LEB18 obtained from Dr. G.G. Churchward (Churchward and Holland, 1976a; Meacock and Pritchard, 1975). LEB16 is F^- *lacZ str thyA drm*, the mutation at the latter locus enabling the strain to grow in low concentrations of thymine. LEB18 is a spontaneous Thy^+ revertant of LEB16, isolated by P.A. Meacock.

For some experiments described in Chapter 5 *E.coli* K12 strains AB2847 and VR42/B9 were used, obtained from Dr. V. Braun (Hancock *et al.*, 1976). AB2847 is *aroB thi tsx* λ^r; VR42/B9 is a derivative of AB2847 with additional characters *feuB cir*.

For the control experiment described in section 17, below, strain LEB500 (rRM100) was used. This strain was obtained from R. Iyer and is a derivative of LEB18 which lacks the 36.5K porin (Iyer and Adshead, 1977).

2. CHEMICALS

Apart from those listed below all chemicals were analytical reagent grade, obtained from Fisons Ltd., Loughborough. Dimethyl sulphoxide, isopropanol and acetic acid obtained from this source and when used for processing of polyacrylamide gels were standard laboratory reagent grade.

L-proline, L-alanine, L-leucine, L-methionine, thymine, chloramphenicol, Tris, mercaptoethanol, α,α'dipyridyl and thiamine,

were obtained from Sigma Laboratories, London.

Acrylamide was obtained from Eastman Kodak and was purified by activated charcoal extraction before use. Electrophoresis grade ammonium persulphate, sodium dodecyl sulphate, Coomassie brilliant blue, and N,N'-methylene-bis-acrylamide (Bis) were obtained from Bio-Rad Laboratories. N,N,N',N'-tetramethylethylenediamine (TEMED) was obtained from Canalco Ltd.

Sarkosyl NL-97 was obtained from Geigy U.K. Ltd. Nalidixic acid was obtained from Boehringer Ltd. Casamino acids was obtained from Difco Ltd.

1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) was obtained from Nuclear Enterprises Ltd. 2(4'-t-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) was obtained from Koch-Light Laboratories Ltd. Biosolv solubiliser was obtained from Beckman Ltd. NCS tissue solubiliser was obtained from Amersham/Searle Corporation.

Radiochemicals were obtained from the Radiochemical Centre, Amersham.

3. MEDIA

For experiments with LEB16, the minimal salts medium used was that described by Helmstetter (1967), containing per litre of distilled water : 2g NH_4Cl, 6g Na_2HPO_4, 3g KH_2PO_4, 3g NaCl, and 0.25g $MgSO_4$ added separately from a 200x stock after autoclaving. Proline and alanine were each added at a final concentration of 0.04% w/v as carbon sources, and thymine was added at the concentration indicated in each experiment (usually 20 µg/ml). This growth medium gave generation times of 65-70 minutes at 37°C.

For most of the experiments with LEB18, M9 minimal salts medium

was used, containing per litre of distilled water : 1g NH_4Cl, 6g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, and 11 mg $CaCl_2$ and 246 mg $MgSO_4.7H_2O$ added separately from a sterile 100x stock after autoclaving. For carbon source, either 0.04% w/v proline/alanine, or 0.4% w/v glucose (final concentration) was added.

For the experiments described in Chapter 8 the following carbon sources were added to M9 minimal salts; doubling times are indicated in brackets : 0.04% w/v alanine (110 minutes); 0.2% w/v glycerol (55 minutes); 1% w/v casamino acids (35 minutes); and 0.4% w/v glucose plus 1% casamino acids (25 minutes).

The E.coli K12 strains AB2847 and VR42/B9 were grown in M9 minimal salts medium with 0.4% w/v glucose as carbon source, supplemented as follows : tyrosine 40 µg/ml, tryptophan 20 µg/ml, phenylalanine 40 µg/ml, shikimic acid 40 µg/ml, vitamin B1 2 µg/ml.

Minimal agar plates were prepared with M9 minimal salts and solidified with 15g/l of Davis New Zealand agar. LEB16 was always kept on proline/alanine plates supplemented with 20 µg/ml thymine; upon subculturing it was routinely checked for thymine requirement.

4. GROWTH OF BACTERIA IN LIQUID CULTURE

The procedure routinely adopted to obtain cultures in balanced exponential growth was as follows. Firstly, 10 ml of minimal medium was inoculated with a single bacterial colony from a plate culture, and this small culture was grown overnight at 37°C, into stationary phase. This overnight culture was used to inoculate a larger volume of medium in a conical flask, and this flask culture was grown at 37°C in a New Brunswick gyrotory shaking water bath. To ensure balanced growth was attained before an experiment, cultures were always taken

through at least six generations of growth before use.

5. OPTICAL DENSITY

The absorbance, A_{450}, of cultures was determined by withdrawing a 2 ml sample and measuring in a Gilford Microsample spectrophotometer at 450 nm.

6. BACTERIAL CELL NUMBERS

These were determined using a Model B Coulter Counter fitted with a 30 μm orifice. For all experiments in which cell numbers were to be determined, minimal salts medium was filtered to remove dust particles, before autoclaving. Samples of cells were diluted into an equal volume of filtered 0.9% w/v saline containing 0.8% w/v formaldehyde to fix the cells, and then further diluted to an appropriate density for counting in 0.9% saline. The instrument settings used for proline/alanine grown cells were as follows: lower threshold 5.5, upper threshold 100, 1/aperture current = 1, 1/amplification = 1/8.

7. FILTRATION AND THYMINE STARVATION OF BACTERIAL CULTURE

Removal of medium from growing cultures was achieved by filtration through a nitrocellulose filter (Sartorius, 0.45 μm pore size) which was always washed immediately before use by passage of 100 ml of pre-warmed medium. Cells bound to the filter were washed several times with appropriate prewarmed growth medium and then resuspended in fresh prewarmed growth medium; for thymine starvation, cells of LEB16 were washed and resuspended in thymineless medium.

8. DETERMINATION OF RNA AND PROTEIN CONTENT OF CELLS

To determine macromolecular composition of cells at different growth rates the following procedure was adopted (von Meyenburg, 1971). A 4.0 ml sample of a culture at a known density ($A_{450} \simeq 0.3$) was taken into a centrifuge tube containing 0.75 ml of 1.2M perchloric acid (PCA). After standing on ice for 20 minutes the precipitate was isolated by centrifugation in the Sorvall SS-34 rotor at 10,000 rpm for 5 minutes. The precipitate was washed once in 5 ml of 0.2M PCA.

For determination of RNA content, the washed PCA precipitate was resuspended in 2.0 ml of 0.3M NaOH and hydrolysed at 37°C for 2 hours. The hydrolysate was cooled and reprecipitated by addition of 1.0 ml of 1.2M PCA and standing on ice for 20 minutes. The precipitate was pelleted as before and a sample of the supernatent containing hydrolysed RNA was removed. The RNA content of the hydrolysate was then estimated by measuring A_{260} against a reagent blank, and subtracting A_{320}. The extinction coefficient of a mixture of ribonucleoside monophosphates corresponding to E.coli ribosomal RNA (weighted mean molecular weight in polymer = 324.2) was taken to be 10.5 $mmol^{-1}$.

For determination of protein content from the same sample the PCA-precipitated material from the alkaline hydrolysate was dissolved in 1.0 ml of 0.1M NaOH, 3% w/v Na_2CO_3. Duplicate 250 µl aliquots of this solution were assayed for protein content using a modification of the method of Lowry et al. (1951), as follows. Two reagents were freshly prepared : reagent I consisted of 48 ml of alkaline Na_2CO_3 (see above) plus 1 ml of 4% w/v sodium potassium tartrate plus 1 ml of 2% w/v $CuSO_4$ $5H_2O$; reagent II was 3.5 ml of Folin-Ciocalteux reagent diluted by addition of 7 ml of distilled water. The assay

procedure was as follows : to 250 µl of protein solution was added 2.5 ml of reagent I; this mixture stood at room temperature for 10 minutes when 0.25 ml of reagent II was added with vortexing. After 30 minutes A_{660} was measured in a Gilford Microsample spectrophotometer. A standard curve was prepared at the same time, using 0-250 µl of a stock solution of bovine serum albumin 0.5 mg/ml.

9. SYNTHESIS OF PROTEIN

Synthesis of protein in bacterial cultures was measured as the incorporation of either L- U-$[^{14}C]$-leucine or L-$[^{35}S]$-methionine into ice-cold 5% w/v trichloroacetic acid (TCA) precipitable material. Details of labelling conditions are given in figure legends. TCA precipitates were collected on to 27 mm membrane filters (Sartorius, 0.45 um pore size) by suction, and washed extensively with several 5-10 ml volumes of ice-cold 5% TCA. Filters were dried under an infra-red lamp and transferred to plastic scintillation vials. To each vial was added 0.5-1 ml of non-aqueous scintillation fluid containing per litre of toluene : 33 mg of dimethyl POPOP and 5g of 2,5-diphenyloxazole (PPO). The plastic vials were stoppered and placed in standard Packard glass scintillation vials, and the samples counted in a Packard Liquid Scintillation Spectrophotometer.

In some experiments, indicated in figure legends, the total radio-activity in SDS lysates of washed cells was used as a measure of protein synthesis. Small volumes (5-10 µl) of lysates were transferred to plastic scintillation vials and 1 ml of aqueous scintillation fluid containing, per litre : toluene, 850 ml, Biosolv solubiliser 150 ml, butyl-PBD 8g was added. The vials were then stoppered and counted as above.

Pulse-labelling of Exponential Cultures

In many experiments the instantaneous rate of synthesis of protein was measured by pulse-labelling at 37°C. Samples of the culture, usually 1.0 ml, were transferred to 25 ml beakers (acid cleaned) containing 1.0 ml of prewarmed labelling medium from a stock of medium containing [^{14}C]-leucine or [^{35}S]-methionine. After a fixed period of 2-5 minutes 2.0 ml of an ice-cold solution was added, containing chloramphenicol (final concentration, 300 µg/ml) and leucine or methionine as appropriate (final concentration 1.875 mg/ml). Labelled samples were kept on ice until processed.

In experiments in which membranes were not to be prepared, pulse-labelling was carried out in centrifuge tubes, thus facilitating the preparation of cell lysates.

10. SYNTHESIS OF DNA

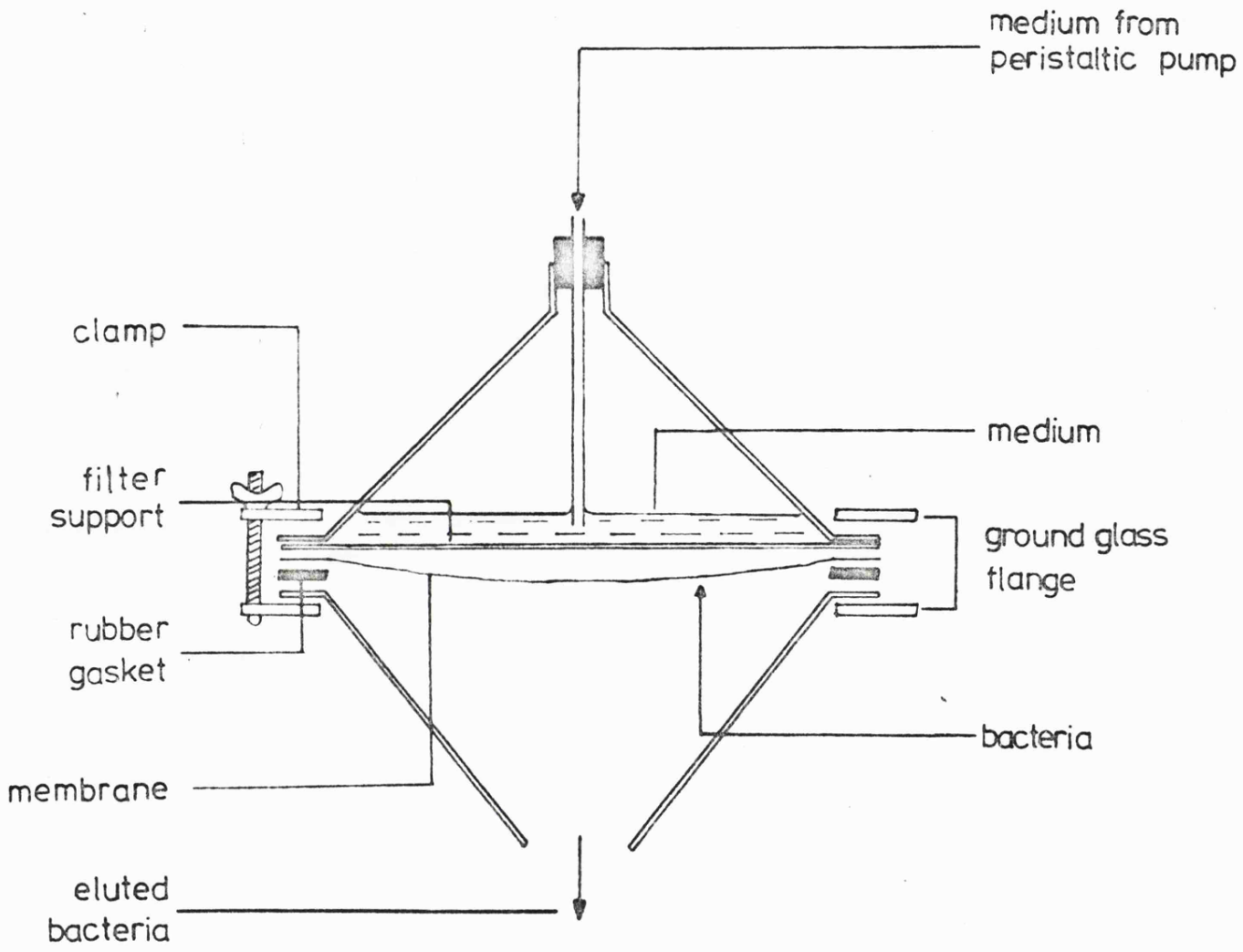
Synthesis of DNA was measured as the incorporation of [methyl-^{3}H] -thymine into 5% TCA-insoluble material. Samples were processed as described above for protein, except that the precipitates were washed with distilled water at 90-100°C.

11. AGE FRACTIONATION OF BACTERIAL CULTURES

Age fractionation of bacterial cultures was achieved by the membrane elution method described by Helmstetter (1967), and was carried out entirely in a 37°C constant temperature room. A 100 ml exponential culture of E.coli B/r LEB16 in proline/alanine medium, at a density of 10^8 cells/ml (A_{450}=0.125), was bound by suction to a nitrocellulose filter (Millipore, grade GS, 0.22 µm pore size) held in the apparatus shown in Figure 2.1. The bacteria were washed with 2x100 ml of medium,

Figure 2.1: MEMBRANE ELUTION APPARATUS (DRAWN BY G.G. CHURCHWARD)

The figure is not drawn to scale.


medium from
peristaltic pump
clamp
medium
filter
support
ground glass
flange
rubber
gasket
bacteria
membrane
eluted
bacteria

with care taken to keep the filter moist, and then the filtration apparatus was inverted. Fresh medium was pumped into the upper reservoir using a peristaltic pump; for the first 6 minutes the flow rate was adjusted to about 20 ml/minute and the apparatus was shaken by hand to remove unbound cells; the flow rate was then decreased to 6 ml/minute and the eluate from the filter was collected over 4 minute periods into large test tubes. Bacterial cell number in each fraction was measured by taking 2.0 ml samples and proceeding as described above.

Pulse-labelling for Membrane Elution

To measure the rates of synthesis of protein fractions and individual proteins in different age fractions the initial exponential culture was labelled by addition of $[^{14}C]$-leucine or $[^{35}S]$-methionine; after a short period (usually 4 or 5 minutes) the culture was bound to a nitrocellulose filter for age fractionation as described above; labelling was thus terminated by filtration and washing. Precise details of labelling procedures used are given in figure legends.

For measurement of total cellular protein synthesis a sample of each eluate fraction, usually 1.0 or 2.0 ml (i.e. 10^6-10^7 cells), was taken into an equal volume of 10% w/v TCA, and the samples were processed as described above.

For measurement of protein synthesis in membrane fractions, samples of eluate fractions, (5.0 or 10.0 ml), were transferred to 25 ml beakers containing chloramphenicol (final concentration 250 μg/ml) and membrane fractions were prepared as described below.

For the analysis of cell lysates by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), samples of eluate (5.0 or 10.0 ml) were

transferred to centrifuge tubes containing chloramphenicol (as above), and the cells lysed as described below.

12. PREPARATION OF CELL LYSATES FOR SDS-PAGE

Cells (10^7-10^8) were transferred to centrifuge tubes and [^{3}H]-leucine-labelled cells were added (10^9 per sample) to provide carrier material and an internal standard (see section 16, below). In experiments in which an internal standard was not required, unlabelled carrier cells (10^9 per sample) were added. The cells were harvested and washed twice in ice-cold 10 mM sodium phosphate buffer, pH7.2, by successive centrifugations in the Sorvall SS34 rotor (10,000 rpm, 5 minutes, 4°C). The washed cell pellet was resuspended in 50 µl of phosphate buffer, transferred to an Eppendorf vial, and 50 µl of SDS electrophoresis sample buffer was added. The samples were immediately boiled for 5-10 minutes to complete cell lysis and to solubilise protein, and then the samples were vortexed to shear DNA. Cell lysates were stored at -20°C and were always reboiled before electrophoresis.

13. PREPARATION OF BACTERIAL CELL ENVELOPES FROM SONIC LYSATES

The basic procedure was that described by Churchward and Holland (1976a), except that $MgSO_4$ was omitted from the buffer. Cells (10^7-10^8) labelled with [^{14}C]-leucine or [^{35}S]-methionine, were transferred to 25 ml beakers and, in many experiments, approximately 3×10^8 [^{3}H]-leucine-labelled cells were added to provide an internal standard (see section 16). Unlabelled carrier cells, from an exponential phase culture of LEB16 or LEB18, grown to an A_{450}=0.5 in the same medium as that used in the experiment, were added to each sample to give approximately 2×10^{10} cells per sample.

The combined sample (final volume adjusted to at least 10 ml by addition of ice-cold 10 mM sodium phosphate buffer, pH7.2), was sonicated for three 30 second intervals, with 30 second cooling periods, using the $\frac{3}{4}$ inch end diameter probe in a 150 watt M.S.E. ultrasonic disintegrator. This and all subsequent operations were carried out at 4°C in sodium phosphate buffer.

Sonicated samples were transferred to centrifuge tubes and centrifuged in the Sorvall SM24 rotor at 7,000 rpm for 5 minutes to remove unlysed cells. The cleared lysates were transferred to another centrifuge tube and centrifuged in the same rotor at 18,000 rpm for 30 minutes. The pellet of cell envelope material was resuspended in 1 ml of buffer and transferred to a polycarbonate centrifuge tube, followed by the addition of 5 ml of buffer. The envelopes were repelleted in a Beckman ultracentrifuge using either the Type 40 rotor (35,000 rpm, 30 minutes) or the Type 35 rotor (30,000 rpm, 30 minutes). The washed envelope pellet was drained, the tube walls dried carefully, and then the pellet was resuspended in 50 µl of phosphate buffer, transferred to an Eppendorf vial, and solubilised by addition of 50 µl of electrophoresis sample buffer. Alternatively the envelope was fractionated into outer and cytoplasmic membrane material as described below.

14. SEPARATION OF OUTER AND CYTOPLASMIC MEMBRANES BY USE OF SARKOSYL

The method used was based upon the report of Filip *et al.*, (1973). Washed envelope pellets, isolated as described above, were resuspended (still in polycarbonate tubes) in 100 µl of 0.5% w/v sarkosyl NL97, and incubated for 30 minutes at room temperature. The outer membrane, which is insoluble in this detergent, was recovered by centrifugation

in a Beckman ultracentrifuge using either the Type 40 rotor (39,000 rpm, 2 hours) or the Type 35 rotor (33,000 rpm, 2 hours). The supernatents, representing the cytoplasmic membrane, were transferred to Eppendorf vials. The walls of the centrifuge tubes were dried to remove traces of supernatent, and then the pellets were resuspended in 50 µl of phosphate buffer and transferred to Eppendorf vials. Both membrane fractions were prepared for SDS-PAGE by addition of 50 µl of electrophoresis sample buffer and boiling for 5 minutes. Samples were stored at -20°C and were always reboiled before electrophoresis.

Radioactivity in membrane fractions was measured by taking samples into aqueous (Biosolv) scintillation fluid.

15. PREPARATION OF CELL ENVELOPES BY OSMOTIC LYSIS OF SPHEROPLASTS

The basic method used was that of Osborn et al. (1972a), and was performed at 4°C. Cells from a 175 ml exponential culture (2×10^8 cells/ml) were harvested by centrifugation in a Sorvall GSA rotor (7,000 rpm, 10 minutes) and resuspended in 6 ml of ice-cold 10 mM Tris-HCl, pH7.8, containing 0.75M sucrose. Egg white lysozyme was added, from a stock prepared in 10 mM sodium phosphate buffer, pH7.2, to a final concentration of 100 µg/ml, and the suspension incubated for 3 minutes on ice, with continuous slow stirring. This was followed by very slow, dropwise addition of 2 volumes of 1.5 mM EDTA pH7.5. The suspension was incubated, stirring, on ice for 10-15 minutes until conversion to spheroplasts was $>$ 95% (as monitored by phase contrast microscopy), and then the spheroplasts were lysed by the addition of 3 volumes of ice-cold distilled water. The lysate was transferred to centrifuge tubes, vortexed to shear DNA, and then cell envelopes were

isolated by the centrifugation procedure described in section 13 above, except that the Sorvall SS34 rotor was used throughout.

Separation of Outer and Cytoplasmic Membranes on Sucrose Gradients

The washed envelope pellet was resuspended in 1.0 ml of 25% w/w sucrose containing 5 mM EDTA pH7.5. Gradients were prepared by layering successive 2.1 ml volumes of 50, 45, 40, 35 and 30% w/w sucrose over a cushion of 1.5 ml of 55% w/w sucrose; all these solutions contained 5 mM EDTA, pH7.5. The resuspended envelope material was layered onto the gradient, and then centrifuged in a Beckman ultracentrifuge using the SW40 rotor (35,000 rpm, 14 hrs).

Fractions (15 drops) were collected from the bottom of the gradient by puncturing the cellulose nitrate centrifuge tube with a fine needle. To accelerate the procedure, air was pumped into the top of the tube by means of a peristaltic pump. The refractive index of every fifth fraction was measured in a refractometer (Bellingham and Stanley Ltd., London). For determination of radioactivity in each fraction, 5 µl samples were taken and counted in aqueous scintillation fluid. For isolation of membrane material, fractions from the various peaks of radioactivity were transferred to polycarbonate centrifuge tubes. The tubes were filled up with 10 mM sodium phosphate buffer, pH7.2, and membrane material isolated by centrifugation in the Type 40 rotor (35,000 rpm, 2 hrs). This pellet was resuspended in 100 µl of phosphate buffer; 50 µl of this suspension was removed to an Eppendorf vial for preparation of the samples for SDS-PAGE. For determination of the proportions of sarkosyl-soluble and insoluble material in each density fraction, 50 µl of 1% sarkosyl was added to the remaining suspension in the centrifuge tube and the insoluble material recovered as described in section 14.

16. USE OF AN INTERNAL STANDARD OF [^{3}H]-LEUCINE-LABELLED CELLS

In many of the experiments described, incorporation of radioactive amino acids into cell protein fractions and individual gel bands was measured. A method was devised to obviate the need for reproducible quantitative recovery from sample to sample. A 30 ml exponentially growing culture (A_{450}~0.1) was labelled by addition of 100-200 µCi of [^{3}H]-leucine (53 Ci/mmol). After two generations of growth unlabelled leucine was added (final concentration 20 µg/ml) and the cells chilled.

To samples of cells labelled with [^{14}C]-leucine or [^{35}S]-methionine was added a constant volume of the ^{3}H-leucine-labelled culture, immediately prior to preparation of cell lysates or envelopes. The ratio of ^{14}C or ^{35}S/^{3}H radioactivities in any protein fraction or gel band derived from these samples was thus a measure of the relative amounts of ^{14}C or ^{35}S radioactivity in the whole of the original samples.

Double-labelled samples were processed for determination of radioactivity by the normal procedures described elsewhere. Counting was performed using the ^{14}C/^{3}H setting of a Packard Model 3255 Liquid Scintillation Spectrophotometer. Samples labelled with each of the isotopes alone were always processed in parallel with experimental samples for measurement of cross-channel spillover. The spillover from the ^{3}H channel to the ^{14}C channel was always $< 1\%$ and was disregarded; spillover in the opposite direction was 40-100%, and ^{3}H cpm were always corrected for this spillover before calculation of isotope ratios. The ratio ^{3}H radioactivity/^{14}C radioactivity in samples was usually $>$ 10:1, so that the spillover correction was $< 10\%$ of total ^{3}H radioactivity.

Table 2.1

SOLUTIONS AND BUFFERS USED IN ELECTROPHORESIS

A. SEPARATING GEL BUFFER:

0.75M Tris HCl, pH8.8, 0.2% w/v SDS.

B. STACKING GEL BUFFER:

0.25M Tris HCl, pH6.8, 0.2% w/v SDS.

C. ACRYLAMIDE SOLUTION:

I 30% w/v acrylamide, 0.8% w/v N;N'-methylene-bis-acrylamide (bis)

II 44% w/v acrylamide, 0.8% w/v bis

III 44% w/v acrylamide, 0.3% w/v bis

D. ELECTROPHORESIS BUFFER:

0.125M Tris, 0.192M glycine, 0.1% w/v SDS

(gives pH - 8.3 with no adjustment)

E. SAMPLE BUFFER:

0.0625M Tris HCl pH6.8, 20% w/v glycerol, 4% w/v SDS, 5% w/v mercaptoethanol

11% SEPARATING GEL:

20 ml buffer A

9.4 ml acrylamide CII or CIII

8.4 ml distilled water

0.95 ml ammonium persulphate (10 mg/ml)

0.075 ml TEMED

17. POLYACRYLAMIDE GEL ELECTROPHORESIS

The basic procedure and buffer system was that of Laemmli (1970). The constitution of the various buffers and solutions used is given in Table 2.1. For some experiments acrylamide solution CI was used to prepare a 12.5% separating gel and a 5% stacking gel. Better overall resolution was obtained by use of acrylamide solution CII, and this was used routinely. For some experiments in which extra resolution of high molecular weight bands was desired, acrylamide CIII was used (Hancock et al., 1976). After mixing, gel solutions were deaerated for several minutes before addition of catalysts and final pouring. Freshly prepared ammonium persulphate solution was always used. Electrophoresis was carried out using a Bio-Rad slab gel system (Model 220). Samples were prepared as described elsewhere. For the amounts of material described in methods the following sample volumes were loaded : cell lysates, 25 µl (≃20 µg protein); outer membrane preparations, 25 µl (≃15 µg protein); and cytoplasmic membrane preparations, 40 µl, (≃30 µg protein). Electrophoresis was carried out at a constant current of 25 mA per gel, until the tracking dye was 1 cm from the bottom of the gel (about 4 hours). Gels were stained overnight in 300 ml 10% v/v acetic acid, 25% v/v isopropanol, 0.05% w/v Coomassie brilliant blue. Diffusion destaining was carried out by shaking the gel in 300 ml of 10% v/v acetic acid, 10% isopropanol for two three hour periods. Gels were stored in 10% v/v acetic acid and were photographed on 4"x5" Ilford FP4 film plates using background illumination and a deep orange filter.

Fluorography

Radioactive gels were dehydrated by two successive washes in dimethylsulphoxide, followed by impregnation with PPO exactly as

described by Bonner and Laskey (1974). The gels were then dried on to a sheet of Whatman No.17 Chromatography Paper in a Bio-Rad gel drying unit. The dried gels were then placed in a Kodak X-Ray cassette with a Kodak R.P.R X-Omat X-ray film plate and exposed at -80°C. Fluorographs and photographic negatives were scanned using a Joyce-Loebl microdensitometer.

18. DIRECT MEASUREMENT OF RADIOACTIVITY IN INDIVIDUAL GEL BANDS

The method used was that of Ames (1974). Stained radioactive gels were dried onto a sheet of Whatman No.4 Chromatography Paper. Bands of interest were cut out carefully using small scissors, and placed in plastic scintillation vials. A 50 µl aliquot of distilled water was added to each vial, with care being taken to ensure that the gel slices were thoroughly wetted. After 10 minutes, 5 ml of scintillation fluid was added, containing per litre 923 ml of toluene, 77 ml of NCS solubiliser, 3.75 g of PPO, and 56 mg of dimethyl POPOP. The vials were stoppered, vortexed, and incubated in a 37° constant temperature room overnight. The vials were cooled, vortexed and then counted as described elsewhere.

This band cutting procedure was used in two different types of experiment. For the determination of the proportion of total radioactivity in the 36.5K band (see Chapter 8) the method was used to recover the band quantitatively. Analysis of triplicate samples revealed that the method gave extremely reproducible values (coefficient of variation $<5\%$). In most experiments, however, samples were double-labelled and it was only necessary to measure the ratio of ^{14}C or $^{35}S/^{3}H$ in the bands analysed.

Table 2.2

PORIN-SPECIFIC MATERIAL IN 36.5K GEL BAND

Cultures of LEB16 and LEB500(rRM100) were grown in proline/alanine medium (+ 20 µg/ml thymine) to an $A_{450} \simeq 0.2$. A 1 ml sample of each culture was pulse-labelled with 5 µCi $[^{35}S]$-methionine (33 µCi/ug) for 2 minutes. To each pulse-labelled sample was added an aliquot of unlabelled cells of LEB16 as carrier. SDS lysates were prepared and analysed by SDS-PAGE : since both samples contained mostly protein from LEB16, each gave a major 36.5K band. The 36.5K and 44K (EF-Tu) bands were cut out from each slot for determination of band radioactivity, and then the remainder of each slot was cut into small pieces for determination of total radioactivity.

Strain	36.5K porin / total protein %	EF-Tu / total protein %
LEB16	4.0	2.9
LEB500(rRM100)	1.3	3.0

Proportion of material in 36.5K band which is porin-specific

In many experiments the rate of porin synthesis was measured by analysing the major 36.5K band in a gel of cell lysates. To determine the proportion of material in this band which is specific to the 36.5K porin, a simple control experiment was performed. The proportion of total ^{35}S-methionine radioactivity recovered in the 36.5K band in lysates of proline/alanine-grown cells of LEB16 and LEB500(rRM100) was measured; the latter strain specifically lacks the 36.5K porin. The results of this experiment are presented in Table 2.2. It is clear from these measurements that at least 70% of the material in the 36.5K band is porin-specific. This estimate is based on the assumption that LEB500(rRM100) has no residual level of the porin, and is, therefore, possibly an underestimate of the homogeneity of the 36.5K band. In any case, I conclude that analysis of the 36.5K band in cell lysates yields a reasonably accurate measurement of the 36.5K porin.

19. PULSE-CHASE ANALYSIS OF OUTER MEMBRANE PROTEIN SYNTHESIS

For the experiments described in Chapter 10 the following procedure was adopted. A 40 ml exponentially-growing culture of LEB18 in M9-glucose medium at 30°C or 25°C (A_{450}=0.1) was labelled by addition of 200 µCi of [^{3}H]-leucine (53 Ci/mmol). After one hour, unlabelled leucine was added (final concentration 100 µg/ml) to reduce residual labelling to a minimum. After a further 15 minutes, 25 ml of this culture was transferred to a 100 ml beaker in a 37°C water bath. The culture was then labelled by the addition of 50 µCi of [^{35}S]-methionine (680 Ci/mmol). After 30 seconds of labelling unlabelled methionine was added (final concentration 150 µg/ml) and then samples (approximate volume 1 ml) were withdrawn at frequent

intervals into 10 ml of ice-cold M9 medium (without glucose) containing chloramphenicol (final concentration approximately 600 μg/ml) and methionine (final concentration approximately 4 mg/ml).

Duplicate 100 ul aliquots of each sample were withdrawn for determination of TCA-precipitate radioactivity. Aliquots (3 ml) were transferred to centrifuge tubes for preparation of cell lysates. The remainder of each sample was used to prepare membrane fractions.

CHAPTER 3

THE SEPARATION OF THE CYTOPLASMIC AND OUTER MEMBRANES USING SARKOSYL NL-97

I. Introduction

The most commonly used method of separating the cytoplasmic and outer membranes of gram negative bacteria is that of Osborn et al. (1972a). In this procedure (described in detail in Chapter 2) cells are resuspended in an osmotically-protective buffer and subjected to treatment with EDTA and lysozyme. EDTA treatment disrupts the outer membrane, exposing the peptidoglycan to the degradative action of lysozyme, which in turn leads to the formation of osmotically fragile spheroplasts. These spheroplasts may be lysed by dilution (osmotic lysis), by sonication, or by a combination of these two procedures. Crude cell envelopes are isolated from the lysate by centrifugation and loaded onto a sucrose density gradient into which the dense outer membrane material penetrates much further than does the cytoplasmic membrane material. The whole procedure is time-consuming and because of the use of density gradients it is not conveniently used for experiments in which large numbers of samples are to be processed.

Apart from its greater density the outer membrane has another property which enables it to be separated from the cytoplasmic membrane; this is its insolubility in non-ionic detergents such as sarkosyl NL-97 (Filip et al., 1973). Thus, crude envelope preparations obtained from sonic lysates of whole cells may be resuspended in a solution of the detergent whereupon the cytoplasmic membrane is solubilised; the insoluble outer membrane may be recovered by centrifugation, whilst the cytoplasmic membrane material remains in solution in the supernatent.

Table 3.1

YIELDS OF CELL ENVELOPE MATERIAL FROM SONIC AND OSMOTIC LYSATES

(a) A culture of LEB18 (A_{450} = 0.1) growing in minimal glucose medium was labelled by the addition of 100 μCi of [^{35}S]-methionine (450 Ci/mmol). Cells were harvested at A_{450} = 0.5 and aliquots were processed, as described in Chapter 2, for whole cell lysates; sonic lysis; and spheroplasting/osmotic lysis. Washed cell envelopes were prepared from sonic and osmotic lysates, and samples of envelope material were treated with 0.5% sarkosyl, as described in Chapter 2. All yields are calculated on the basis of recovery of radioactive material counted in aqueous (Biosolv) scintillation fluid.

(b) A sample of washed cell envelope material prepared from the osmotic lysate was loaded on to a sucrose density gradient (see Figure 3.2). Peak fractions were pooled, and then membranes were isolated and treated with 0.5% sarkosyl. Yields were measured as above.

(a)

method of preparation	$\frac{\text{envelope protein}}{\text{total protein}}$ %	$\frac{\text{sarkosyl-insoluble protein}}{\text{envelope protein}}$ %	$\frac{\text{sarkosyl-insoluble protein}}{\text{total protein}}$ %
sonic lysis of whole cells	6	45	2.7
osmotic lysis of spheroplasts	19	29	5.5

(b)

peak (see Figure 3.2)	peak density (g/ml)	% of material sarkosyl-insoluble
A	1.24	66
B	1.20	49
C	1.17	7
D	1.14	< 1

This method of membrane isolation is convenient since sonic lysis of whole cells is a relatively rapid process and detergent separation of the two membranes allows large numbers of samples to be processed. In the present study I have used this latter method of membrane preparation almost exclusively. However, since many other workers use a gradient separation method, I carried out an experiment to compare the two methods for yield and protein composition of the membrane fractions recovered.

II. Comparison of Cell Envelopes Obtained from Osmotic and Sonic Lysates

The data presented in Table 3.1(a) show the yield of envelope proteins obtained from sonic lysates of whole cells and from osmotic lysates of spheroplasts; also shown is the distribution of this envelope material between sarkosyl-soluble and - insoluble fractions. These various fractions were also analysed by SDS-PAGE, shown in Fig. 3.1, slots 2-5. The envelope material obtained from the two lysates was very similar in polypeptide composition, and there was no significant contamination of the envelope preparations by major soluble proteins such as elongation factor (EF)-Tu or the β and β' subunits of RNA polymerase (compare slot 1). It seems, therefore, that sonic lysis of whole cells leads to the isolation of envelope and sarkosyl-insoluble envelope fractions qualitatively similar to those obtained by osmotic lysis of spheroplasts. Considerably less material is recovered from sonic lysates however, with the yield of sarkosyl-soluble material being especially reduced (see Table 3.1).

Figure 3.1: ANALYSIS OF VARIOUS MEMBRANE FRACTIONS BY SDS-PAGE

Various membrane fractions from the experiment described in the legends to Table 3.1 and Figure 3.2 were subjected to SDS-PAGE. The figure is a photograph of the stained gel.

slot 1 : whole cell lysate

slot 2 : cell envelope material from osmotic lysate

slot 3 : cell envelope material from sonic lysate

slot 4 : sarkosyl-insoluble envelope material from osmotic lysate

slot 5 : sarkosyl-insoluble envelope material from sonic lysate

slot 6 : peak A from gradient shown in Figure 3.2

slot 7 : " B " " " "

slot 8 : " C " " " "

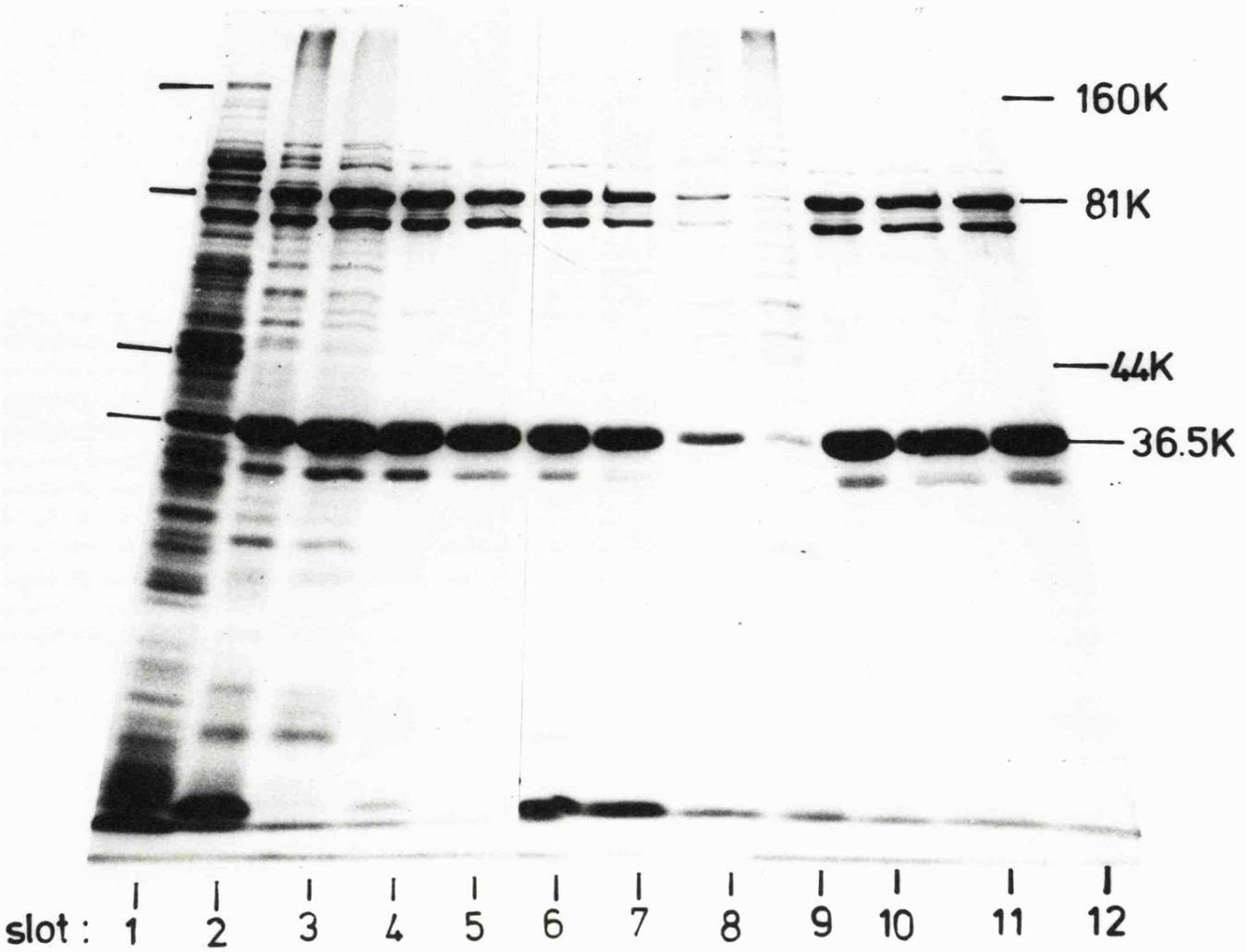
slot 9 : " D " " " "

slot 10 : sarkosyl-insoluble material from peak A

slot 11 : " " " B

slot 12 : " " " C

N.B. The heavily-staining band running just behind the gel front in slots 2, 6 and 7 is lysozyme, added during the preparation of spheroplasts.

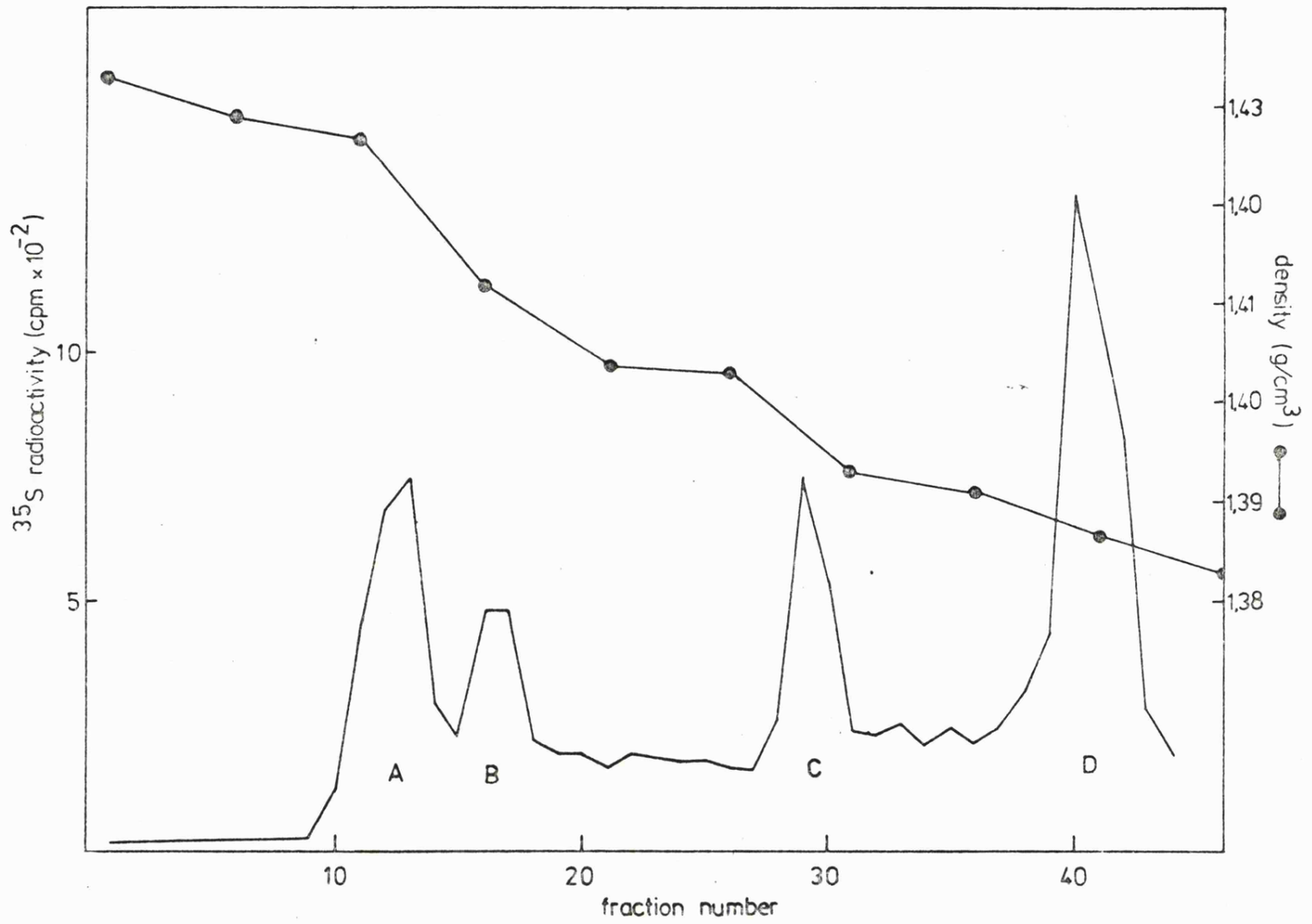

160K
81K
44K
36.5K
slot : 1 2 3 4 5 6 7 8 9 10 11 12

III. Comparison of Sarkosyl Treatment and Density Gradient Centrifugation as Methods of Separating Cytoplasmic and Outer Membranes

In order to demonstrate the correlation between the density of a membrane fraction on a sucrose density gradient and its insolubility in sarkosyl, crude envelope material from the osmotic lysate was subjected to sucrose density gradient centrifugation, as shown in Figure 3.2. Material from each peak was analysed by SDS-PAGE (Fig. 3.1; slots 6-9) and, in addition, the proportion of material which was sarkosyl-insoluble was determined (Table 3.1(b)). This sarkosyl-insoluble material was also analysed by SDS-PAGE (Fig. 3.1; slots 10-12). Of the material present in the densest peak, (A), which is normally found to be outer membrane, almost 70% was sarkosyl-insoluble and qualitatively similar to the sarkosyl-insoluble material obtained directly from either osmotic or sonic lysates. The less dense peaks B, C and D were found to contain progressively less sarkosyl-insoluble material, which was nevertheless qualitatively similar to other insoluble fractions. Those polypeptides which were selectively solubilised from peaks A, B and C by sarkosyl can be seen in Fig. 3.1 to be those polypeptides enriched in peak D, which are presumably cytoplasmic membrane proteins. It seems, therefore, that the four peaks of membrane recovered from the gradient represent four different mixtures of sarkosyl-insoluble and sarkosyl-soluble material. It should be noted that although no sarkosyl-insoluble material was recovered from peak D it nevertheless contained significant amounts of the 36.5K porin, a major outer membrane protein.

Figure 3.2: SUCROSE GRADIENT ANALYSIS OF MEMBRANES OF LEB18

Washed cell envelopes prepared from an osmotic lysate of LEB18 (see Table 3.1) were loaded on to a 25-55% w/w sucrose gradient. Centrifugation, fractionation, and measurement of refractive indices were as described in Chapter 2.


35S radioactivity (cpm x 10-2)
10
5
1,43
1,40
1,41
1,40
1,39
1,38
density (g/cm3)
A
B
C
D
10
20
30
40
fraction number

IV. Quantitation of the Amount of Presumptive Outer Membrane Protein in the Sarkosyl-Soluble Fraction of Crude Envelopes

Although the data presented above indicate that the sarkosyl-insoluble envelope fraction contains only outer membrane proteins, they do not demonstrate the nature of the soluble fraction. The gel shown in Fig. 3.3 is of sarkosyl fractions of cell envelopes isolated from a sonic lysate. It is clear that the sarkosyl-soluble fractions contain significant levels of the 36.5K porin, a protein which is thought to be specifically associated with the outer membrane. Although it cannot be ruled out that this sarkosyl-soluble porin has biological significance it seems more likely that it simply reflects the imperfection of the sarkosyl fractionation; this latter interpretation is supported by the obvious increase in the level of soluble porin produced by use of a higher detergent concentration. The data presented in Table 3.2 show the proportions of the porin present in the sarkosyl fractions shown in Fig. 3.3, as determined by microdensitometry. Treatment with 0.5% sarkosyl led to the appearance of almost 25% of the total envelope porin in the supernatent; when the concentration of detergent used was 1% the soluble fraction of the porin increased to almost 40% of the total. The final column of Table 3.2 shows the proportion of sarkosyl-soluble envelope protein which is actually outer membrane specific, calculated by assuming that all outer membrane proteins fractionate in the same way as the porin. In most of the experiments described in subsequent chapters the concentration of detergent used was 0.5%; under these conditions the sarkosyl-soluble fraction comprises about 87% cytoplasmic membrane protein and 13% outer membrane protein.

Figure 3.3: EFFECT OF SARKOSYL CONCENTRATION UPON FRACTIONATION

Washed cell envelopes were prepared from a sonic lysate of cells of LEB16 labelled with [^{3}H]-leucine (53 Ci/mmol) and harvested at A_{450} = 0.2. Aliquots of the cell envelope suspension were treated with 0.5% or 1% sarkosyl. Soluble and insoluble fractions were isolated, and equal volumes from each fractionation were analysed by SDS-PAGE. The figure shows a photograph of the stained gel.

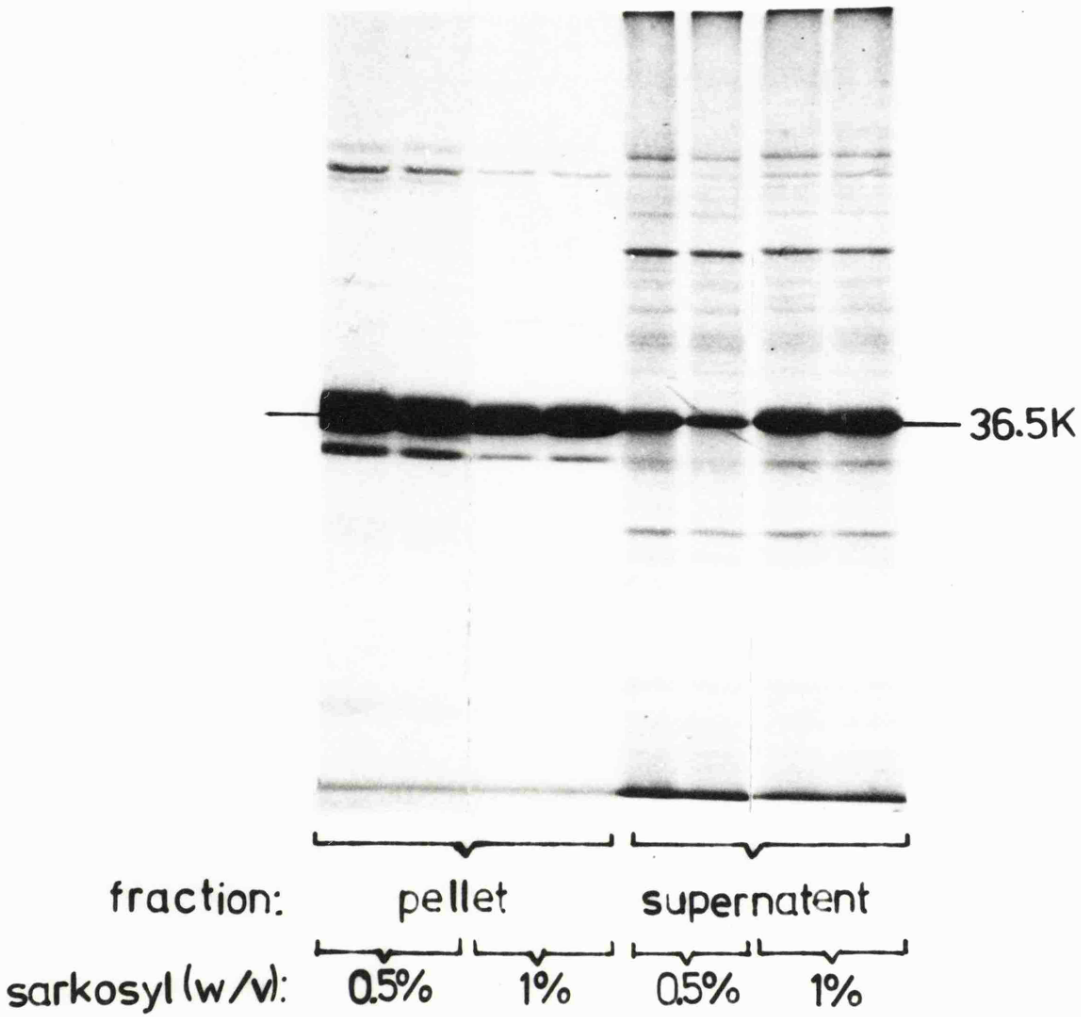

36.5K
fraction:
pellet
supernatent
sarkosyl (w/v):
0.5%
1%
0.5%
1%

Table 3.2

THE EFFECT OF SARKOSYL CONCENTRATION UPON THE DISTRIBUTION OF ENVELOPE PROTEINS BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS

The photographic negative of the gel shown in Figure 3.3 was scanned using a Joyce-Loebl microdensitometer. The peak corresponding to the 36.5K porin band was cut out and weighed; these data were used as estimates of the amounts of porin in each sample (see Chapter 4). This enabled calculation of the proportion of total porin present in the detergent-soluble fraction of each sample (column 2).

The distribution of envelope proteins between detergent-soluble and -insoluble fractions (column 3) was calculated from measurements of radioactivity. Sarkosyl-solubility and -insolubility are equated with location in the cytoplasmic and outer membranes respectively. A corrected distribution of envelope protein between the outer and cytoplasmic membranes was calculated (column 4), assuming that all 36.5K porin is located in the outer membrane, and that the partial sarkosyl solubility of the porin is representative of all outer membrane proteins. The following equation was used:

$$\text{corrected outer membrane \%} = \frac{\text{apparent outer membrane \%} \times 100\ \%}{100 - \text{soluble porin}}$$

From this corrected distribution of envelope material, the proportion of sarkosyl-soluble protein presumed to be specific to the outer membrane (column 5) was calculated as

$$= \frac{\text{apparent cytoplasmic membrane \%} - \text{corrected cytoplasmic membrane \%}}{\text{apparent cytoplasmic membrane \%}} \times 100\%$$

(1)	(2)	(3)		(4)		(5)
sarkosyl concentration (% w/v)	sarkosyl-soluble porin / total porin %	apparent envelope composition		corrected envelope composition		proportion of sarkosyl-soluble material which is presumptive outer membrane %
		outer membrane %	cytoplasmic membrane %	outer membrane %	cytoplasmic membrane %	
0.5	23	30	70	39	61	13
1.0	38	22	78	35	65	17

V. Discussion

Both the generation of osmotically-lysed spheroplasts, and the subfractionation of crude cell envelopes into outer and cytoplasmic membranes using sucrose density gradients are time-consuming procedures. By comparison, sonic lysis of whole cells followed by sarkosyl subfractionation of crude cell envelopes is a rapid process which also allows larger numbers of samples to be easily processed. It is for these reasons that the latter procedure has been used in the present study.

I conclude from the data presented in this chapter that sonic lysis of whole cells gives a lower yield of envelope material than does osmotic lysis of spheroplasts, and that it leads to a differential loss of cytoplasmic membrane. Osmotic lysis is taken as a standard for comparison since it is the gentler of the two lysis procedures. Treatment of crude envelope material with 0.5% sarkosyl yields outer and cytoplasmic membrane protein fractions which are reasonably free of cross-contamination and which are identical in polypeptide composition to those obtained by sucrose density gradient subfractionation. Thus the preparative methods used in the present study yield membrane fractions which are representative of outer and cytoplasmic membranes as defined by gradient methods.

CHAPTER 4

THE USE OF MICRODENSITOMETRY AND FLUOROGRAPHY TO QUANTITATE GEL BANDS

I. Introduction

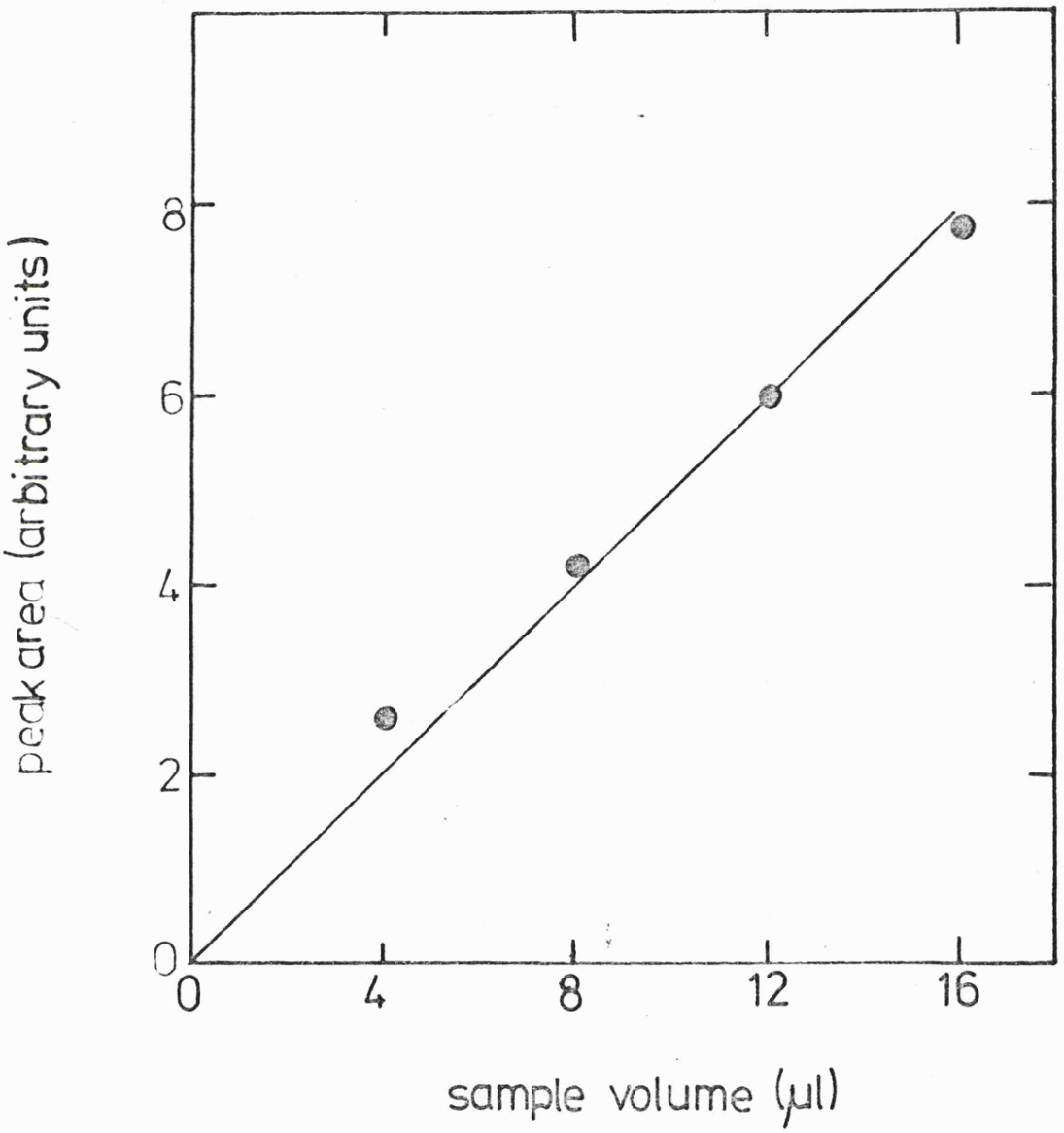
Much of the data presented in this thesis is derived from quantitative analysis of bands on polyacrylamide gels. Such quantitation is often achieved by microdensitometric scanning of gels; this is a potentially powerful technique since a single scan of a gel slot contains a large amount of information. Because of its convenience, microdensitometry was used in a previous study of envelope protein synthesis to measure bands in photographic negatives of Coomassie-blue-stained gels, and in fluorographs of radioactive gels (Churchward, 1975; Churchward and Holland, 1976a). As part of the present study, control experiments were carried out to ascertain the validity of the assumption that these procedures yield true measures of the amounts of material in gel bands.

II. Microdensitometry of Photographic Negatives

Microdensitometry of a photographic negative of a stained gel was used to obtain data presented in Chapter 3 pertaining to the use of sarkosyl to separate the outer and cytoplasmic membranes of the E.coli cell envelope. This use of microdensitometry was based on the assumption that the area under a peak in a densitometric scan is directly proportional to the amount of protein in the corresponding gel band. To test this assumption a simple control experiment was carried out in which different known volumes of the same envelope

Figure 4.1: MICRODENSITOMETRY OF PHOTOGRAPHIC NEGATIVES

Different known volumes of a single preparation of cell envelopes from LEB16 were subjected to SDS-PAGE. The stained gel was photographed on to an Ilford FP4 film plate. The resulting negative was scanned using a Joyce-Loebl micro-densitometer, and the peak corresponding to the 36.5K porin band was cut out and weighed, as a measure of the area under the peak. The data are plotted with the line corresponding to the theoretical ideal response curve drawn in.


peak area (arbitrary units)
0
2
4
6
8
0
4
8
12
16
sample volume (µl)

protein sample were subjected to SDS-PAGE, the stained gel was photographed, and the photographic negative was scanned for the major porin band. The data presented in Figure 4.1 show that the peak area is indeed a reasonable measure of the relative amounts of protein in these gel bands. It should be noted that in such a photographic negative the intensity of the porin band is probably constant, and so the effect of increasing the amount of protein loaded onto the gel is merely to widen the band. This means that the corresponding peaks in the densitometric scan will be of similar height but of variable width.

III. Microdensitometry of Fluorographs of Radioactive Gels

In the study made by Churchward and Holland (1976a) of envelope protein synthesis in E.coli, the rates of synthesis of various polypeptides were deduced by estimating the amount of radioactive material in specific gel bands. This was achieved by impregnating polyacrylamide gels with the scintillant PPO, drying the gels, and then exposing X-ray film to the scintillant-impregnated gels, a process known as fluorography (Bonner and Laskey, 1974), and described in detail in Chapter 2. Churchward and Holland used microdensitometry of fluorographs to estimate radioactivity in gel bands. A control experiment was performed by Churchward (1975) in which different volumes of a single radioactively-labelled membrane preparation were analysed by SDS-PAGE, and a fluorograph of the resulting gel was scanned. Under these conditions the peak area in the scan was found to be directly proportional to the amount of labelled protein in the gel band. This control is not relevant to the experimental system used by Churchward and Holland, however, since in the control experiment the amount of

Figure 4.2: EFFICIENCY OF FLUOROGRAPHIC DETECTION OF RADIOACTIVITY

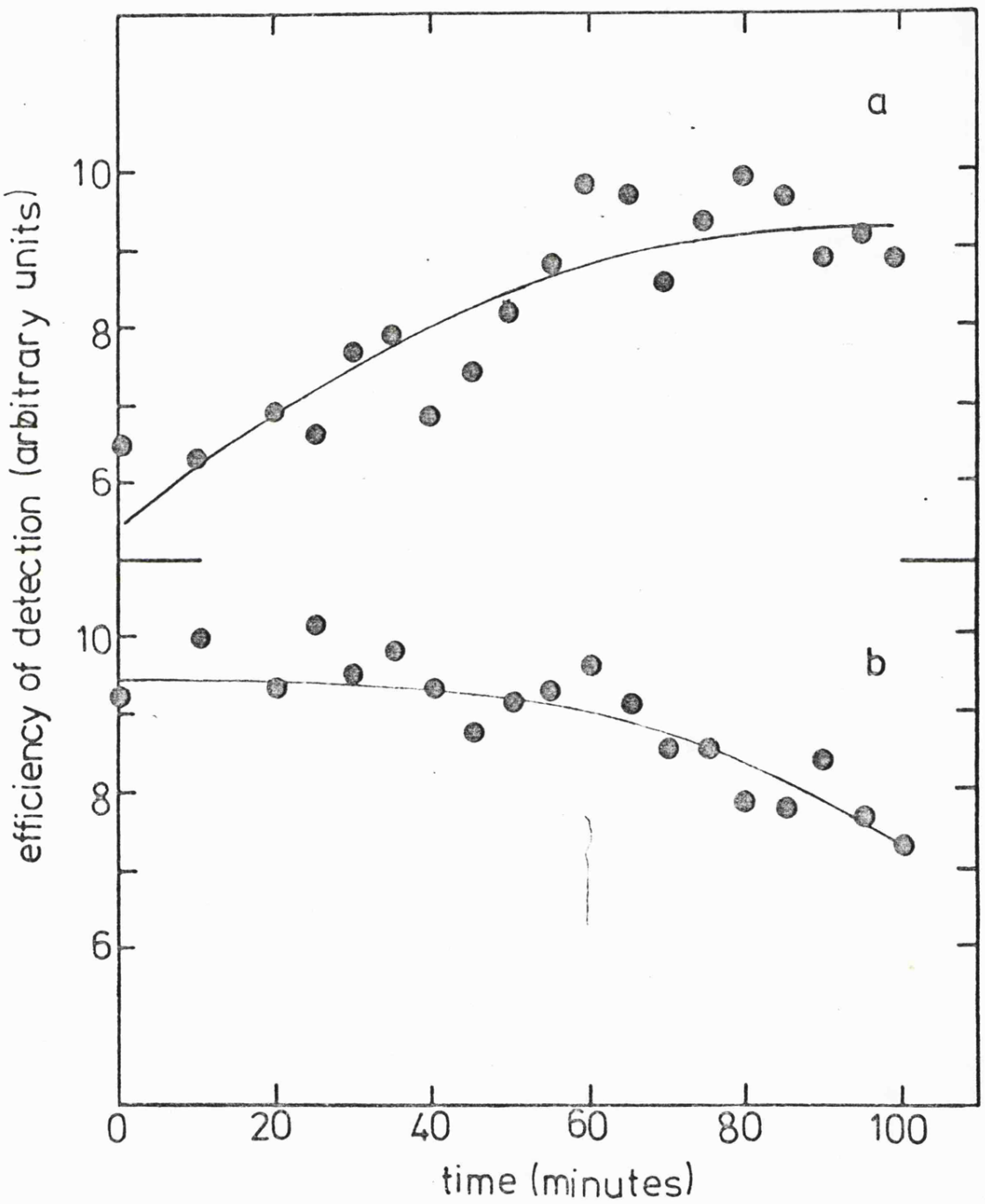
One ml aliquots of a growing culture of LEB16 in proline/alanine medium, supplemented with 20 μg/ml thymine, were pulse-labelled for 4 min with [^{14}C]-leucine (311 mCi/mmol); sampling was carried out over a 100 min period. A constant amount of unlabelled cells was added to each pulse-labelled sample and cell envelopes were prepared. Known volumes of these envelope preparations were analysed by SDS-PAGE. The gel was processed for fluorography and X-ray film plates were exposed to the processed gel as described in Chapter 2. The resultant fluorographs were scanned using a Joyce-Loebl micro-densitometer and the area under the peak formed by the 36.5K porin was determined by cutting and weighing; total radioactivity in each envelope sample was measured by counting an aliquot in Biosolv scintillation fluid. The efficiency of fluorographic detection of the porin band was defined as

$$\text{efficiency of detection} = \frac{\text{porin peak area}}{\text{cpm loaded onto gel}}$$

; this is based upon the assumption that labelled porin accumulates as a constant fraction of total cell envelope radioactivity. The values of efficiency of detection are shown plotted against the time of pulse-labelling; this time is a convenient measure of the specific activity of the porin band since each sample had a constant amount of unlabelled material added.

(a) 3 day exposure

(b) 12 day exposure


a
b
10
8
6
10
8
6
0
20
40
60
80
100
efficiency of detection (arbitrary units)
time (minutes)

radioactive protein of constant specific activity was varied, whereas the experimental procedure generated samples containing a constant amount of protein, but of variable specific activity. Thus in the control experiment the gel band width would be expected to be the major variable, whereas in the actual experiments bands of constant width but of variable intensity would have been obtained in fluorographs. Laskey and Mills (1975) have reported that this parameter, the absorbance of the fluorographic image, is not proportional to the concentration of radioactivity in the gel bands, with small amounts of radioactivity producing disproportionately faint images.

The data shown in Figure 4.2 summarise an experiment performed in the present study in which the response of fluorography was determined over a range of band specific activities. When X-ray film was exposed to the treated radioactive gel for a relatively short time it was found that the radioactivity in the samples of lower specific activity was detected with a lower efficiency than that in samples of higher specific activity (Fig. 4.2(a)). This result is in accordance with the report of Laskey and Mills referred to above. With a longer exposure time this effect was abolished (Fig. 4.2(b)), but instead the most highly radioactive samples were detected with lower efficiency, presumably because the film emulsion was saturated at high levels of exposure.

IV. Discussion

I conclude from the data presented in Section II above that microdensitometry of photographic negatives can be used to measure the amounts of protein in bands on Coomassie-stained gels. This method was used to derive some of the data presented in Chapter 3.

The data shown in Section III indicate that, in contrast, microdensitometry of fluorographic images is not a straight-forward measure of the amounts of radioactivity in gel bands. In the simple experiment which was performed two problems were highlighted : these were (1) inefficient detection at low band density and (2) saturation of the film emulsion with longer exposure times. Careful control experiments would clearly be necessary before fluorography could confidently be used for quantitative experiments. Laskey and Mills (1975) have reported that short pre-exposure of X-ray film to light can sensitise the film to low intensity radiation. This technique could presumably be used to make quantitative fluorography feasible.

As part of their study of envelope protein synthesis in the cell cycle of E.coli Churchward and Holland (1976a) reported that whilst one class of envelope proteins exhibited an abrupt doubling in rate of synthesis, a second class exhibited a tripling in rate at the same time in the cell cycle. These data were derived from microdensitometric analysis of fluorographs. It is clear from the data presented here that this apparent tripling in rate of synthesis is probably due to an erroneous measurement of a doubling in rate of synthesis of proteins in low intensity bands. This type of artifact must be borne in mind by anyone using fluorography in quantitative experiments.

Because of this problem with use of fluorography, alternative methods of quantitation of bands on gels were sought in the present

study. The method used in most of the experiments described below is based on the direct measurement of radioactivity in bands cut from gels, a method described in Chapter 2.

CHAPTER 5

THE PERIODIC PROTEIN

I. Introduction

There have been two independent reports that the synthesis of an envelope protein of *E.coli* B/r is restricted to a brief period of the cell cycle. Gudas *et al.* (1976) used sucrose density gradient fractionation of an exponential culture to generate a synchronous culture and demonstrated that the synthesis of an outer membrane protein of approximate molecular weight 80K, designated protein D, was restricted to one part of the cell cycle. This periodic synthesis of protein D coincided with the initiation of DNA replication at two growth rates. Further evidence for a link between initiation of DNA replication and protein D synthesis came from the demonstration that when a culture of bacteria was treated with nalidixic acid to inhibit DNA synthesis and then the antibiotic was removed by filtering the culture, a burst of protein D synthesis accompanied the rapid reinitiation of DNA synthesis. On the strength of this evidence Gudas *et al.* proposed that synthesis of protein D is a cell cycle event associated with initiation of DNA replication.

Churchward and Holland (1976a) found a similar periodic pattern of synthesis for an envelope protein of approximate molecular weight 76K in synchronous cultures of *E.coli* LEB16, generated by the membrane elution procedure described by Helmstetter, (1967) (see Chapters 2 and 6). In this case, at one growth rate, the periodic synthesis of the protein occurred late in the cycle, around the time of termination of DNA replication. Churchward and Holland also found that if an

exponential culture was pulse-labelled and then fractionated by membrane elution into cells of different age classes, the periodic synthesis of this envelope protein was not detected; this was explained by postulating that the protein was periodically synthesised and then rapidly degraded. Synthesis of this periodic protein was also found to be induced by thymine starvation of E.coli LEB16 (Churchward and Holland, 1976b), and in conjunction with the timing of periodic synthesis in the cell cycle this led to the proposition that synthesis of the protein is linked to termination of replication, or to any other event which causes a cessation of DNA synthesis.

Despite the differences in detail it seems probable that the same phenomenon was being observed in both laboratories. A protein of similar molecular weight to the periodically synthesised protein was subsequently found associated with a membrane-bound chromosome preparation (Portalier and Worcel, 1976), and this rather circumstantial evidence strengthened the case for an involvement of the protein with DNA replication.

II. Synthesis of the 76K Protein is Induced by Filtration of Cultures of E.coli B/r

I originally intended to investigate the role of the 76K protein in the cell cycle as part of the present study. Before embarking upon this investigation I decided, however, to perform a control experiment for the effect of thymine starvation. In this experiment a culture of LEB16 was filtered as for thymine starvation but was resuspended in medium containing normal levels of thymine. The result of this experiment is illustrated in Figure 5.1; these data show clearly that filtration of the culture is sufficient to induce the synthesis of an

Figure 5.1: EFFECT OF FILTRATION UPON OUTER MEMBRANE PROTEIN SYNTHESIS

A culture of LEB16 was grown in proline/alanine medium (+ 20 μg/ml thymine) to an A_{450} = 0.2, when the cells were taken on to a nitrocellulose filter, and washed and resuspended in prewarmed complete medium. This manipulation did not disturb overall growth. Samples were withdrawn at intervals for pulse-labelling (4 minutes) with 0.8 μCi [^{14}C]-leucine (311 mCi/mmol). Outer membranes were prepared from each sample, and analysed by SDS-PAGE. The figure shows a fluorograph of the resulting gel.

slot 1 : 15 minutes before filtration

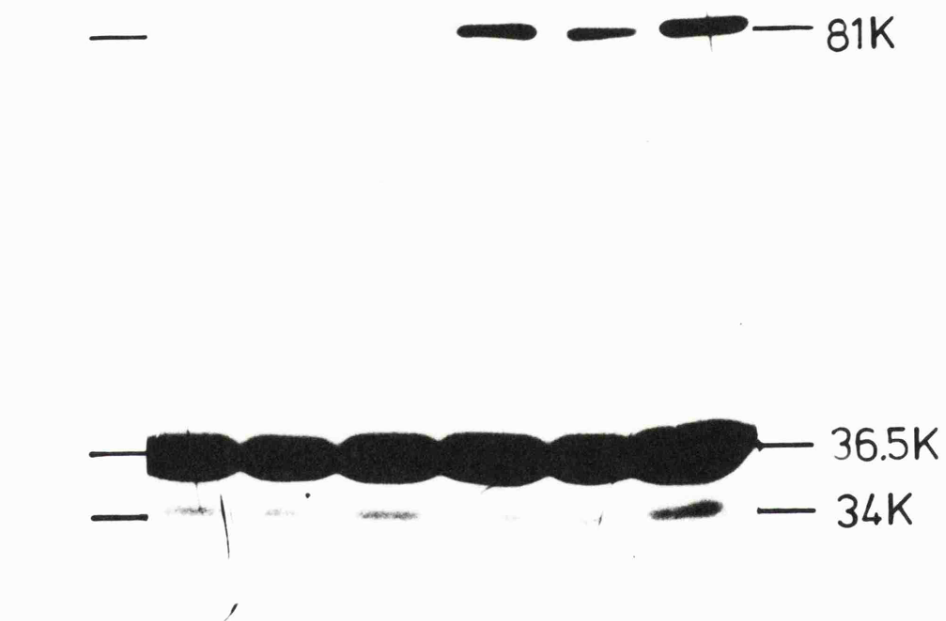
slot 2 : 5 minutes before "

slot 3 : 5 minutes after "

slot 4 : 10 " " "

slot 5 : 20 " " "

slot 6 : 30 " " "



slot: 1 2 3 4 5 6

envelope protein of similar molecular weight to the 76K periodic protein. This induced protein was found to be insoluble in sarkosyl, indicating that it is an outer membrane protein. The molecular weight of this filtration-induced protein makes it virtually certain that it is identical to the periodic protein observed by Churchward and Holland, whilst its location in the outer membrane strengthens the conclusion that it is the same as protein D of Gudas et al. Close examination of Figure 5.1 reveals that two other outer membrane proteins, of molecular weight slightly less than that of the major induced species, appeared after filtration; such minor changes could not have been easily observed in the experiments of Churchward and Holland since an unfractionated envelope preparation was analysed and the resolution of their gels was not as high as that achieved in the present study. It is interesting to note that James (1975) has reported that treatment of cells with the antibiotic mecillinam induces synthesis of protein D; in fact close examination of his data reveals that this treatment also causes induction of other outer membrane proteins. This point will be returned to in the Discussion at the end of this chapter.

The result shown in Figure 5.1 was confirmed and extended by the experiment shown in Figure 5.2, in which a culture of LEB16 was filtered as before and the pattern of outer membrane proteins synthesised was analysed over the subsequent two generations of growth. The data are presented in the form of a microdensitometric analysis of a fluorograph. Data were presented in Chapter 4 to show that fluorography is an unreliable way of detecting radioactivity in gels; however in this experiment the method was used solely to obtain a qualitative picture of the induction process. It is clear from Figure 5.2 that there was no second peak of filtration-induced outer membrane protein

Figure 5.2: PATTERN OF INDUCTION OF OUTER MEMBRANE PROTEINS

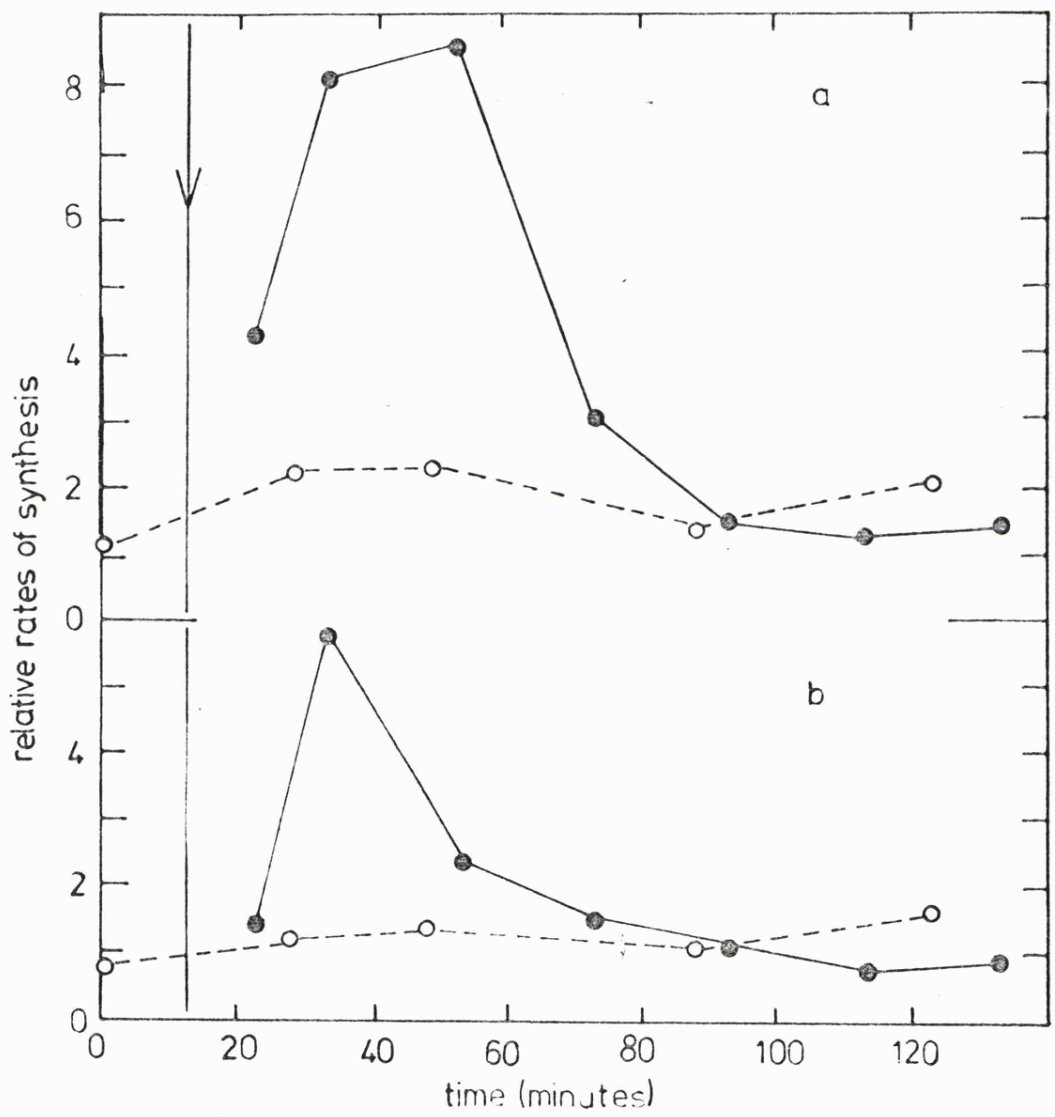
A culture of LEB16 was grown in proline/alanine medium (+ 20 μg/ml thymine) to an $A_{450} \simeq 0.1$, when a 1.0 ml sample was removed for pulse-labelling (4 minutes) with 1 μCi $[^{14}C]$-leucine (311 mCi/mmol). Thirteen minutes later, half of the culture was filtered and resuspended. Samples were subsequently taken for pulse-labelling from both cultures during two generations of exponential growth. Outer membranes were prepared from the pulse-labelled samples and analysed by SDS-PAGE. A fluorograph of the gel was scanned using a Joyce-Loebl microdensitometer, and peaks corresponding to the 81K and 76K induced bands, and also to an uninduced 70K band, were cut out and weighed. The ratios of 81K and 76K/70K were taken as measures of the relative rates of synthesis of the corresponding polypeptides.

O———O : control culture

●———● : filtered culture

(a) 81K polypeptide

(b) 76K polypeptide


relative rates of synthesis
8
6
4
2
0
a
b
4
2
0
0
20
40
60
80
100
120
time (minutes)

synthesis; this is in contrast to the finding by both Gudas et al. (1976) and Churchward and Holland (1976b) that in synchronous cultures a second peak of periodic protein synthesis occurred one generation after the first peak of synthesis. This discrepancy will be considered in the Discussion.

III. Attempts to Repress Filtration-Induced Synthesis by the Use of Conditional Medium and Ferric Chloride

The molecular weights of the group of filtration-induced outer membrane proteins shown in Figure 5.1 are similar to those of a group of inducible outer membrane proteins known to be involved in the transport of chelated ferric iron (Braun et al., 1976; see Section IV of Chapter 1). One of these iron-uptake proteins is the feuB gene product, an outer membrane protein which is necessary for the transport of the ferric form of the chelator enterochelin. Of the three known chelators utilised by E.coli, enterochelin is the only one which is produced by the cells themselves; it is clearly, therefore, the most important chelator to cultures of E.coli growing in defined minimal medium.

In view of the similarity between the filtration-induced outer membrane proteins and the chelated-iron uptake proteins it seemed possible that removal of cells from established culture medium, which presumably contains levels of enterochelin essential for growth, followed by resuspension in fresh medium lacking enterochelin, might be producing transient iron deprivation and consequent induction of the synthesis of uptake systems. To test this hypothesis filtration experiments were performed and the bacteria were resuspended in fresh conditioned medium prepared by filtration or centrifugation of

Figure 5.3: FAILURE OF CONDITIONED MEDIUM TO SUPPRESS INDUCTION OF OUTER MEMBRANE PROTEINS

Cultures of LEB16 were grown in proline/alanine medium (+ 20 µg/ml thymine) to an A_{450} = 0.15, when the cells were filtered as before, except that resuspension was in prewarmed conditioned medium. Conditioned medium was prepared by growing cultures of LEB16 in proline/alanine medium (+ 20 µg/ml thymine) to an A_{450} of 0.15 and then removing the cells from the medium by filtration (a), or by centrifugation (b).

(a) Filtered medium

Samples were withdrawn at intervals for pulse-labelling (4 minutes) with 1 µCi $[^{14}C]$-leucine (311 mCi/mmol). Outer membranes were prepared from each sample and analysed by SDS-PAGE. The Figure shows a fluorograph of the resulting gel.

slot 1 : 20 minutes before filtration

slot 2 : 10 minutes before filtration

slot 3 : 10 minutes after filtration

slot 4 : 20 minutes after filtration

(b) Centrifuged medium

Samples were withdrawn at intervals for pulse-labelling (4 minutes) with 7 µCi of $[^{35}S]$-methionine (290 Ci/mmol). Outer membranes were prepared from each sample, and analysed by SDS-PAGE. The figure shows a fluorograph of the resulting gel.

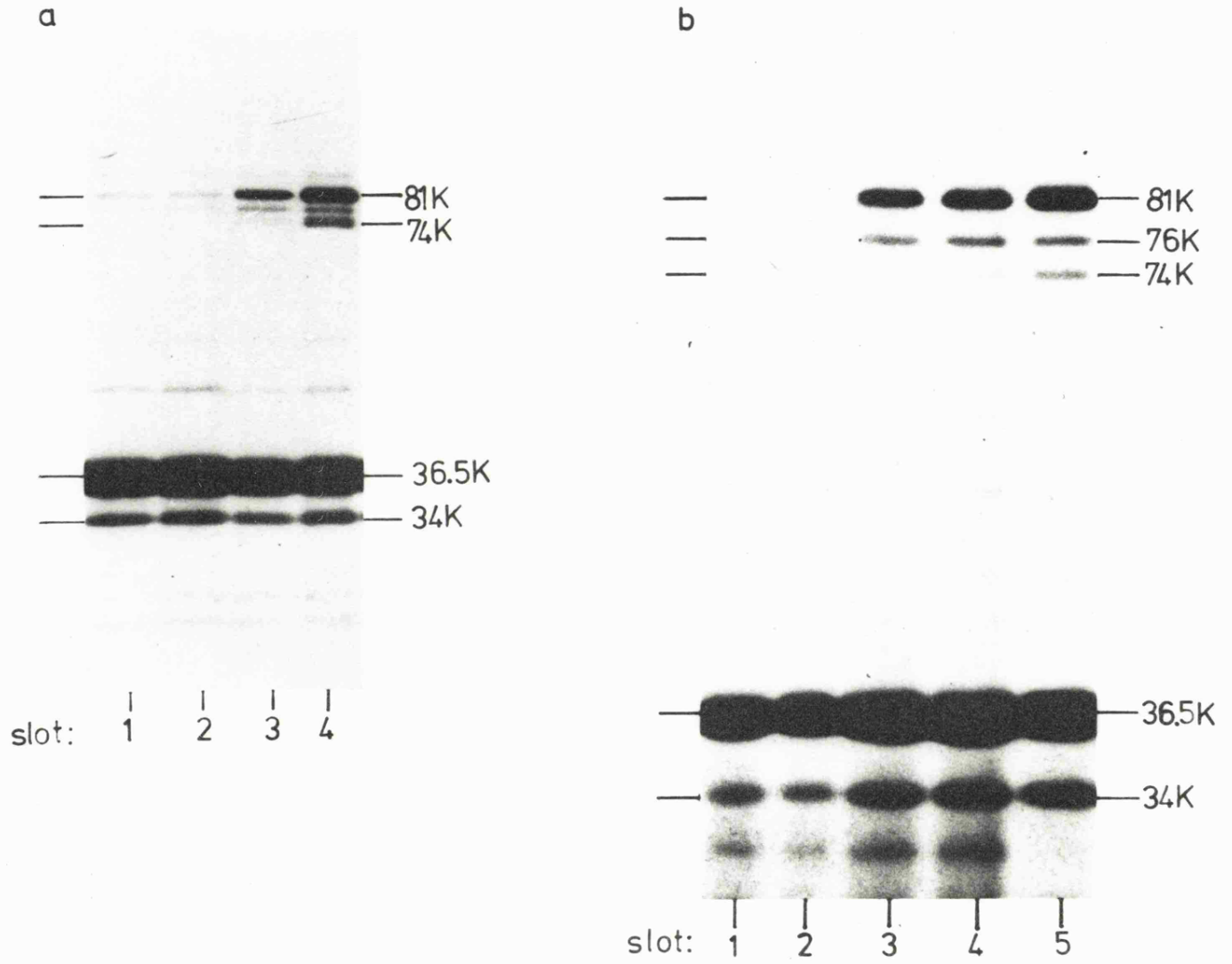
slot 1 : 20 minutes before filtration

slot 2 : 10 minutes before filtration

slot 3 : 10 minutes after filtration

slot 4 : 20 minutes after filtration

slot 5 : 30 minutes after filtration


a
81K
74K
36.5K
34K
slot: 1 2 3 4
b
81K
76K
74K
36.5K
34K
slot: 1 2 3 4 5

exponentially-growing cultures of the same strain in mid-log phase. The results of these experiments are shown in Figure 5.3; neither type of conditioned medium suppressed induction of synthesis of the outer membrane proteins, indicating that induction does not occur merely as a result of the removal of an extracellular component from the growth medium.

The hypothesis that filtration-induced synthesis of outer membrane proteins is due to an iron deficit was further tested by gauging the effect of a high concentration of ferric ions upon the induction process. High levels of iron in the medium are able to enter the cell by low affinity channels which bypass the inducible outer membrane systems. The result of this experiment is shown in Figure 5.4. A filtered culture of LEB16 was resuspended in fresh medium and then half of this resuspended culture was supplemented with 100 μM ferric chloride. As can be seen in the Figure the induction of outer membrane protein synthesis seen in the unsupplemented half of the filtered culture was completely and specifically suppressed by the presence of ferric ions in the unsupplemented half of the culture; in fact the level of synthesis of the proteins was depressed below that in the uninduced culture (compare slots 1 and 3). This result strongly suggests that the phenomenon of filtration-induction is due to some sort of iron starvation.

Figure 5.4: FERRIC CHLORIDE SUPPRESSES INDUCTION OF OUTER MEMBRANE PROTEINS

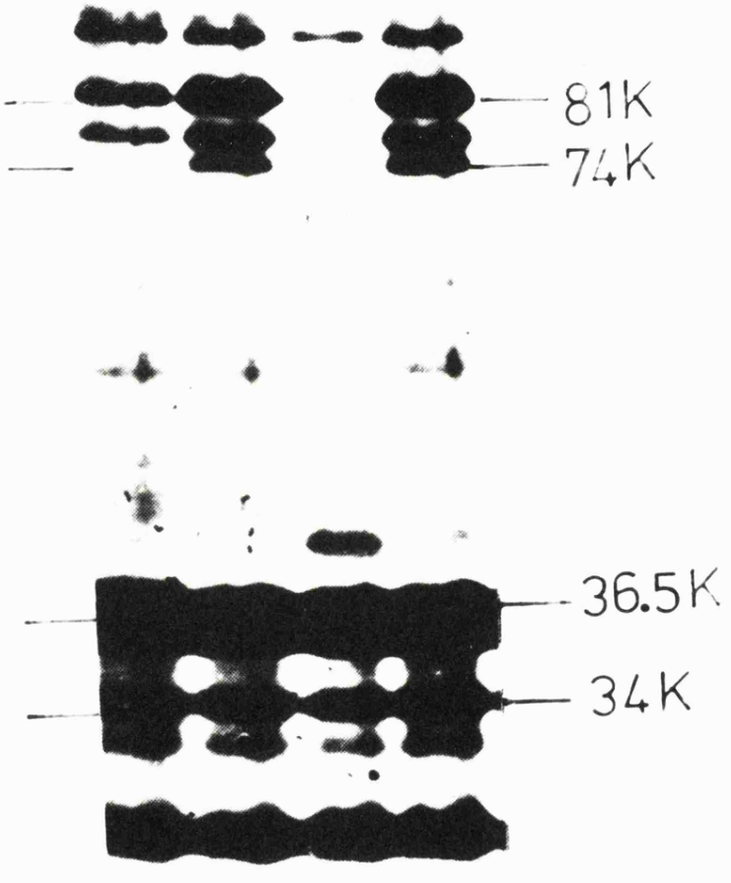
A culture of LEB16 was grown in proline/alanine medium to A_{450} = 0.15, when the cells were filtered, washed and resuspended as before. Half of the resuspended culture was immediately transferred to a flask containing $FeCl_3$ (final concentration 100 μM). Samples were withdrawn at intervals for pulse-labelling (4 minutes) with 8.5 μCi [^{35}S]-methionine (290 Ci/mmol). Outer membranes were prepared from each sample and analysed by SDS-PAGE. The figure shows a fluorograph of the resulting gel.

slot 1 : 10 minutes before filtration

slot 2 : 20 minutes after filtration

slot 3 : 20 minutes after filtration + $FeCl_3$.

slot 4 : 30 minutes after filtration


81K
74K
36.5K
34K
slot:
1
2
3
4

IV. Identification of the Induced Proteins as the Products of the *feuB*, *tonA* and *cir* Loci

The suppression of the filtration phenomenon by the addition of ferric chloride, together with the molecular weights of the filtration-induced proteins, suggested very strongly that the proteins were indeed identical to the group of outer membrane proteins concerned with transport of chelated iron. This identity was confirmed by the experiment summarised in Figure 5.5. In this experiment the iron-chelating compound α,α'-dipyridyl was used to lower the concentration of available iron in growth medium and so produce high rates of synthesis of chelated-iron transport proteins in various bacterial strains. These dipyridyl-induced proteins were compared to those induced in *E.coli* B/r by filtration.

By comparing slots 3, 4 and 5 in Figure 5.5 it can be seen that in *E.coli* B/r the filtration-induced proteins are indeed identical to those induced by 100 μM dipyridyl. The observation of filtration induction in the strain LEB18, which is thy^+, also confirms that the phenomenon is not related to thymine starvation in any way.

Slots 1, 2 and 3 show the induction of synthesis of outer membrane proteins by dipyridyl in a K12 strain, *E.coli* M26. There are clearly four induced proteins, and three of these are apparently identical to those induced by dipyridyl or filtration in *E.coli* B/r. The identification of these proteins is completed by comparing slots 8, 9 and 10. Slot 8 shows the dipyridyl-induced proteins of *E.coli* AB2847 and slot 9 shows the corresponding proteins in *E.coli* AB2847 (*feuB cir*), a mutant strain which lacks the outer membrane protein products of the *feuB* and *cir* loci (Hancock *et al.*, 1976); the missing

Figure 5.5: IDENTIFICATION OF THE FILTRATION-INDUCED OUTER MEMBRANE PROTEINS IN E.COLI B/r

A culture of E.coli K12 M26 was grown in minimal glucose medium to an A_{450} = 0.15, when α,α' dipyridyl (DP) was added to a final concentration of 100 μM. Samples were pulse-labelled (5 minutes) with 15 μCi [^{35}S]-methionine (590 Ci/mmol).

A culture of LEB18 was grown in proline/alanine medium to an A_{450} = 0.15 and then filtered. A sample of the filtered culture was pulse-labelled (5 minutes) with 8 μCi [^{35}S]-methionine (570 Ci/mmol).

Cultures of LEB18, M26, E.coli K12 AB2847 and E.coli K12 AB2847 (feuB cir) were grown in minimal medium in the presence of 100 μM DP or 100 μM citrate, to an A_{450} = 0.5. Samples containing approximately $2x10^{10}$ cells were taken for the preparation of outer membranes.

Outer membranes were prepared in all of the above experiments, and analysed by SDS-PAGE on a single gel. The left-hand part of the figure shows a fluorograph of the radioactive section of the gel, while the right-hand part shows stained material. The discontinuity between the two parts is merely a photographic artefact - the two slots bracketed together as slot 4 are in fact images of the same slot. No other slot is presented in both parts of the figure.

slot 1 : M26, 10 minutes before DP addition (fluorograph)

slot 2 : M26, 10 minutes after DP addition (fluorograph)

slot 3 : M26, 20 minutes after DP addition (fluorograph)

slot 4a : LEB18 + DP (fluorograph)

slot 4b : LEB18 (stained gel; carrier not DP-induced)

slot 5 : LEB18 + DP (stained gel)

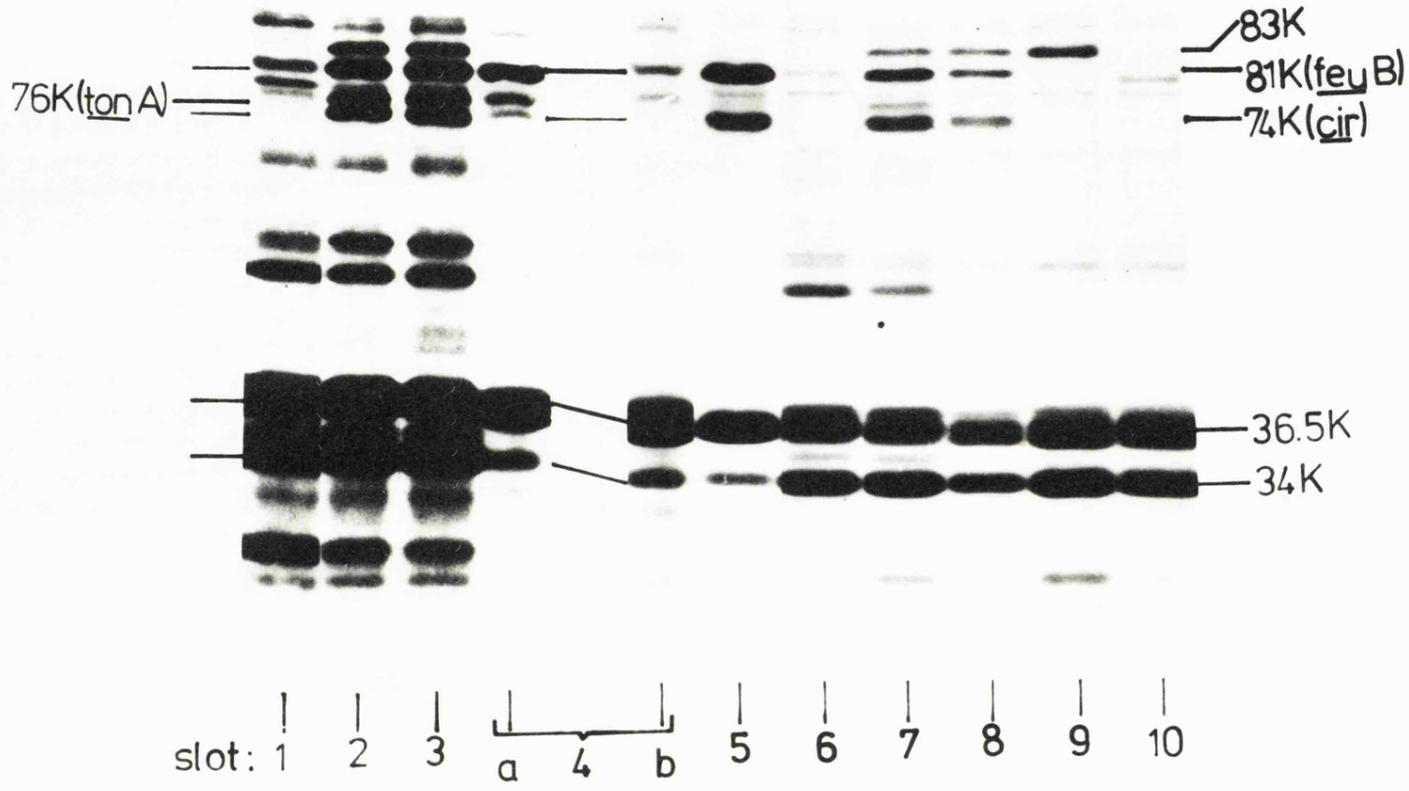
slot 6 : M26 (stained gel)

slot 7 : M26 + DP (stained gel)

slot 8 : AB2847 + DP (stained gel)

slot 9 : AB2847 (feuB cir) + DP (stained gel)

slot 10 : AB2847 (feuB cir) + citrate (stained gel)


76K(ton A)
83K
81K(feu B)
74K(cir)
36.5K
34K
slot: 1 2 3 a 4 b 5 6 7 8 9 10

bands are easily identified by comparison with slot 8. Slot 10 shows AB2847 (*feuB cir*) grown in the absence of dipyridyl, but with 1 mM citrate added to the medium. In comparison with slot 9 there is a protein missing at 83K, and a new protein at 81K. The protein of molecular weight 83K is also induced by dipyridyl in *E.coli* M26 (slots 1 and 2) but is not seen in *E.coli* B/r under any circumstances. The new protein, of molecular weight 81K is presumably the *cit* gene product, a protein involved in transport of ferric citrate which is induced only by the presence of citrate in the medium, not by iron deprivation (Hancock *et al.*, 1976).

In summary these data show clearly that two of the three outer membrane proteins induced by filtration in *E.coli* B/r are the *feuB* and *cir* gene products. The induced protein of intermediate molecular weight is presumably the *tonA* gene product, although this is not proven by these data. The relationship of these induced proteins to the periodically synthesised outer membrane proteins observed by Churchward and Holland (1975) and by Gudas *et al.* (1976) will be discussed below.

V. Discussion

The data presented in this chapter demonstrate clearly that filtration of cultures of *E.coli* B/r induces synthesis of three outer membrane proteins which are involved in the uptake of chelated iron. The reason for this phenomenon is not clear, although the data suggest that it is the act of taking cells onto a nitrocellulose filter *per se* which causes induction, rather than the nature of the resuspension medium. Presumably the physical manipulation of the cells in some way disrupts envelope function, causing a block in iron uptake and a fall in intracellular levels of iron which triggers the induction process.

There is evidence that the process of chelated iron uptake involves a complex interaction between the outer membrane and the electrochemical gradient of protons across the cytoplasmic membrane (Pugsley and Reeves, 1977). It also seems possible that a specific protein, the tonB gene product, is involved in making cytoplasmic membrane energy available to the outer membrane. Thus tonB mutants are specifically blocked in the uptake of chelated iron via the enterochelin, ferrichrome and citrate systems (Braun et al., 1976). The nature and cellular location of the tonB gene product are unknown, but its postulated function makes it possible that it is periplasmic, or only peripherally associated with one or both membranes. Physical damage to the outer membrane caused by binding cells to a nitrocellulose filter could cause leakage of such a molecule, or cause disruption of subtle outer membrane-cytoplasmic membrane interactions.

Relating the filtration-induction phenomenon to the observations made of periodic envelope protein synthesis relies upon tentative extrapolations of my data. The first point in this extrapolation is the deduction that any experiment in which cells are pulse-labelled after a manipulation involving the binding of cells to a nitrocellulose filter would be expected to result in induction of the iron uptake proteins. Experiments which can readily be put under this heading are the thymine starvation experiment of Churchward and Holland (1976b) and the nalidixic acid removal experiment of Gudas et al. (1976). The periodic synthesis of the 76K protein observed by Churchward and Holland (1976a) can also be explained in these terms since the generation of synchronous cultures by the membrane elution technique used in that study involved collection of the eluate from a filter-bound culture, followed by a further filtration to concentrate the eluted cells before

resuspension in fresh warm medium and regrowth as a synchronous culture for pulse-labelling. Thus the periodic 76K protein is identified as the feuB gene product. It is also possible to explain why periodic synthesis was not seen in the age-fractionation experiments described by Churchward and Holland (1975); in this case cells were pulse-labelled before being bound to a filter, so that any proteins induced by the treatment would obviously not be labelled.

The periodic synthesis of protein D reported by Gudas et al. (1976) is more difficult to bring into line with this view. Firstly, the data presented here do not formally prove that the periodically synthesised protein D is the feuB gene product (the major filtration-induced species) although this conclusion seems warranted since Gudas et al. identified the protein induced by removal of nalidixic acid (i.e. by filtration) as being identical to protein D. The synchronous cultures used by Gudas et al. were generated by fractionation of a culture on a sucrose density gradient. The details of the method were not published so that it is not clear whether a filtration step was involved, to wash the cells free of sucrose for example. The fractionated cells will certainly have experienced severe plasmolysis however, and it is possible that this treatment produces disruption of envelope structure similar to that caused by filtration. Indeed it is possible that many treatments may mimic the effect of filtration; one such is treatment with mecillinam, a β-lactam antibiotic which causes cells to become spherical and in addition induces the synthesis of a group of outer membrane proteins, claimed by Gudas et al. to include protein D.

The only aspect of the periodic syntheses of protein D and the 76K protein which cannot be easily explained in terms of the filtration-

induction is the second peak of synthesis which was detected in both of the previous studies. In contrast, my data demonstrate that filtration does not result in a second peak of outer membrane protein synthesis after a further generation of growth.

CHAPTER 6

ENVELOPE PROTEIN SYNTHESIS IN THE CELL CYCLE OF E.COLI B/r

I. Introduction

The pattern of synthesis of cell envelope proteins in the cell cycle of E.coli B/r has been described in two recent reports in the literature. The study of this problem by Churchward and Holland (1976a) showed that in contrast to total cell protein, which accumulates exponentially during the cell cycle, the envelope fraction accumulates at a constant rate which doubles at a point in mid-cell cycle. This latter accumulation pattern will henceforth be referred to as a linear accumulation. Although envelope proteins were not separated into cytoplasmic and outer membrane fractions by Churchward and Holland, individual proteins identified by SDS-PAGE were analysed by micro-densitometry of fluorographs and all seemed to accumulate at a constant rate, although some seemed to undergo a tripling in rate of synthesis. This aspect of the data of Churchward and Holland was discussed in Chapter 4; the apparent tripling in rate of synthesis of some proteins was almost certainly due to a fluorographic artifact. There is no reason to think, however, that the use of fluorography could create linear accumulation patterns from an underlying exponential pattern. Thus, the simplest interpretation of the data of Churchward and Holland is that all envelope proteins accumulate linearly. In agreement with these data is the finding by Hakenbeck and Messer (1977) of a linear pattern of accumulation for both cytoplasmic membrane protein and outer membrane protein fractions, although in this case the doubling in rate occurred later in the cycle.

II. Analysis of the Bacterial Cell Cycle by Use of the Membrane Elution Technique

The method which was used to analyse the cell cycle in the study by Churchward and Holland, and in the present study, was the membrane elution technique described in detail by Helmstetter (1967). This procedure does not involve the generation of synchronous cultures; rather, an exponentially growing culture is pulse-labelled with a radioactive precursor of the macromolecule of interest and then the labelled culture is fractionated according to the age of cells at the time of labelling. The amount of radioactive label per cell in each age fraction is taken as a measure of the rate of macromolecular synthesis at that particular cell age. It is important to note that all culture manipulations occur after the radioactive labelling has been completed. Details of the membrane elution procedure which was used in the present study are to be found in Chapter 2. Briefly, the pulse-labelled cells are immediately bound to a nitrocellulose membrane filter, which is then inverted and eluted with a continuous flow of fresh medium. The cells continue to grow and divide on the filter, and growth is essentially unperturbed since the entire experiment is conducted in a 37°C room. When a filter-bound cell divides it sheds a daughter cell into the eluate; those cells about to divide when labelled and bound to the filter will almost immediately contribute a cell to the eluate, whilst cells which were newborn in the original culture will shed a daughter cell after one generation time. Thus, the earliest eluate fractions contain cells from late in the cell cycle, and subsequent fractions contain cells from progressively earlier points in the cycle.

Figure 6.1(a) shows the theoretical pattern of cell concentrations in the eluate from a membrane elution experiment, as predicted from the age distribution of exponentially growing cells (Powell, 1956); note that in such a culture there are twice as many newborn cells as there are cells about to divide. In Figure 6.1(b) is shown the curve expected for the rate of synthesis of a macromolecule which accumulates exponentially as a function of cell age. In experimental terms this rate is measured as the amount of radioactivity per cell in the eluate. In Figure 6.1(c) is shown the corresponding curve for the rate of synthesis of a macromolecule which accumulates at constant rate, with a doubling in rate occurring at cell age 0.5. Finally, Figure 6.1(d) shows the curve which results if curve (c) is divided by curve (b). If, for example, curve (b) represented the rate of synthesis of total cell protein and curve (c) represented the rate of synthesis of an individual protein species, then curve (d) would represent the relative rate of synthesis of that individual protein species. The relative rate curve clearly has the same shape as the cell number profile, but is displaced along the abscissa according to the timing of the doubling in rate.

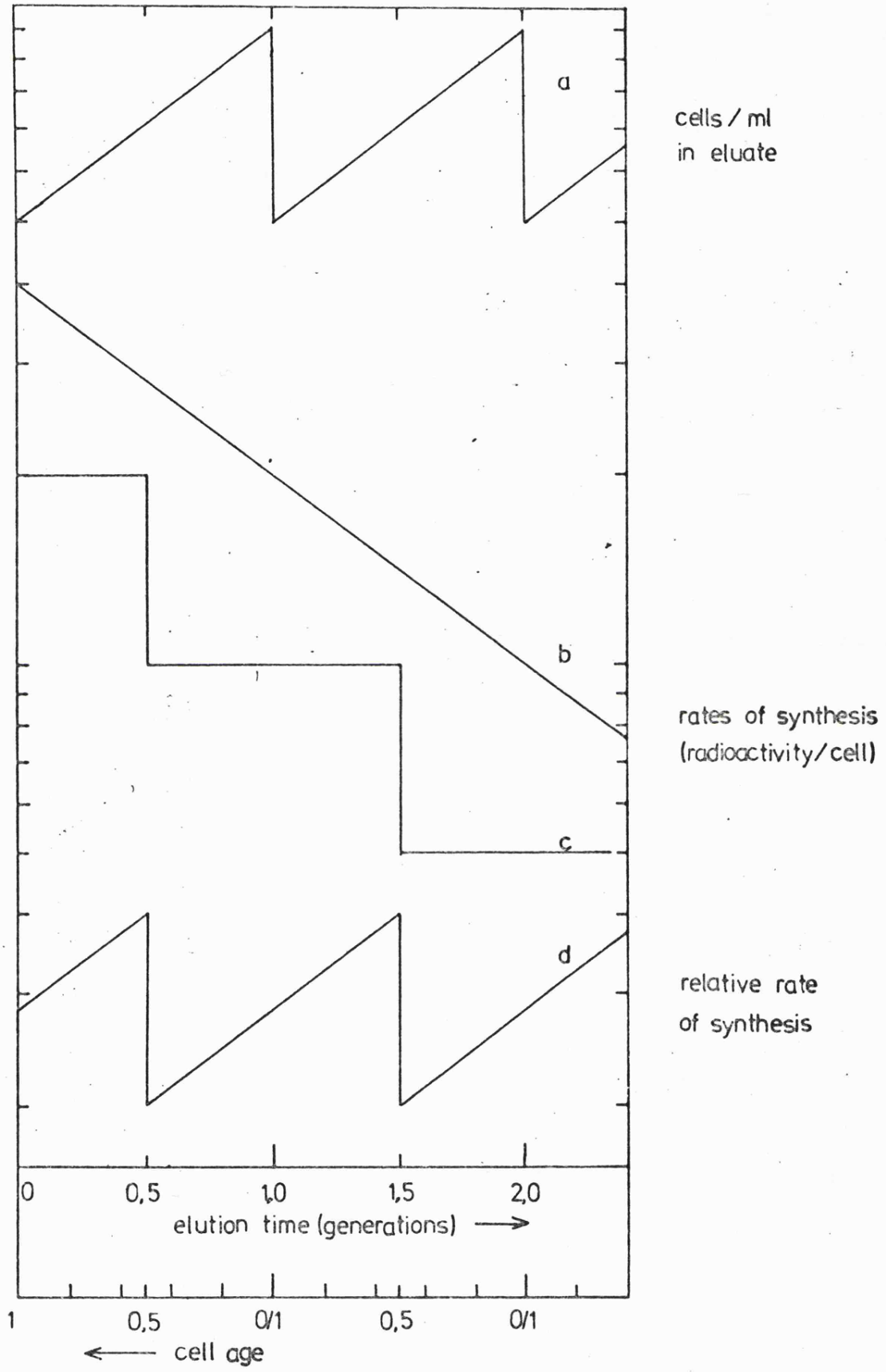
Figure 6.1: THEORETICAL RESULTS FROM A MEMBRANE ELUTION EXPERIMENT

(a) Concentration of cells in eluate.

(b) Rate of synthesis of a cell component which accumulates exponentially.

(c) Rate of synthesis of a cell component which accumulates linearly.

(d) Relative rate of synthesis (curve (c)/curve (b)).


a
b
c
d
cells / ml
in eluate
rates of synthesis
(radioactivity/cell)
relative rate
of synthesis
0
0,5
1,0
1,5
2,0
elution time (generations)
1
0,5
0/1
0,5
0/1
cell age

III. The Rates of Synthesis of Cytoplasmic Membrane and Outer Membrane Protein Fractions in the Cell Cycle

As part of this study of membrane protein synthesis I set out to confirm and extend the findings of Churchward and Holland (1976a), discussed above. The strain of E.coli B/r used in the present study, LEB16, has been studied in some detail : Meacock and Pritchard (1975) determined the pattern of DNA synthesis in the cell cycle in proline/alanine medium (τ = 65-70 min), and Churchward and Holland (1976a) used the same strain and growth medium in their study. This background provides a useful framework into which other studies of the LEB16 cell cycle may be fitted.

Turning now to my experimental findings, Figure 6.2 shows the result of a membrane elution experiment in which the rates of synthesis of cell protein fractions during the cell cycle were measured. The observed cell number profile (Figure 6.2(e)) deviates considerably from the theoretical curve shown in Figure 6.1; this is due to the variation in individual cell cycle times (Schaechter et al., 1962), and clearly limits the resolution of this method of cell cycle analysis. As previously reported, (Dennis and Young, 1975; Churchward and Holland, 1976a; Meacock, 1975), the rate of synthesis of total cell protein can be seen from the data in Figure 6.2(a) to increase exponentially as a function of cell age. The other data shown in Figure 6.2 represent measurements of the rates of synthesis of membrane protein fractions. These data demonstrate that whilst the rate of synthesis of cytoplasmic membrane protein increases exponentially with cell age, the rate of synthesis of outer membrane protein remains constant through much of the cycle, doubling abruptly late in the cycle, around the time of termination of DNA replication, as deduced from the data of Meacock

Figure 6.2: MEMBRANE PROTEIN SYNTHESIS IN THE CELL CYCLE OF LEB16

A membrane elution experiment was carried out at 37°C, as described in Chapter 2. The initial culture of LEB16, growing exponentially in proline/alanine medium (+ 20 µg/ml thymine) was pulse-labelled with 50 µCi of [^{14}C]-leucine (311 mCi/mmol) for 5 minutes. From each eluate sample, aliquots were removed for determination of cell number and total TCA-precipitable radioactivity. An aliquot of each eluate sample was also removed for the preparation of membrane fractions; an internal standard of [^{3}H]-leucine-labelled cells was incorporated into each membrane sample, as described in Chapter 2. Data for membrane fractions are presented as ^{14}C/^{3}H ratio, or relative radioactivity. Radioactivity in membrane fractions was measured in Biosolv scintillation fluid.

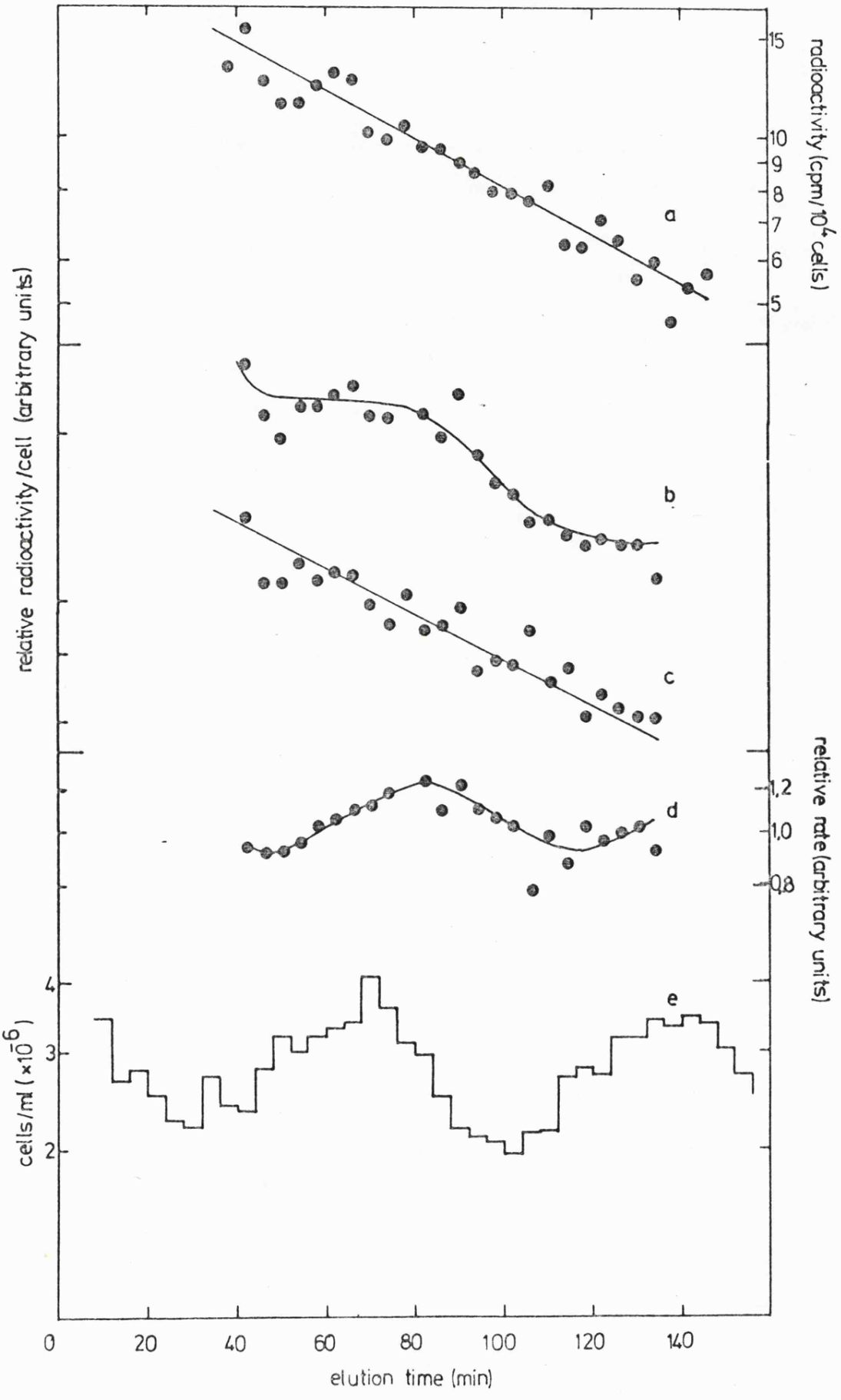
(a) Total cell protein.

(b) Sarkosyl-insoluble envelope protein (outer membrane).

(c) Sarkosyl-soluble envelope protein (cytoplasmic membrane).

(d) Outer membrane/cytoplasmic membrane.

(e) Cell concentration.


relative radioactivity/cell (arbitrary units)
radioactivity (cpm/10⁴ cells)
relative rate (arbitrary units)
cells/ml (×10⁻⁶)
elution time (min)
a
b
c
d
e
15
10
9
8
7
6
5
1.2
1.0
0.8
4
3
2
0
20
40
60
80
100
120
140

and Pritchard (1975). As explained above, rate curves are generated by determining radioactivity (or relative radioactivity) per cell in the eluate; the data obtained in this way contain the errors inherent in determination of both the radioactivity and the cell concentration. In comparison, the curve showing relative rate of synthesis of the two membrane protein fractions (Figure 6.2(d)) is derived independently of the cell number determinations and, consequently, contains less scatter. This curve clearly shows a fluctuation in the relative rate of synthesis of the two protein fractions during the cell cycle, and confirms the interpretations of the rate data shown in curves (b) and (c). As shown in Figure 6.1, the theoretical shape of this relative rate curve is the same as that of the cell number profile, but it is displaced along the abscissa according to the timing of the rate doubling. It should be noted, however, that the relative rate curve shown in Figure 6.2 is much flatter than the cell number profile. Possible reasons for this aspect of the data will be considered in the discussion at the end of this chapter.

IV. Rates of Synthesis of Individual Proteins in the Cell Cycle

The data presented above indicate that outer membrane proteins accumulate linearly in the cell cycle. To extend this observation an experiment was performed in which the rate of synthesis in the cell cycle of the major outer membrane porin (molecular weight 36.5K) was determined directly by analysis of the cognate band in an SDS-polyacrylamide gel of total cell protein. As shown in Figure 6.3 this polypeptide forms the heaviest-staining band in a gel analysis of total cell protein of *E.coli* B/r grown in proline/alanine medium.

Figure 6.3: RATES OF SYNTHESIS OF INDIVIDUAL PROTEINS IN THE CELL CYCLE

A membrane elution experiment was carried out exactly as described in Chapter 2 and the legend to Figure 6.2. An aliquot of each $[^{14}C]$-labelled eluate sample was removed for the preparation of whole cell lysates, and an internal standard of $[^{3}H]$-leucine labelled cells was incorporated. Cell lysates were analysed by SDS-PAGE and the 36.5K (porin) and 44K (EF-Tu) bands were cut out from the gel for determination of $^{14}C/^{3}H$.

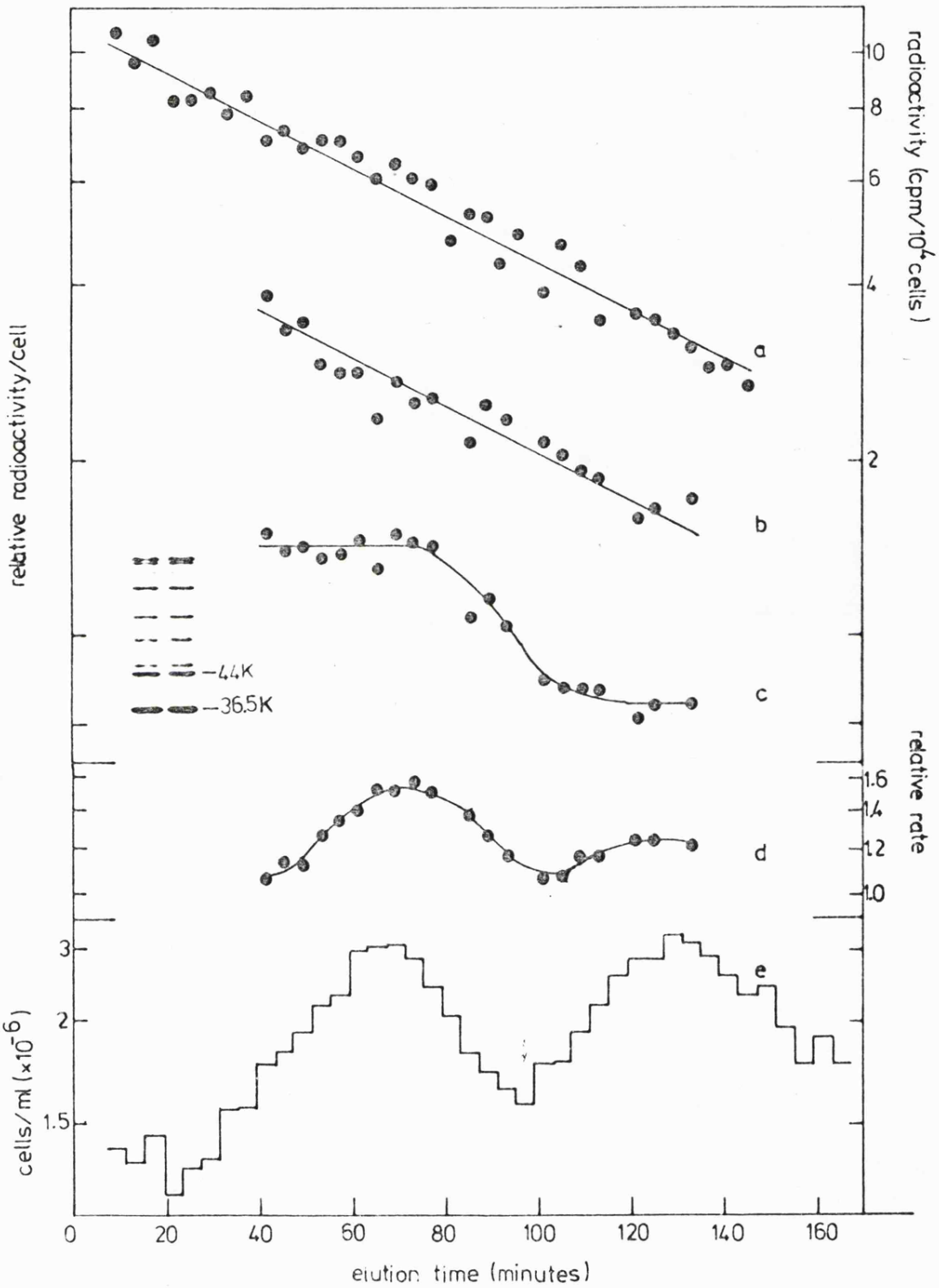
(a) Total cell protein.

(b) 44K band (EF-Tu).

(c) 36.5K band (porin).

(d) Rate of synthesis 36.5K/rate of synthesis 44K.

(e) Cell concentration.


relative radioactivity/cell
radioactivity (cpm/10⁴ cells)
relative rate
cells/ml (×10⁻⁶)
elution time (minutes)
a
b
c
d
e
44K
36.5K
0
20
40
60
80
100
120
140
160
1.5
2
3
1.0
1.2
1.4
1.6
2
4
6
8
10

Furthermore, a control experiment described in Chapter 2 showed that at least 70% of the material in this band is specific to the 36.5K porin; it can thus be estimated that in proline/alanine grown cultures there are some 10^5 copies of this protein molecule per cell. Another major band in the gel analysis shown in Figure 6.3 is formed by elongation factor (EF)-Tu, molecular weight 44K, of which there are about 7×10^4 copies per cell in slow-growing E.coli K12 (Pedersen et al., 1978). It is likely, therefore, that most of the material present in the 44K band in my gels is specific to EF-Tu. In fact, unless any contaminating polypeptides display a cell cycle pattern of accumulation different from that of EF-Tu, this question is irrelevant to the present study.

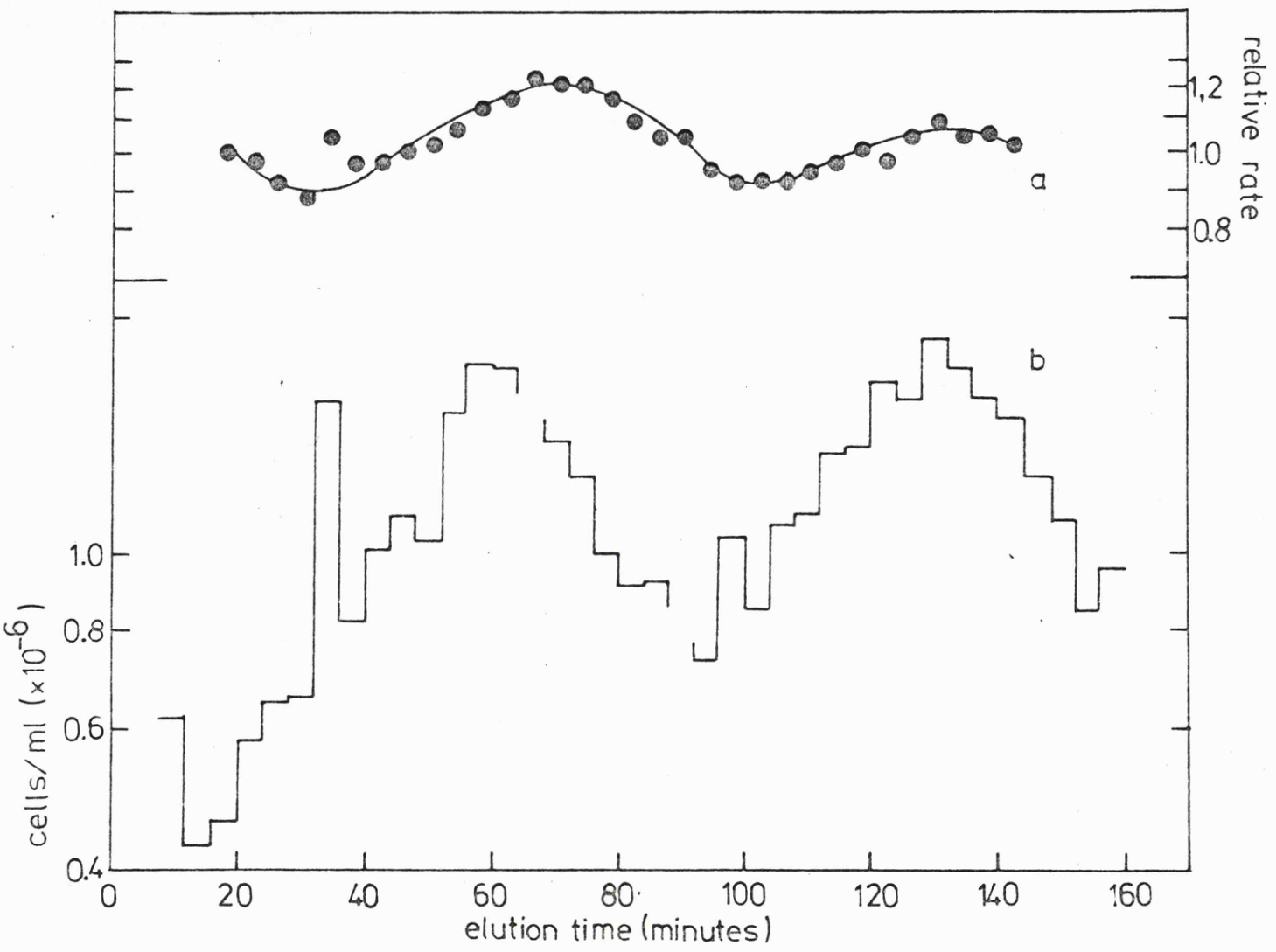
The data shown in Figure 6.3 summarise the results of a membrane elution experiment in which the 36.5K and 44K bands were analysed directly by cutting them from the gel and measuring their radioactivity. The rate of synthesis of total cell protein was also determined and as can be seen in Figure 6.3(a), it increased exponentially with cell age, as expected. The analysis of the 44K band revealed the same exponential increase (Fig. 6.3(b)) indicating that the majority of the polypeptides in this band, including EF-Tu,show the same cell cycle accumulation as total cell protein. In contrast, the material in the 36.5K band (predominantly the outer membrane porin) is synthesised at a constant rate which doubles late in the cycle. This pattern of synthesis is very similar to that found for bulk outer membrane protein, of which the 36.5K porin constitutes 35% in proline/alanine medium. The rate of synthesis of the porin relative to that of EF-Tu is shown in Figure 6.3(d); as explained above this measurement is free from scatter generated by the cell number determination. This relative rate curve

Figure 6.4: PORIN SYNTHESIS IN THE CELL CYCLE

Cells, pulse-labelled with [^{14}C]-leucine, were eluted from a membrane filter as before. Samples of eluate were taken, and an internal standard of [^{3}H]-leucine labelled cells was added before preparation of SDS lysates. Samples of lysates were analysed by SDS-PAGE and the 36.5K and 44K bands were cut out of the gel for determination of $^{14}C/^{3}H$.

(a) Rate of synthesis 36.5K/rate of synthesis 44K.

(b) Cell concentration.


relative rate
1.2
1.0
0.8
a
b
cells/ml (x10⁻⁶)
1.0
0.8
0.6
0.4
0
20
40
60
80
100
120
140
160
elution time (minutes)

shows clearly the same fluctuation as found previously for the rate of synthesis of bulk outer membrane protein relative to cytoplasmic membrane protein. In Figure 6.4 are shown the results of an experiment similar to that shown in Figure 6.3. The rate of synthesis of porin relative to EF-Tu shows exactly the same fluctuation during the cell cycle as that shown in Figures 6.2 and 6.3.

V. Discussion

The data presented above demonstrate that in proline/alanine medium outer membrane proteins are synthesised in the cell cycle at a constant rate, which doubles 5-15 min before division. I will now discuss this interpretation of the data and attempt to place them in the context of the findings of Churchward and Holland (1976a) and Hakenbeck and Messer (1977).

The aspect of the data that I wish to discuss is the shape of the relative rate curves shown in Figures 6.2 - 6.4. These curves deviate markedly from the theoretical sawtooth curve shown in Figure 6.1. In fact, the curves display two types of deviation from the theoretical curve. The first of these is a decrease in amplitude, or flattening. The theoretical fall from peak to trough is 50%; the experimental values of this parameter are 24% for the data of Figure 6.2; 31% in Figure 6.3; and 27% in Figure 6.4. One source of this flattening is clearly the imperfection of the age fractionation technique itself. The particular eluate fractions containing cells whose age at time of labelling corresponded to the trough of the relative rate curve will, because of the variation in the interdivision times of individual cells, contain cells which were slightly younger and slightly older

than this critical age at the time of labelling; thus the apparent relative rate at the trough will be greater than the true relative rate. The same argument can be applied to the peak value of the relative rate. A second potential source of flattening is heterogeneity of the protein fractions analysed. Thus, for example, it was estimated in Chapter 2 that the 36.5K porin band analysed in Figures 6.3 and 6.4 may contain 30% contamination from other polypeptides of similar molecular weight. I have calculated that this level of contamination by an exponential component would cause a decrease in the amplitude of the relative rate curve from 50% to 38%. The relatively low amplitude of the curves for relative rate of synthesis of the porin can thus be readily explained by the combination of the effects of imperfection of the age fractionation and of heterogeneity of the 36.5K band. The amplitude of the curve for the rate of synthesis of outer membrane protein, relative to cytoplasmic membrane protein, is even lower (24% in Figure 6.2). In this case the heterogeneity resides in the cytoplasmic membrane fraction. It was shown in Chapter 3 that the sarkosyl-soluble fraction of envelope protein contains 13% of presumptive outer membrane protein. I have calculated that this level of contamination of the cytoplasmic membrane fraction by a linear component would decrease the amplitude of the relative rate curve from 50% to 44%. On the other hand, there is no reason to suspect that the sarkosyl-insoluble fraction contains significant contamination from, for example, cytoplasmic membrane proteins. If the combination of imperfect age fractionation and the heterogeneity of the sarkosyl-soluble envelope protein fraction is insufficient to explain the low amplitude of the relative rate curve then the possibility must be entertained that not all of the sarkosyl-

insoluble outer membrane proteins accumulate linearly. Until the contributions of the various factors to the flattening of the relative rate curves can be determined accurately, however, this possibility remains a mere speculation.

The second deviation of the relative rate curves from the theoretical sawtooth pattern lies in the spreading of the curve, seen in the relative gradients of the rising and falling phases. It can easily be shown that this is not affected at all by the heterogeneity of protein fractions. The most obvious source of this spreading is again the spreading inherent in the age fractionation itself. Examination of the cell number profiles in the various figures shown above reveals that these are virtually symmetrical, with only a slight tendency for the falling phase of the curve to be steeper than the rising phase. This probably accounts for much of the spreading in the relative rate curves.

One other factor which could affect the shape of the relative rate curves should also be borne in mind. The membrane elution technique fractionates a bacterial culture according to cell age at the time of labelling; cells of a particular cell age may, however, have a range of sizes or densities, so that if a cell cycle event such as the rate doubling was linked to attainment of a critical size or density then the variance of these parameters with cell age would manifest itself in an age fractionation experiment by both flattening and spreading the relative rate curve. Koch and Blumberg (1976) have pointed out that since cell cycle events may indeed be 'sized' rather than 'timed', so a size fractionation of cultures by, for example, density gradient centrifugation, might be better suited to cell cycle analysis than is an age fractionation technique such as membrane elution.

Having said all of this, the simplest interpretation of the data presented is that outer membrane proteins are synthesised at a constant rate in the cell cycle which doubles 5-15 minutes before division, i.e. around the time of termination of DNA replication. This result differs from that of Churchward and Holland (1976a) in two respects. Firstly, Churchward and Holland detected a doubling in rate of total envelope protein synthesis. I estimate that 40% of total envelope protein is specific to the outer membrane. It is surprising that Churchward and Holland were able to detect a rate doubling over a background of 60% exponential accumulation, but it is clearly not impossible. Churchward and Holland also found that their rate doubling occurred earlier in the cycle than that reported here; this discrepancy is completely inexplicable since the same strain and growth conditions were used in the two studies.

Hakenbeck and Messer (1977) have reported that both cytoplasmic membrane and outer membrane protein bulk fractions accumulate linearly in the cell cycle, with a rate doubling occurring around the time of termination of replication. Whilst this timing agrees well with the data presented here, their data are in conflict with mine over the pattern of accumulation of cytoplasmic membrane protein. Their experimental technique was, however, based upon the generation of synchronous cultures and the separation of cytoplasmic and outer membranes on sucrose density gradients. These differences may be responsible for the different results obtained in the two studies; moreover because of the method of membrane separation which was used fewer data were analysed and the degree of resolution attained was thus lower than that in the present study.

CHAPTER 7

LACK OF COUPLING BETWEEN OUTER MEMBRANE PROTEIN SYNTHESIS AND DNA REPLICATION

I. Introduction

The growth of the cell surface of E.coli in relation to the cell cycle was discussed in Section V of Chapter 1. It will be recalled that consideration of the variations in cell shape with growth rate has led to the postulate that the cell surface is synthesised linearly in the cell cycle, with a doubling in rate occurring during the cycle (Pritchard, 1974; Rosenberger et al., 1978). Since the peptidoglycan forms the rigid layer of the cell envelope it has been this layer which has been suggested to grow linearly; because of the plasticity of membranes it might not be expected that the synthesis of membrane components would be constrained to match that of the peptidoglycan. Nevertheless there is evidence which suggests that at least some membrane proteins do accumulate linearly in the cell cycle (Churchward and Holland, 1976a; Hakenbeck and Messer, 1977). In the preceding chapter I presented evidence that bulk outer membrane protein is synthesised linearly. There are two possible reasons for this : firstly, that the rate of synthesis of bulk outer membrane protein may be for some reason directly constrained in this way, independently of the growth of other surface layers such as the peptidoglycan; or, secondly, that the rate of synthesis of bulk outer membrane protein may be directly dependent upon the rate of growth of the peptidoglycan layer. In either case the underlying trigger for the doubling in rate could be the replication of a specific region of the chromosome, as suggested in the surface growth

models of Previc (1970) and Pritchard (1974). This hypothesis, that the rate of synthesis of bulk outer membrane protein is tightly coupled to DNA replication, is the subject of this chapter.

II. Effects of Inhibition of DNA Synthesis Upon Membrane Protein Synthesis

One prediction of any model of cell growth which includes the postulate of a tight coupling between chromosome replication and the rate of synthesis of the cell surface is that inhibition of DNA synthesis in an exponentially growing culture should fix every cell at its particular rate of surface synthesis - either pre - or post-doubling - and should thus fix the rate of surface synthesis in the culture as a whole. To test this hypothesis a culture of LEB16, a thymine auxotroph, was starved of thymine to block DNA synthesis. Meacock (1975) has determined that the residual synthesis of DNA in LEB16 under these conditions is less than 7% of normal. The effect of thymine starvation upon the rate of synthesis of membrane proteins was measured, as shown in Figure 7.1. After thymine starvation growth continued for about one mass doubling, with a detectable deviation from exponential growth after about half a mass doubling. Because of this rather drastic effect of thymine starvation upon growth in LEB16, conclusions can only be drawn from the early period of the experiment, when the rate of total protein synthesis continued to increase exponentially. It is clear that during this period the rate of synthesis of both outer and cytoplasmic membrane protein fractions continued to increase in parallel with that of total protein. There is, therefore, no indication from this experiment that chromosome replication is necessary for any increase in the rate of outer membrane protein synthesis.

Figure 7.1: THYMINE STARVATION OF LEB16

A culture of LEB16 was grown in proline/alanine medium (+ 20 µg/ml thymine) to an $A_{450} \simeq 0.15$. One ml samples of the culture were removed at intervals for pulse-labelling (4 min) with [^{14}C]-leucine (0.6 µCi/pulse; 311 mCi/mmol). At an $A_{450} \simeq 0.23$ the culture was starved for thymine as described in Chapter 2; a dilution was included at the resuspension step. During the subsequent period of growth 1.0 ml samples were again removed for pulse-labelling. To each pulse-labelled sample was added a constant volume of [^{3}H]-leucine labelled cells, as internal standard. Aliquots of samples were removed for determination of total TCA-precipitable radioactivity, and then membrane fractions were prepared. Radioactivity in membrane fractions was measured in Biosolv scintillation fluid. Isotope ratio, $^{14}C/^{3}H$ is plotted as a normalised measure of the rate of synthesis of protein fractions.

(a) Sarkosyl-insoluble envelope material (outer membrane)

(b) Sarkosyl-soluble envelope material (cytoplasmic membrane)

(c) Total protein.

(d) Optical density.

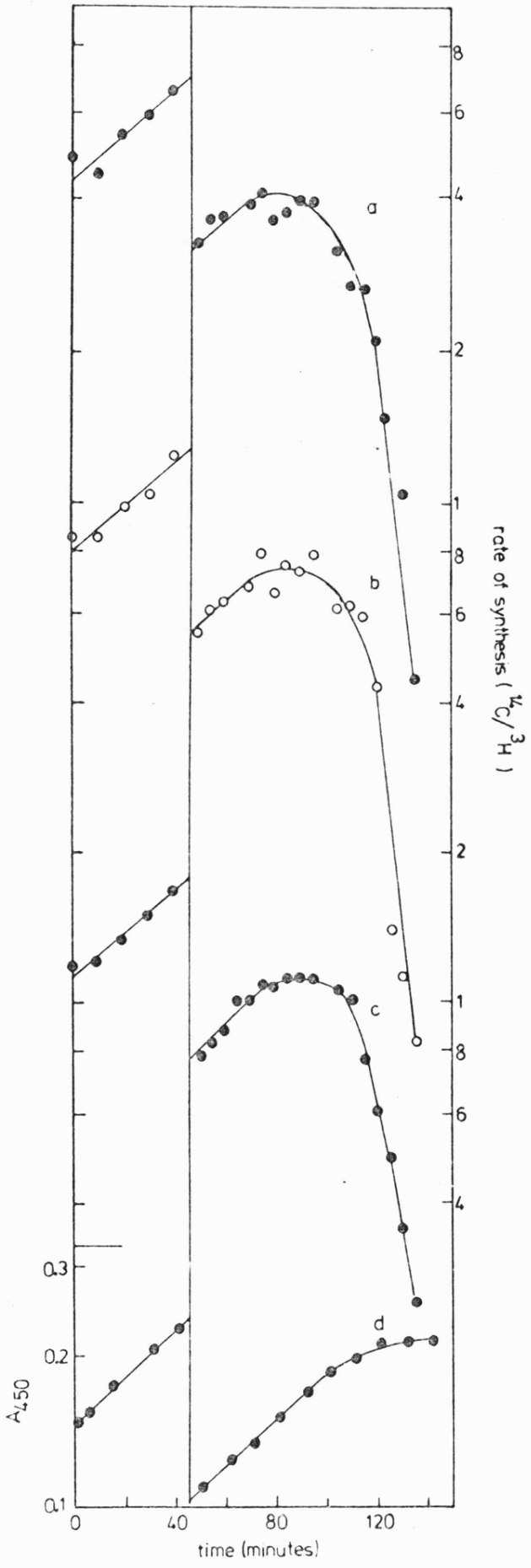

a
b
c
d
8
6
4
2
1
8
6
4
2
1
8
6
4
0.3
0.2
0.1
A_{450}
0
40
80
120
time (minutes)
rate of synthesis ($^{14}C/^{3}H$)

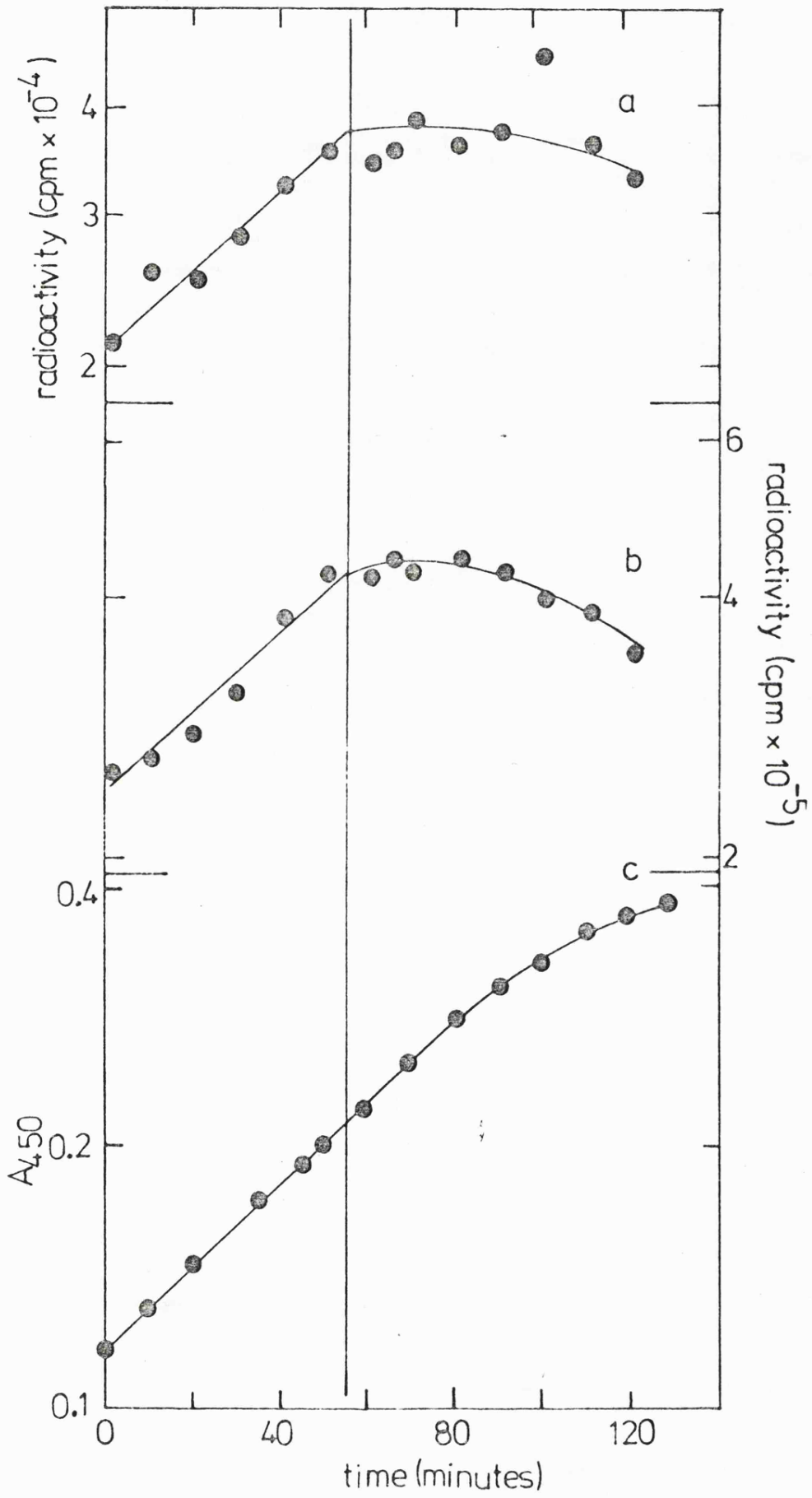
Figure 7.2: NALIDIXIC ACID TREATMENT OF LEB16

A culture of LEB16 was grown in proline/alanine medium (+ 20 µg/ml thymine) to an A_{450} = 0.11. One ml samples of the culture were removed at intervals for pulse-labelling (4 min) with [^{14}C]-leucine (1 µCi/pulse; 311 mCi/mmol). At an A_{450} = 0.21 nalidixic acid was added to a final concentration of 20 µg/ml. During the subsequent period of growth 1.0 ml samples were again removed for pulse-labelling. Aliquots of each pulse-labelled sample were removed for determination of total TCA-precipitable radioactivity, and then cell envelopes were prepared. Radioactivity in envelope preparations was measured in Biosolv scintillation fluid.

(a) Envelope protein.

(b) Total cell protein.

(c) Optical density.


radioactivity (cpm × 10⁻⁴)
radioactivity (cpm × 10⁻⁵)
A450
time (minutes)
a
b
c
4
3
2
6
4
2
0.4
0.2
0.1
0
40
80
120

As an alternative to thymine starvation, the antibiotic nalidixic acid was used to block DNA synthesis, and the effect of this treatment upon the rate of synthesis of membrane proteins was measured, as shown in Figure 7.2. No conclusions about the regulation of membrane protein synthesis could, however, be drawn from this experiment since the nalidixic acid treatment inhibited any further increase in the rate of total protein synthesis.

III. Effects of Thymine Limitation Upon Membrane Protein Synthesis

It is well-established that lowering the concentration of exogenous thymine supplied to certain thymine auxotrophs of E.coli, including LEB16, causes a decrease in replication velocity (Pritchard and Zaritsky, 1970; Zaritsky and Pritchard, 1971), and thereby alters the steady state pattern of gene concentrations in a culture, without affecting growth (Chandler and Pritchard, 1975). This technique thus provides another means of testing the hypothesis that the rate of synthesis of outer membrane protein is determined by the concentration of some gene. An experiment in which the concentration of thymine is raised from a limiting value to a non-limiting value is called a thymine step-up. The effect of a thymine step-up upon DNA synthesis in LEB16 is shown in Figure 7.3. The pre-step concentration of thymine (2 μg/ml) is low enough to be rate-limiting for DNA synthesis, and causes a decreased replication velocity, without affecting the rate of initiation of replication; addition of excess thymine allows all the existing replication forks to accelerate, producing a transient increase in differential rate of DNA synthesis, as previously reported by Zaritsky and Pritchard (1971). Figure 7.4 shows the results of an

Figure 7.3: DNA SYNTHESIS DURING A THYMINE STEP-UP IN LEB16

A culture of LEB16 was grown in proline/alanine medium supplemented with [^{3}H]-thymine, 2 µg/ml (0.02 µCi/ug) to an A_{450} = 0.13. At intervals 1.0 samples were removed into 1.0 ml of ice-cold TCA. At an A_{450} = 0.23 [^{3}H]-thymine of identical specific activity to that already present in the culture was added to bring the concentration of thymine in the culture up to 20 µg/ml. Samples were then taken into TCA as before.

(a) DNA.

(b) Optical density.

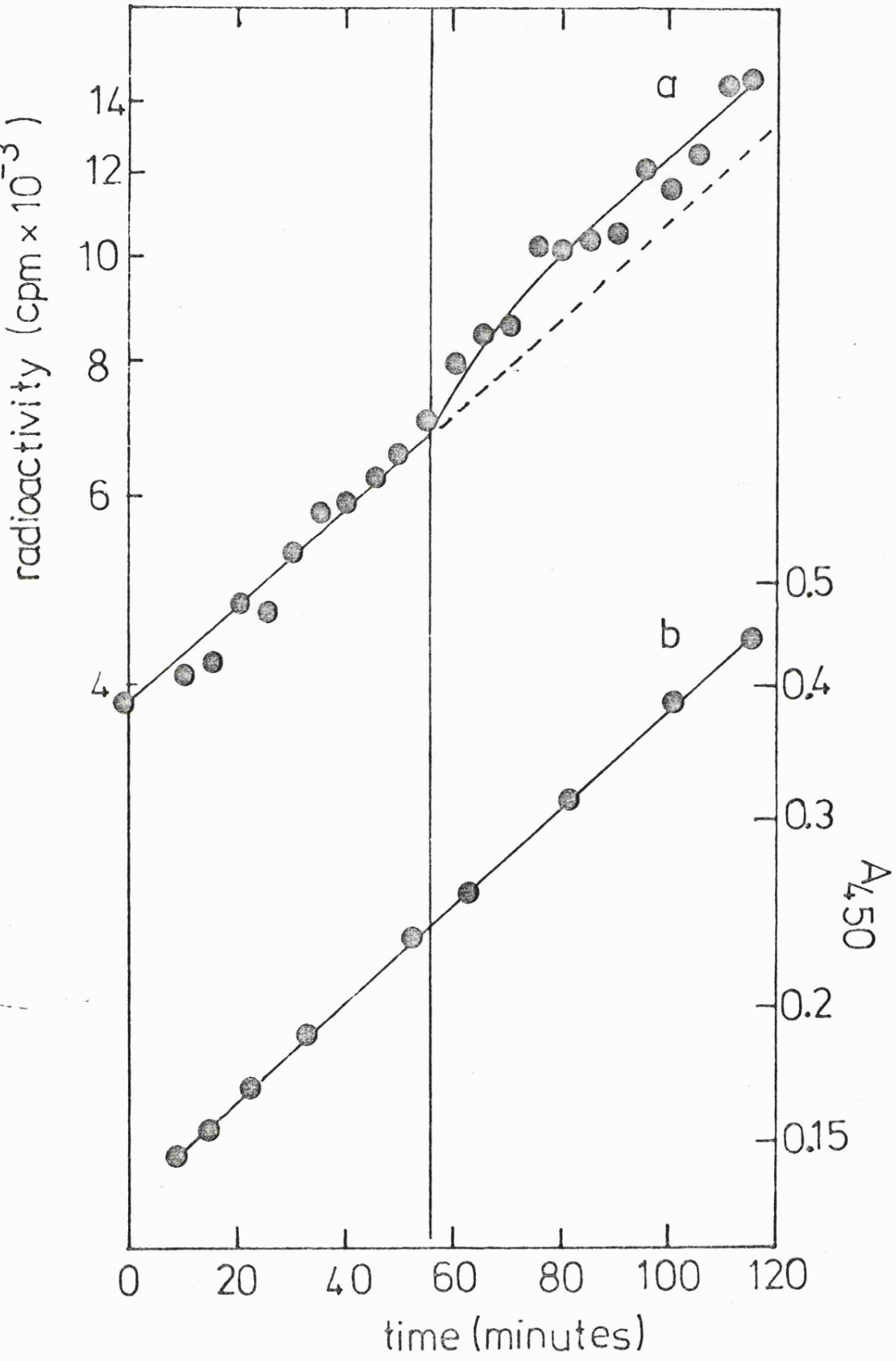

radioactivity (cpm × 10⁻³)
14
12
10
8
6
4
a
b
0.5
0.4
0.3
0.2
0.15
A450
0
20
40
60
80
100
120
time (minutes)

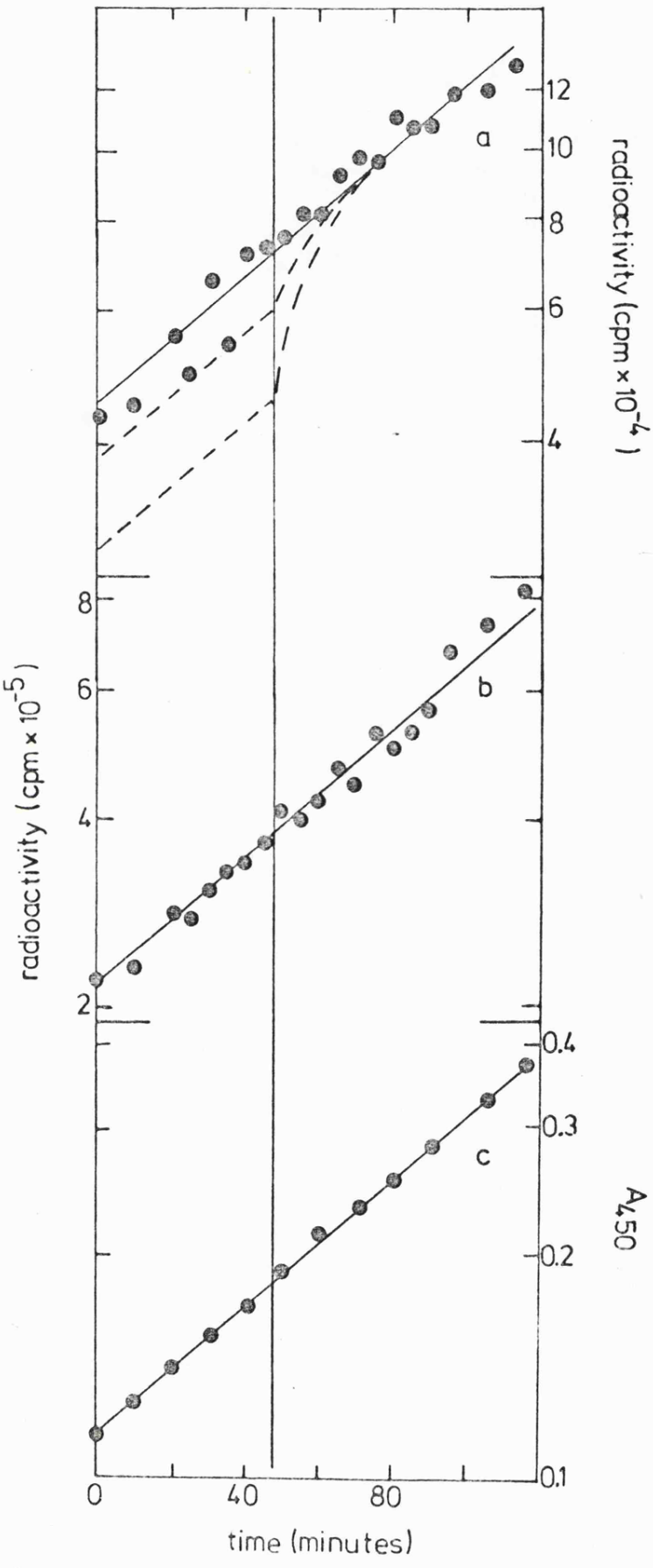
Figure 7.4: ENVELOPE PROTEIN SYNTHESIS DURING A THYMINE STEP-UP IN LEB16

A culture of LEB16 was grown in proline/alanine medium (+ 2 μg/ml thymine), to an A_{450} = 0.12. One ml samples were removed at intervals for pulse-labelling with [^{14}C]-leucine (1 μCi/pulse; 311 mCi/mmol). At an A_{450} = 0.19, thymine was added to a final total concentration of 20 μg/ml, and sampling was continued. Aliquots of each pulse-labelled sample were removed for determination of total TCA-precipitable radioactivity, and then cell envelopes were prepared. Radioactivity in envelope preparations was measured in Biosolv scintillation fluid.

(a) Envelope protein. The three curves drawn are extrapolations back from the data for times greater than C = 40 minutes after the thymine step, when a new steady state should be reached. The solid line is the predicted curve if the rate of synthesis of envelope protein is linked to replication of a gene near the origin of replication (or is independent of DNA replication). The upper and lower broken lines represent the corresponding curves if the rate of synthesis of envelope protein is linked to the replication of genes lying midway along the chromosome, and lying at the terminus of replication, respectively. If only a fraction of envelope protein was subject to this type of regulation then the magnitude of the effect measured in cell envelope preparations would be correspondingly reduced.

(b) Total cell protein.

(c) Optical density.


radioactivity (cpm × 10⁻⁴)
radioactivity (cpm × 10⁻⁵)
A450
time (minutes)
a
b
c
12
10
8
6
4
8
6
4
2
0.4
0.3
0.2
0.1
0
40
80

experiment in which the effect of this thymine step-up upon the rate of envelope protein synthesis was measured. If the rate of synthesis of a membrane protein fraction was somehow limited by the number of copies of a particular gene in the culture, then the step-up, by transiently increasing the differential rate of replication of that gene, would be expected to increase the differential rate of synthesis of envelope protein, i.e. the rate of synthesis relative to the rate of mass increase. The data of Figure 7.4 show no effect of the thymine-step upon the differential rate of synthesis of envelope protein.

In addition to following membrane protein synthesis during a transition between two steady states of growth I determined the composition and size (but not shape) of cells growing in steady state at the two concentrations of thymine. These measurements are presented in Table 7.1, together with a measurement of the parameter surface area/mass of the cells taken from the data of Meacock (1975). The measurements of growth rate and macromolecular composition indicate the specificity of the effect of thymine limitation upon DNA and surface synthesis; that the limitation did have the expected effect upon cell shape (as reported by Zaritsky and Pritchard, 1973; Meacock, 1975) is suggested by the increased mean cell mass. In contrast, thymine limitation did not affect the differential rate of synthesis of outer membrane protein. Also shown in the table are results of an experiment in which the effect of thymine limitation upon the differential rate of synthesis of the 36.5K porin was measured. In direct contrast to the result for bulk membrane protein fractions, thymine limitation reduced the differential rate of synthesis of the porin by 11%.

Table 7.1

EFFECT OF THYMINE CONCENTRATION UPON MEMBRANE PROTEIN SYNTHESIS

Bulk membrane fractions

A single batch of proline/alanine medium was prepared, supplemented with 5 μg/ml [^{14}C]-leucine (0.2 μCi/ug). Two cultures of LEB16 were grown in aliquots of this medium supplemented with 2 or 20 μg/ml thymine. The cultures were grown through at least five generations to an $A_{450} \simeq 0.12$. Samples were taken at intervals for determination of cell number, and for preparation of membrane fractions. Aliquots of each sample were removed for determination of TCA-precipitable radioactivity, and then a constant aliquot of [^{3}H]-leucine labelled cells was added to all samples (from both cultures) before preparation of membrane fractions. Radioactivity in membrane fractions was measured in Biosolv scintillation fluid.

The quotient $\frac{^{14}C/^{3}H \text{ in membrane fraction}}{^{14}C \text{ in total protein}}$

was then used as a measure of the relative compositions of cells growing in the two concentrations of thymine. Values are expressed relative to those in 20 μg/ml thymine.

Macromolecular composition of cells

Samples of cultures of LEB16 growing in proline/alanine medium supplemented with 2 or 20 μg/ml thymine were taken for estimate of RNA and protein content as described in Chapter 2.

Porin content of cells

The proportion of total cell protein recovered in the 36.5K gel band was measured as described in the legend to Table 8.1. Porin/mass was then calculated from the tabulated values of protein/mass.

thymine concentration (μg/ml)	growth rate (doublings/hr)	protein $\mu g/A_{450}$	RNA $\mu g/A_{450}$	cell mass $A_{450}/10^9$ cells	surface / mass *	cytoplasmic membrane / total protein	outer membrane / total protein	36.5K porin / total protein %	porin / mass $\mu g/A_{450}$
20	0.91	107	28.5	1.07	1.0	1.0	1.0	6.4	68.1
2	0.91	108	27.9	1.23	0.87	1.01	0.98	5.7	51.6

* Data taken from Meacock (1975);
surface/mass in 20 μg/ml thymine
is arbitrarily set as 1.0.

IV. Discussion

The hypothesis being tested in the experiments described above is that the cell cycle pattern of bulk outer membrane protein synthesis reflects a link with DNA replication. This postulated link could arise as a result of direct regulation of outer membrane protein synthesis by the number of copies of a specific chromosomal locus; alternatively it could be another surface component, such as the peptidoglycan, whose rate of synthesis might be related to DNA replication, with outer membrane proteins being constrained to follow this pattern for some structural reason. In the latter case the link between DNA replication and outer membrane protein synthesis would be indirect, and might be over-ridden under certain circumstances. Bearing this reservation in mind I will now discuss some implications of the data presented in this chapter.

The data of Section II clearly demonstrate that thymine starvation has no differential effect upon outer membrane protein synthesis. Under these conditions at least, replication of DNA is not essential for an increase in the rate of synthesis of bulk outer membrane protein. This experiment is, however, subject to two strong criticisms. Firstly, the period of growth after removal of thymine cannot be assumed to be representative of cell growth in general : growth stops rapidly, and it is clear that many regulatory mechanisms which operate in normal growth might break down under these experimental conditions. In fact, attempts were made in the present study to measure the effect of thymine starvation upon the induced rate of β-galactosidase synthesis in a *lac*$^+$ revertant of LEB16 : inhibition of DNA synthesis is expected to fix the fully induced (i.e. gene-limited) output of any gene. The

data obtained (not shown) indicated that the treatment was producing other effects upon the output of this gene, since the rate of enzyme synthesis fluctuated between wide limits. This result probably arose from cAMP-mediated effects upon lacZ gene output; whatever the reason, the result demonstrates that detailed knowledge of the system being investigated is required before the effects of thymine starvation can be interpreted with confidence.

The second criticism of the experimental approach is more specific : thymine starvation of LEB16 was achieved by taking growing cells on to a nitrocellulose filter and resuspending them in thymineless medium. As described in Chapter 5, this filtration treatment induces the synthesis of a group of outer membrane proteins concerned with the transport of chelated iron into the cell. The possible effects of this induction upon the results of the thymine starvation are difficult to evaluate; this point will be considered in more detail in Chapter 9. For the present it is sufficient to note that the hypothesis being tested here concerns a regulatory mechanism acting upon bulk outer membrane protein synthesis, and does not allow for any special class of outer membrane proteins escaping this regulation.

Thymine starvation has been used by others to test the postulated link between DNA replication and surface growth : thus Donachie et al. (1976) reported that thymine starvation did not prevent an increase in the rate of cell elongation in synchronous cultures; in contrast, Sargent (1975) has reported that in B.subtilis there is a linear accumulation of membrane protein in the cell cycle, and that thymine starvation does, in this case, fix the rate of synthesis of membrane protein in an exponential population.

It was because of the criticisms which could be levelled at the thymine starvation experiment that experiments were performed in which the supply of thymine to LEB16 was not completely removed, but merely limited. Chandler and Pritchard (1975) have demonstrated that the rates of processes which are limited by gene concentration are indeed altered in a predictable fashion by thymine limitation; the magnitude of such effects depends upon the chromosomal location of the gene in question, being zero for a gene at the origin, and maximal for a gene at the terminus.

The data presented here relating to the effect of thymine limitation upon bulk outer membrane protein synthesis may be viewed in two ways. First of all it is clear, both from the measurements made upon the steady state cultures and those made during a thymine step-up, that an altered pattern of gene concentrations has no effect upon bulk outer membrane protein synthesis. It therefore seems unlikely that the linear mode of synthesis of outer membrane protein in the cell cycle, described in Chapter 6, reflects a link with replication of a specific region of the chromosome. Although, as discussed above, little change in concentration of early-replicated genes is produced by this procedure, the timing of the cell cycle doubling in rate of outer membrane protein synthesis is, in any case, coincident with replication of late regions of the chromosome.

On the other hand, the data must also be considered in terms of the hypothesis that the pattern of outer membrane protein synthesis simply reflects the underlying pattern of synthesis of the peptidoglycan. Thymine limitation of cultures of LEB16 undoubtedly causes changes in cell shape (Meacock, 1975), although the interpretation of these changes as reflecting an effect of DNA replication upon surface synthesis

(Pritchard, 1974) has now been revised in the light of data which show that changes in gene concentration produced by other means do not affect cell shape (Pritchard et al., 1978; Zaritsky and Woldringh, 1978). Thus it now seems likely that the shape changes which occur in thymine-limited cultures reflect a role of a thymidine-linked sugar in biosynthesis of surface components; this may either be a direct role as a precursor molecule for LPS synthesis, or a less direct, regulatory role (Hosono et al., 1975; Ohkawa, 1977). Whatever the reason for the effect upon cell shape it is nevertheless clear that in the thymine-limited cultures the reduction in surface area/mass does not affect the differential rate of synthesis of bulk outer membrane protein; instead there is an increase in the amount of outer membrane protein per unit surface area. The measured values correspond to a 15% increase in protein/surface area, with no effect upon the growth of the culture. In contrast, thymine limitation did cause a decrease in differential rate of 36.5K porin synthesis such that the parameter porin/surface area only increased by 4%. Another set of circumstances under which this parameter is held fairly constant will be described in Chapter 8.

It should be noted that it is not known whether the amount of peptidoglycan per unit surface is affected in thymine-limited cultures; thus the relationships discussed here between outer membrane proteins and surface area cannot be assumed to reflect relationships between protein and peptidoglycan. It must also be borne in mind that the effects of thymine limitation upon the cell surface are not understood, so that it is possible that the relative amounts of the molecular components of the outer membrane change - for example, a decrease in the LPS content might allow an increased amount of protein to be

incorporated into the membrane. This type of effect might also be responsible for the discrepancy between the effects of thymine limitation upon bulk outer membrane protein synthesis and upon 36.5K porin synthesis. These various points will be reconsidered in Chapter 11.

Despite all of these reservations about the experimental methods used, I feel that I am justified in concluding, from the evidence presented, that the rate of synthesis of bulk outer membrane protein is not strictly coupled to DNA replication. It seems unlikely, therefore, that the doubling in rate of synthesis of these proteins in the cell cycle is triggered by the replication of any region of the chromosome.

CHAPTER 8

RELATIONSHIP BETWEEN PORIN SYNTHESIS AND GROWTH RATE : CORRELATIONS WITH GENE CONCENTRATION AND CELL SURFACE AREA

I. Introduction

At one point during the development of this project the available evidence seemed to point to a special regulatory mechanism governing the synthesis of the 36.5K porin. This evidence was as follows : firstly, the rate of synthesis of the protein is constant in the cell cycle, with a doubling in rate occurring late in the cycle (see Chapter 6); secondly, the rate of synthesis of the protein appeared to be frozen by thymine starvation of an exponential culture (to be discussed in Chapter 9); and thirdly, the differential rate of synthesis of the porin is reduced somewhat by thymine limitation of cultures in steady state growth. It therefore seemed possible that the 36.5K porin is the product of a constitutive gene; this would explain both the cell cycle pattern of synthesis, and the apparent effect of thymine starvation, since the rate of synthesis of the protein would always be determined by the concentration of the corresponding structural gene. In this view the cell cycle pattern of synthesis of bulk outer membrane protein would simply reflect domination of the fraction by the porin. In view of the possibility that the porin might be synthesised constitutively I decided to measure the differential rate of synthesis of the porin at various growth rates. The theoretical reasoning behind this approach is discussed below.

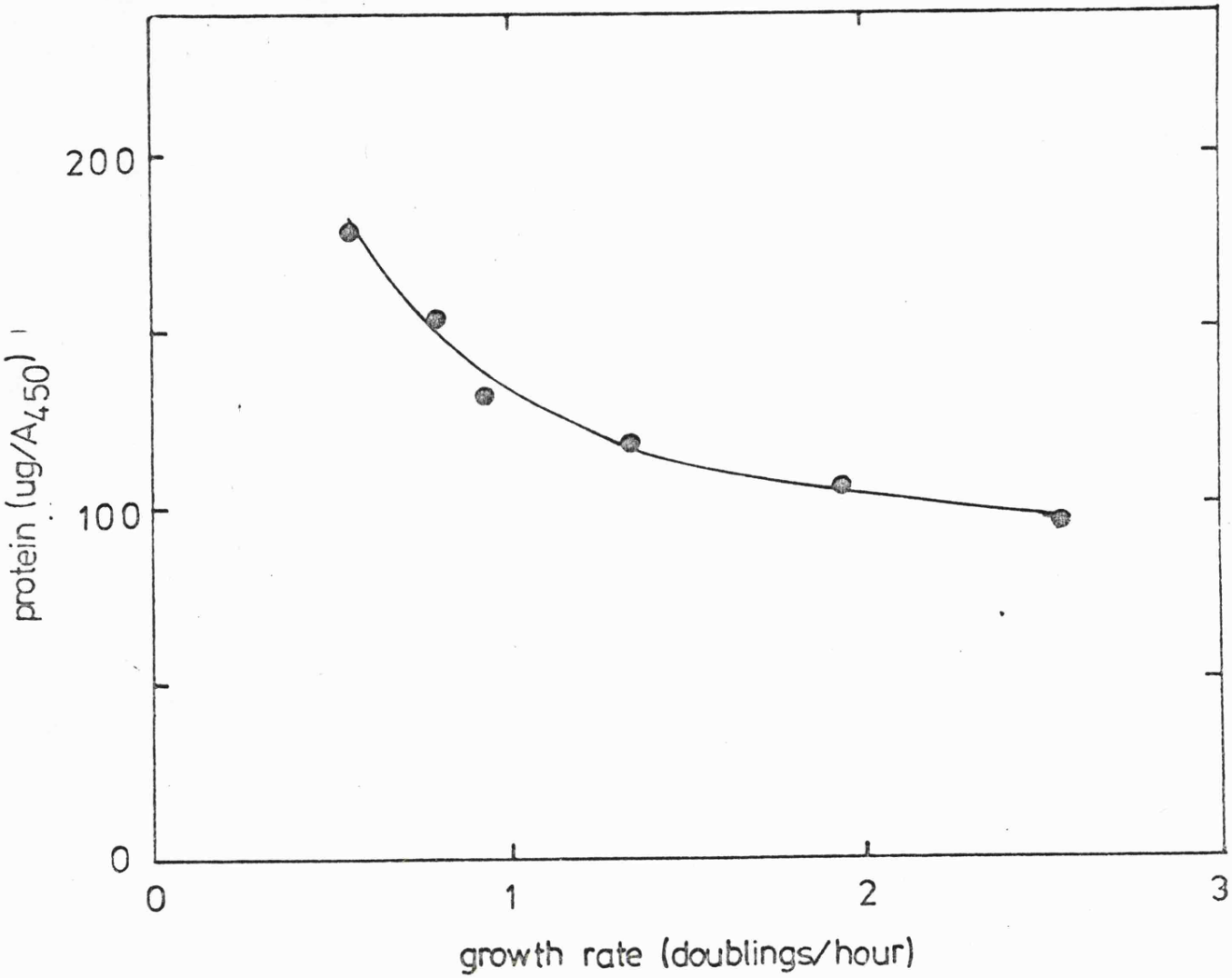
II. Relationship Between Growth Rate and Synthesis of the 36.5K Porin

The parameter porin/mass in cultures of LEB18 growing at various rates was determined as follows . Firstly, the amount of porin was measured as a proportion of total cell protein at various growth rates. This was achieved by measuring the proportion of total protein radioactivity which was recovered in the 36.5K band cut out from an SDS-PAGE analysis of total cell protein. Although these data are presented as percentages in Table 8.1, they can only be regarded as relative values since total and porin radioactivity were determined under different conditions. Next, the parameter total protein/cell mass was measured at various growth rates; these data are shown in Figure 8.1. Protein/mass clearly decreases as a complex function of growth rate, as reported by Maaløe and Kjeldgaard (1966). This change in cell composition with growth rate reflects the increased ratio of RNA/protein occurring in richer medium as an increased proportion of the cellular economy is directed towards ribosome synthesis.

Finally, the porin/protein data were multiplied by interpolated values of protein/mass taken from Figure 8.1, to derive the values of porin/mass listed in Table 8.1. I will now describe two different interpretations of these porin/mass data.

Figure 8.1: EFFECT OF GROWTH RATE UPON PROTEIN CONTENT OF LEB18

Cultures of LEB18 were grown in media containing the carbon sources listed in Table 8.1. Samples of cultures were taken for determination of protein content as described in Chapter 2.


200
100
0
protein (ug/A450)⁻¹
0
1
2
3
growth rate (doublings/hour)

III. Correlation Between Porin/Mass and Gene Concentration

It has been demonstrated by Chandler and Pritchard (1975) that under conditions of thymine limitation, the differential output of an unregulated gene is directly proportional to the concentration of that gene (i.e. to the number of copies of that gene per unit mass). In Chapter 7, I presented some evidence that thymine limitation produces an 11% decrease in the differential rate of porin synthesis, as would be expected if the porin was encoded by a constitutive gene; since values of C were not measured in the thymine limitation experiment it is not however, possible to calculate a location for the hypothetical constitutive gene. As discussed in Section V of Chapter 1, an increasingly steep gradient of gene concentrations, from early to late genes, similar to that induced by growth in conditions of thymine limitation, can also be produced by increasing the growth rate. The changes in relative concentration of any gene can readily be calculated, and the corresponding theoretical lines for a gene at the origin and a gene at the terminus are shown in Figure 8.2.

Unfortunately, in contrast to thymine limitation, which causes changes in gene concentration without any other changes in the metabolic state of the cells, deliberate variation of growth rate may cause changes in the availability of other factors contributing to gene output, making it difficult to predict the precise effects upon constitutive gene output. Despite this objection to a simplistic analysis of growth rate data, I have also plotted in Figure 8.2 the values for porin/mass listed in Table 8.1. The parameter porin/mass is a direct measure of the differential output of the porin gene since, in an exponentially growing culture, the rate of synthesis of any component relative to mass is directly proportional to the concentration of that component. As is

Table 8.1

EFFECT OF GROWTH RATE UPON PORIN CONTENT OF LEB18

Cultures of LEB18 were grown in media containing the indicated carbon sources. Those cultures not containing casamino acids were supplemented with 5 µg/ml [^{14}C]-leucine (0.02 µCi/µg); to those cultures containing casamino acids, 0.8 µCi/ml of [^{14}C]-leucine (311 mCi/mmol) was added to produce an unknown final specific activity. At $A_{450} \simeq$ 0.2 samples were removed for preparation of whole cell lysates. Total radioactivity in the lysates was measured by taking an aliquot into Biosolv scintillation fluid. Duplicate aliquots of lysates were subjected to SDS-PAGE and radioactivity in the 36.5K band was measured as described in Chapter 2.

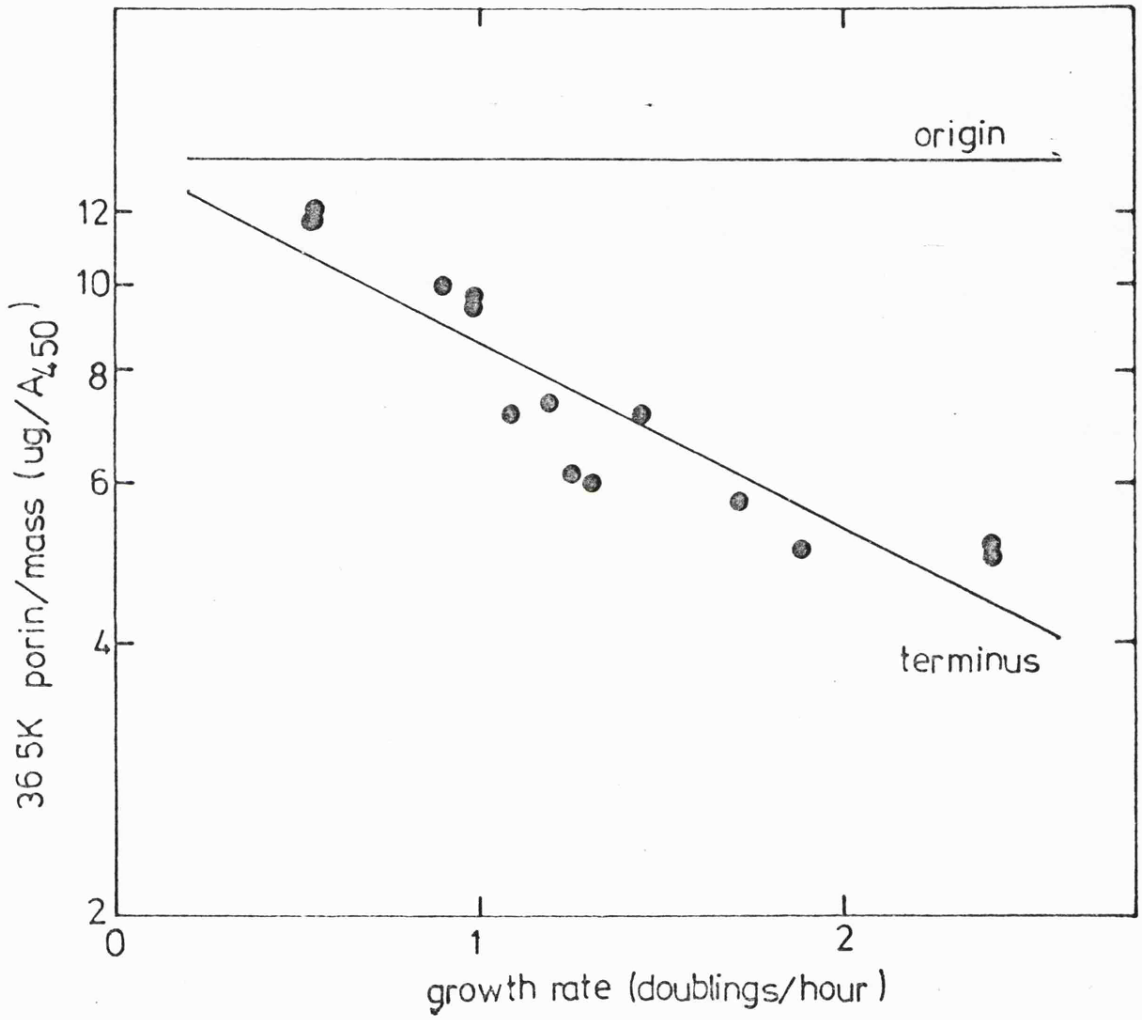
The data shown in column 1 represent the proportion of total radioactivity recovered in the 36.5K band. These were used to derive the values of porin/mass shown in column 2 by multiplying them by interpolated values for total protein/mass taken from Figure 8.1. Finally, values of porin/surface area were derived as described in the text.

Since 36.5K band radioactivity and total protein radioactivity were measured in different scintillation systems the figures for porin/total protein may only be regarded as relative values.

carbon source	growth rate (doublings/hour)	(1) porin / total protein %	(2) porin / mass ($\mu g/A_{450}$)	(3) porin / surface area (arbitrary units)
0.04% alanine	0.55	6.90	11.7	1.24
	0.55	6.88	11.7	1.24
0.04% alanine	0.90	7.28	9.83	1.17
+	0.98	7.23	9.40	1.14
0.04% proline	0.98	7.29	9.48	1.15
0.2% glycerol	1.07	5.69	7.11	0.89
	1.18	6.02	7.28	0.95
0.4%	1.25	5.20	6.14	0.82
glucose	1.30	5.14	5.96	0.81
	1.43	6.32	7.08	1.00
1% casamino	1.71	5.30	5.72	0.89
acids	1.88	4.77	5.01	0.82
1% casamino acids	2.40	5.20	5.15	1.00
+	2.40	5.03	4.98	0.97
0.4% glucose				

Figure 8.2: CORRELATION BETWEEN PORIN CONTENT AND NUMBER OF CHROMOSOME TERMINI

The data were derived as described in the legend to Table 8.1. The two lines drawn in the figure are the theoretical curves of gene/mass for the origin and terminus of replication - these have been aligned with the experimental data so that the terminus line passes through the mean of the data. The regression line through the data is indistinguishable from the line drawn.


origin
terminus
12
10
8
6
4
2
0
1
2
36 5K porin/mass (ug/A450)
growth rate (doublings/hour)

clear from Figure 8.2, the values of porin/mass fit closely to the line calculated for concentration of a terminally-located gene. In fact I have calculated the linear regression line through the porin/mass data (correlation coefficient = 0.91) and have found that the slope of this regression line differs from that of the terminus/mass line by only 2%. Thus, if the output of a constitutive gene at different growth rates is indeed determined by gene concentration, then the 36.5K porin could be the product of such a gene located near the terminus of replication.

IV. Correlation Between Porin and Cell Surface Area

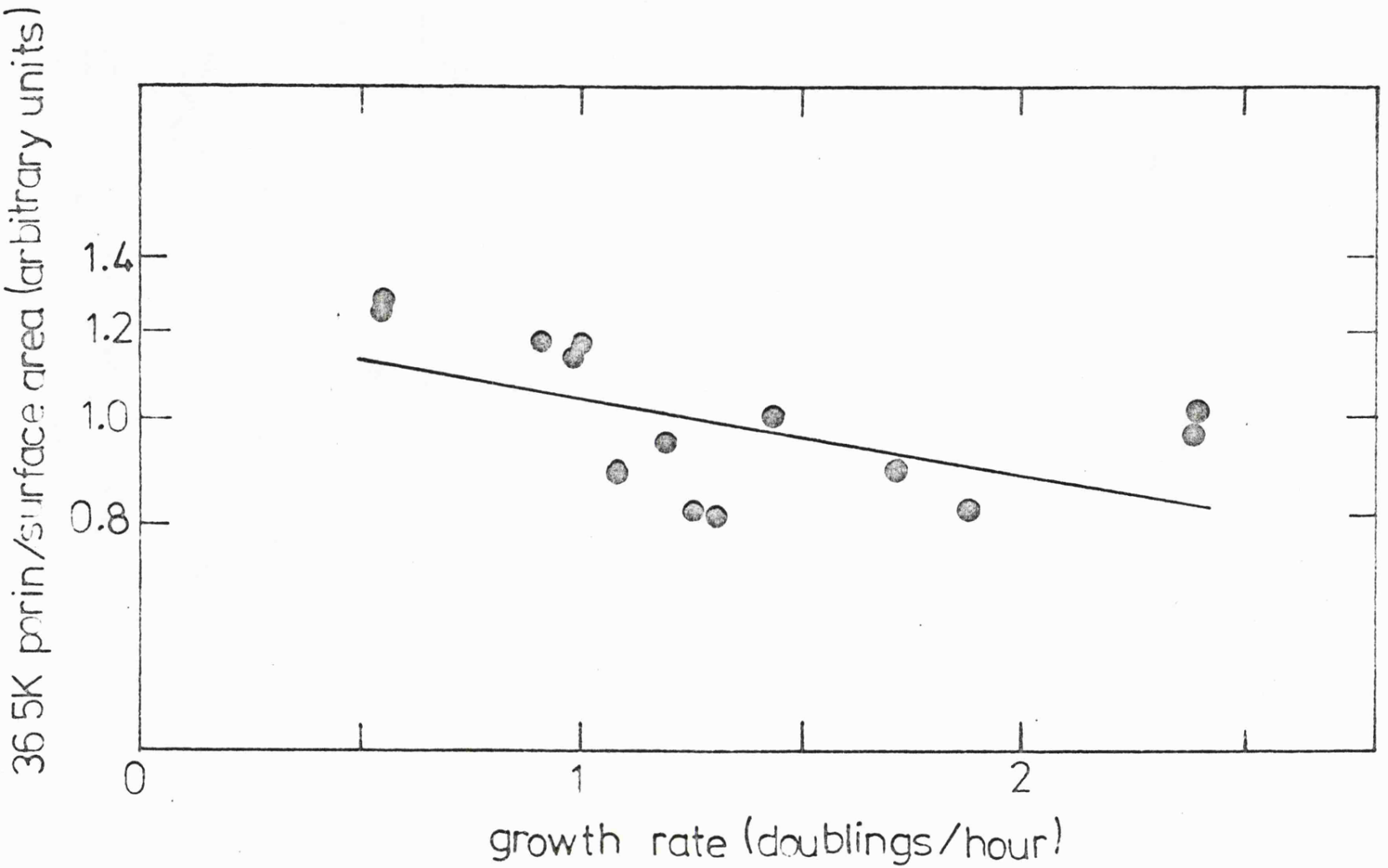
The porin/mass data of Table 8.1 were also used to derive a set of values for the amount of porin per unit surface area over the range of growth rates, as follows. From data of R.F. Rosenberger (personal communication), an empirical relationship was derived relating mean surface area/volume of cells to growth rate. This relationship, derived by least squares linear regression, is

$$\text{surface/volume} = 11.35 \times e^{-0.33/\tau}\ \mu m^{-1}$$

and was used to generate values of surface area/volume for the growth rates for which porin/mass had been measured. These two sets of figures were then combined to produce values for the parameter porin/surface area, plotted in Figure 8.3.

Figure 8.3: VARIATION IN SURFACE DENSITY OF THE 36.5K PORIN WITH GROWTH RATE

The data presented in Table 8.1 are plotted in the figure.


36.5K prin/surface area (arbitrary units)
1.4
1.2
1.0
0.8
0
1
2
growth rate (doublings/hour)

V. Discussion

I have measured the parameter 36.5K porin/mass for LEB18 over a range of growth rates. This measurement was derived by assuming that there is no growth rate dependent variation in the proportion of the 36.5K band which is porin-specific.

It is clear from the plot of Figure 8.2 that the variation in porin/mass with growth rate correlates well with the number of chromosome termini per unit mass. If the output of a constitutive gene at different growth rates is determined solely by gene concentration, then this correlation is consistent with the presence of a constitutively expressed porin gene located near the terminus. As discussed in Section III of Chapter 1, the location of the porin genes of *E.coli* K12 has not been unambiguously established. The best candidate for the gene encoding protein b (equivalent to the 36.5K porin of *E.coli* B/r) is, however, the *tolF* locus located at 22 minutes. This map position corresponds to a fractional distance of 0.2 from the chromosomal terminus. Within the limits of resolution of the experimental methods used, this location is consistent with the porin/mass data of Figure 8.2. However, despite this close correlation between cellular levels of porin and the number of chromosome termini, other evidence to be presented in Chapter 9 weakens the case for constitutive expression of the porin gene. The idea that the cell cycle pattern of outer membrane protein synthesis may merely reflect constitutive synthesis of the 36.5K porin will therefore be discussed further in Chapter 11, in the light of all available data.

The derivation of the values of porin/surface area (surface density of the porin) shown in Figure 8.3 is based upon the assumptions that

mean cell density is independent of growth rate (Kubitschek, 1974), and that the surface/volume data derived from measurements of the dimensions of E.coli B/r substrain H at various growth rates can be applied to LEB18 (substrain F). With these reservations in mind, the data of Figure 8.3 are consistent with a progressive fall in the surface density of the porin with growth rate. This result has implications for any attempt to relate the cell cycle pattern of porin synthesis, and indeed that of bulk outer membrane protein, to the linear growth of the cell surface postulated by Rosenberger et al. (1978). I will return to this point in Chapter 11.

CHAPTER 9

EVIDENCE FOR A FACTOR WHICH LIMITS OVERALL SYNTHESIS OF BULK OUTER MEMBRANE PROTEIN

I. Introduction

A general hypothesis which can explain the cell cycle pattern of synthesis of bulk outer membrane protein (described in Chapter 6) is that there is a limiting factor involved in outer membrane protein synthesis whose availability doubles at the point in the cycle corresponding to the doubling in rate of outer membrane protein synthesis. In fact, this hypothesis merely defines the problem : what is the limiting factor, specific to outer membrane protein synthesis, whose availability doubles at a fixed cell cycle point? In Chapter 7 I presented data from experiments designed to test the hypothesis that the availability of the limiting factor is determined by the number of copies of a specific region of the chromosome : these data indicate that, in fact, the rate of synthesis of outer membrane protein is independent of chromosome replication. In this chapter I will present more direct evidence that a limitation upon the overall rate of synthesis of outer membrane protein does exist, which may be responsible for the cell cycle pattern of synthesis.

II. An Apparent Effect of Thymine Starvation Upon the Rate of Synthesis of the 36.5K Porin

As part of my investigation of the effect of thymine starvation upon outer membrane protein synthesis, discussed in Chapter 7, experiments were performed in which the rate of synthesis of the major 36.5K porin of LEB16 was measured during thymine starvation. The results of these experiments are shown in Figure 9.1. Although bulk outer membrane protein synthesis is unaffected by thymine starvation (see Figure 7.1), it is clear from Figure 9.1 that there is, nevertheless, a decreased relative rate of synthesis of the 36.5K porin (which comprises 35% of total outer membrane protein being synthesised before starvation). At first this result was taken to indicate a link between DNA replication and porin synthesis, perhaps due to constitutive synthesis of the protein. It was realised, however, that the crucial point arising from these experiments, taken together with the evidence presented in Chapters 5 and 7, was that during thymine starvation, despite this reduced rate of porin synthesis, and despite the presumed induction of the outer membrane iron transport proteins by the filtration manipulation used in the thymine starvation experiments (see Chapter 5), the overall rate of synthesis of bulk outer membrane protein synthesis was not detectably affected (see Figure 7.1). To confirm this, another thymine starvation experiment was performed in which all of these changes were measured, shown in Figure 9.2. In fact, the data show an abrupt fall in differential rate of synthesis of bulk outer membrane protein which occurred with thymine starvation in this particular experiment. This was also seen in the corresponding curve for cytoplasmic membrane protein and was presumably due to a labelling artifact in this experiment,

Figure 9.1: APPARENT EFFECT OF THYMINE STARVATION UPON PORIN SYNTHESIS

The data are from four independent experiments. Cultures of LEB16 were grown in proline/alanine medium (+ 20 µg/ml thymine) to an $A_{450} \simeq 0.1$. Samples were withdrawn at intervals for pulse-labelling (2 min) with 10 µCi $[^{35}S]$-methionine (specific activity = 50 µCi/ug). Thymine starvation was carried out as described in Chapter 2 (i.e. by filtration) and pulse-labelling was continued during subsequent growth. The kinetics of mass increase always closely resembled those shown in Figure 7.1. Cell lysates were prepared from pulse-labelled samples, and total and 36.5K gel slice radioactivity were determined as usual. The data are presented as relative rates of porin synthesis, i.e. 36.5K incorporation/total incorporation in pulse.

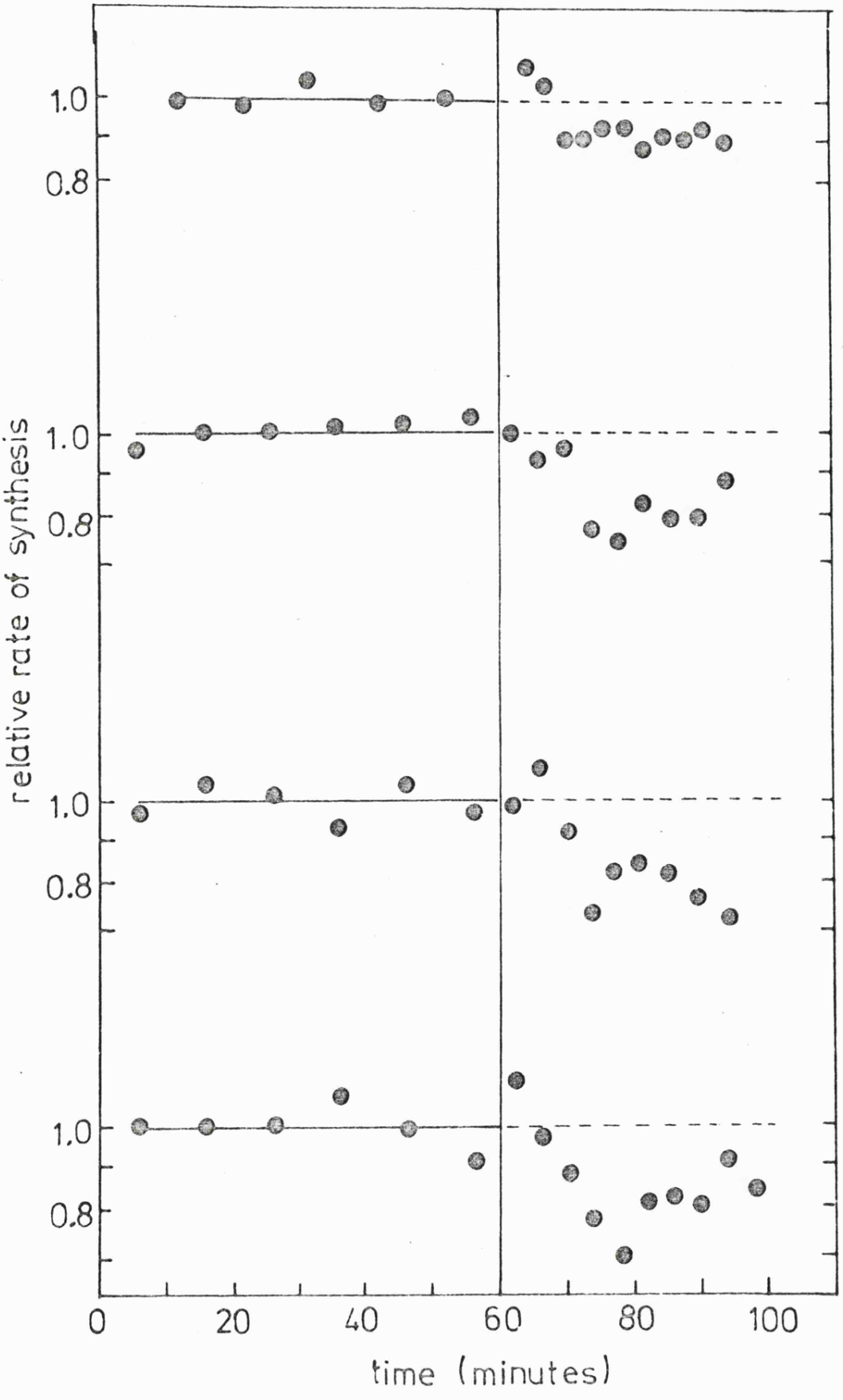

relative rate of synthesis
1.0
0.8
1.0
0.8
1.0
0.8
1.0
0.8
0
20
40
60
80
100
time (minutes)

Figure 9.2: MULTIPLE EFFECTS OF THYMINE STARVATION UPON OUTER MEMBRANE PROTEIN SYNTHESIS

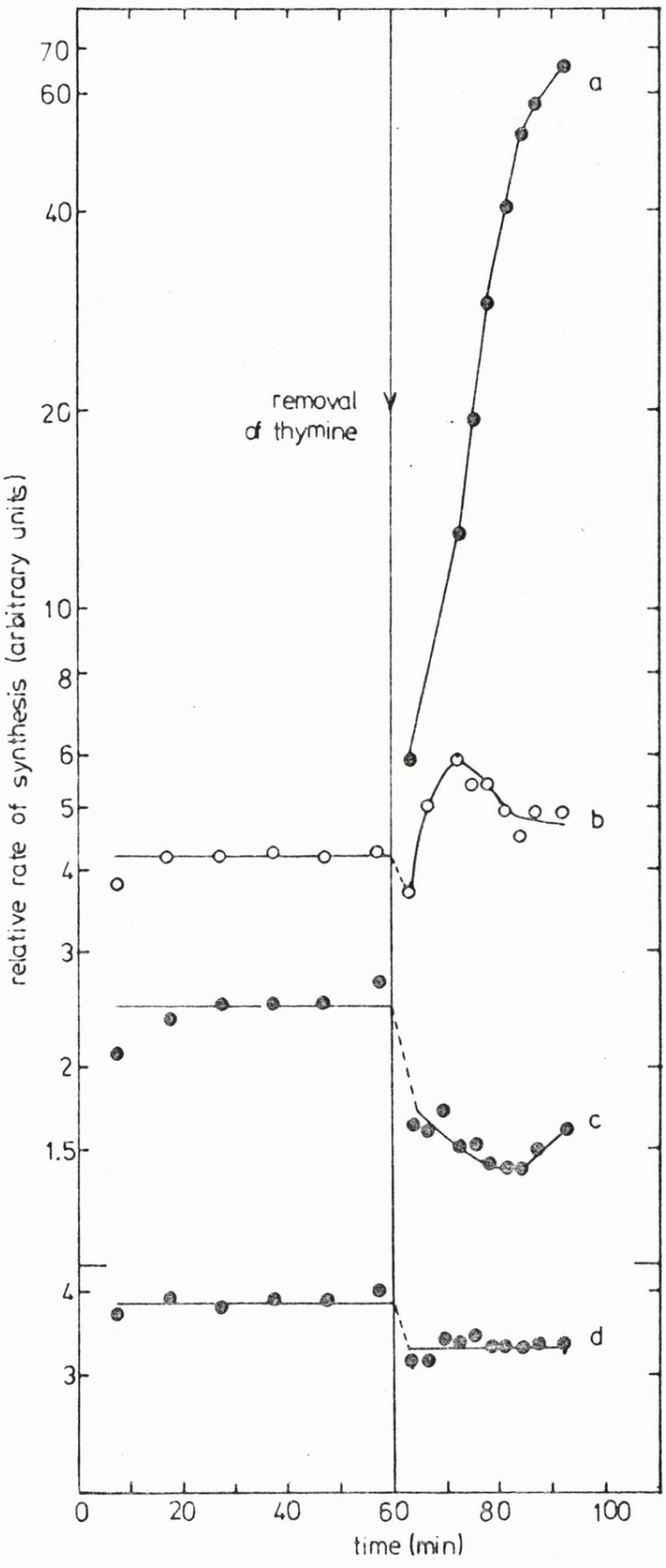
A thymine starvation experiment was performed as described in the legend to Figure 9.1, except that the concentration of methionine in the pulse-medium was < 0.075 μg/ml. An internal standard of [^{3}H]-leucine-labelled cells was added to pulse-labelled samples before determination of TCA-precipitable radioactivity and membrane preparation. Radioactivity in outer membrane protein was measured in Biosolv scintillation fluid. Samples of outer membrane preparations were also analysed by SDS-PAGE and radioactivity in the 36.5K, 34K and 81K bands was measured. The data are presented as rates of synthesis relative to total cell protein.

(a) 81K band (*feuB* protein) : radioactivity in this band before thymine starvation was too low to be determined with any accuracy.

(b) 34K band

(c) 36.5K band

(d) bulk sarkosyl-insoluble protein (outer membrane)


70
60
40
20
10
8
6
5
4
3
2
1.5
4
3
removal
of thymine
a
b
c
d
relative rate of synthesis (arbitrary units)
0
20
40
60
80
100
time (min)

arising from the use of a very low concentration of radioactive methionine (< 0.075 μg/ml), in combination with the two-fold dilution which accompanied thymine starvation. In any case the important conclusions to be drawn from the data of Figure 9.2 are derived solely from the post-starvation period, when it is clear that despite the fall in differential rate of synthesis of the porin; despite the induction of the feuB protein (estimated from data presented below to be increased to at least 10% of total outer membrane protein being synthesised at the peak of induction); and despite an effect of the experimental procedure upon the rate of synthesis of the 34K major outer membrane protein d (around 5% of total outer membrane protein in LEB16), the differential rate of synthesis of bulk outer membrane protein remained constant throughout the starvation period. It therefore seemed possible that the apparent effect of thymine starvation upon the rate of porin synthesis was in fact caused by the induction of the iron transport proteins, and reflected an overall limitation upon the rate of synthesis of bulk outer membrane protein.

III. The Effect of α, α' Dipyridyl Upon Outer Membrane Protein Synthesis

In order to test the hypothesis that induction of synthesis of the outer membrane iron transport proteins could affect the synthesis of the 36.5K porin, an experiment was performed in which the effects of α,α' dipyridyl (DP) upon outer membrane protein synthesis were measured. As described in Chapter 5, this compound chelates ferric ions, thereby reducing the amount of iron available in the growth medium. Cells of E.coli respond to this iron stress by increasing

their rate of synthesis of the chelator enterochelin and of specific outer membrane proteins which transport ferric enterochelin and other ferric chelate complexes into the cell (discussed in Chapter 1, Section IV).

The results of an experiment in which a culture of LEB18 was exposed to DP are shown in Figure 9.3. At the level of DP used, (50 μM), there was a small transient effect upon growth lasting for 5-10 minutes, after which growth rapidly resumed its former rate. This effect upon growth is reflected in the data showing the rate of synthesis of total protein in the culture. The data relating to synthesis of bulk membrane fractions, the 36.5K porin, and the feuB protein are plotted as rates of synthesis relative to that of total cell protein. Addition of DP clearly had no effect upon the relative rate of synthesis of bulk cytoplasmic membrane protein; in the case of bulk outer membrane protein synthesis, however, there was an early transient increase in relative rate of synthesis during the period when overall growth was affected. The treatment clearly induced the synthesis of the feuB gene product; this protein, of molecular weight 81K, is the outer membrane protein responsible for transport of ferric enterochelin, and is, together with the products of the ent gene cluster (responsible for biosynthesis of enterochelin), induced by iron deficit. In this experiment the rate of synthesis of the feuB protein was increased 14-fold. In contrast, the DP treatment reduced the relative rate of synthesis of the 36.5K porin measured both in outer membrane preparations and also in an SDS-PAGE analysis of total cell protein; this latter measurement indicates that the rate of synthesis of the porin was indeed reduced, not merely the rate of insertion into the outer membrane. The data shown in Table 9.1 are from the same

Figure 9.3: EFFECTS OF DIPYRIDYL UPON OUTER MEMBRANE PROTEIN SYNTHESIS

A culture of LEB18 was grown in proline/alanine medium to an A_{450} = 0.12, and samples of culture were pulse-labelled for 2 minutes with 5 µCi [^{35}S]-methionine (17 µCi/ug). At an A_{450} = 0.15, dipyridyl was added to a final concentration of 50 µM and then samples were again withdrawn for pulse-labelling. To each pulse-labelled sample was added a constant aliquot of [^{3}H]-leucine-labelled cells which had been labelled during growth in 50 µM dipyridyl. Aliquots of each sample were then removed for the determination of TCA-precipitable radioactivity, for preparation of SDS lysates, and for preparation of membrane fractions. Radioactivity in membrane fractions was determined in Biosolv scintillation fluid. Cell lysates and outer membrane preparations were analysed by SDS-PAGE. The 36.5K band was cut out from both gels, and the 81K (*feuB*) band was cut out from the gel of outer membrane proteins. All data are presented as relative rates of synthesis (^{35}S/^{3}H in band or fraction/^{35}S/^{3}H in total protein).

(a) 81K band (*feuB* protein)

(b) 36.5K band analysed in cell lysates

(c) 36.5K band analysed in outer membrane preparations

(d) sarkosyl-insoluble envelope material (outer membrane protein)

(e) sarkosyl-soluble envelope material (cytoplasmic membrane protein)

(f) rate of synthesis of total cell protein

(g) optical density

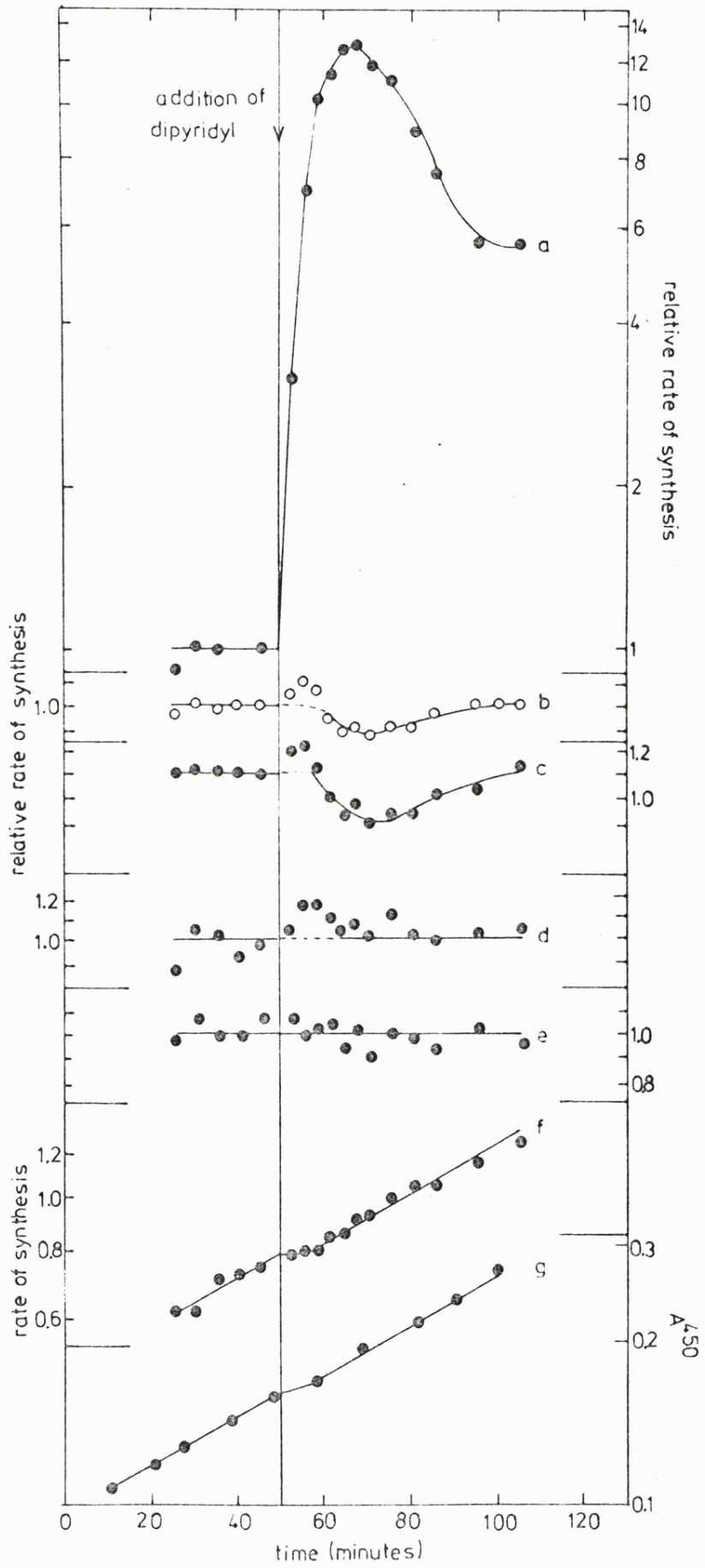

addition of
dipyridyl
a
b
c
d
e
f
g
relative rate of synthesis
relative rate of synthesis
rate of synthesis
A450
time (minutes)
0
20
40
60
80
100
120

Table 9.1

EFFECT OF DIPYRIDYL UPON OUTER MEMBRANE COMPOSITION

Sarkosyl-insoluble membrane proteins from uninduced and maximally induced samples from the experiment shown in Figure 9.2 were analysed by SDS-PAGE. The bands corresponding to the 81K *feuB* protein and the 36.5K porin were cut out to derive the data shown in Figure 9.2. The region of the gel containing the other induced proteins (74 - 81K) was cut out and then the rest of the gel was cut into small pieces; the radioactivity in each piece was then measured in NCS scintillation fluid. The total ^{35}S radioactivity in each slot was thus determined and the proportions of pulse-labelled outer protein formed by the 36.5K porin and the iron transport proteins was calculated.

protein species	fraction of total outer membrane protein	
	uninduced %	induced %
iron transport proteins	2.8	17.1
36.5K porin	35.0	27.2
other	62.2	55.7

experiment as that of Figure 9.3, and represent measurements, in pre- and post-treatment samples, of the proportions of pulse-labelled outer membrane protein formed by the 36.5K porin and the induced iron transport proteins. These data show an approximately 6-fold induction of the combined iron transport proteins lying in the molecular weight range 74-81K. This is lower than found for the individual *feuB* protein, a difference which probably reflects the presence in that region of the gel (74K-81K) of polypeptides whose synthesis is not induced by DP treatment.

IV. Discussion

The experiment of Figure 9.3 was designed to test the hypothesis that there is an overall limitation upon the rate of synthesis of bulk outer membrane protein in an exponential culture : synthesis of a new class of outer membrane proteins was induced, and the effects of this induction upon the rates of synthesis of bulk outer membrane protein, and of an individual outer membrane protein, were measured. The predictions of a hypothesis of overall limitation are that the rate of bulk outer membrane protein synthesis should be unaffected by the induction of a new class of outer membrane proteins, but that the rates of synthesis of other outer membrane proteins should be correspondingly reduced as the new class of proteins competes for a share of the limiting factor. The results of the experiment were in general agreement with these predictions. First of all, it is clear that the DP treatment had very little effect upon the overall rate of synthesis of bulk outer membrane protein. The early, transient, increase in differential rate may indicate a relative insensitivity of outer membrane protein synthesis to the transient inhibition of growth; this

hypothesis is supported by the fact that the same effect is seen in the relative rate curve for the 36.5K porin. The transient effect of DP upon the kinetics of mass increase and rate of total cell protein may reflect a brief inhibition of transcription; in this case the relative rate of synthesis of outer membrane proteins, whose mRNAs have been reported to be unusually long-lived, (Hirashima et al., 1973) might be expected to behave in just this manner. Whatever the explanation of this early effect, it is clear that in the samples corresponding to the peak of induction of the feuB protein, the relative rate of synthesis of bulk outer membrane protein is not significantly different from that in the period before addition of DP.

Turning now to the effect of the induction upon the synthesis of the 36.5K porin, it is clear that there is a strong reciprocity between this rate of synthesis and that of the feuB protein. At the time corresponding to the peak of induction of the feuB protein, the relative rate of synthesis of the porin is reduced by about 20%. From the data of Table 9.1 it can be calculated that the predicted reduction in relative rate, assuming an absolute limitation upon overall synthesis, is 15% if all outer membrane proteins compete on an equal footing. This discrepancy probably reflects inaccuracy of measurement, but could be due to the derepression of other outer membrane proteins, either as a direct result of DP treatment or because their rate of insertion into the outer membrane is strictly maintained. It should be noted that if there was an overall limitation upon total envelope protein synthesis, (i.e. cytoplasmic membrane and outer membrane protein) the effect upon rate of synthesis of the porin would be expected to be much lower.

In summary, I wish to conclude from the data presented in this

chapter that there is, indeed, an overall limitation upon the rate of synthesis of bulk outer membrane protein in an exponential culture, such that if synthesis of a new class of outer membrane proteins is induced, then the rate of synthesis of other outer membrane proteins is correspondingly reduced. This explains the apparent effect of thymine starvation upon porin synthesis.

A discontinuous increase in the availability of this limiting factor during the cell cycle would explain the observed linear accumulation of outer membrane protein in the cycle. I will defer speculation as to the nature of the limiting factor until Chapter 11.

CHAPTER 10

AN ANALYSIS OF THE KINETICS OF SYNTHESIS AND INSERTION OF OUTER MEMBRANE PROTEINS

I. Introduction

The route by which outer membrane proteins reach their final location is completely obscure. Section IV of Chapter 1 contained some discussion of evidence which suggests that outer membrane proteins may be synthesised as nascent preproteins, with presumptive amino-terminal signal sequences determining at least some stages of their insertion into the cell envelope. The general role of such signal sequences in membrane protein synthesis was discussed in detail in Section II of Chapter 1.

Apart from attempts to identify preprotein forms of outer membrane proteins, investigations of the problem of outer membrane protein synthesis have been restricted to simple experiments, designed basically to characterise the kinetics of movement of proteins into the outer membrane, and to detect any intermediates in the cytoplasmic membrane. Thus Lee and Inouye (1974) performed a pulse-chase experiment in which they attempted to detect the movement of pulse-labelled proteins from the cytoplasmic membrane to the outer membrane, having separated the two membranes by sucrose density gradient centrifugation. Lee and Inouye concluded that outer membrane proteins are inserted directly into the outer membrane, since no cytoplasmic membrane intermediates were detected. More recently Ito *et al.* (1977) have carried out a somewhat more

thorough analysis of pulse-chase experiments, and have measured the kinetics of synthesis and insertion of protein into the cytoplasmic and outer membranes, again separated on a density basis. Ito et al. concluded that the movement of proteins into the outer membrane occurred more slowly than did that of proteins into the cytoplasmic membrane; these conclusions were, however, based upon measurements of bulk membrane material, which are, as I will demonstrate below, extremely difficult to interpret. I therefore set out to define more carefully the kinetics of outer membrane protein synthesis, with the additional intention of measuring an individual polypeptide species - the 36.5K porin.

II. Rationale of the Pulse-Chase Experiments

The pulse-chase technique used was designed to detect short-lived kinetic intermediates of outer membrane proteins. The details of the experiments are described in figure legends, and in Chapter 2. Briefly, cells were pulse-labelled with radioactive methionine and the pulse was terminated by addition of an excess of unlabelled methionine. During the ensuing chase period samples of labelled cells were taken, chilled immediately, and the kinetics of chasing of radioactivity into various cell fractions and gel bands were analysed. It is clear that at the time when chasing commences, many unfinished labelled polypeptides will be present; as time of chasing elapses these molecules will be completed, and will gradually contribute to the total radioactivity of a given polypeptide species, analysed as a band of known molecular weight on a gel. Thus the time taken for the radioactivity in an individual polypeptide to reach a plateau value during the chase period (referred to from now on as run-out time) is a direct measure of the

time taken to synthesise that part of the polypeptide lying between the first methionine residue and the carboxyl terminus of the protein. Since many polypeptides, including the 36.5K porin, have their amino-terminal methionine residue removed, the run-out time is likely to be an underestimate of the time taken to synthesise the complete polypeptide chain. In experiments in which it was vital to have an accurate measure of translation times the comparitively rare amino acid methionine would not be the label of choice; it would be better to use either a common amino acid such as leucine, or a mixture of amino acids. In fact this problem does not affect the main conclusions to be drawn in this study.

The general principle of the pulse-chase procedure is illustrated by the autoradiograph shown in Figure 10.1. In this experiment samples were taken at increasing times of chase and cell lysates were analysed by SDS-PAGE. Since equal amounts of radioactive material were loaded in each slot of the gel, the increasing intensity of specific bands in progressively later samples reflects the run-out of those polypeptides which were incomplete at the start of the chase period. It should also be noted that if a complete preprotein precursor form of the 36.5K porin existed *in vivo* (as opposed to a preprotein which was processed during translation) then the precursor might have been seen in this experiment, as a band of approximate molecular weight 39K, visible in the earliest samples but disappearing as chase time elapsed. Since there is no obvious candidate for such a band in Figure 10.1, it seems likely that the complete preprotein form of the porin detected in toluenised cells (Sekizawa et al., 1977) does not exist *in vivo*.

Figure 10.1: COMPLETION OF INDIVIDUAL POLYPEPTIDES IN A PULSE-CHASE EXPERIMENT

A culture of LEB18 was grown at 30° in minimal glucose medium to a A_{450} = 0.2, and then pulse-labelled by the addition of 50 µCi of [^{35}S]-methionine (680 Ci/mmol). After 30 seconds of labelling, unlabelled methionine was added to a final concentration of 150 µg/ml, and samples were withdrawn at frequent intervals into ice-cold medium lacking glucose but containing chloramphenicol (final concentration - 600 µg/ml). SDS lysates were prepared and analysed by SDS-PAGE. The gel was dried down and autoradiographed (no fluorography).

slot 1 : 0 seconds of chase

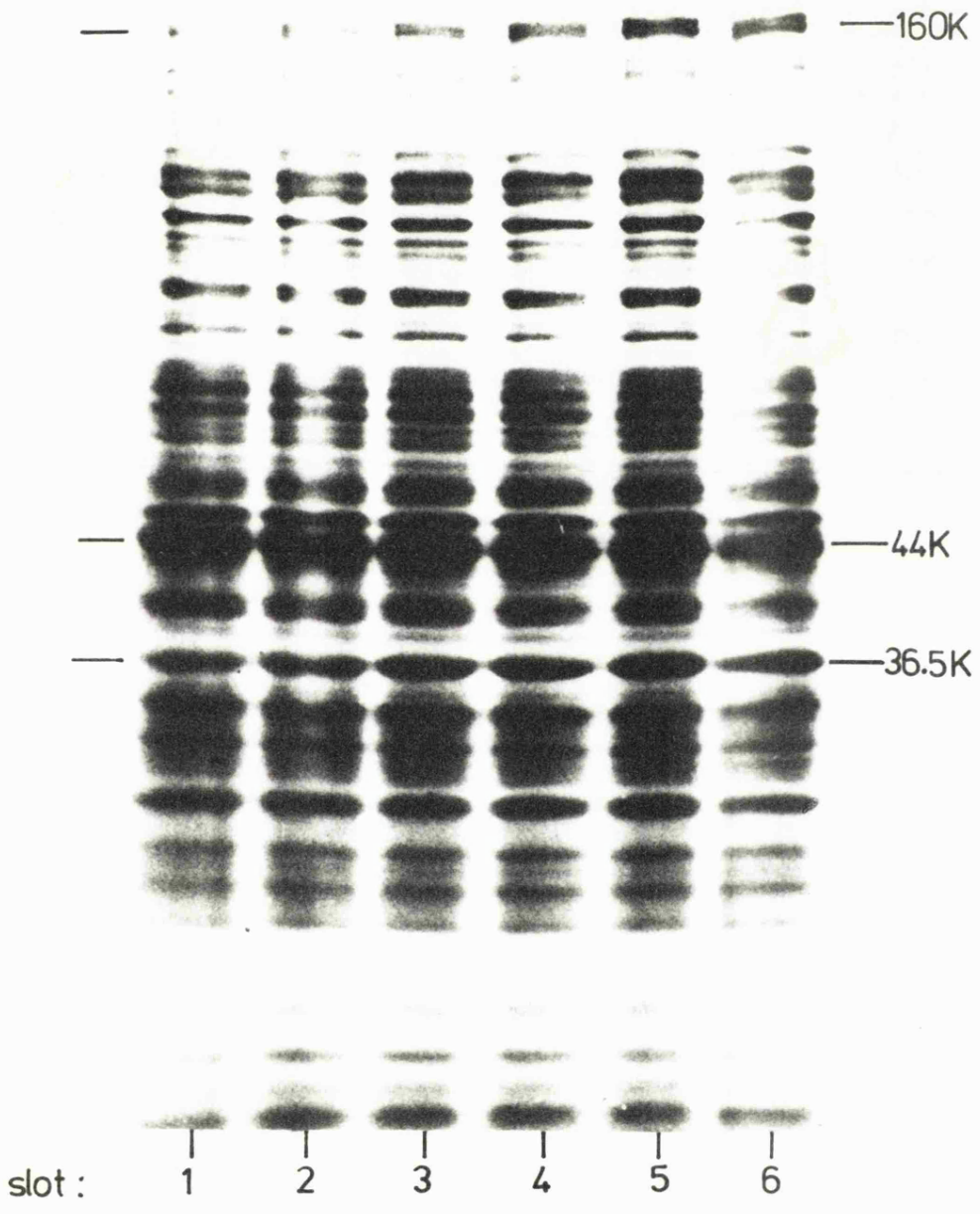
slot 2 : 15 " " "

slot 3 : 30 " " "

slot 4 : 60 " " "

slot 5 : 90 " " "

slot 6 : 210 " " "


160K
44K
36.5K
slot :
1
2
3
4
5
6

III. Kinetics of Synthesis and Insertion of the 36.5K Porin

The run-out time for a given polypeptide analysed in a cell lysate represents the point when the last labelled copy of that polypeptide is completed. If, however, we consider the biosynthesis of an envelope protein, then it is clear that if the kinetics of synthesis of that polypeptide are measured in envelope fractions, the run-out time will be equal to the sum of the translation time and any time elapsing before insertion of the completed protein into the envelope. The analysis of run-out kinetics in various cell fractions thus provides a means of defining some features of outer membrane protein synthesis and insertion.

The data shown in Figure 10.2 are from a pulse-chase experiment, carried out at 30°C, in which the kinetics of appearance of complete 36.5K porin were analysed in total cell lysates and in outer membrane fractions. Also shown are the kinetics of appearance of completed molecules of the RNA polymerase subunits β and β', which provide a readily identifiable high molecular weight band for analysis (Iwakura et al., 1974); the two polypeptides actually form a doublet in the gel, but were cut out as a single band for this analysis. From the run-out time for the ββ' doublet, and from the approximate mean molecular weight of the two proteins (160K) it is possible to calculate a value for polypeptide chain growth rate assuming that one or both of the two polypeptides has a methionine residue at or near the amino terminus. This value for polypeptide chain growth rate, shown in Table 10.1, was used to calculate a theoretical translation time for the 36.5K porin, indicated in Figure 10.2. Despite the uncertainties inherent in the method of analysis, this time agrees fairly well with the observed run-out time. It should be noted that the existence of an amino-terminal

Table 10.1

POLYPEPTIDE CHAIN GROWTH RATE AT VARIOUS TEMPERATURES

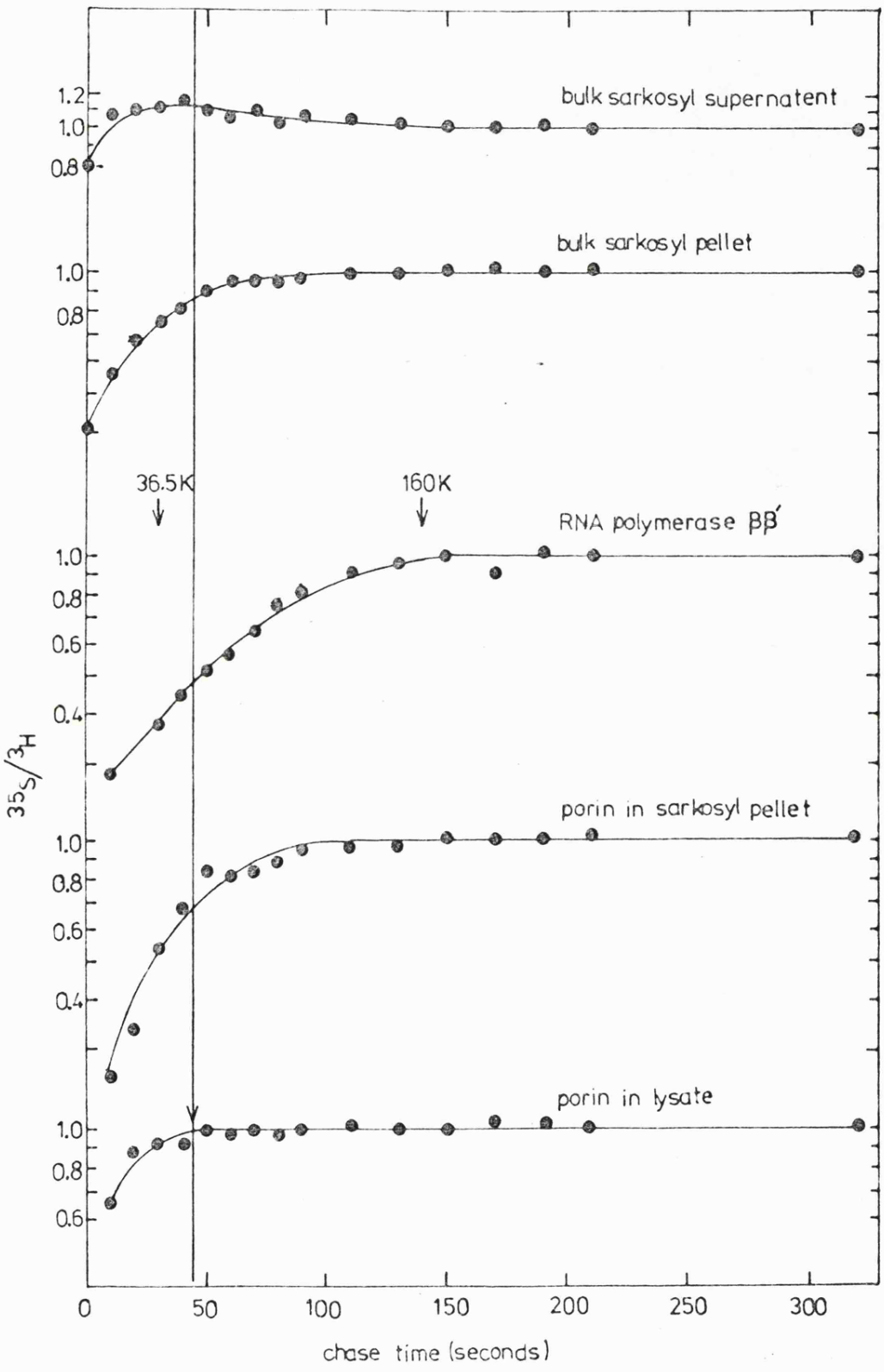
The run-out times for the RNA polymerase ββ' gel band in the experiments of Figures 10.2 - 10.4 were used to calculate polypeptide chain growth rates, assuming a mean molecular weight of 120 for amino acids. The chain growth rate at 37° was taken from data of Dennis and Bremer (1974).

temperature (°C)	polypeptide chain growth rate (amino acids/sec/ribosome)
* 37	13.5
30	9.5 ; 11.1
25	6.7

Figure 10.2: KINETICS OF OUTER MEMBRANE PROTEIN SYNTHESIS

A culture of LEB18 was grown at 30°C in glucose medium supplemented with 1 μg/ml [^{3}H]-leucine (4 μCi/μg), to an $A_{450} \simeq 0.15$, when 25 ml of culture was transferred to a 100 ml beaker. The cells were pulse-labelled by the addition of 50 μCi of [^{35}S]-methionine (680 Ci/mmol); after 30 seconds unlabelled methionine was added (final concentration 150 μg/ml) and then samples (≃ 1 ml) were withdrawn at frequent intervals into 10 ml of ice-cold M9 medium (without glucose) + chloramphenicol (final concentration - 600 μg/ml). Aliquots of each sample were removed for the preparation of SDS lysates, and then membrane fractions were prepared from the remainder of each sample. SDS lysates and outer membrane material were analysed by SDS-PAGE. The 36.5K band was cut out from both gels, and the RNA polymerase ββ' doublet was cut out from the gel of SDS lysates. Radioactivity in membrane fractions was determined in Biosolv scintillation fluid. All data are presented as $^{35}S/^{3}H$ in the corresponding band or fraction, normalised to the final plateau value.

The predicted run-out time for a 36.5K polypeptide is marked, as calculated from the estimated run-out time for the ββ' doublet (160K, also marked). The vertical line drawn in the figure passes through the estimated run-out point of the 36.5K porin analysed in the cell lysate.


bulk sarkosyl supernatent
bulk sarkosyl pellet
36.5K
160K
RNA polymerase ββ'
porin in sarkosyl pellet
porin in lysate
35S/3H
chase time (seconds)
0
50
100
150
200
250
300

signal sequence in the nascent porin polypeptide would not affect this analysis as long as it was proteolytically removed during translation, as is suggested by the data of Figure 10.1.

Turning to the analysis of the entry of the porin into the outer membrane, it is clear from the data of Figure 10.2 that there is a lag of at least 40 seconds between run-out in the cell lysate and run-out into the outer membrane. This is direct evidence that the protein does not immediately become associated with the sarkosyl-insoluble outer membrane upon completion of translation. This finding was confirmed and extended by the experiment shown in Figure 10.3. In this case the run-out kinetics of the porin were also measured in a total cell envelope fraction. Uncertainty in the data makes it difficult to determine with confidence the point of run-out in the lysate analysis; comparison with the kinetics measured in the cell envelope is therefore difficult, but it seems that if there is any lag between completion of a porin molecule and its appearance in the envelope, then it must be very short. In contrast, a clear lag before run-out into the outer membrane was again obtained, lasting at least 25 seconds in this experiment. It must be emphasised that since the 36.5K gel band is analysed in these experiments, this data constitutes evidence for a sarkosyl-soluble intermediate form of the mature porin, not of an unprocessed preprotein (presumptive molecular weight, 39K).

In an effort to obtain a clearer measurement of this phenomenon, and to determine whether or not there is a delay between completion of the porin and its appearance in the cell envelope, I repeated the experiment once more, this time with cells grown at 25°C; the results of this experiment are presented in Figure 10.4. These data confirm the previous findings : firstly the run-out time for the porin analysed

Figure 10.3: KINETICS OF OUTER MEMBRANE PROTEIN SYNTHESIS

A second pulse-chase experiment was carried out at 30°C as described in the legend to Figure 10.2, except that unlabelled leucine (final concentration 100 μg/ml) was added to the culture 15 minutes before labelling with [^{35}S]-methionine, to abolish residual [^{3}H]-leucine incorporation during the chase period.

Aliquots of each sample were removed for determination of total TCA-precipitable radioactivity, and for the preparation of SDS lysates and then membrane fractions were prepared from the remainder of each sample. The 36.5K band was cut out from SDS-PAGE analyses of SDS lysates, cell envelopes and sarkosyl-insoluble outer membranes. All other procedures were as before.

Theoretical and estimated run-out times are marked as explained in the legend to Figure 10.2.

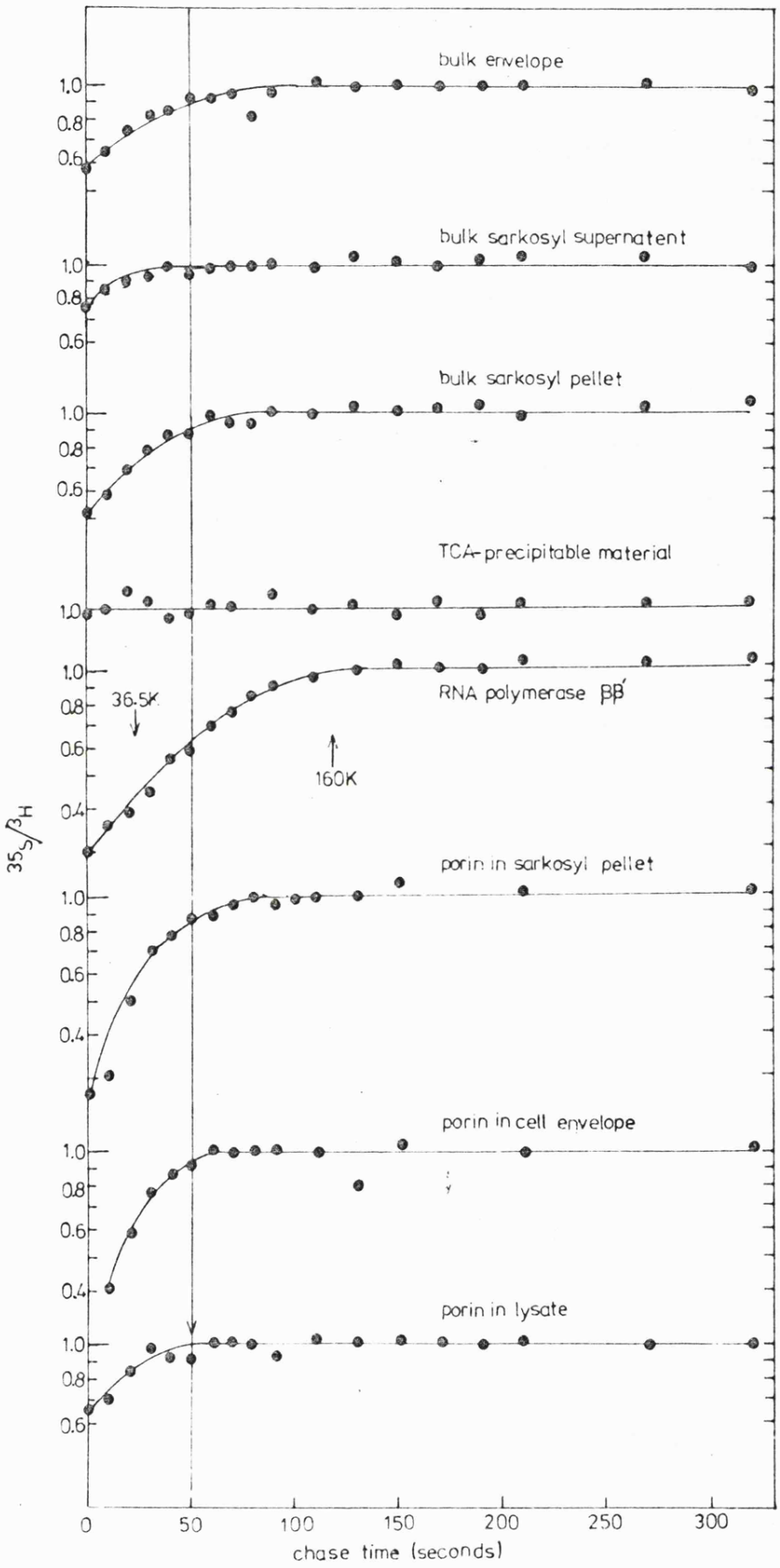
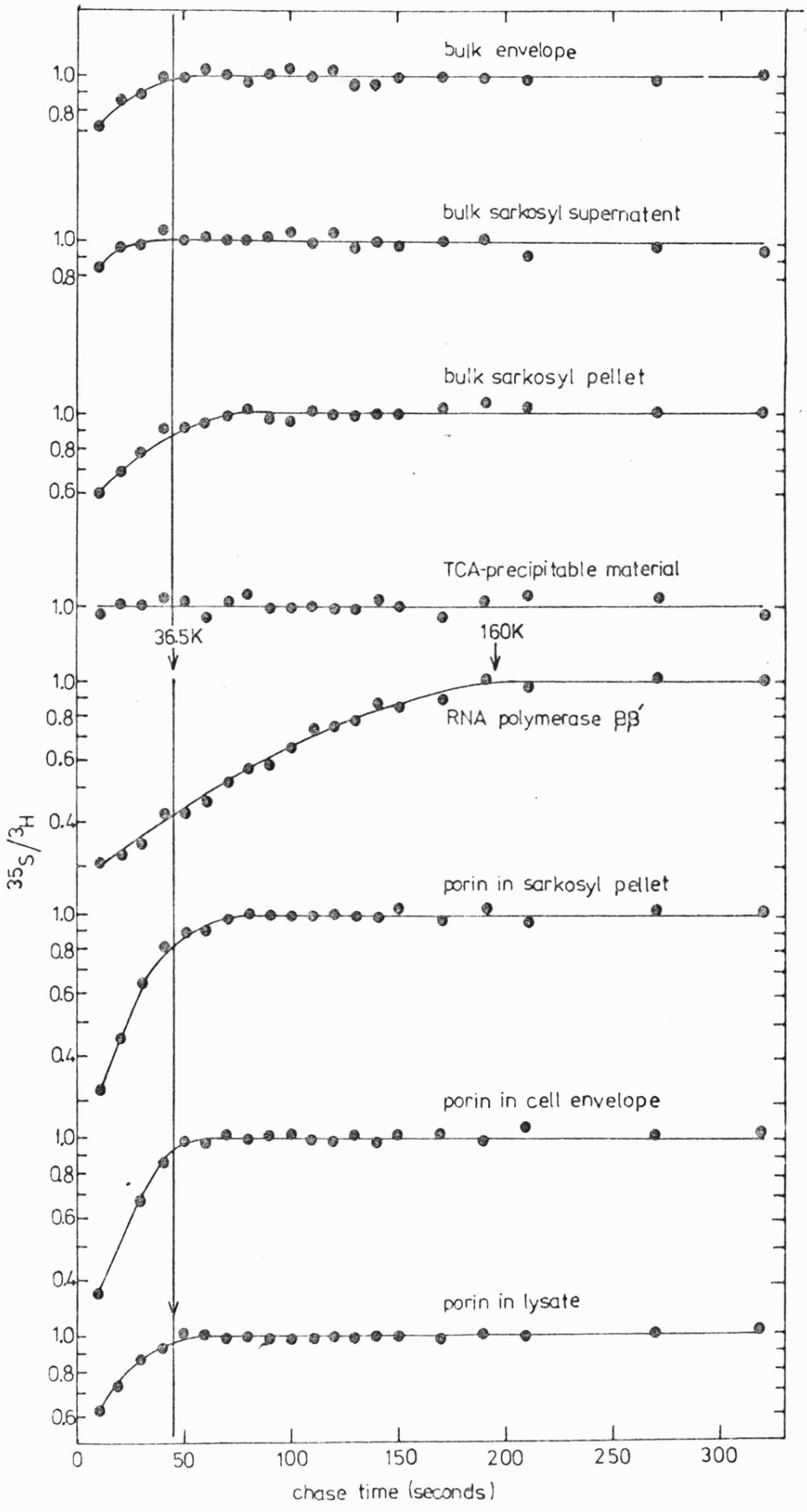

bulk envelope
bulk sarkosyl supernatent
bulk sarkosyl pellet
TCA-precipitable material
RNA polymerase ββ'
36.5K
160K
porin in sarkosyl pellet
porin in cell envelope
porin in lysate
1.0
0.8
0.6
0.4
$^{35}S/\beta_H$
0
50
100
150
200
250
300
chase time (seconds)

Figure 10.4: KINETICS OF OUTER MEMBRANE PROTEIN SYNTHESIS AT 25°C

A culture of LEB18 was grown at 25°C in glucose medium to $A_{450} \simeq$ 0.1, when 200 µCi of [^{3}H]-leucine (53 Ci/mmol) was added. After 60 minutes of labelling, unlabelled leucine was added (final concentration (100 µg/ml); after a further 15 minutes [^{35}S]-methionine was added for pulse-labelling as before. All subsequent procedures were as described in the legends to Figures 10.2 and 10.3.

Theoretical and estimated run-out times are marked as explained in the legend to Figure 10.2.


bulk envelope
bulk sarkosyl supernatent
bulk sarkosyl pellet
TCA-precipitable material
36.5K
160K
RNA polymerase ββ'
porin in sarkosyl pellet
porin in cell envelope
porin in lysate
35S/3H
chase time (seconds)
0
50
100
150
200
250
300

in cell lysates closely matched the calculated translation time; secondly there was no detectable lag before run-out into the cell envelope; and thirdly there was a 30 second lag before run-out into the sarkosyl-insoluble outer membrane.

The data of Figures 10.2 - 10.4 thus demonstrate that the 36.5K porin is associated with the cell envelope at the time when its translation is complete, and is at this point present as mature porin; it then enters a sarkosyl-soluble pool from where it rapidly moves to the sarkosyl-insoluble membrane fraction.

IV. Kinetics of Run-Out in Bulk Membrane Fractions

In the experiments described above, as well as analysing the kinetics of the individual 36.5K polypeptide, I determined the run-out kinetics of bulk membrane fractions. It is important to be aware from the outset, however, that the interpretation of such data is not straightforward since total radioactivity in the envelope fraction may include completed proteins and membrane-bound polysomes bearing incomplete polypeptides.

In Figures 10.2 - 10.4 are shown the run-out kinetics of bulk envelope, bulk cytoplasmic membrane and bulk outer membrane measured in the three experiments already discussed, and based upon the use of sarkosyl to fractionate the cell envelope. Considering firstly the kinetics of bulk outer membrane material, it is clear that since completed polypeptides do not immediately enter the sarkosyl-insoluble fraction, it is unlikely that the bulk outer membrane fraction contains any incomplete polypeptides - thus the bulk kinetics might be expected to reflect the summation of the run-out kinetics of each individual

polypeptide species in the outer membrane. Putting this another way, we might expect that the run-out time for the sarkosyl-insoluble outer membrane fraction would be determined by the run-out time of the largest polypeptide which forms a significant proportion of the total fraction. Since the run-out time for the bulk outer membrane is not markedly different from that of the 36.5K porin in Figures 10.2 - 10.4, it must be concluded that the bulk kinetics are dominated by the porin.

Interpretation of the kinetics of run-out into the sarkosyl-soluble fraction of the cell envelope is difficult. The data of Figures 10.2 - 10.4 indicate that this fraction has a very short run-out time. It is, however, clear from the foregoing discussion that this fraction must be very heterogeneous, containing some, or all of the following classes of material:

(1) membrane-bound polysomes containing incomplete polypeptides destined for the cytoplasmic membrane, the periplasm, and the outer membrane;

(2) complete cytoplasmic membrane proteins;

(3) complete outer membrane proteins passing through the sarkosyl-soluble pool; and

(4) general cross-contamination due to the imperfection of the sarkosyl fractionation. In view of this extreme heterogeneity it is not, I believe, possible to draw any meaningful conclusions from the run-out kinetics of the sarkosyl-soluble fraction.

Finally, it is clear that since the run-out curves for bulk envelope protein shown in Figures 10.3 and 10.4 contain a rising phase, then some incomplete envelope polypeptides are not recovered with the envelope fraction i.e. not all membrane-bound polysomes are recovered

by the experimental procedure used. To recover all membrane-bound polysomes it may be necessary to use much gentler lysis procedures (e.g. osmotic lysis of spheroplasts) and carefully formulated buffers.

V. Discussion

Even if it is assumed that the possession of amino-terminal signal sequences is a general property of outer membrane proteins (evidence discussed in Section IV of Chapter 1), the question remains : what determines whether a particular protein will move to the cytoplasmic membrane, to the periplasm, or to the outer membrane. The biosynthesis of periplasmic and cytoplasmic membrane proteins can be envisaged in the terms of the extended signal hypothesis proposed by Rothman and Lenard (1977), and discussed in detail in Section II of Chapter 1. Thus we might imagine that the synthesis of both of these classes of proteins is initiated upon free, cytoplasmic ribosomes, but that the nascent product of translation, possessing an amino-terminal signal sequence of predominantly hydrophobic amino acids, directs the association of the translational complex with the cytoplasmic membrane, to produce extrusion of the growing polypeptide chains across the bilayer. In the case of a periplasmic protein, such as alkaline phosphatase, this process continues until the entire polypeptide has been extruded into the periplasm; for integral membrane proteins it is thought that the primary sequence of these polypeptides determines that they become stably associated with the bilayer during extrusion.

But what of outer membrane proteins? It is possible to imagine many schemes to describe how these proteins reach their destination. Firstly, we may assume that the possession of a signal sequence designates the nascent outer membrane protein as envelope material; it

is from this point on that the hypothetical routes into the outer membrane diverge. I will now list several major possibilities.

(i) Initial Synthesis as a Protein of the Cytoplasmic Membrane

The protein enters the cytoplasmic membrane as an integral component which may or may not be proteolytically processed; a subsequent interaction, perhaps with LPS, produces movement into the outer membrane by an unknown mechanism - possibly at the LPS insertion points or other special regions of the envelope. This hypothesis thus predicts : the existence of a cytoplasmic membrane kinetic intermediate, perhaps in precursor form; that outer membrane specificity may reside in sequences of the protein other than the signal sequence; and that final insertion into the outer membrane should occur at specific sites.

(ii) Initial Synthesis as a Protein of the Periplasm

The protein enters the periplasm - perhaps as a soluble molecule? Subsequent interaction either with preexisting outer membrane bilayer or at special sites (e.g. peptidoglycan growth zones) produces insertion. This hypothesis predicts : the existence of a soluble kinetic intermediate; that specificity could reside either in a special class of signal sequence or in other sequences of the protein; that insertion might be restricted to specific sites or might be randomly distributed over the whole surface.

(iii) Direct Extrusion Across Bilayers

This is envisaged specifically as a special signal sequence mechanism. The nascent preprotein is not recognised by the protease which normally processes secreted proteins. Thus the growing chain is extruded into the periplasm in possession of an amino-terminal signal sequence capable of a hydrophobic interaction with the outer membrane bilayer. Proteolytic processing would occur in the outer membrane.

This hypothesis thus predicts : that proteins are essentially inserted directly into the outer membrane with no kinetic intermediate in the cytoplasmic membrane; that specificity resides at least in part in the signal sequence; and that intercalation could occur at random over the whole surface.

(iv) Special Structures

This is in essence a special case of hypothesis (iii); a special class of signal sequence is seen as directing insertion at special protein-specified sites in the cytoplasmic membrane from which the outer membrane bilayer is directly accessible.

This list of possibilities is by no means exhaustive, and each hypothesis includes postulates which could be modified. This proliferation of models arises from the paucity of direct evidence relating to biosynthesis of outer membrane proteins. I will now discuss the evidence which I have presented above, and other evidence which may help to narrow down the choice of models.

Firstly, from the finding that there is close agreement between run-out time for the 36.5K porin analysed in cell lysates and the calculated translation time, I conclude that proteolytic removal of the presumptive signal peptide identified by Sekizawa et al. (1977) occurs during translation. Secondly, from the finding that the 36.5K porin is stably associated with the cell envelope when translation is completed, I conclude that there is no soluble intermediate form of the porin. This rules out all hypotheses in which the protein passes through the periplasm in soluble form, such as (ii) above.

The third, and potentially most important finding is that there is a sarkosyl-soluble intermediate form of the mature 36.5K porin.

It is important to note that this intermediate could not be directly demonstrated in a cytoplasmic membrane fraction, since this always contains significant cross-contamination from outer membrane protein which would mask the intermediate. It should also be noted that the extent of this contamination is proportional to the concentration of sarkosyl used, and that the amount of porin found in the sarkosyl soluble fraction is too great to be easily accounted for by the soluble kinetic intermediate. The general problem of cross-contamination of outer and cytoplasmic membrane fractions may account for the failure of Lee and Inouye (1974) to detect any intermediate in the cytoplasmic membrane. In any case the density fractionation used by those authors may not be sensitive enough to resolve the kinetic intermediate detected here.

It is tempting to identify the sarkosyl-soluble porin with the cytoplasmic membrane intermediate envisaged in hypothesis (i) above. It is, however, just as likely that the existence of the soluble intermediate reflects either the insertion of the protein into regions of the outer membrane with atypical properties (assuming translocation occurs at specific sites), or the slow acquisition of stable interactions with surrounding membrane components (assuming random intercalation over the entire surface). If the porin molecules do indeed enter atypical regions of the outer membrane, from where they move, by diffusion, into more typical regions stabilised by extensive intermolecular interactions, then the lack of an effect of temperature upon the process (cf. Figures 10.2 - 10.4) may reflect a buffering of the fluidity of the outer membrane. Indeed, Rottem et al. (1978) have reported that in Proteus mirabilis, cultivation at a low temperature produces the appearance of

an unsaturated fatty acid in the lipid A moiety of the LPS, indicating that there is some physiological control over the fluidity of the outer membrane.

Turning to consideration of the kinetics of bulk membrane fractions, the measurements made upon bulk envelope material indicate that not all envelope-designated, incomplete polypeptides were recovered by the procedures used. In general this could result from a non-specific loss of membrane-bound polysomes engaged in the synthesis of both classes of membrane proteins or, more speculatively, from a specific lability of one class of polysomes e.g. those synthesising outer membrane proteins at the special sites envisaged in hypothesis (iv) above. It is interesting to compare the run-out kinetics of bulk membrane fractions presented here with those of Ito et al. (1977), and it is encouraging that the density gradient fractionation used by Ito et al, and the detergent fractionation of envelope material used here, yield broadly similar data. In the light of my data concerning porin synthesis, however, the 'slow assembly' of outer membrane proteins inferred by Ito et al. can be seen to merely reflect the requirement for completion of translation before stable association with the outer membrane.

The other aspect of outer membrane protein biosynthesis, not approached directly in the present study, is the topography of insertion of proteins into the surface : do these enter at specific sites, or are they intercalated randomly over the entire surface? Once again there is very little direct evidence on this point. Smit and Nikaido (1978) grew cultures of S.typhimurium under conditions where the synthesis of one major porin was temporarily eliminated; they then induced porin synthesis, and used specific ferritin-labelled antibody directed against the porin to detect, in electron micrographs,

the emergence of the porin at the cell surface. Many discrete clusters of porin appeared, which Smit and Nikaido interpreted as demonstrating localised insertion of the protein at approximately 300 sites per cell.

In summary, the available evidence is consistent with the idea that newly-synthesised proteins enter the outer membrane at discrete sites, rather than being randomly intercalated over the entire surface. These sites may be identical to the LPS insertion sites discussed in Section IV of Chapter 1. The sarkosyl-soluble kinetic intermediate identified in the present study may merely reflect the atypical structure of those regions of the outer membrane in the immediate vicinity of the insertion sites. In addition, my data demonstrate that there is no soluble intermediate form of the porin, but rather that the protein is stably associated with the cell envelope upon completion of translation. There is nothing in my data which enables a definite choice to be made between the hypothesis that outer membrane proteins are initially synthesised as proteins of the cytoplasmic membrane, and the hypothesis that they are inserted directly into the outer membrane at special sites.

CHAPTER 11

GENERAL DISCUSSION

I. The Cell Cycle Pattern of Synthesis of Outer Membrane Protein

I embarked upon this project in the hope that a further study of the linear accumulation of envelope protein, originally reported by Churchward and Holland (1976a), might yield information about the mode of growth of the cell surface of E.coli B/r. Using the data which I have presented above, I shall develop the argument that the cell cycle pattern of synthesis of outer membrane protein may indeed give some insight into cell growth, and, in particular, I hope to show that it supports the hypothesis that the cell surface grows linearly.

The data which I have presented in Chapter 6 represent a refinement of the data of Churchward and Holland (1976a), in that they clearly demonstrate that the linear pattern of accumulation of envelope protein is in fact restricted to the sarkosyl-insoluble fraction, i.e. to the outer membrane proteins. The doubling in rate observed in these experiments occurs late in the cell cycle, 5-15 minutes before division. This result must be considered in the context of other studies of surface growth : several authors have proposed that E.coli surface growth is a linear process (Pritchard, 1974; Rosenberger et al., 1978) and, as discussed in Section V of Chapter 1, there is a diverse body of evidence to support this hypothesis (Hoffman et al., 1972; Donachie et al., 1976; Cullum and Vicente, 1978; Poole, 1977; Churchward and Holland, 1976a; Hakenbeck and Messer, 1977; Rosenberger et al., 1978). It is tempting to speculate that the pattern of synthesis of outer

membrane protein may be related to this postulated linear surface growth. There is some experimental evidence that the peptidoglycan sacculus grows linearly (Hoffman et al., 1972; Koppes et al., 1978), and on the strength of this it is usually assumed that the growth of the rigid peptidoglycan layer determines the overall pattern of growth of the cell surface. The outer membrane, however, has several initimate interactions with the peptidoglycan, (discussed in Section III of Chapter 1), so that it is possible that these two layers are constrained to grow in synchrony. Indeed, it may be desirable for the rate of synthesis of outer membrane protein to be matched to that of peptidoglycan for some structural reason; in this case synthesis of both surface components might be subject to a single regulatory mechanism, or, alternatively outer membrane protein synthesis might be directly constrained by the growth of the peptidoglycan layer. These ideas are discussed further below.

II. Limitation of Outer Membrane Protein Synthesis

The cell cycle phenomenon described above, i.e. the linear accumulation of outer membrane protein, may be envisaged in general terms as follows : there is a factor, required for synthesis of all outer membrane proteins, whose availability limits the overall capacity of the cell for synthesis of this class of proteins. The linear accumulation of outer membrane protein may then be explained by postulating that the level of this hypothetical factor is constant during the cell cycle, with a doubling in this level occurring at a fixed cell cycle point.

The evidence which I have presented in Chapter 9 demonstrates that in an exponentially growing culture of E.coli B/r the capacity of the

cells for synthesis of outer membrane protein is indeed saturated. Thus, when the synthesis of a new class of outer membrane proteins - the iron transport proteins - is induced, then the rates of synthesis of other outer membrane proteins, including the 36.5K porin, are reduced. The agreement in that experiment between the predicted and the observed effect upon the relative rate of porin synthesis argues strongly that the rate of synthesis of the majority, if not all of the outer membrane proteins, but of no other class of proteins, is limited by the availability of some factor. It is possible that it is the extremely high rate of synthesis of the 36.5K porin which is responsible for this saturation effect. Whatever the reason, if the availibility of the limiting factor increased discontinuously during the cell cycle this would clearly account for the cell cycle pattern of synthesis of outer membrane protein.

What might this limiting factor be? I will now discuss several possibilities and also what evidence there is to help to choose between them.

(a) Limitation at the Transcriptional Level

In this view, the capacity of the cell for outer membrane protein synthesis would be limited by the level of a factor engaged uniquely in the transcription of those genes encoding outer membrane proteins; such a factor might be, for example, a special RNA polymerase subunit. This hypothesis would also require that the corresponding promoters should have a special feature in common, and this raises the problem of outer membrane proteins being synthesised from polycistronic mRNAs encoding other proteins not associated with the outer membrane. There are three possibilities : firstly all outer membrane proteins could be synthesised from monocistronic messengers; secondly those outer

membrane proteins lying in polycistronic units of transcription might not adhere to the linear pattern of accumulation; and thirdly, those proteins of the cytoplasm and of the cytoplasmic membrane whose genes are cotranscribed with a gene encoding on outer membrane protein, might accumulate linearly. Since the gene encoding at least one outer membrane protein (the malB gene product) lies in a transcriptional unit which includes genes specifying proteins of the periplasm and of the cytoplasmic membrane then the first possibility listed above is not generally true. It does not seem likely that the malB gene product would be exempt from the postulated general regulatory mechanism since it is present as a major protein of the outer membrane under certain growth conditions. It also seems unlikely that other proteins not associated with the outer membrane would be unnecessarily constrained by the mechanism; because of these drawbacks to regulation at the level of transcription I shall not consider this hypothesis any further.

(b) Limitation at the Translational Level

In this view the limiting factor would be uniquely involved in translation of those mRNAs encoding outer membrane proteins. Thus, genes whose rate of transcription increased continuously during the cell cycle might nevertheless exhibit a linear pattern of output because of cell age dependent variations in the efficiency of translation of the available mRNA. This type of regulatory mechanism again raises the problem of polycistronic units of transcription encoding both outer membrane proteins and other classes of protein : in the case of a translational regulatory mechanism, however, it is possible to imagine independent units of translation being carried on the same mRNA molecule.

Apart from the rather unlikely hypothesis that there are special ribosomes responsible for the translation of mRNAs encoding outer membrane proteins, I believe that there are two possible regulatory mechanisms which could operate at the level of translation. Both of these mechanisms have the additional feature that they arise directly from specific features of outer membrane structure and biogenesis. The first mechanism may be conveniently described as a limitation by the availability of membrane sites. I have described, in Chapters 1 and 10, the molecular aspects of the biosynthesis of outer membrane proteins, and whatever the exact mechanism, it is clear that they are synthesised by membrane-bound ribosomes (Randall et al., 1978). Some sort of membrane site must, therefore, be involved in translation of the corresponding mRNAs. In fact, since the limited capacity for outer membrane protein synthesis observed in the present study does not extend to proteins of the cytoplasmic membrane, these limiting membrane sites would have to be special sites engaged solely in the synthesis and translocation of outer membrane proteins. The number of these sites would thus limit the maximum overall rate of synthesis of outer membrane protein, and the cell cycle doubling in rate would be determined by a duplication of these sites at a specific point in the cycle.

The second regulatory mechanism which I shall consider invokes a structural limitation upon the rate of outer membrane protein synthesis, determined by the rate of growth of the peptidoglycan. To be more specific, I envisage the peptidoglycan as the foundation or template upon which outer membrane proteins are assembled; thus it would be the rate of expansion of this foundation layer which would determine the rate of provision of surface for assembly of outer membrane proteins.

In this case the cell cycle doubling in rate of synthesis of outer membrane proteins would be directly determined by the underlying pattern of synthesis of the peptidoglycan. It must be emphasised that this hypothesis does not exclude the possibility that outer membrane proteins are synthesised and translocated at special sites but merely requires that the number of these sites is not limiting for overall synthesis. I believe that this hypothesis is more attractive than one in which the number of translocation sites is limiting since it does not require that a large number of such presumably complex sites should be synchronously duplicated during the cell cycle.

Whether the limitation lies at the level of translocation or of assembly, it is clear that there are two possibilities for the way in which the rate of translation itself might be regulated. Thus, there could be a direct feedback mechanism, so that mRNAs are only translated when a site of translocation or space for assembly becomes available; or alternatively, mRNAs translated in the absence of an available site might give rise to cytoplasmic forms of outer membrane polypeptides, which would presumably be rapidly degraded. The latter possibility seems less likely on economic grounds; thus there may be some mechanism which ensures that translation does not proceed past an early stage until a membrane site becomes vacant, and which tightly couples translation to translocation and assembly at the cell surface.

The above constitutes, I believe, a comprehensive list of the possible reasons for the limitation upon synthesis of outer membrane proteins. I will now consider the mechanism by which the cell cycle doubling in rate of synthesis might be timed.

III. Relationship Between Outer Membrane Protein Synthesis and DNA Replication

It is tempting to link any cell cycle event to replication of a specific region of the chromosome, since the replicative process provides a ready-made clock in the cycle. Thus, in the terms of the various limitation hypotheses discussed above, a burst of synthesis of special RNA polymerase subunits, of special ribosomal proteins, or of membrane sites, would double the capacity of the cell for outer membrane protein synthesis; and this burst of synthesis would be in some way triggered at a specific stage of the replication cycle. In the terms of the hypothesis of direct constraint by the rate of growth of the sacculus, the underlying doubling in rate of peptidoglycan synthesis would be triggered by a replication event.

A special case of this model linking the linear pattern of synthesis of outer membrane protein to the replication cycle is that the pattern of synthesis might be dominated by several major outer membrane proteins which are the products of unregulated genes. The rate of synthesis of these proteins would thus be expected to double when the corresponding genes were replicated. In proline/alanine medium, the three major protein species of the outer membrane of E.coli B/r are the 36.5K porin, protein d, and the lipoprotein, together making up at least 50% of total outer membrane protein (Inouye, 1975). The equivalent proteins of E.coli K12 are thought to be encoded respectively by the tolF (21 min), ompA (21 min), and lpo (34 min) genes; these three loci all lie near enough to the terminus of replication (≃30 min) to give rise to the observed timing of the rate doubling, if they were expressed constitutively. This hypothesis of constitutive synthesis of outer

membrane proteins does not, however, explain the effect of the induction of the outer membrane iron transport proteins. In such experiments the rate of synthesis of the 36.5K porin was reduced by 20%, demonstrating that the rate of synthesis of this protein is not solely determined by the number of copies of the corresponding structural gene. In addition, in E.coli K12, the rate of synthesis of the equivalent protein, porin b, is reduced by increasing the osmolarity of the growth medium, (Van Alphen, W. and Lugtenberg, 1977). It is thus not possible to sustain the argument that the cell cycle pattern is due to constitutive synthesis of major outer membrane proteins.

I will now return to the more general hypothesis that the doubling in the cells capacity for synthesis of bulk outer membrane protein is triggered at a specific point in the replication cycle. It is clear, from the timing of the doubling in rate of synthesis observed in the experiments described in Chapter 6, that the chromosomal locus in question would have to lie very near to the terminus of replication. If the rate doubling is causally and tightly related to replication of a gene, then it would be expected that inhibition of DNA synthesis, by fixing the number of copies of this hypothetical gene, would freeze the rate of synthesis of outer membrane protein in a culture. Whilst the data presented in Chapter 7 must be viewed with caution, they show that the rate of synthesis of outer membrane protein continues to increase in the absence of DNA synthesis. In a complementary experiment, also described in Chapter 7, in which the number of copies per unit mass of terminally located genes was decreased in steady state growth by thymine limitation, there was again no evidence that the overall rate of outer membrane protein synthesis is determined by the number

of copies of such a gene. I conclude that the cell cycle doubling in rate of synthesis of bulk outer membrane protein is not triggered by replication of any gene.

IV. Relationship Between Outer Membrane Protein Synthesis and Surface Growth

I have argued above that the cell cycle pattern of synthesis of outer membrane protein may be related to the postulated linear growth of the peptidoglycan layer of the cell envelope. In view of this possible relationship between the patterns of growth of the two outer layers of the envelope, it is not surprising to find that just as my data discussed above indicate no causal link between DNA replication and outer membrane protein synthesis, so several lines of evidence have shown that overall surface growth is not linked to replication. Thus changes in DNA concentration induced by dnaA and repA mutations do not cause changes in surface area/mass of cells, (Pritchard et al., 1978; Zaritsky and Woldringh, 1978). Similarly, thymine starvation does not prevent an increase in the rate of cell elongation in synchronously growing cultures (Donachie et al., 1976). In view of these results Rosenberger et al. (1978) have proposed a model in which a doubling in rate of surface growth is triggered, not by a gene doubling mechanism, but by the attainment of a critical cell density. As discussed in Section V of Chapter 1, the combination of an exponentially increasing mass with a linearly increasing surface (and thus volume) would be expected to result in age dependent variations in cell density. Is it possible that the cell cycle pattern of synthesis of outer membrane protein reported in the present study is related to this linear growth

of the cell surface postulated by Rosenberger *et al.* (1978). Analysis of the growth rate dependence of the cell dimensions of *E.coli* B/r, substrain H , has led to the postulate that the rate of surface growth doubles at a fixed time d (= 45 minutes) before cell division. In contrast, my data show a doubling in rate of synthesis of outer membrane protein occurring 5-15 minutes before division. Furthermore, the data presented in Chapter 8 are consistent with the idea that this timing is adhered to over a wide range of growth rates : thus, the parameter porin/mass decreases with growth rate in parallel with the parameter terminus/mass. If it is accepted that the rate of porin synthesis is unrelated to DNA replication, then this correlation is consistent with a doubling in rate occurring at a fixed cell cycle point D ($\simeq$ 20 minutes) before division, which is not, however, causally linked to termination. Indeed, since the porin constitutes a large proportion of bulk outer membrane protein, and since physiological variations in the relative amounts of the various outer membrane proteins with growth rate are likely to be quite small, then these data are also consistent with a doubling in the rate of synthesis of bulk outer membrane protein occurring approximately D minutes before cell division over a wide range of growth rates. Direct measurements of the pattern of synthesis of bulk outer membrane protein in membrane elution experiments,conducted at various growth rates, would be necessary to demonstrate this point conclusively. Nevertheless, it seems clear that all of my data are consistent with a doubling in the rate of synthesis of outer membrane protein which occurs much later in the cell cycle, nearer division, than does the doubling in rate of surface synthesis deduced by Rosenberger *et al.* (1978). This difference is also

reflected in the growth rate dependent changes in the level of porin per unit surface area shown in Figure 8.3. One possible explanation for this apparent discrepancy between the surface growth hypothesis of Rosenberger et al. and my data is that the hypothesis is based upon measurements made upon E.coli B/r substrain H, whereas in the present study E.coli B/r substrain F was used. The various substrains of E.coli B/r have been shown to differ significantly in the lengths of their D periods (Helmstetter and Pierucci, 1976), and it seems possible, therefore, that the parameter d, proposed by Rosenberger et al. may also vary from strain to strain. In this context it should be noted that Hoffman et al. (1972) have reported a doubling in rate of synthesis of peptidoglycan occurring 10-20 minutes before cell division in cells of E.coli B/r, (of unknown substrain), growing with a mean generation time of 41 minutes; this timing is also inconsistent with the hypothesis of Rosenberger et al. It is clearly necessary that measurements of cell dimensions, and of the cell age dependency of the rates of synthesis of peptidoglycan and of outer membrane should be made upon the same bacterial strain before the relationships between these various phenomena can be clarified. It is implicit in the above discussion that if the rates of synthesis of the two outer layers of the cell envelope are synchronised, then the amount of outer membrane protein per unit surface area should remain constant under all conditions. I have argued above that the growth rate dependent changes in the cellular levels of the porin may reflect just such a relationship, but that the necessary measurements of cell surface area have not been made in LEB16. Changes in cell surface area have, however, been measured by Meacock (1975) for LEB16 growing under conditions of

thymine limitation, as discussed in Chapter 7. I have measured the cellular levels of bulk outer membrane protein under the same conditions, and as shown in Chapter 7, thymine limitation has no effect upon this parameter, despite the fact that it causes changes in cell surface area/mass. It seems, therefore, that under conditions of thymine limitation at least, the cellular surface area does not limit the amount of outer membrane protein which can be incorporated into the cell envelope. The effects of thymine limitation upon the structure and composition of the outer layers of the cell envelope are, however, by no means clear. The effects of thymine limitation upon cell shape have led to the proposal that a thymidine-linked sugar may have a regulatory role in peptidoglycan synthesis (Pritchard et al., 1978); furthermore, a thymidine-linked sugar is also involved in LPS biosynthesis, although the significance of this latter role is unclear since, in the rough strains of E.coli used in the laboratory, the sugar in question, rhamnose,is not incorporated into the LPS. It is quite possible, therefore, that thymine limitation produces such profound changes in envelope structure that extra protein can be accommodated in the outer membrane. Another indication that the effects of thymine limitation upon outer membrane protein synthesis may be complex, is to be seen in the finding that although the relative rate of synthesis of bulk outer membrane is unaffected, that of the 36.5K porin is affected to almost the same extent as cell surface area. I feel that these effects of thymine limitation upon outer membrane protein synthesis must be investigated in more detail before they can be interpreted with any confidence.

Despite the various conflicting pieces of evidence discussed above,

I believe that the most coherent interpretation of my data is that the cell cycle pattern of outer membrane protein synthesis is related to overall linear growth of the cell surface. I also believe, as I have argued above, that the outer membrane is constrained to grow in step with a linearly-growing peptidoglycan layer by a rather passive mechanism whereby proteins are only capable of reaching the outer membrane when vacant space for assembly is formed by the expansion of the peptidoglycan layer. I have argued elsewhere that it might not be expected that the plastic membrane layers of the cell envelope should be constrained to grow in step with the peptidoglycan, and so an explanation for the different behaviour of the outer membrane and the cytoplasmic membrane must be looked for in their different structures, and in their different relationships with the peptidoglycan. Thus, the cytoplasmic membrane is a typical, fluid-mosaic membrane with no known strong interactions with the peptidoglycan sacculus; in contrast, as discussed in Section III of Chapter 1, the outer membrane is characterised by an unusual level of molecular organisation and also has several interactions with the peptidoglycan. It is these features of the structure of the outer membrane which must, I believe, determine its pattern of cell cycle growth. I therefore conclude that the cell cycle pattern of synthesis of outer membrane protein provides further evidence that the cell surface of E.coli B/r grows linearly. The reasons for linear surface growth remain obscure; it is possible that the molecular nature of the peptidoglycan dictates that the sacculus should be synthesised at a constant rate at growth zones, and it is also possible that the fluctuations in relative rates of formation of cell surface and cell mass may provide a mechanism by which the process of cell division can be initiated.

CHAPTER 12

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