FUNCTION OF OUTER MEMBRANE PROTEINS IN *Escherichia coli* K12

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ABSTRACT

The outer membrane of *Escherichia coli* contains proteins which can act as pores for a wide variety of molecules, and proteins which facilitate the transport of specific classes of molecules.

This thesis demonstrates that the LamB protein which is a specific pore for maltose and maltodextrins, derives its specificity in part, from the periplasmic maltose binding protein (MalE protein). This was demonstrated by looking at the ability of an *ompB* mutant which lacks the major porin, to transport various substrates through the LamB pore, when this protein is substituting for the major porin. It was found for a variety of substrates that in the presence of a *malE* mutation (MalE protein deficient) the alleviating effect of LamB protein in an *ompB* mutant was enhanced over strains carrying *ompB* alone. It was postulated that the MalE protein associates with the LamB protein in such a way as to "gate" the pore to molecules other than maltose or maltodextrins, and that removal of the MalE protein allows enhanced diffusion of molecules other than maltose or maltodextrins. There does, however, exist a class of substrates for which the *malE* mutation has no such alleviating effect. This may be due to imperfect "gating" of the pore by the MalE protein, or to the molecules being of sufficiently small size to squeeze past the gate. An attempt was made to directly

demonstrate the association of the MalE protein with the LamB protein; however this was not successful using the methods employed. It appeared likely that the MalE protein exists in two populations; "free" and "bound", possibly to the peptidoglycan. The MalE protein also seems to be associated with the membrane, although there is no correlation of this to the presence or absence of LamB protein.

The ideas postulated for the LamB protein were tested for the Tsx and PhoE proteins which are specific nucleoside and (possibly?) phosphate transport proteins. It was demonstrated that the Tsx protein appears to be a pore, similar to LamB, in that it is possible for the Tsx protein to alleviate an *ompB* mutation; the Tsx protein also shows some selectivity in the transport of these "non specific" substrates.

The PhoE protein, a porin under phosphate control, and the phosphate binding protein (PhoS protein) were also examined. No evidence suggests that the PhoS protein could gate the PhoE protein in a similar fashion to the MalE and LamB proteins.

During the course of these experiments it was found when selecting mutants resistant to bacteriophage TC45 (which uses PhoE protein as a receptor) approximately 50% are LPS mutants. These LPS mutants result in a general decline in the amount of PhoE protein in the membrane.

The OmpA protein which is thought not to be a porin, interacts with both the major porins and also the LamB protein, possibly through protein-protein interactions, so as to modify their ability to act as porins. Thus the *ompA* mutation results in a decline of transport of some substrates through OmpC and OmpF proteins, but causes a slight enhancement of transport through the LamB porin.

STATEMENT

I state that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

Michael W. Heuzenroeder

August, 1981

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The gram negative bacterial cell, of which *Escherichia coli* is an example is surrounded by two membranes, an inner or cytoplasmic membrane and an outer membrane. These two membranes display unique properties and functions. The inner cytoplasmic membrane contains the enzyme systems of the respiratory chain, oxidative phosphorylation, active transport and the biosynthetic machinery for various membrane and cell wall components such as phospholipid, peptidoglycan, lipopolysaccharide (LPS) and capsular polysaccharides.

The outer membrane in contrast to the cytoplasmic membrane exhibits only a very limited range of enzymic activities; phospholipase A1, lysophospholipase (Scandella and Kornberg, 1971; Osborn *et al.*, 1972) and some processing enzymes (Inouye and Beckwith 1977; Pagès *et al.*, 1978). The outer membrane contains less and different phospholipids, fewer proteins and a carbohydrate component, the lipopolysaccharide (LPS), (Osborn *et al.*, 1972). These two membranes of the gram negative cell define an extra cytoplasmic, but not extra-cellular region known as the periplasmic space, which also contains the peptidoglycan layer which gives the cell its structural integrity. Also this space contains various hydrolytic enzymes, membrane derived oligosaccharides, and specific binding proteins required for the transport of various substrates such as sugars and amino acids.

The periplasm can occupy as much as 20-40% of the total cell volume (Stock *et al.*, 1977) and can best be described as a prokaryatic equivalent to the lysosome; it is a compartment where potentially hazardous reactions may take place without detriment to the cell.

This chapter is concerned primarily with the function of the outer membrane, or more specifically the functions of the proteins within that membrane, and their interactions with other components of the cell evelope.

1.1 COMPONENTS OF THE OUTER MEMBRANE

1.1.1 Phospholipids

The outer membrane as stated previously differs from the cytoplasmic membrane in the composition and amount of lipid present. Osborn *et al.*, (1972) reported that the outer membrane has a higher proportion of phosphatidylethanolamine (PE) than the inner membrane and was deficient in the other two phospholipids common to *E. coli* and *Salmonella typhimuruim;* phosphatidylglycerol (PG) and cardiolipin (CL). The outer membrane also appears to be enriched in fatty acids (Lugtenberg and Peters, 1976).

1.1.2 Lipopolysaccharide (LPS)

Mühlradt and Golecki (1975) have demonstrated that LPS is present in only the outer-leaflet of the outer membrane. LPS is a molecule with a highly

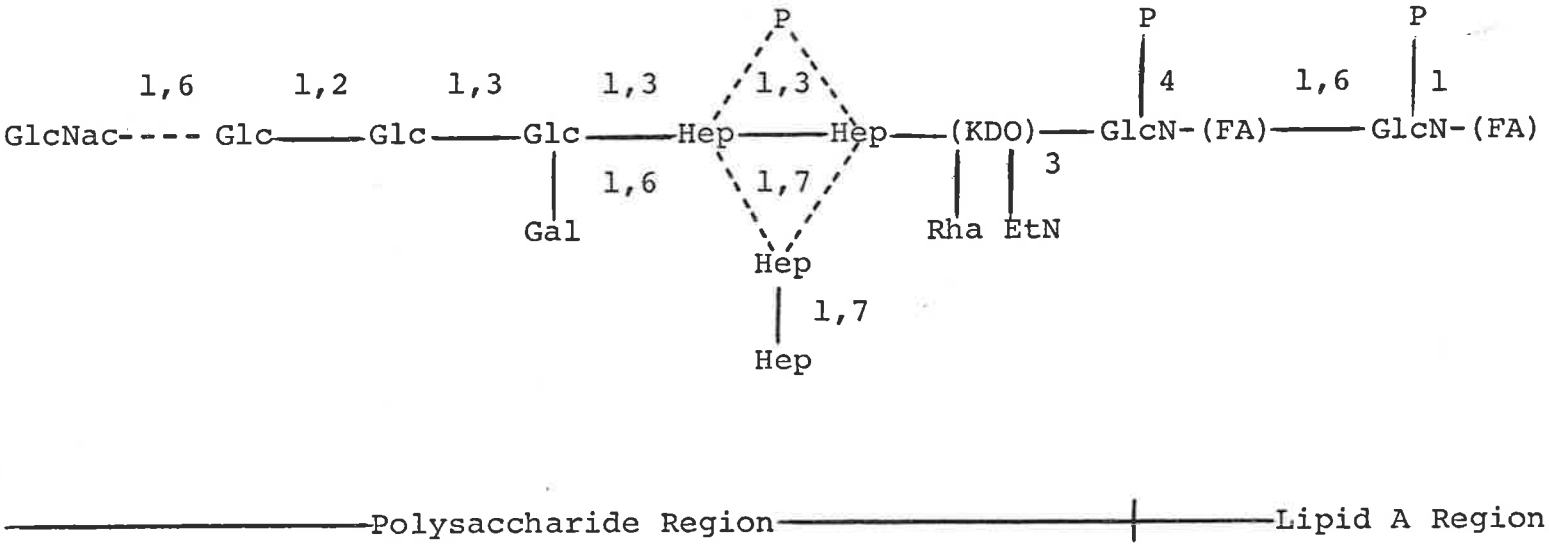
variable structure, it consists of three regions: a hydrophobic lipid containing portion (lipid A), an oligosaccharide portion (the core) and a polysaccharide portion (the O-antigen). The core and the lipid A regions are very similar amongst the Enterobacteria, however the O-antigen side chain which *E. coli* K12 lacks is subject to considerable variation (Luderitz *et al.*,1971). The exact structure of LPS (see figure 1.1) in *E. coli* is unclear and many models for LPS structure have been proposed (Boman and Monner, 1975; Hancock and Reeves, 1976; Prehm *et al.*,1976(b); Havekes *et al.*, 1977; Picken and Beacham, 1977). The lipid A is a glycolipid, in which fatty acids are linked to usually two sugars. These fatty acids are predominantly saturated and of short chain length C10-C14 in comparison to phospholipid. The sugar or core region of LPS contains carbohydrates, KDO (3-deoxy-D-mannooctulosonic acid) and HEP (L- glycero-D-mannoheptose).

It has been postulated by Braun (1978) that the lipid A region of LPS plays an important role in the formation of the lipid bilayer which forms the basis of the outer membrane. The number of fatty acids provided by the LPS lipid A equals the number of phospholipid molecules. Kamio and Nikaido (1976) showed that PE head groups are not hydrolysed by externally added phospholipase C and they do not become linked to cyanogen bromide activated dextran

Figure 1.1

Tentative structure of *E. coli* K-12 LPS, this structure is that presented by Coleman and Leive, 1979. Symbols: GlcNaC, N-acetyl-glucosamine; Glc, glucose; Gal, galactose; HEP, L-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctulosonic acid; Rha, rhamnose; EtN, ethanolamine; GlcN, glucosamine; P, phosphate; and FA, fatty acid.

Figure 1.1


P
P
P
1,6
1,2
1,3
1,3
1,3
4
1,6
1
GlcNac---- Glc—— Glc—— Glc—— Hep—— Hep—— (KDO)—— GlcN-(FA)—— GlcN-(FA)
1,6
1,7
3
Gal
Rha
EtN
Hep
1,7
Hep
Polysaccharide Region
Lipid A Region

suggesting that they are not exposed on the cell surface. It is possible therefore that the inner leaflet of the outer membrane is composed of phospholipid and the outer leaflet lipid is composed of fatty acids furnished by the LPS. In addition, Mühlradt *et al.*, (1974) showed the lateral diffusion constant for LPS to be some five times lower than that for phospholipid, indicating that LPS molecules are not immersed as single molecules in a highly liquid phase.

The lipopolysaccharide plays an important role in the functioning of the outer membrane; low amounts of altered LPS leads to increased susceptibility to a number of antibiotics (Tamaki and Matsahashi, 1973), detergents (Luderitz, Westphal *et al.*, 1971; Wu, 1972), phospholipase (Verkleij *et al.*, 1977), lysozyme (Birdsell and Cota-Robles, 1967) and lysis by complement (Reynolds and Pruul, 1971). The LPS is thought to act as a barrier to these agents by the mass of polysaccharides which project from the membrane. The LPS has been likened to a "picket fence" by Costerton *et al.*, (1974) which excludes high molecular weight molecules which may be detrimental to the cell.

1.1.3 The Outer Membrane Proteins

The outer membrane in contrast to the cytoplasmic membrane, contains a number of proteins which occur in large amounts (Schnaitman, 1970b; Osborn *et al.*,

1972; Henning and Haller, 1975; Lugtenberg *et al.*, 1975; Mizushima and Yamada, 1975). Some of the outer membrane proteins are tightly but not covalently associated with the peptidoglycan. These are stable in 2% SDS at 60°C but dissociate upon boiling (Rosenbusch, 1974). Many of the outer membrane proteins may act as receptors for colicin and bacteriophage. Thus, by selecting colicin and bacteriophage resistant mutants it is possible for outer membrane protein deficient mutants to be readily produced. Such mutants are extremely useful for functional and genetic investigations.

It was reported by Schnaitman (1970) that some 70% of the outer membrane protein was composed of a single protein of apparent molecular weight of 44K. This protein was designated the "major outer membrane protein". It was later found through the application of improved SDS gel electrophoresis systems that this apparently single major protein of *E. coli* K12 could be resolved into four major protein species. These four bands have been given various nomenclatures by different authors. This will be discussed below.

1.2 NOMENCLATURE OF THE MAJOR OUTER MEMBRANE PROTEINS

The identification and genetic analysis of outer membrane proteins in *E. coli* was carried out by many groups simultaneously. In many cases they used different SDS gel systems, different strains and a large variety of bacteriophage, colicin and toxic

agents for the selection of mutants. This resulted in a rather confusing array of nomenclatures which are given in table 1.1. It is now possible to give the major outer membrane protein a uniform system of nomenclature. The structural and genetic determinants of the major proteins are now well characterized. It has been proposed by Reeves (1979), Lee *et al.*, (1979) and Wu and Osborn (1980) that the proteins should be named in accordance with the standard nomenclature established for their structural genes in the current linkage map (Bachmann and Low, 1980). For example the product of the *ompA* gene is known as the OmpA protein. This system of nomenclature will be used in this thesis and is summarized in comparison with the other systems for major proteins in table 1.1.

1.3 FUNCTIONS OF OUTER-MEMBRANE PROTEINS

The outer membrane proteins can act as receptors for bacteriophage and colicins. These functions can be regarded as secondary or incidental properties, since they are not immediately "useful" to the individual cell. It can be argued that genetic exchange can take place via the bacteriophage-mediated process of transduction which is a "useful" process, however, this benefits a population of cells rather than the individual. This section will mainly concentrate on the primary functions of the outer-membrane proteins.

The outer membrane proteins can be divided into

TABLE 1.1 Summary of the nomenclature of the major outer membrane proteins of *E. coli* K12.

Investigator	Proteins				
Lugtenberg	a	b	c	d	e
Henning		Ia	Ib	II*	Ic
Mizushima	0-7	0-9	0-8	0-10	
Rosenbusch		Matrix Protein			
Schnaitman	3b	1a	1b	3a	NmpA/B
Proposed Systematic Nomenclature		OmpF	OmpC	OmpA	PhoE

two groups: 1. Those which have as their primary function the transport of either a wide range of or specific substrates, 2. Those which have a function other than transport.

1.4 PORE FORMING PROTEINS

It is now generally accepted that the permeability properties of the outer-membrane are determined by various proteins which form trans-membrane channels of varying specificity. The outer membrane serves as a permeability barrier for most molecules above a certain size but allows rapid diffusion of hydrophilic molecules up to 600 daltons. This observation led Decad and Nikaido (1976a,b) refer to the outer membrane as a molecular sieve. The outer membrane proteins which are involved in permeation can be divided into three classes.

1. The so called "porins", Nakae, (1976a,b) which form general hydrophilic pores, whose selectivity is based largely upon the size of the substrate molecule.

2. The specific porins, which allow diffusion of specific classes of substrates, but do not appear to strongly bind their substrate.

3. Proteins which interact with the substrate directly, and which take part directly in substrate transport.

1.5 NON SPECIFIC PORINS - OmpC AND OmpF PROTEINS

The hypothesis that outer membrane proteins constitute pores in the outer membrane was first tested by Nakae (1976) who made liposomes in the presence of outer membrane proteins. Membrane vesicles were reconstituted by adding an aqueous suspension of protein and LPS to a film of phospholipids. If the reconstitution medium contained (^{3}H) dextran and (^{14}C) sucrose both were present in both inside and outside the vesicles. These vesicles were then separated from the medium by filtration, during which a large proportion of the (^{14}C) sucrose diffused out of the protein containing vesicle resulting in the $^{3}H/^{14}C$ ratio of the filtered liposome preparation being several times higher than in the initial reconstitution. A control mixture containing no protein produced essentially identical $^{3}H/^{14}C$ ratios as the initial concentration. It was concluded from these results that outer membrane proteins form water filled pores. Further work by (Nakae, (1976a,b), using similar *in vitro* techniques and purified proteins, established that OmpC and OmpF proteins constitute the major porins. It was found that the pores that were formed were non-specific and excluded molecules of molecular weight above 600 daltons. More recently Benz *et al.*, (1978), using black lipid membranes were able to show that membrane conductance increases by many orders of magnitude when protein is present, consistent with the view that the proteins from aqueous trans-membrane channels.

These *in vitro* studies are also supported by other work conducted *in vivo*. Von Meyenberg (1971) described mutants of *E. coli* which were defective in the transport of many low molecular weight substrates. Beacham *et al.*, (1973) described mutants which were "cryptic" for certain periplasmic enzymes; this means that the enzyme in question (alkaline phosphatase or 5' nucleotidase) are present in normal amounts in the periplasm, but show reduced ability to degrade their substrates. It was later found that the mutants of Von Meyenberg and Beacham *et al.*, were missing major outer membrane proteins, in particular the OmpC and OmpF proteins. The genetics of OmpC and OmpF proteins were for some years quite confusing and has only recently become clear. Mutants lacking the major porins of *E. coli* can readily be selected by resistance to specific phage or tolerance to colicins. Genetic analysis of such mutants have discovered three loci designated *ompB*, *ompC* and *ompF* (Bachmann and Low, 1980). Mutations at *ompF* (21 mins, previously *tolF* Foulds, 1976 , *kmt* Bavoil *et al.*, 1977 and *cry* Beacham *et al.*, 1977) result in an $OmpC^{+}OmpF^{-}$ protein phenotype. Mutations at *ompC* (47 mins, formerly *par* Bassford *et al.*, 1977 and *meoA* Verhoef *et al.*, 1977) show the reverse phenotype $OmpC^{-}OmpF^{+}$. A third class of mutants could also be isolated was found to map at 74 min, at the *ompB* locus (Sarma and Reeves, 1977). Different *ompB* mutants may show a phenotype in which either or both

OmpC or OmpF proteins are lacking.

It has been shown by Ichihara and Mizushima (1978) that both OmpC and OmpF proteins show marked differences in amino acid sequence and are clearly derived from different genes. The evidence for *OmpC* being a structural gene is strong, since altered OmpC protein mutants mapping at the *ompC* locus have been isolated by Van Alphen *et al.*, (1977). Hall and Silhavy (1979) fused *ompC* to the *lac* operon and showed OmpC protein production to be under *lac* control. The evidence for *ompF* being a structural gene is based mainly upon the work of Sato and Yura, (1979) in which merodiploids of *Salmonella typhimurium* carrying an *E. coli* F'-*ompF*$^+$ have been shown to express *E. coli* OmpF protein in addition to the usual *S. typhimurium* outer membrane proteins, recently a specialised λ transducing phage was constructed carrying the *ompF* gene (Mutoh *et al.*, 1981).

The role of the *ompB* locus, though still not clear, would appear to be a regulatory function. Hall and Silhavy (1979) using *ompB::*Mu*cts* insertions and *ompC-lac* fusions have shown that *ompB* results in a decrease in β-galactosidase enzyme activity, and that *ompB*::Mu*cts* alleles of the OmpC$^-$OmpF$^-$ phenotype results in the maximal decrease in enzyme levels. The effect with OmpC$^-$OmpF$^+$ was somewhat less. These authors suggest that *ompB* specifies a diffusable positive regulatory element acting at the transcriptional level, and that the locus codes for two functions; one

to switch on the structural genes and another regulates the amounts of protein present in response to environmental factors, such as ion concentration.

The *ompB* region has recently been shown to be even more complex than described above. Wanner *et al.*, (1979) reported the isolation of a pleiotropic mutant designated *perA*, that produces reduced amounts of some periplasmic proteins including alkaline phosphatase and three outer membrane proteins, including OmpF protein. OmpC was over produced. The *perA* mutation is linked some 97-98% to *ompB*. Since *ompB* mutations do not usually affect alkaline phosphatase it was concluded that *perA* is probably a separate gene.

More recently Wandersman *et al.*, (1980) described mutants in the *ompB* region which affect the expression of outer membrane proteins other than OmpC and OmpF. These mutants were isolated by virtue of their resistance to bacteriophage TP1. This phage is quite novel in that it can use either LamB protein (λ-receptor) or OmpF protein as a receptor (Wandersman and Schwartz, 1978). The mutation maps very close to *ompB*, and results in the loss of OmpF protein and reduces the amount of LamB in non induced cells. It is possible that the TP1-resistant mutants may be analagous to the *perA* mutants.

1.5.1 PhoE protein (New membrane protein)

Mutants deficient in OmpC and OmpF proteins

exhibit multiple transport defects. Pseudorevertants of these mutants result in the expression of an electrophoretically distinct new outer membrane protein (Henning *et al.*, 1977; Van Alphen *et al.*, 1977 and Foulds and Chai, 1978). This new protein has also been shown to exhibit porin properties (Lugtenberg *et al.*, 1978; Pugsley and Schnaitman, 1978 and Van Alphen *et al.*, 1978). It was initially suggested that there existed a strong structural relationship between the new protein and OmpF as suggested by the similarity of the N-terminal octapeptides (Henning *et al.*, 1977). Lee *et al.*, (1979) have shown them to be quite distinct; they differ in isoelectric point, apparent molecular weight, peptide patterns and phage inactivation. It has recently been shown that the new membrane protein can be induced by phosphate limitation (Overbeeke and Lugtenberg, 1980). Mutations resulting in constituitive synthesis of the protein have been isolated in either of two loci; first called *nmpA* and *nmpB* which map at minute 83 and minute 9 respectively (Foulds and Chai, 1978; Lee *et al.*, 1979; Pugsley and Schnaitman, 1978). Argast and Boos (1980) found that the new membrane protein was co-regulated with alkaline phosphatase. Tommassen and Lugtenberg (1980) demonstrated that *nmpA* mutants are either *phoS*, *phoT* or *pst*, which are involved in phosphate uptake and *nmpB* is identical to *phoR* which is involved in regulating the *pho* operons. More recently Tommassen and Lugtenberg

(1981) mapped the structural gene for the new membrane protein at minute 6 and designated the locus *phoE* and its product is therefore called the PhoE protein. It was possible to isolate electrophoretically altered PhoE protein mutants in both *nmpA* and *nmpB* backgrounds. This result clarifies the position with respect to how many loci code for the outer membrane protein.

The PhoE protein is as indicated above, a porin, but little has been done to determine its substrate specificity. It has been included in this section since the available data suggested it to form a general pore analogous to OmpC and OmpF. In view of its association with the phosphate regulon, it is not unreasonable to assume it may be a specific porin for phosphates.

1.6 PORINS FOR SPECIFIC CLASSES OF MOLECULES

In general, these proteins are present in the outer membrane in low amounts, and their presence only becomes critical at low substrate concentrations, or the molecular weight of the substrate.

1.6.1 LamB protein

The outer membrane protein known as the receptor for phage λ and as a pore for maltose and higher maltodextrins is coded for by the *lamB* gene (Thirion and Hofnung, 1972; Randall-Hazebauer and Schwartz, 1973; Hofnung *et al.*, 1976; Braun-Breton and Hofnung, 1977).

The *lamB* gene maps in the malB region at around minute 90. The malB region has been shown to consist of two divergent operons (Hofnung *et al.*, 1974 and Hofnung, 1974). The genes contained in these operons *malG*, *malE* and *malF* for one and *malK lamB* for the other operon, are all involved in the transport of maltose and maltodextrins across the bacterial cell wall (Kellerman and Szmelcman 1974; Szmelcman and Hofnung, 1975; Szmelcman *et al.*, 1976; Schwartz *et al.*, 1976; Ferenci *et al.*, 1977). (See figure 1.1). The *malG* gene product has not yet been identified; the MalF and MalK proteins are known to be resident in the cytoplasmic membrane (Silhavy *et al.*, 1976, 1977; Bavoil *et al.*, 1980). The MalE protein is periplasmic and has affinity for maltose and maltodextrins (Kellerman and Szmelcman, 1974; Schwartz *et al.*, 1976). The gene products of *malE*, *-F*, *-G* or *-K* all show a Mal$^-$ phenotype and are essential for maltose transport. For a detailed discussion of the physical and genetic map of the malB region see Raibaud *et al.*, (1979a and b) and Silhavy *et al.*, (1979).

In addition to the malB region there also exists the malA region which maps at minute 74. This region encodes three genes *malQ*, *malP* and *malT*. *MalP* and *malQ* code for proteins involved in maltose and maltodextrin metabolism (amylomaltase and maltodextrin phosphorylase). The *malT* gene product acts as a positive control on the three regulons (Hofnung *et al.*,

1971, 1974; Thirion and Hofnung, 1972). See figure (1.2). *LamB* mutants have been selected as resistant to phage λ (Hofnung *et al.*, 1971); Schwartz, 1967), phage TP1 (Wandersman and Schwartz, 1978) or more recently to phage K10 (Roa, 1979), while retaining the ability to grow well on maltose at concentrations above 10μM. Absence of the λ receptor in *lamB* mutants result in inability to grow on maltohexaose, maltopentaose and maltotetraose as well as less marked growth effects on maltose and maltotriose at low concentrations (Szmelcman *et al.*, 1976; Wandersman *et al.*, 1979). The LamB protein has been isolated and purified (Randall-Hazelbauer and Schwartz 1973). The protein has been reconstituted into artificial bilayer membranes (Boehler-Kohler *et al.*, 1979) and into outer membrane vesicles (Nakae, 1979; Luckey and Nikaido, 1980). The LamB protein formed aqueous pores analagous to the OmpC, OmpF proteins. It has been claimed by Boehler-Kohler *et al.*, (1979) that the LamB protein forms larger diameter pores than the OmpC or OmpF proteins in black lipid membranes. This could explain why malto-oligosaccharides the size of maltoheptaose (molecular weight 1152) are able to diffuse through the membrane, when the OmpC and OmpF proteins have an exclusion limit around 600 daltons. *In vitro* data of Tokunaga *et al.*, (1979) and Luckey and Nikaido (1980) suggest that the LamB protein does have specificity for maltodextrin permeability. Ferenci and co-workers (Ferenci, 1980

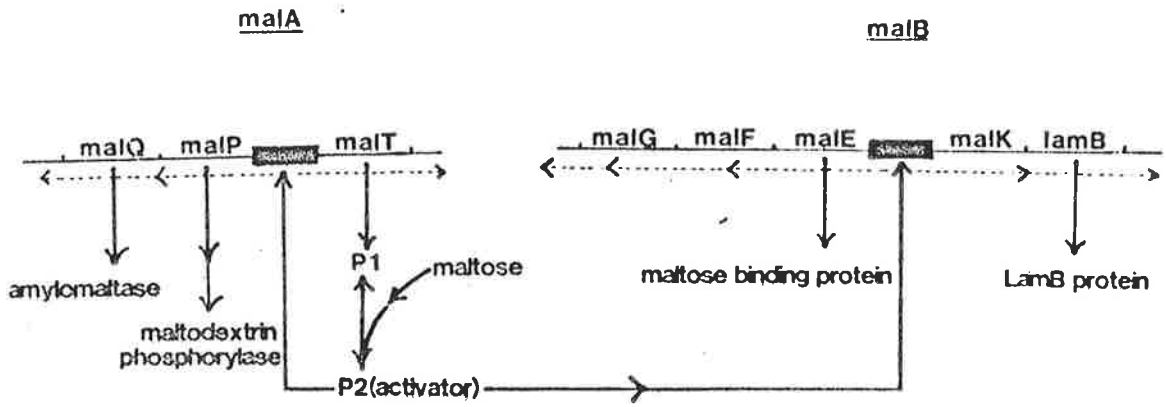


Fig. 1.2

The maltose regulon in *E. coli*, *malT* is a positive regulator gene, the product of which in the presence of maltose activates the expression of the three maltose operons. The directions of transcription of the three *mal* operons are indicated by broken arrows.

Adapted from Raibaud *et al.*, (1979).

and Ferenci *et al.*, 1980), were able to demonstrate that the LamB protein does have a binding capacity for maltodextrins, moreover it was shown that cells possessing the LamB protein could bind maltooligosaccharides up to 2500 molecular weight. The high affinity binding of these substrates was shown to be dependent upon both the LamB protein and the MalE protein (periplasmic maltose binding protein). The mechanisms of specificity of the LamB protein and the other specific pores for its substrates will be discussed later in this chapter.

1.6.2 Tsx protein

Genetic evidence based upon the existence of mutants resistant to both colicin K and bacteriophage T6 led to the postulate that colicin K and phage T6 shared a common receptor (Fredricq, 1949). It was found more recently that most T6 resistant mutants lack an outer membrane protein of around 25,000 molecular weight which is the product of the *E. coli tsx* gene (Manning and Reeves, 1976). This protein was found to be able to inactivate both colicin K and phage T6 and is the receptor for both agents (Manning and Reeves, 1978). It was shown later that Tsx protein promotes the transport of nucleosides across the outer membrane (McKeown *et al.*, 1976; Munch-Petersen *et al.*, 1979; Krieger-Brauer and Braun, 1980). The Tsx protein has many properties similar to the LamB protein. The Tsx

protein is co-regulated with the other components of nucleoside uptake (Krieger-Brauer and Braun, 1980). The dependence of nucleoside uptake on Tsx protein is only apparent at low substrate concentrations. There is no competitive inhibition of the absorption of T6 by the substrate (Krieger-Brauer and Braun, 1980) indicating that the Tsx protein could be a passive pore. At present no *in vitro* evidence is available to support this concept. It is not known whether a periplasmic binding protein is required for transport of nucleosides, however Munch-Petersen *et al.*, (1979) have presented some circumstantial evidence that a binding protein exists; specifically, nucleoside transport was decreased when cells were subjected to osmotic shock; shock sensitive transport systems usually involve a binding protein.

1.7 NON PORIN OUTER MEMBRANE SPECIFIC TRANSPORT PROTEINS

The LamB protein and, by analogy the Tsx protein are considered to be water filled pores which transport specific classes of substrates. There does exist however a class of outer membrane proteins which transport molecules too big for the general pores that have strong recognition sites on the outer membrane. These proteins bind strongly to their substrates and pass them on to the next component in the transport process. The transport processes

mediated by this class of proteins exist for: vitamin B_{12}, ferrienterochelin, ferrichrome and citrate. In all cases the binding step is not energy dependent, but subsequent steps require an energised membrane and are dependent upon the *tonB* gene product (Pugsley and Reeves, 1977; Bradbeer and Woodrow, 1976; Bassford *et al.*, 1976).

1.7.1 The BtuB protein

It was found by White *et al.*, (1973) that vitamin B_{12} binding capacity was confined to the outer membrane. Later Di Masi *et al.*, (1973) demonstrated that the outer membrane receptor for B_{12} uptake is identical to the receptor for colicins E2, E3 and bacteriophage BF23. Sabet and Schnaitman (1973) purified the protein and found it to be a 60,000 dalton polypeptide, with the structural gene for this protein located at *btu*B which maps at minute 88 (Bachmann and Low, 1980). The evidence that BtuB protein is a receptor rather than a porin arises from the following evidence. It was shown by Sabet and Schnaitman (1971) that vitamin B_{12} is able to protect cells from colicins E1 and E3. In addition the colicins and phage BF23 competitively inhibit the binding of B_{12} to whole cells, outer membrane and purified receptor (Di Masi *et al.*, 1973; Bradbeer *et al.*, 1976).

1.7.2 Iron uptake systems: FepA protein

Bacteria require iron in trace amounts for many metabolic processes: because iron in the ferric state has a very low solubility, bacteria growing under normal conditions find very little that can be easily taken up. *E. coli* has evolved systems in which low molecular weight siderophores (iron chelators) are produced which are taken up by the cell in complex with the iron. *E. coli* produces a phenolate-type siderophore called Enterochelin (Pollack and Neilards, 1970). Enterochelin uptake is the major system of iron uptake used by cells at low iron concentrations. *E. coli* can also take up hydroxamate type siderophores made by other bacteria and fungi. Finally there is an iron transport system which uses citrate as a chelator.

Cox *et al.*, 1970 and Langman *et al.*, 1972 described mutants that are unable to assimilate ferric enterochelin. This mutation was found to map at minute 13 and is referred to as *fepA* (Wookey and Rosenberg, 1978). This mutation was previously known as *feuB* (Hancock *et al.*, 1976), *cbr* (Pugsley and Reeves, 1976). Enterochelin has been shown to afford cells protection against certain of the B group colicins. Guterman (1973) was able to show that enterochelin acts as an inhibitor of colicin adsorption. Hollifield and Neilands (1978) have provided evidence that solubilized outer membrane extracts prepared from colicin B sensitive cells contain a component for which colicin B

and enterochelin compete for binding. If *E. coli* is shifted from a high iron medium to a low iron medium which results in derepression of enterochelin production, this also leads to the production of three high molecular weight polypeptides in the outer membrane. Hantke and Braun (1975), using colicin B as a selective agent were able to isolate mutants defective in enterochelin uptake. This mutant does not absorb colicin B and lacks an outer membrane polypeptide of molecular weight 81,000, the abundance of which depends upon iron concentration in the external medium (Hancock *et al.*, 1976; Ichihara and Mizushima, 1978). Pugsley and Reeves (1976), were also able to isolate similar mutants. It would appear that the *fepA* gene controls the production of an outer membrane protein of 81,000 molecular weight that serves as a receptor for colicin B and is involved in the uptake of ferric-enterochelin.

1.7.3 TonA Protein

E. coli in addition to the uptake system for enterochelin also possesses an uptake system for the hydroxamate-type siderophore, ferrichrome, which is produced by other organisms. It was proposed some years ago that bacteriophages T1 and T5 and colicin M share the same receptor, which is encoded by the *tonA* gene at minute 3 (Fredricq, 1951; Fredricq and Smarda, 1970; Bachmann and Low, 1980). It was later shown

that phage ϕ80 also shares this same receptor (Davies and Reeves, 1975; Hancock and Braun, 1976). Wayne and Neilands (1975) subsequently showed that ferrichrome protects cells from ϕ80 phage adsorption but other iron ligands did not afford this same protection. Pugsley *et al.*, (1975) were able to provide evidence for competition between T5 and ferrichrome and their outer membrane receptor. Protection against colicin M by Ferrichrome could also be shown (Hantke and Braun, 1975; Wayne *et al.*, 1976). Furthermore Hantke and Braun (1975) showed that *tonA* mutants defective in uptake of ferrichrome lacked a single outer membrane protein (mol. wt. 78,000) which is present in wild-type and in *tonA*$^+$ revertants. It is apparent that the *tonA* gene specifies a protein that functions in ferrichrome transport and that this protein actively binds its substrate.

1.7.4 Fec protein

It was found by Cox *et al.*, (1970) that citrate stimulates iron uptake and growth in *fepA* mutants. It was later shown that mutants defective in both *fepA* and *tonA* functions are able to accumulate iron by a third uptake system; by way of citrate, and that this system is physiologically and genetically distinct from the other two (Cox *et al.*, 1970; Langman *et al.*, 1972; Young *et al.*, 1967; Hantke and Braun, 1975). Woodrow *et al.*, (1978) isolated mutants in the citrate uptake system which they designated *fec* which could be mapped

at minute 6. Hancock *et al.*, (1976) have shown that strains growing in the presence of citrate possess an outer membrane protein in large amounts with an apparent molecular weight of 81,000. It is not known at present the precise role this protein plays in citrate transport, or if it binds to its substrate in the same fashion as the *fepA* or *tonA* gene products.

1.8 PERIPLASMIC BINDING PROTEINS: Involvement in transport

These proteins bind with their respective substrates but do not catalyze any chemical change. Genetic and biochemical evidence suggests that these proteins play an essential role in transport in the whole cell (Rosen and Heppel, 1973). The binding proteins can be removed from the cell envelope by EDTA-osmotic shock treatment with a concomittant loss of transport function. Binding proteins have been isolated for most shock sensitive transport systems. Both binding protein and the specific transport system exhibit similar substrate affinities; inhibitors for the binding protein usually inactivate the transport system. Mutants lacking a binding protein are unable to transport and the genetic expression of the binding protein and transport system is coregulated.

The method of EDTA-osmotic shock has been used to isolate a number of binding proteins such as those for sulphate, phosphate and histidine (Oxender and Quay,

1976), and several of the proteins have been crystallized and sequenced (Langridge *et al.*, 1970; Hogg and Hermodson, 1977; Quiocho *et al.*, 1979).

An important consideration in trying to assess the role of these proteins is the determination of their location within the cell envelope. It is generally accepted that periplasmic binding proteins are located outside the cytoplasm, however, it is not known whether the binding proteins exist in free or bound form within the cell envelope. Some evidence exists which implies that binding proteins may in fact be exposed on the cell surface (Lo, 1979).
The peptidoglycan, with which a number of outer membrane proteins are associated is the major structure within the periplasmic space (Rosenbusch, 1974; Hasegawa *et al.*, 1976). It is also possible that some periplasmic proteins may be anchored to the peptidoglycan.

1.9 NON TRANSPORT OUTER MEMBRANE PROTEINS

1.9.1 Protein 3b

This 40,000 dalton polypeptide is expressed at fairly high levels in *E. coli* K12 but its precise functions and genetics are still rather unclear. The protein has been mapped to minute 12.5 by Earhart *et al.*, (1979), however, no designation has been given to the locus so it will be referred to as protein 3b in this thesis in accordance with the nomenclature of Schnaitman (1974). Fiss *et al.*, (1979) present

evidence that 3b is a protease that catalyses the conversion of FepA protein from an apparent molecular weight 81,000 to 74,000. Gayda and Markovitz (1978) suggest that 3b inhibits the synthesis of capsular polysaccharide. These workers have cloned the fragment of DNA that specifies 3b. Plasmid mutants showing depressed capsule synthesis were deficient in 3b synthesis and two other low molecular weight polypeptides specified by the DNA fragment. These results indicate the 3b may be involved in capsule synthesis. It has been known for some time that the synthesis of 3b is dependent upon temperature (Lugtenberg *et al.*, 1976; Manning and Reeves, 1977), so that it is possible that the mucoid phenotype of *E. coli* at low temperatures is due to low levels of protein 3b at these temperatures. Furthermore Gayda *et al.*, (1979) suggest that the conversion of the precursor form of protein 3b is dependent upon the *capR* gene.

1.9.2 OmpA protein

The ompA protein of *E. coli* is a major outer-membrane protein. Recent evidence has established that the ompA protein is a transmembrane protein (Enderman *et al.*, 1978). The protein also acts as a receptor for bacteriophages TuII* (Henning and Haller, 1975; Henning *et al.*, 1976) and K3 (Manning and Reeves, 1975; Manning *et al.*, 1976; Van Alphen *et al.*, 1977).

The physiological functions of OmpA protein are still not understood. *OmpA* mutants are defective in F-pilus mediated conjugation (Skurray *et al.*, 1974). The purified OmpA protein has been shown to inhibit conjugation (Schweizer and Henning, 1977; Van Alphen *et al.*, 1977), it has now been demonstrated that OmpA protein stabilizes mating pairs: Achtman *et al.*, (1978) showed that if matings were allowed to occur on a solid matrix the defect in conjugation could be almost totally reversed. This also suggests that the OmpA protein has no direct effect on the organization of the outer membrane.

It has been recently suggested that the OmpA protein plays a significant role in the maintenance of cellular morphology and outer membrane integrity possibly through interactions with peptidoglycan (Sonntag *et al.*, 1978). It has been reported that *ompA* mutations affect outer membrane functions; reducing ferrichrome mediated iron transport (Coulton and Braun, 1979), nucleoside transport (Krieger-Brauer and Braun, 1980) and amino acid transport (Manning *et al.*, 1977). This observation is rather controversial since the amino acid concentration used in these experiments was far above the concentrations for which the outer membrane is considered limiting. The result obtained may be explained by the lower viability and growth rates of the *ompA* mutants described in the same publication.

It is well established that the *ompA* locus at

minute 21.5 is the structural gene for the outer membrane protein (Foulds, 1974; Manning *et al.*, 1976; Datta *et al.*, 1976). Recently the *omp*A region has been cloned and the regulatory region of the gene sequenced (Movva *et al.*, 1980). In addition the purified protein has also been recently sequenced (Chen *et al.*, 1980). The protein was found to be of 325 amino acid residue with a molecular weight of 35,159. The transmembrane portion of the protein was found to be between residues 1-177, and a lipophilic structure of 27 residues in an α-helical configuration was found in this region. It was also found that the OmpA protein bears little similarity to the major porins.

1.9.3 The Murein Lipoprotein

The murein lipoprotein is a small protein of only 58 amino acid residues and is thought to be the most abundant protein in the cell ($7x10^5$ molecules). Two thirds of it exist in a free form, and the other third in a form which is covalently linked to peptidoglycan via the ε-amino group of its C-terminal lysine (Inouye *et al.*,1972). Its N-terminal amino acid, cysteine carries an amino acid as well as a diglyceride group. This region is thought to be embedded in the outer membrane (Braun, 1975). The structural gene for this protein, *lpp*, is located at minute 36.5 on the chromosome (Bachmann and Low, 1980). The protein seems to be highly conserved among the *Enterobacteriaceae* (Nakamura *et al.*, 1979), giving some

credence to its role as a primary structural protein.

The role postulated for the lipoprotein is that it plays a primary role in the stabilization of the cell envelope. The mutant in which the *lpp* gene was isolated by Hirota *et al.*, (1977) showed increased blebbing of the outer-membrane, alterations in barrier function, increased sensitivity to EDTA and some defect in septa formation during cell division (Suzuki *et al.*, 1978). This phenotype appears to arise mainly from a loss of the bound form of the protein since a missense mutant that contains greatly reduced amounts of bound lipoprotein shows the same properties (Wu *et al.*, 1977; Yem and Wu, 1978). The *lpp* mutation seems to have no obvious effect on porin assembly or function (Endermann and Henning, 1979; Nikaido *et al.*, 1977).

In summary the lipoprotein appears to play a structural role, its C-terminus is linked to the peptidoglycan and its lipophilic N-terminus embedded in the outer membrane. This configuration would therefore suggest that the primary role of the lipoprotein is to anchor the outer membrane to the peptidoglycan. This is considered to be the primary function of the lipoprotein by most workers.

1.10 CONCLUDING REMARKS

This thesis will examine the function of the outer membrane proteins of *E. coli*. It will primarily

concentrate on the specific uptake systems involving outer membrane proteins. The mechanism of specificity of substrate transport will be studied with emphasis on the relationship of the outer membrane protein with other components of the cell wall, such as periplasmic binding proteins.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 MATERIALS AND METHODS

2.1 MEDIA

Nutrient broth (Difco, 0003) was prepared double strength plus 5mg/ml NaCl; Nutrient agar was blood agar base (Difco, 0045) and prepared as directed without the addition of blood; soft nutrient agar was prepared by mixing equal volumes of molten nutrient agar and nutrient broth.

Minimal medium was prepared according to Davis and Mingioli (1950); minimal agar was prepared by the addition of 20g/litre agar to liquid medium. Glucose was added as a carbon source to a final concentration of 5mg/ml. Glycerol to a final concentration of 1% (vol/vol). Growth supplements were added to give a final concentration of 20µg/ml. Soft agar overlays have an agar concentration of 1%. Luria broth was prepared according to Miller (1972). Luria broth agar plates contained agar at 15g/l. Tetrazolium agar was prepared by the addition of $2x10^{-5}$% (w/v) 2,3,5-triphenyl tetrazolium chloride to luria broth agar. The sugar to be tested was added to give 1% final concentration. Tetracycline plates were made by the addition of tetracycline to a final concentration of 16µg/ml to nutrient agar plates.

2.2 BACTERIAL STRAINS

The bacterial strains used in this study are listed in table 2.1; all strains used were constructed

TABLE 2.1 Bacterial Strains

Strain	Genotype	Source
AB1297(CGSC1972)	*metA28, argH1, purF1, xyl-7, supE44* HfrP012	B. Bachmann
AB2847(CGSC2847)	*aroB malA* F^-	A.J. Pittard
pop3127	*araD139, rpsL, malT^C, Δ(lacA-lacJ)*	M. Schwartz
pop1753	*his, thi, malE11*	G. Hazelbauer
pop1754	*his, thi, malE12*	G. Hazelbauer
pop1755	*his, thi, malE13*	G. Hazelbauer
pop1756	*his, thi, malE14*	G. Hazelbauer
P1831	*tsx-354, ompB101, malT354*	V. Sarma, 1978
P2222	*supE44, purF1, xyl-7, zij::tn10-9, argH1 metA28*	This thesis
P2335	*tsx-354, ompB101, malT^C, zij::tn10-9, metA28, lamB 24*	This thesis
101a CGSC5656	*phoT32, pit-10, relA1, tonA22,* $T2^R$	H. Rosenberg
C78 CGSC5651	*phoS28, pit-10, relA1, tonA22,* $T2^R$	H. Rosenberg
P400	*thr, leu, proA, argE, thi, rpsL, lacY, galK, ara, mtl, xyl, supE, non*	Hancock & Reeves, 1975
P407	*tsx-200* derivative of P400	R.E.W. Hancock, 1974
P433	*tsx-201* derivative of P400	R.E.W. Hancock, 1974
P1578	*ompB105* derivative of P400	P.A. Manning, 1977
P1602	*tsx-203* derivative of P1578	P.A. Manning, 1977
P1848	*tonA208* derivative of W1485	V. Sarma, 1978
P1926	*tsx-10* derivative of P1848	V. Sarma, 1978
P1930	*tonA208, ompB106* derivative of P1848	V. Sarma, 1978
P2024	*tonA208, ompB106, tsx232* derivative of P1848	V. Sarma, 1978
P460	*ompA1* derivative of P400	R.E.W. Hancock, 1974
P1665	*ompA9* derivative of P400	P.A. Manning *et al.*, 1976
P1667	*ompA11* derivative of P400	P.A. Manning *et al.*, 1976
P1744	*tsx-206* derivative of P1665	P.A. Manning & Reeves, 1978

Table 2.2 Colicinogenic Strains

Strain	Colicin produced	Source
E. coli CA42	E2	a
E. coli K-12 W3100 (colE3-CA38, I-CA38)	E3[b]	a
Serratia marcescens JF246	L	c

a. Davies (1974) and Davies and Reeves (1975).

b. Produces no detectable colicin I.

c. J. Foulds (1972).

in this laboratory unless otherwise stated. Colicogenic strains have been listed in table 2.2 and have been described previously (Davies and Reeves, 1975; Foulds, 1972).

2.3 BACTERIOPHAGE

All bacteriophage used were laboratory stocks and are listed in table 2.3. The phage were usually propagated on P400 and maintained as high titre lysates at 4°C.

2.3.1 Selection of bacteriophage resistant mutants

All phage resistant mutants were selected using the following method; an early log phase culture (0.1ml) of the cells to be treated was mixed with an bacteriophage (m.o.i. = 10 approx.) in a 3ml soft nutrient agar overlay. The plates were incubated (18hr; 37°C). The phage resistant mutants were purified by single colony isolations and their resistance pattern checked by cross streaking against the appropriate bacteriophage.

2.3.2 Routine screening for bacteriophage and colicin resistance

Using a sterile swab, a phage stock of high titre was streaked across a nutrient agar plate. After drying the plate, a stationary phase culture in nutrient broth of the appropriate strain was streaked with a swab at right angles to the phage streak. After incubation

Table 2.3 Bacteriophage used in this study

P1*cml c1r100*

P1*kc*

λ*vir*

λNK55

TC45

U3

C21

T6

K3

K3*hr*1

Tula

Tulb

T4

T7

K10

(18hr; 37°C) the plates were examined for lysis.

To test for resistance to colicins, a culture of the appropriate colicinogenic strain was streaked onto a nutrient agar plate. This was then incubated (18hr; 37°C); the bar of growth on the plate was killed by exposure to chloroform for approximately 30 minutes. The plate was then overlayed with a thin layer of nutrient agar, dried, and the strains to be tested were streaked with a swab at right angles to the colicin streak. The plates were then incubated (18hr; 37°C).

2.3.3 Preparation of a P1 bacteriophage lysate

Lysates to be used in P1 transduction were prepared in the following manner. Early log phase cultures ($OD_{625n.m.}$ = ∿0.2) of the donor strain were grown in nutrient broth containing 0.01M $MgSO_4$ and 0.001M $CaCl_2$ at 37°C with shaking. To this culture 0.1ml of P1*kc* (Lennox, 1955) of titre ∿10^9 p.f.u./ml was added. After incubation at 37°C with shaking for 2-3 hours lysis usually occurs. Chloroform was added and a further 15 minutes incubation. The lysate was then centrifuged in a bench centrifuge and the supernatant stored at 4°C until use.

2.3.4 Transduction with bacteriophage P1*kc*

10ml log phase cultures ($OD_{625n.m.}$=1.0) were grown in nutrient broth at 37°C with shaking. Cells were harvested by centrifugation for 10 minutes on a

bench centrifuge. The pellet was resuspended in 1ml of a solution containing 0.1M $MgCl_2$ and 0.005M $CaCl_2$. The cells (0.1ml) were then added to 0.1ml of the neat donor phage stock. This mixture was then incubated without shaking for 20 minutes at 37°C. The mixture of phage and cells was then plated (0.1ml) onto the appropriate selection plate. The method above is a modification of Miller (1972).

2.4 TRANSPOSON METHODS

2.4.1 Construction of Tn10 random integrates

The method used is essentially that of Kleckner *et al.*, (1977). The desired strain is grown in 10mls of nutrient broth and 0.2% maltose to mid log phase. The cells were centrifuged (7000xg; 10 min) and resuspended in nutrient broth (0.3ml) containing 20mM $MgCl_2$. λNK55 (0.3mls; $5x10^{10}$p.f.u./ml) is added and allowed to incubate (10mins; 42°C). Nutrient broth was then added (6mls; prewarmed 42°C) and incubation continued for 20 minutes. The cell-bacteriophage mixture is centrifuged (7000xg; 10 min) and resuspended in nutrient broth (0.3ml). This was then plated (0.1ml) onto nutrient agar tetracycline plates containing 0.01M tetra-sodium pyrophosphate. The plates were then incubated (18hr; 42°C). The tetracycline resistant colonies were then harvested by the addition of nutrient broth (4ml) and the re-suspended colonies added to a further 10ml of nutrient

broth. The cells were centrifuged (7000xg; 10 min), resuspended in 0.5ml nutrient broth plus 0.5ml 80% glycerol and frozen at -20°C until further use.

2.4.2 Insertion of *tn10* near to *metA28 (zij::tn10)*

The method described below is essentially that of Kleckner *et al.*, 1977.

The random mix method described in section 2.4.1 was applied to P1848. The P1848*::tn10* random mix was then used to propagate bacteriophage P1*cm1c1r100*. The P1848*::tn10* random mix was diluted ($5x10^8$ organisms/ml), this was added (0.1ml) to 9.1ml of P1 bacteriophage ($5x10^9$p.f.u./ml), poured in a soft nutrient agar overlay (plus 10mM $CaCl_2$). This was sufficient to give confluent lysis. The plates were then incubated for 7-8 hours at 37°C. The overlay containing confluent plaques was scraped off into 5ml of nutrient broth and centrifuged (7000xg;10min). The titre of the phage in this stock was approximately 10^{10}p.f.u./ml. The stock was stored with chloroform (to ensure sterility) at 4°C.

The P1 phage stock propagated on P1848*::tn10*, random mix was then used to transduce AB1297 to *metA*$^+$ *tet*^{R} selecting on minimal tetracycline (8µg/ml) plates containing all supplements except methionine. The plates were incubated (18h; 37°C). Three of the *met*$^+$ *ter*^{R} colonies were picked and purified. These colonies were used as hosts to grow three separate phage P1 stocks. It was possible to determine the

co-transduction frequency of *tn10* with *metA28* (if any). The phage stocks propagated on the *metA*$^{+}$ *tet*^{R} strains were used to transduce AB1297 selecting on nutrient agar and tetracycline plates. A *metA*$^{+}$ *tet*^{R} strain having 67% cotransduction of the two markers was chosen. This strain was assumed to have *tn10* integrated close to *metA28*. In the transduction step above some colonies (23%) show the following phenotype Met^{-}Tet^{R}, colonies of this type were selected and P1 phage propagated on them. It is possible to use the P1 phage grown on these stocks to transduce the *metA28* marker to any *metA*$^{+}$ strain using *tet*^{R} as a selection. The *metA28zij::tn10-9* derivative of AB1297 was designated P2222 and used as a source of the *metA28* allele linked closely to *tn-10* for subsequent P1 transduction into AB2847 to give a *metA28* derivative of this strain.

2.5 MEASUREMENT OF SENSITIVITY TO TOXIC AGENTS

2.5.1 Toxic peptides

Growth inhibition was measured essentially by the method of De Felice *et al.*, (1973). Minimal plates containing glycerol as carbon source were overlaid with 3ml of soft agar innoculated with 0.2ml of nutrient broth grown culture of the strain to be tested. Filter paper discs (9mm) were laid on the plates, and 10µl of a 5mg/ml solution of the peptide is added to the disc. The diameter of the inhibition zone was measured after overnight incubation.

2.5.2 Antibiotics and Detergents

Overnight cultures (standing, nutrient broth grown) of the strains to be tested are streaked across nutrient agar plates. The agent to be tested are impregnated into filter paper strips (10x100mm) which were laid at right angles to the bacterial streaks. The zones of inhibition are measured after overnight incubation at 37°C.

The test strips are constructed in the following fashion: The sterile filter paper strips were soaked with 200µl of a solution of the agent to be tested. The strips were then dried (60 minutes at 37°C) and stored at 4°C until use.

2.6 UPTAKE METHODS

2.6.1 In minimal medium

Log-phase cultures (nutrient broth; 37°C; aerated; equivalent to 10^8cells/ml) were used. Inducers were employed where necessary. The cells were washed once in minimal salts containing 1% glycerol and suspended in the same medium containing 0.1mg/ml of chloramphenicol. This suspension was transferred to the uptake vessel, aerated and kept at 37°C or 25°C for 10 minutes before the addition of the radioactive substrate. Samples (0.1ml) were withdrawn at various time intervals, filtered on membrane filters (Gelman HA, 0.45µm pore diameter) on a multiple filtering manifold and washed with 20ml of warm 0.9% saline. The membranes were then dried and

radioactivity counted in a Packard Tri-Carb liquid scintillation counter.

2.6.2 In HEPES buffer

In some cases the uptake experiments were carried out in 10mM HEPES buffer pH7.5 plus 5mM $MgCl_2$ with 0.1mg/ml chloramphenicol. All other conditions are as described in section 2.6 above.

2.7 PROTEIN METHODS

2.7.1 Preparation of periplasmic proteins

The method used was modified after Ito *et al.*, (1977). Cultures (10ml) were grown in nutrient broth to a density of 1.6×10^9 cells/ml with aeration. The cells were washed once in 0.03M Tris-HCl pH8.1. The pellet was resuspended in 0.2ml of 20% sucrose in 0.03M Tris-HCl pH8.1. This resuspension was converted to spheroplasts by the addition of 20µl of 1mg/ml lysozyme in 0.1M EDTA pH7.3 and incubation on ice for 30 minutes. This step should liberate the periplasmic proteins; the mixture was then centrifuged (15,000xg; 15 min), the supernatant containing the periplasmic proteins was collected and the spheroplasted cells either discarded or retained for whole membrane preparation (see below). To the periplasmic protein solution an equal volume of 10% cold TCA (trichloroacetic acid) was added and the resulting suspension of protein centrifuged at 3000xg for 5 minutes. The pellet was then resuspended in acetone,

subjected to a further 3000xg; 5 minute centrifugation and finally dissolved in Lugtenberg's solution (Lugtenberg *et al.*, 1975) in preparation for polyacrylamide gel electrophoresis.

2.7.2 Whole membrane preparation

The following method was used for the rapid preparation of whole membranes: The spheroplasted cells obtained from the periplasmic protein procedure above were frozen, thawed, and resuspended in 3ml of 3mM EDTA (pH7.3); subjected to sonication for 1 minute, the tubes being kept on ice during this procedure. This sonicated suspension was then centrifuged for 60 minutes at 28,000xg. The pellet obtained was dissolved in 0.25ml of Lugtenberg's solution (Lugtenberg *et al.*, 1975) in preparation for electrophoresis.

2.7.3 Tris (hydroxymethyl) aminomethane -HCl pH7.2 buffer treatment of cells

Cells were grown in minimal media and 0.5% glycerol (200ml) to stationary phase overnight. These cultures were then washed once in an equal volume of 10mM Tris-HCl, pH7.2 buffer containing 50µg/ml chloramphenicol and 0.5% glycerol. The pellets were resuspended in 100ml of the same buffer, chloramphenicol and glycerol mixture, allowed to stand (4hrs at 37°C), centrifuged (7000xg; 10 minutes) and the supernatant dialysed against 5mM Tris-HCl (18hr;

4°C). This mixture was then concentrated by lypophylization and the residue dissolved in 1ml of distilled water. This was used for Ouchterlony double gel diffusion or polyacrylamide gel electrophoresis after addition of an equal volume of Lugtenberg's solution.

2.8 POLYACRYLAMIDE GEL ELECTROPHORESIS

2.8.1 Preparation of samples

The preparation of samples for slab gel electrophoresis was carried out according to the methods described by Lugtenberg *et al.*, (1975). The samples were dissolved in Lugtenberg's solution and heated at 100°C for 3-5 minutes immediately prior to loading.

2.8.2 Standard Lugtenberg System

The method used, was essentially identical to that of Lugtenberg *et al.*, (1975). The slabs are stained according to Fairbanks *et al.*, (1971).

2.8.3 Gradient Gels

The method and staining procedure for 11-20% gradient gels is that of Achtman *et al.*, (1978).

2.8.4 4M Urea Gels

The method is essentially that of Achtman *et al.*, (1978) except that the standard 11-20% gradient gels contain urea at a final concentration of 4M. This method was a personal communication of Dr. P.A. Manning.

2.8.5 Protein Estimation

The method of estimating the concentration of protein was that of Schaeterle and Pollack (1973).

2.9 PURIFICATION OF PURE MALTOSE BINDING PROTEIN

Maltose binding protein was prepared by Mr. M.R. Penney by affinity to an amylose column according to the method of Ferenci and Klotz (1978).

2.9.1 Preparation of anti-sera to maltose binding protein

Anti-sera to maltose binding protein was prepared by Mr. M.R. Penney in the following fashion. 1mg of the pure binding protein was injected with Freund's complete adjuvant into rabbits both intra-muscularly and sub-cutaneously. The rabbits were boosted 2 weeks later by a similar regime of injections. At 8 weeks the rabbits were bled and the sera collected.

2.9.2 Ouchterlony double gel diffusion

Ouchterlony double gel diffusion was carried out according to the method of Ouchterlony 1962), using specific anti-sera to the maltose binding protein described above.

CHAPTER 3

SPECIFICITY OF LamB PORE IS IN PART CONFERRED BY THE MalE PROTEIN

3.1 INTRODUCTION

It has been known for some time that the LamB protein is involved in the uptake of maltose and higher maltodextrins (Hazelbauer, 1975; Szmelcman and Hofnung, 1975).

The LamB protein had been considered for some time to have little or no affinity for maltose (Szmelcman *et al.*, 1976). It was recently shown by Ferenci and co-workers (Ferenci, 1980; Ferenci *et al.*, 1980) that the LamB protein has a binding capacity for maltodextrins, but a weaker capacity for maltose. The high affinity binding of maltodextrins *in vivo* was shown to be dependent upon both the LamB protein and the maltose binding protein (MalE protein). Experiments are presented in this chapter that were designed to test the hypothesis that the LamB protein is an open hydrophilic pore with little specificity, but specificity of the pore is due to association with the MalE protein. It is postulated that the association of the MalE protein with the LamB protein impedes the diffusion of most solutes, but the MalE protein is still available for binding with its substrate.

The hypothesis is tested by examining the ability of the LamB protein to substitute the OmpC and OmpF proteins in an *ompB* background. The strains are made $malT^{c}$ to make LamB protein synthesis constituitive, and *malE* so that no MalE protein is produced.

3.2 STRAIN CONSTRUCTION

Strains were made in a *malT*^{c} background containing combinations of *ompB101* or *ompB*$^{+}$, *malE (malE11, malE12, malE13, malE14)* or *malE*$^{+}$ and *lamB24* or *lamB*$^{+}$. The starting strain was AB2847; the *metA28* allele of strain AB1927 was transferred by P1 transduction, using as a donor a derivative carrying transposon *tn10-9* integrated close (67% transduction) to *metA28*. The methods of Kleckner *et al.*, (1977) as described in chapter 2 were used. The AB2847 *metA28, zij::tn10-9* strain derived by transduction using P2222 as a donor for *metA28 zij::tn10-9*, was used as base strain and was sequentially transduced with:

(i) bacteriophage P1 grown on strain pop3127 using maltose fermentation to select *malT*^{c} derivatives.

(ii) phage P1 grown on strain P1831 using selection of *aroB*$^{+}$ followed by screening for resistance to colicin L (Davies and Reeves, 1975; Sarma, 1978) to give *ompB101* and *ompB*$^{+}$ derivatives.

(iii) phage P1 grown on either pop1753, pop1754, pop1755 or pop1756 using selection for *metA*$^{+}$ followed by screening for maltose fermentation to give *malE* and *malE*$^{+}$ derivatives.

Strains carrying the *lamB24* mutation were constructed in parallel to those above; P2335, a *lamB* mutant of strain AB2847 was used as a base strain to construct a parallel series of strains using the same methods as above. All derivatives are now *aroB*$^{+}$

Table 3.1

The relevant genotype and outer membrane proteins present in the strains.

Group	Strains	Genotype			Proteins			
		lamB	*malE*[a.]	*ompB*	LamB	OmpC	OmpF	MalE
1	P2239, P2340 P2341, P2412	+	-	*101*	+	-	-	-
2	P2413, P2414 P2415, P2416	+	-	+	+	+	+	-
3	P2417, P2418 P2342, P2419	+	+	*101*	+	-	-	+
4	P2238, P2239 P2343, P2241	+	+	+	+	+	+	+
5	P2420, P2337 P2421, P2422	*24*	-	*101*	-	-	-	-

a. The *malE* mutation includes the alleles *malE11, 12, 13, 14* respectively.

and retain transposon *tn10-9*.

A summary of the strain types produced is shown in table 3.1. The strains are divided into groups depending upon their genotype.

3.3 SENSITIVITY TO TOXIC PEPTIDES

Strains that lack or have reduced amounts of the major porins (OmpC and OmpF proteins) showed a marked resistance to toxic valine containing di- and tri-peptides. It was concluded that resistance was due to the lack of porin in the outer membrane and a subsequent decrease in outer membrane permeability. The effect of the MalE protein on the sensitivity of an *ompB* strain to toxic peptides is shown in table 3.2. In all cases strains lacking the MalE protein in an *ompB* background have enhanced inhibition zones, indicating increased outer membrane permeability in comparison to those strains carrying *ompB* alone. The strains having *lamB* mutations in addition to *malE* and *ompB* are the most resistant group. The greater sensitivity of the *malE ompB* strain over the *ompB* strains thus appears to be dependent on the presence of the LamB protein.

3.4 GROWTH ON 0.01% LACTOSE PLATES

The set of strains carrying combinations of *ompB*, and *ompB malE* mutations were grown on minimal lactose plates as described in Chapter 2; however lactose is supplied at 0.01%. The rationale behind

Table 3.2

Strain Group*	Genotype	Mean zone of inhibition and standard deviation (mm)	
		GGV[a]	TOR[b]
1	*malE ompB*	34.0 ± 2.10	23.3 ± 0.49
2	*malE*	37.7 ± 2.40	24.4 ± 1.08
3	*ompB*	30.3 ± 1.70	19.6 ± 0.86
4	wild-type	35.4 ± 1.20	24.5 ± 1.33
5	*malE ompB lamB*	30.4 ± 1.94	19.6 ± 1.12

a. glycyl-glycyl-L-valine

b. tri-L-ornithine

* Each value represents the pooled data for four strains of similar genotype as presented in Table 3.1

the experiment is that when grown under limiting conditions, *ompB* strains will produce smaller colonies than wild-type strains, since the uptake of lactose will be markedly reduced due to the lack of porins. However, strains carrying an additional mutation which alleviates the *ompB* defect by provision of other pores should produce larger colonies under these conditions than strains carrying *ompB* alone. The results of this experiment are depicted in table 3.3.

The four members of group 1 were found to have significantly different means ($P<0.05$) but this was due entirely to the *malE13* strain, which was therefore omitted from analysis of inter group variations. There was no significant variation within the other groups. The pooled data for strains of groups 1 and 3 were then compared and the difference between the mean colony diameters found to be significantly different ($P<0.001$). This result indicates that the inclusion of the *malE* mutation in an *ompB* mutant results in a significantly larger colony when grown on low level lactose as sole carbon source. On the basis of these experiments, where the effect of *malE* seemed to be maximized when the *malE13* allele is present it was decided to use *malE13* derivatives for subsequent experiments. P2341 *malE13, ompB101;* P2342 *ompB101;* P2343 'wild-type'; P2421 *malE13, ompB101, lamB24.*

Table 3.3

Strain group	Relevant Genotype	*malE* allele	Mean colony diameter mm
Group 1	*malE ompB*	malE11	0.40
		malE12	0.47
		malE13	0.57
		malE14	0.42
Group 3	*ompB*	malE$^+$	0.33

Mean colony diameters for each relevant strain or strain group grown on 0.01% lactose minimal agar for 36h at 37^{o}C. Each result for group 1 is the average of 40 colony measurements. Group 3 represents the pooled data for 40 colonies each from 3 independent *malE*$^+$ transductants, (i.e. 120 colonies).

3.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

The proteins present in the outer membrane of the strains (P2343, P2342, P2341, P2421) were checked, by running whole membrane preparations on polyacrylamide gels. This is shown in figure 3.1. The LamB protein is seen to be a major outer membrane protein by virtue of the *malT*^{c} mutation. P2343 possesses both OmpC and OmpF proteins whereas those strains carrying the *ompB101* mutation show the expected marked reduction of porin in the outer membrane. Strain P2421 shows the expected complete lack of LamB protein in the membrane, although the OmpA protein appears in larger amounts, perhaps as a compensatory measure for the lack of major porin and LamB protein.

3.5.1 Periplasmic shock proteins

The periplasmic shock proteins were run on polyacrylamide gels. This is shown in figure 3.2 Track A shows the purified MalE protein. The periplasmic proteins of strains P2343 and P2342 which are both *malE*$^{+}$ show large amounts of a protein corresponding to MalE protein. Strains P2341 and P2421 which are *malE13* show no such protein band and thus appear to lack or have substantially reduced amounts of MalE protein. The other *malE* alleles were tested in this way, and all were found to lack detectable MalE protein on polyacrylamide gels.

Figure 3.1

Whole membrane protein preparations.
Equal (15µl) amounts loaded onto 11-20%
gradient polyacrylamide gels.

A; P2343

B; P2342

C; P2341

D; P2421

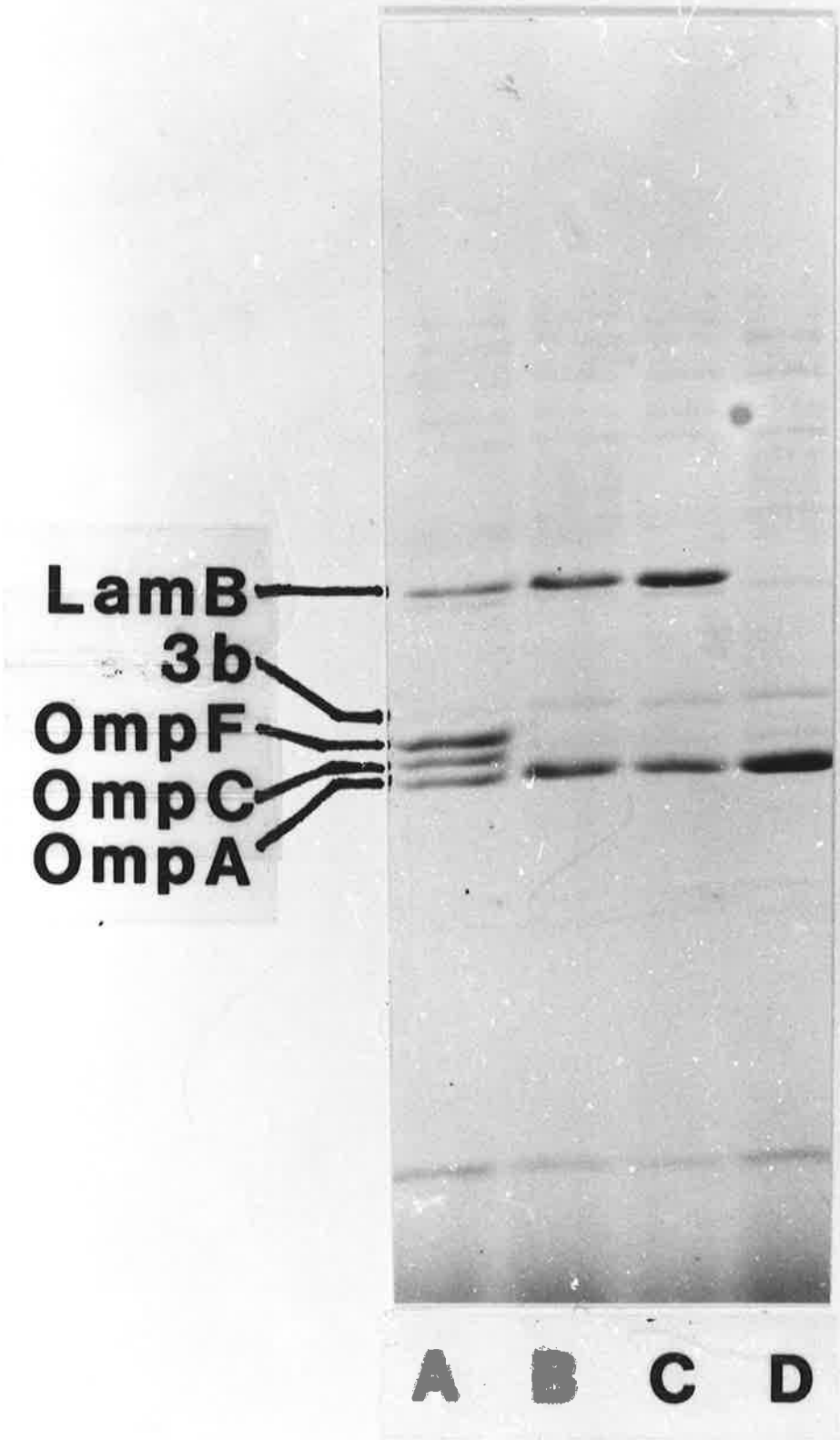

LamB
3b
OmpF
OmpC
OmpA
A
B
C
D

Figure 3.2

Periplasmic shock proteins. Equal (15µl) amounts loaded on to 11-20% gradient polyacrylamide gels.

A : pure MalE protein

B : P2343

C : P2342

D : P2341

E : P2421


MalE
(MALTOSE
BINDING PR.)
A
B
C
D
E

3.6 UPTAKE OF RADIOACTIVE SUBSTRATES

The ability of the strains to take up radioactive substrates was tested, in order to determine if the *malE* mutation has any effect upon the uptake rate in an *ompB* background.

Figs. 3.3 and 3.4 show the uptakes of (^{14}C)-lactose and (^{14}C) mannitol respectively. When the radioactive sugars are supplied to *ompB* mutants at limiting concentrations diffusion across the outer membrane becomes rate limiting. These results confirm this observation. Furthermore as predicted by our hypothesis, the inclusion of the *malE13* mutation substantially restores to strain P2341 the ability to take up both lactose and mannitol. The inclusion of the *lamB* mutation results in a further decrease in uptake rate of an *ompB* strain. This result again demonstrates the restorative effect of the *malE* mutation is dependent upon the presence of an intact LamB protein pore.

3.6.1 Uptake of nucleosides

The four strains P2343, P2342, P2341 and P2421 all carry the *tsx-354* mutation, these strains therefore lack the specific pathway of nucleoside permeation (Hantke, 1976). The uptake of nucleosides in these strains would be expected to depend upon the OmpC and OmpF proteins for outer membrane permeation.

The uptake of (^{14}C)-thymidine is shown in

Figure 3.3

The uptake of (^{14}C) lactose, showing the effect of the *malE* mutation on uptake (37°C; 1μM final concentration). Cells were induced with 10^{-2}M IPTG prior to uptake. P2343, wild-type; P2342, *ompB*; P2341, *malE*, *ompB*; P2421, *malE*, *ompB*, *lamB*.

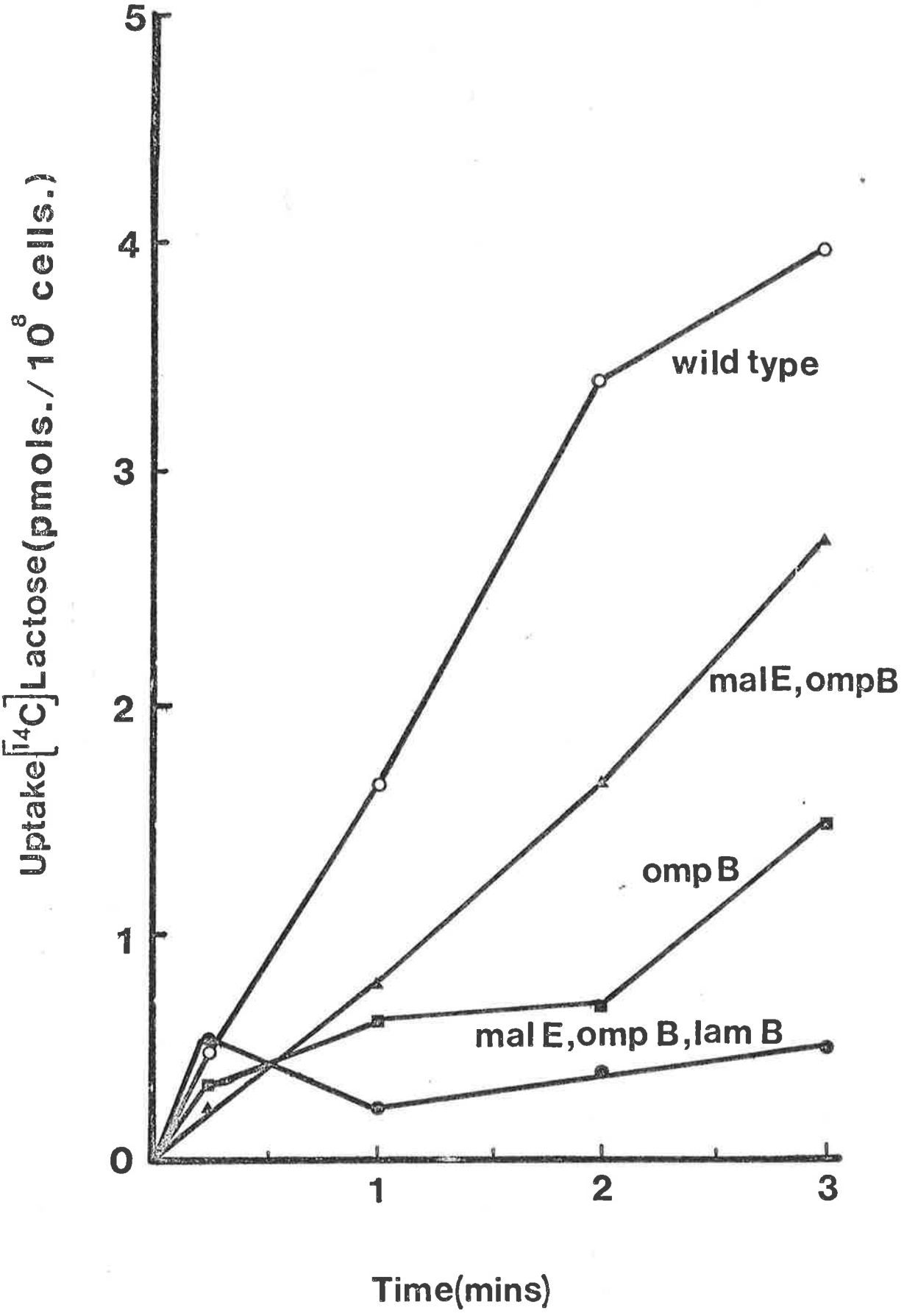

Uptake[^{14}C]Lactose(pmols./10^8 cells.)
5
4
3
2
1
0
1
2
3
Time(mins)
wild type
malE,ompB
ompB
malE,ompB,lamB

Figure 3.4

The uptake of (^{14}C) mannitol, showing the effect of the *malE* mutation on uptake (25^{o}C; 0.2μM final concentration). Cells were induced with 0.2% mannitol prior to uptake. P2343, wild-type; P2342, *ompB*; P2341, *malE*, *ompB*; P2421, *malE*, *ompB*, *lamB*.

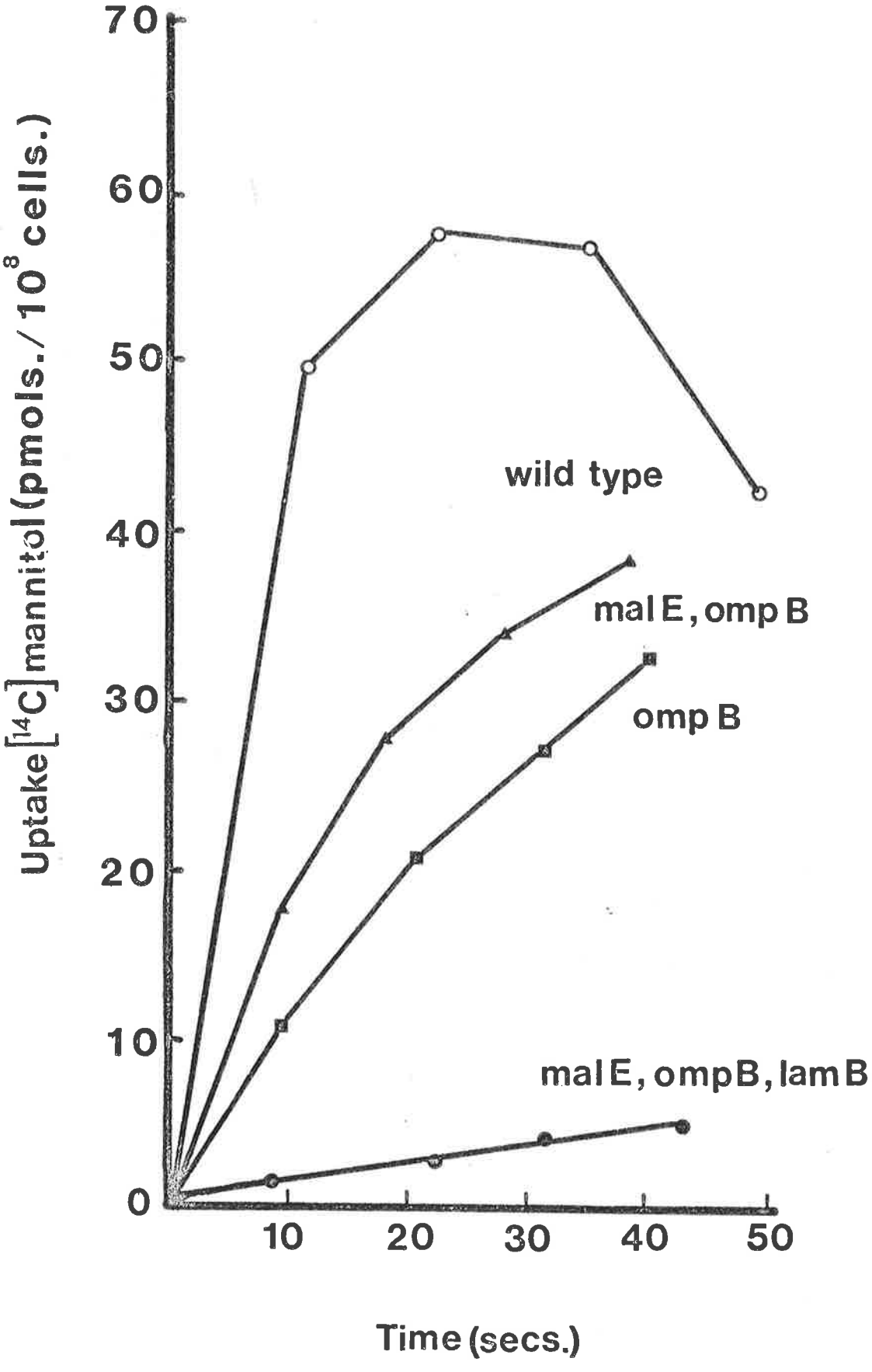

70
60
50
40
30
20
10
0
10
20
30
40
50
Uptake [14C] mannitol (pmols./10^8 cells.)
Time (secs.)
wild type
malE, ompB
ompB
malE, ompB, lamB

Figure 3.5

The uptake of (^{3}H) thymidine, showing the effect of the *malE* mutation on uptake (37^{o}C: 0.08μM final concentration). P2343, wild-type; P2342, *ompB*; P2341, *malE*, *ompB*, P2421, *malE*, *ompB*, *lamB*.

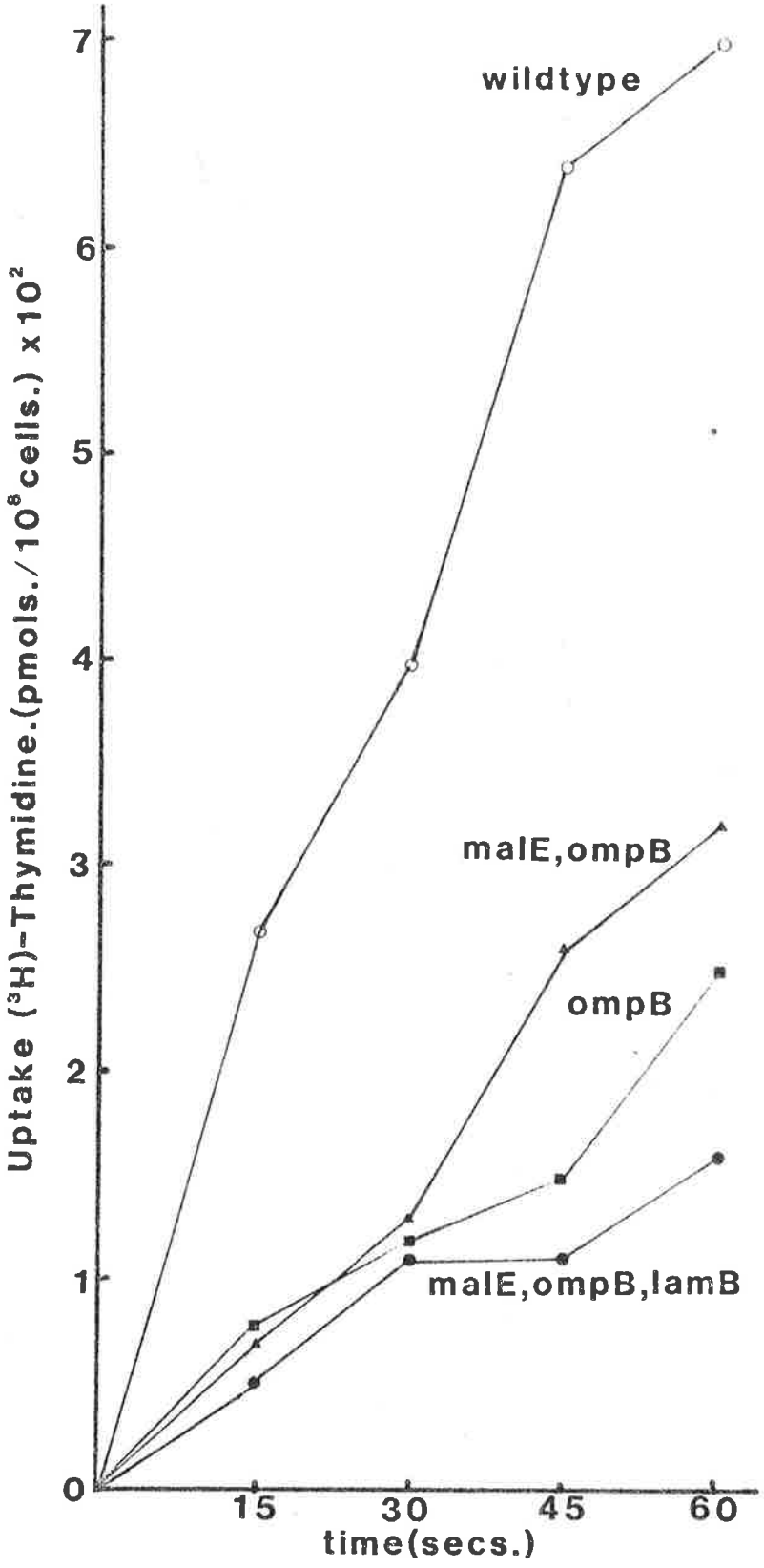

wildtype
malE,ompB
ompB
malE,ompB,lamB
Uptake (^{3}H)-Thymidine.(pmols./10^8cells.) x10^2
time(secs.)
0
1
2
3
4
5
6
7
15
30
45
60

Figure 3.6

The uptake of (^{3}H) adenosine, showing the effect of the *malE* mutation on uptake (37^{o}C; 0.09μM final concentration). P2343, wild-type; P2342, *ompB*; P2341, *malE*, *ompB*; P2421, *malE*, *ompB*, *lamB*.

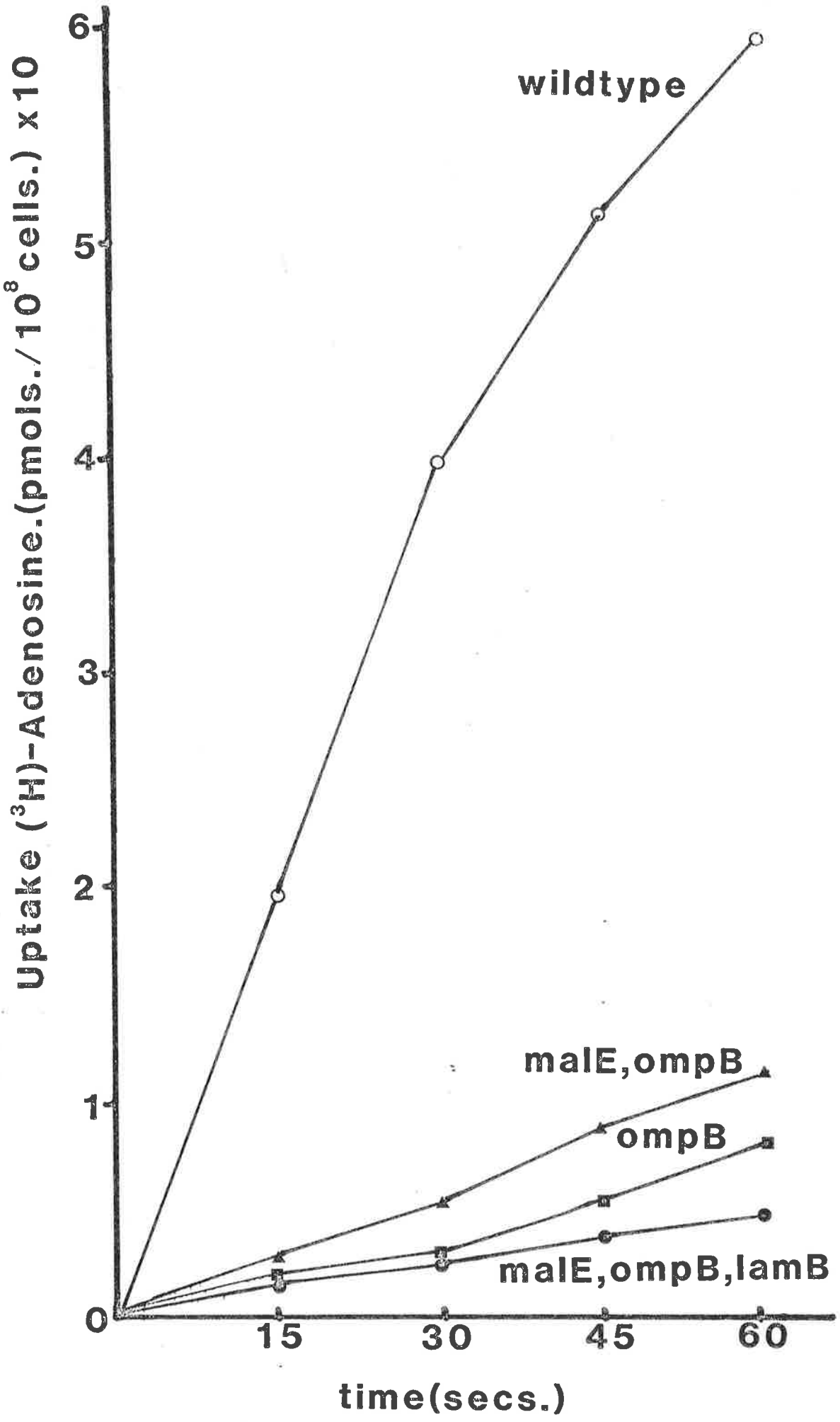

Uptake (^{3}H)-Adenosine.(pmols./10^8cells.) x10
0
1
2
3
4
5
6
15
30
45
60
time(secs.)
wildtype
malE,ompB
ompB
malE,ompB,lamB

fig. 3.5. It is apparent that the *ompB* mutation causes a reduction in thymidine uptake of about 50-60% and the further inclusion of the *lamB* mutation causes a reduction to some 20% of wild-type level. It is also noteworthy that the *malE* mutation results in an enhancement of the ability of the LamB protein to alleviate an *ompB* defect.

Fig. 3.6 shows the uptake of (^{3}H) adenosine. The *ompB* mutation causes a much greater drop in uptake (80-90%) relative to wild type in comparison to thymidine. In molar terms the amount of adenosine transported by all strains is greater than for thymidine. It is apparent that the LamB protein alleviates the *ompB* defect though not to the same extent as for thymidine, the *malE* mutation also has an effect on the level of compensation by *LamB*, although in this case the effect is more marginal.

3.6.2 Uptake of substrates not influenced by *malE*

The *malE* mutation in combination with the LamB protein can alleviate the uptake of some substrates in the presence of an *ompB* mutation. There also exists substrates in which *malE* has no alleviating effect.

Figure 3.7 depicts the uptake of (^{14}C) glucose. The LamB protein restores the uptake of glucose to levels very similar to P2343. This is especially apparent since P2421 which lacks LamB in

Figure 3.7

The uptake of (^{14}C) glucose, a typical result of the type summarized in table 3.4 (37^{o}C with 0.8μM final concentration). P2343, wild-type; P2342, *ompB*; P2341, *malE*, *ompB*; P2421, *malE*, *ompB*, *lamB*.

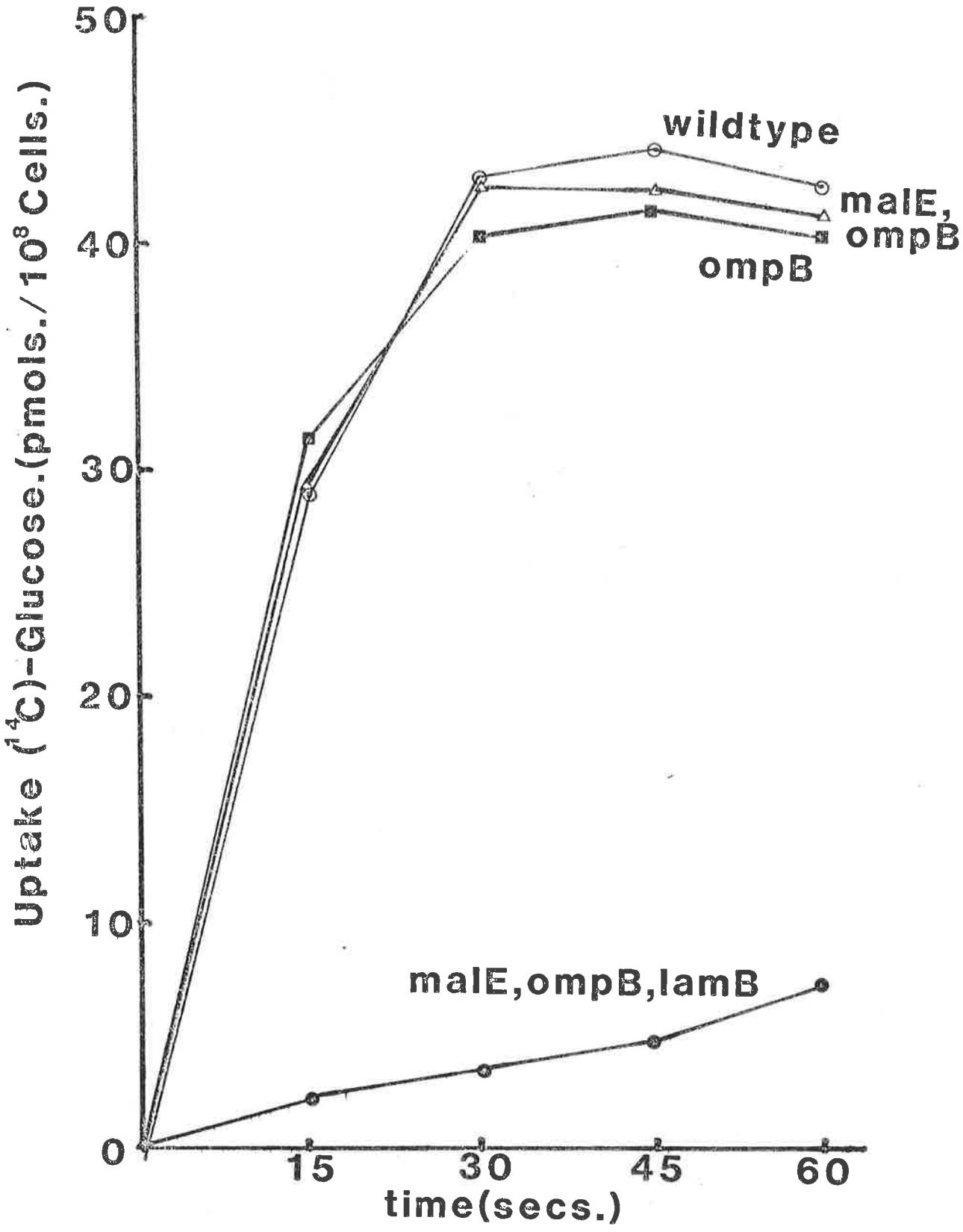

Uptake (^{14}C)-Glucose.(pmols./10^8Cells.)
50
40
30
20
10
0
15
30
45
60
time(secs.)
wildtype
malE, ompB
ompB
malE,ompB,lamB

Table 3.4

	relative uptake rates[a].			
	P2343	P2342	P2341	P2421
Substrate	wild-type	*ompB*	*ompB malE*	*ompB lamB*
Glucose (0.83)[b]	100 (40)	89	96	17
Glycerol(0.63)	100 (168)	72	74	43
Galactose[c] (0.83)	100 (23)	79	79	20
Glucuronic acid[c] (0.5)	100 (0.62)	119	117	59
Glucosamine (0.86)	100 (6.3)	105	99	98

a. Initial rate of uptake expressed relative to wild-type (pmols/10^8 cells).

b. Final concentration (μM).

c. Cells were induced with 0.2% galactose and 0.2% glucuronic acid respectively prior to uptake of these substrates.

addition to major porin, and is severely disadvantaged in its ability to transport glucose. P2341 also does not transport glucose at a rate significantly above P2342; the effect of the MalE protein is negligible in this case.

Table 3.4 summarizes the other substrates tested that exhibit behaviour similar to glucose. In all cases the LamB protein when present alleviates an *ompB* mutation. In the case of glucuronic acid, P2341 and P2342 transport glucuronic acid at a higher rate than P2343.

In all cases there is no significant increase in uptake rate for P2341 in comparison to P2342, indicating the effect of maltose binding protein is not detectable for these substrates.

Glucosamine does not appear to be dependent on either OmpC, OmpF or LamB proteins for diffusion across the outer membrane. It is probable that glucosamine diffuses across the outer membrane by some other means and the presence of this alternative pathway may mask any effect of the LamB protein.

3.7 SUMMARY

OmpB mutants show a decreased rate of uptake for many substrates: the LamB protein can substitute for the OmpC and OmpF proteins. The *malE* mutation can enhance the uptake through the LamB pore for some substrates; lactose, mannitol, thymidine, glycylglycyl-L-valine, tri-L-ornithine and to a lesser extent adenosine.

The *malE* mutation however has no effect upon the transport of glucose, glycerol, galactose and glucuronic acid.

CHAPTER 4

ASSOCIATION OF THE MalE PROTEIN WITH CELL WALL COMPONENTS

4.1 INTRODUCTION

The hypothesis put forward in chapter 3 postulates that the LamB and MalE proteins physically associate and thus impede the passage of molecules other than maltose and maltodextrins, therefore conferring specificity upon the LamB pore.

This chapter describes the attempt to show a physical association between the LamB and MalE proteins.

4.2 ISOLATION OF *lamB* MUTANTS

If a physical association were to exist between the MalE and LamB proteins, it should be possible to demonstrate, given the right conditions. It would be expected that such an association could not be demonstrated in a *lamB* mutant, but should be apparent in a strain having both MalE and LamB proteins. Four *lamB* mutants were isolated from the following strains, P2238, P2239, P2343 and P2421 which are described in table 3.1.

Bacteriophage resistant mutants were isolated as described in chapter 2 using *λvir* phage and plating onto maltose tetrazolium agar. It is possible for λ resistant mutants to arise in three ways: *malT* mutants, *malK* polar mutants or *lamB* mutants (Hofnung *et al.*, 1971, 1974; Thirion and Hofnung, 1974). It is possible to distinguish *lamB* mutants since they retain the ability to ferment maltose. *LamB* mutants from each of the four parental strains were isolated;

Table 4.1

Bacterial strains.

Strain	Description
P2242	*lamB20* derivative of P2238
P2245	*lamB21* derivative of P2239
P2248	*lamB22* derivative of P2343
P2252	*lamB23* derivative of P2241

they are listed in table 4.1. These mutants (with the exception of P2245) show no detectable LamB protein in whole membrane preparations and are typical *lamB* mutants. P2245 which carries the *lamB21* allele, although λ resistant, is also sensitive to bacteriophage K10, which has been shown to be able to use LamB protein as a receptor (Hancock 1974; Roa 1979). Whole membrane preparations from this mutant have a protein on polyacrylamide gels corresponding to the LamB protein. The *lamB21* allele appears to give rise to an altered LamB protein.

4.3 RELEASE OF PERIPLASMIC PROTEINS

During osmotic shock, it would be expected that if MalE protein associates with LamB protein, it should be possible to detect a difference in the relative amounts of MalE protein released by a *lamB* or *lamB*$^+$ strain. It would be expected that *lamB* mutants should release MalE more readily into the external medium. It had been reported by Enderman *et al.*, (1978) that the LamB protein interacts with the peptidoglycan. If the LamB protein interacts with the peptidoglycan it is possible that the MalE protein may also be associated with peptidoglycan. Therefore treatment with lysozyme may cause the release of MalE protein, even when LamB protein is present, thus masking any effect of association between the MalE and LamB proteins.

4.3.1 General outline of experimental procedure

The four *lamB* strains and their respective parental strains were subjected to the standard osmotic shock procedure as described in chapter 2. A second batch of strains were grown under identical conditions and subjected to the osmotic shock procedure, except lysozyme is omitted. The shock fluids for all strains were collected; and the protein concentration determined by the method described in chapter 2. It was hoped that the stained gel from electrophoresis would be able to distinguish between the relative amounts of MalE protein in the shock fluid of a *lamB* and *lamB*$^+$ strain.

4.3.2 Protein concentration in the shock fluids

Table 4.2 shows the relative amounts of protein in the shock fluids of the strains, treated with either lysozyme-EDTA or EDTA alone. There appears to be no significant difference between the amount of protein released, regardless of whether the strain is *lamB* or *lamB*$^+$. The most notable observation is that the treatment with lysozyme results in a release about 3-4 times more protein than treatment with EDTA alone. This effect is not due to lysozyme in the shock fluid since it accounts for <1% of the average total shock fluid protein concentration for all lysozyme treated strains.

Table 4.2

Protein concentrations of periplasmic shock fluids of strains subjected to Lysozyme-EDTA or EDTA treatment.

Strain	Protein conc. of periplasmic shock fluid (µg/ml)	
	Lysozyme-EDTA	EDTA
P2238	1226	390
P2239	1350	296
P2343	1500	358
P2241	1257	296
P2242	1318	327
P2245	1380	358
P2248	1597	265
P2252	1287	327

4.3.3 Polyacrylamide gel electrophoresis

If equal amounts of the shock fluid are loaded onto polyacrylamide gels as shown in figure 4.1, the *malE* protein and other shock proteins are present in low amounts, when lysozyme treatment is omitted. If the amount of shock fluid is adjusted to give equivalent concentrations of protein as shown in figure 4.2 it is apparent that the same amount of MalE protein is released by *lamB* and *lamB*$^+$ cells irrespective of whether lysozyme is present. It is significant that when 2½ times more non-lysozyme treated samples are loaded onto the gel, only 5 major protein bands are in evidence, including the MalE protein. Treatment with lysozyme results in the release of many proteins including the MalE protein which is released in much greater quantity. The MalE protein is also one of the major protein species to be released during treatment with lysozyme. Figure 4.3 shows the whole membrane preparations prepared after osmotic shock either in the presence or absence of lysozyme. It is apparent that lysozyme has no effect upon the relative amounts of major outer membrane proteins, nor is any maltose binding protein (MalE) detectable in these preparations.

4.4 TRIS(HYDROXYMETHYL) AMINOETHANE-MEDIATED RELEASE OF MALTOSE BINDING PROTEIN

If any association between MalE protein and LamB protein occurs, then it must be weak or not

Figure 4.1

Polyacrylamide gel electrophoresis (Lugtenberg) of periplasmic proteins released both in the presence and absence of lysozyme. The first track for each strain is of EDTA-lysozyme treated cells, the second of those treated only with EDTA. Equal (10µl) amounts loaded for each sample.

A : P2343

B : P2242

C : P2248

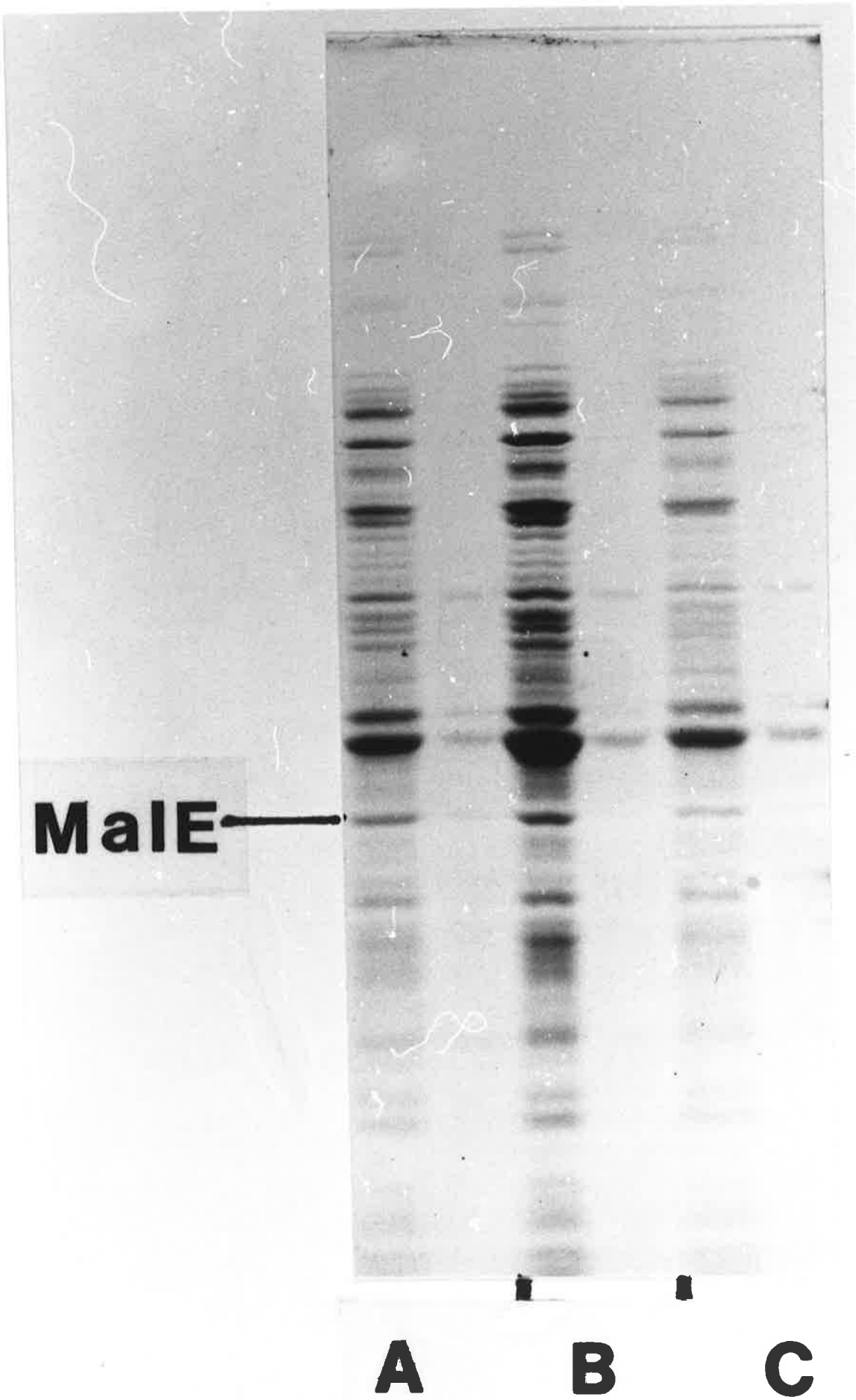

MalE
A
B
C

Figure 4.2

As for figure 4.1, however the amount of protein added for non-lysozyme treated is 2.5 times that of the lysozyme-EDTA treated cells.

A : P2238

B : P2239

C : P2343

D : P2241

E : P2242

F : P2245

G : P2248

H : P2252

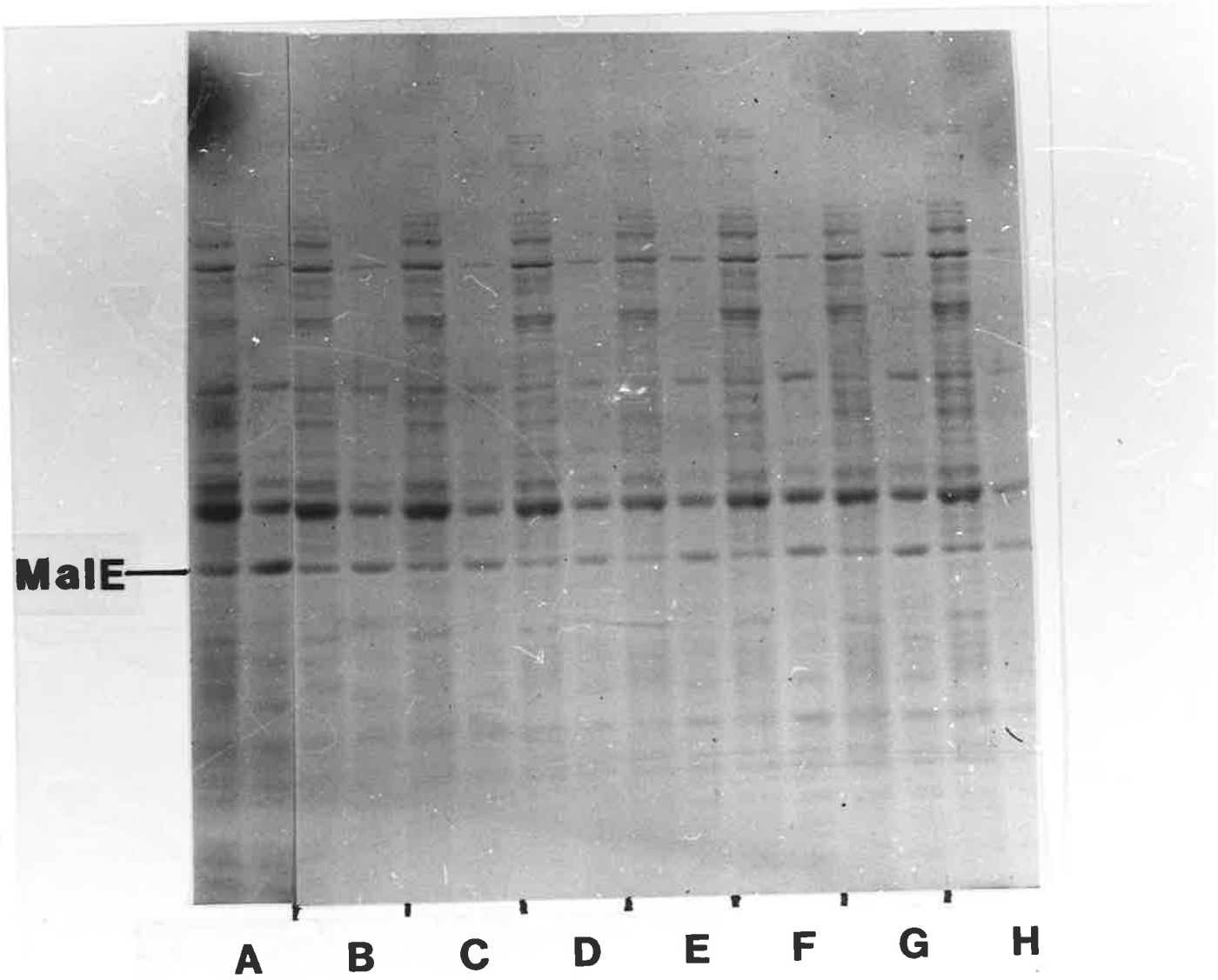

MalE
A
B
C
D
E
F
G
H

Figure 4.3

Polyacrylamide gel electrophoresis (Lugtenberg) of whole membrane preparations prepared from the cells used in figure 4.2. Equal (20µl) amounts loaded for each sample.

A : P2238

B : P2239

C : P2343

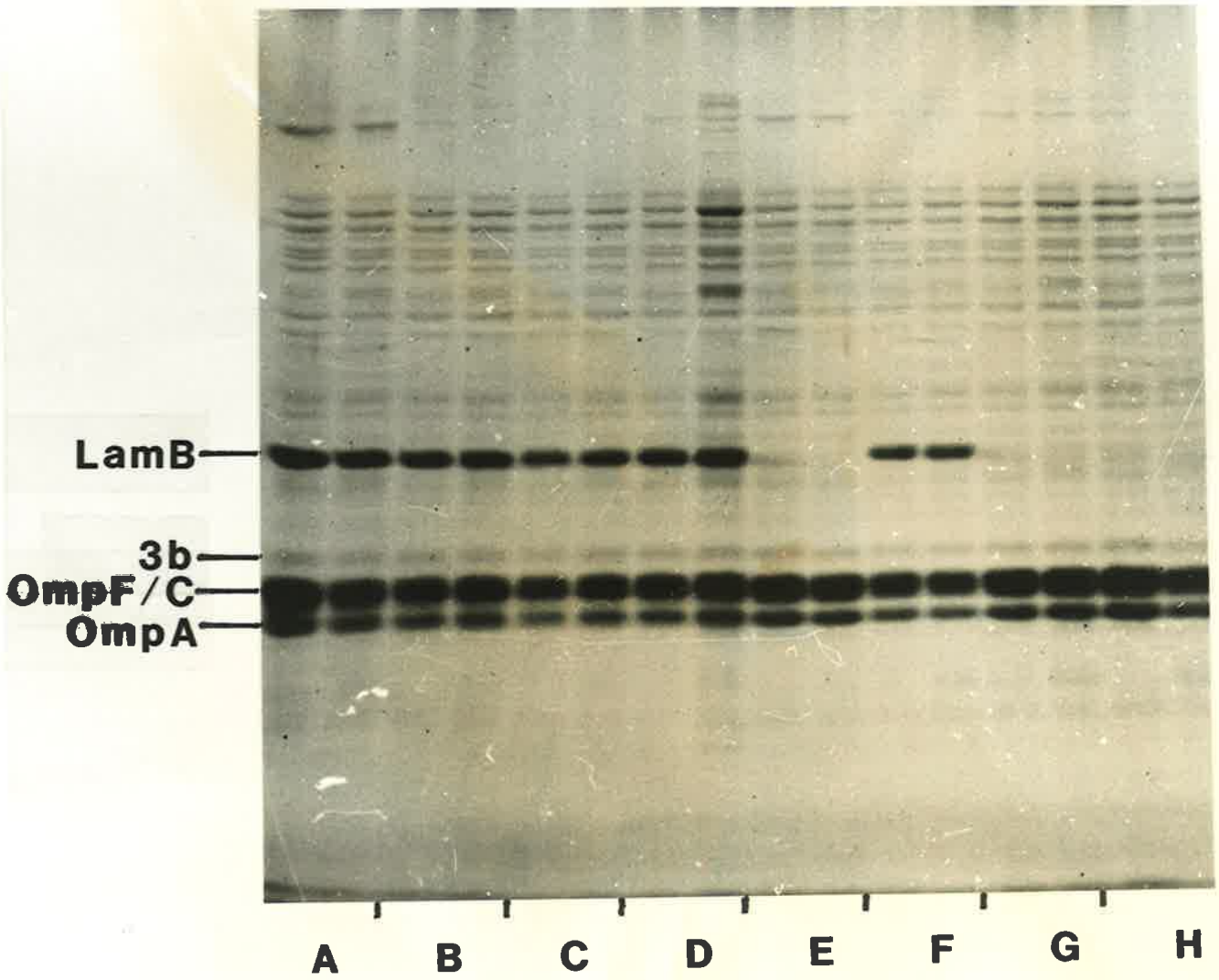
D : P2241

E : P2242

F : P2245

G : P2248

H : P2252


LamB
3b
OmpF/C
OmpA
A
B
C
D
E
F
G
H

maintained after EDTA-lysozyme treatment. The next section utilizes methods which are less disruptive to the outer membrane than EDTA-lysozyme treatment. It has been well documented that incubation of *E. coli* with Tris-buffer results in the release of periplasmic proteins. In this set of experiments cells are incubated with Tris buffer for four hours as described in chapter 2. The cells are removed and the supernatant concentrated by freeze-drying, and the protein species (if any) are examined.

Two methods were used to detect the relative amounts of binding protein released by each strain.

1. Ouchterlony double gel diffusion
2. Polyacrylamide gel electrophoresis

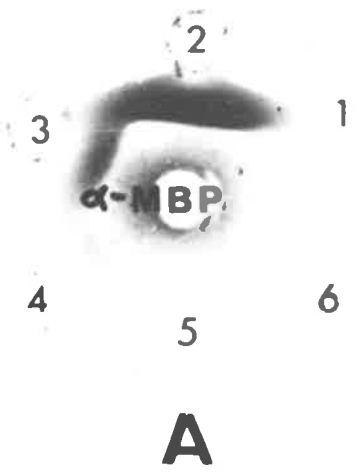
The concentrated proteins produced by Tris treatment were examined by Ouchterlony double gel diffusion against anti-sera to the purified MalE protein. This is presented in figure 4.4. It is apparent that for all strains the dilution of protein to which a precipitin line can be assigned extends to $^{1}/_{32}$. There appears to be no significant difference in the amount of MalE protein released from each strain as measured by this method. It is apparent that the precipitin bands are quite broad, in contrast to the reaction of the anti-sera with pure MalE protein. The broad band of precipitation may be indicative of the association of the MalE protein with other components of the supernatant.

Figure 4.4

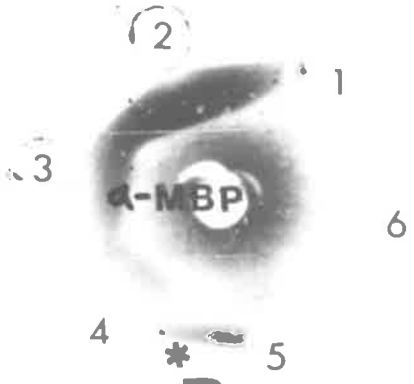
Ouchterlory gel diffusion against anti-maltose binding protein (MalE) of proteins recovered and concentrated from cells incubated with tris buffer.

Well #1 = 5µl undiluted antigen
#2 = 5µl ¼ diluted antigen
#3 = 5µl 1/16 diluted antigen
#4 = 5µl 1/32 diluted antigen
#5 = 5µl 1/64 diluted antigen
#6 = 5µl 1/128 diluted antigen
A = P2238 Tris released protein
B = P2239 Tris released protein
C = P2343 Tris released protein
D = P2242 Tris released protein
E = P2245 Tris released protein
F = P2248 Tris released protein
G = purified MalE protein

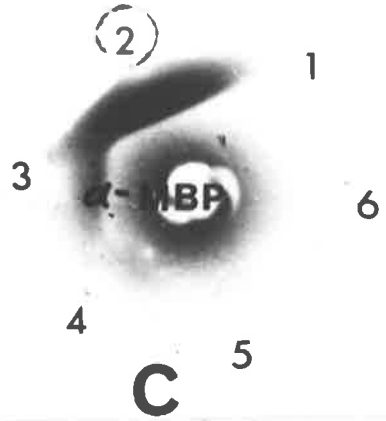
N.B. *spurious bands caused by the diffusion of concentrated antigen from adjacent but unrelated wells. These bands should be ignored.


2
3
1
α-MBP
4
6
5

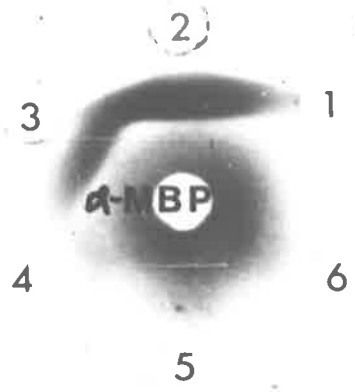
A


2
1
3
α-MBP
6
4
*
5

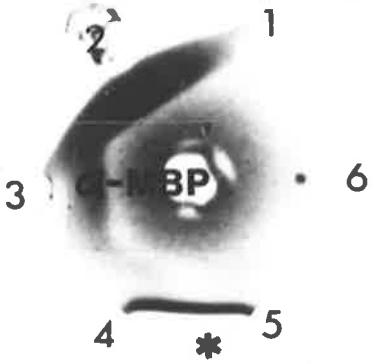
B


2
1
3
α-MBP
6
4
5

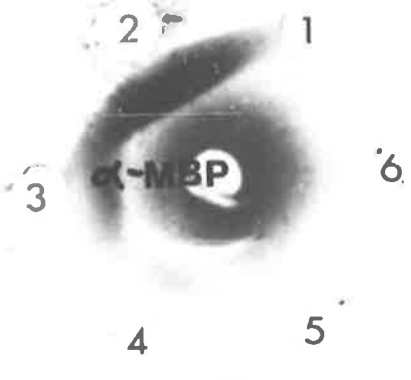
C


2
3
1
α-MBP
4
6
5

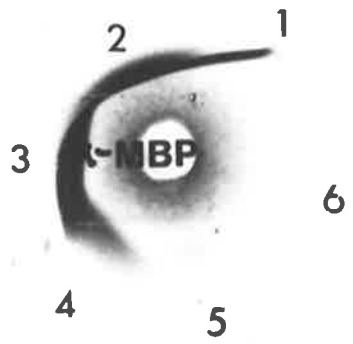
D


2
1
3
α-MBP
6
4
*
5

E


2
1
3
α-MBP
6
4
5

F


2
1
3
α-MBP
6
4
5

G

If the same protein preparations that are used in the gel diffusion test are treated with Lugtenberg's solution and run on polyacrylamide gels, as depicted in figure 4.5, it is apparent that the MalE protein is one of the major protein species that is released by the treatment of cells with tris buffer. It is also seen that there is no detectable difference in the amounts of MalE protein released by each of the strains tested. It is noteworthy that many protein species (possibly periplasmic) are released by this method into the external medium. There also appears to be very little detectable major outer membrane protein present in these preparations.

4.5 ASSOCIATION OF THE MalE PROTEIN WITH THE MEMBRANE

The cells that were treated with tris buffer were further analysed to determine the amount of MalE protein remaining with the cells after tris-treatment. The tris-treated cells were treated as depicted in figure 4.6. The treatment of the cells with EDTA results in the release of membrane fragments into the external medium. The results of this treatment are depicted in figures 4.7 and 4.8. Figure 4.7 is a standard Lugtenberg gel. The MalE protein is seen to run very close to the major proteins in the 40K molecular weight region, and it is difficult to easily distinguish the MalE protein. If the cell membrane fractions are subjected to electrophoresis

Figure 4.5

Polyacrylamide gel electrophoresis (Lugtenberg) of proteins released from cells treated with tris buffer. Equal (10µl) amounts loaded for each sample.

A : P2238

B : P2239

C : P2343

D : P2242

E : P2245

F : P2248

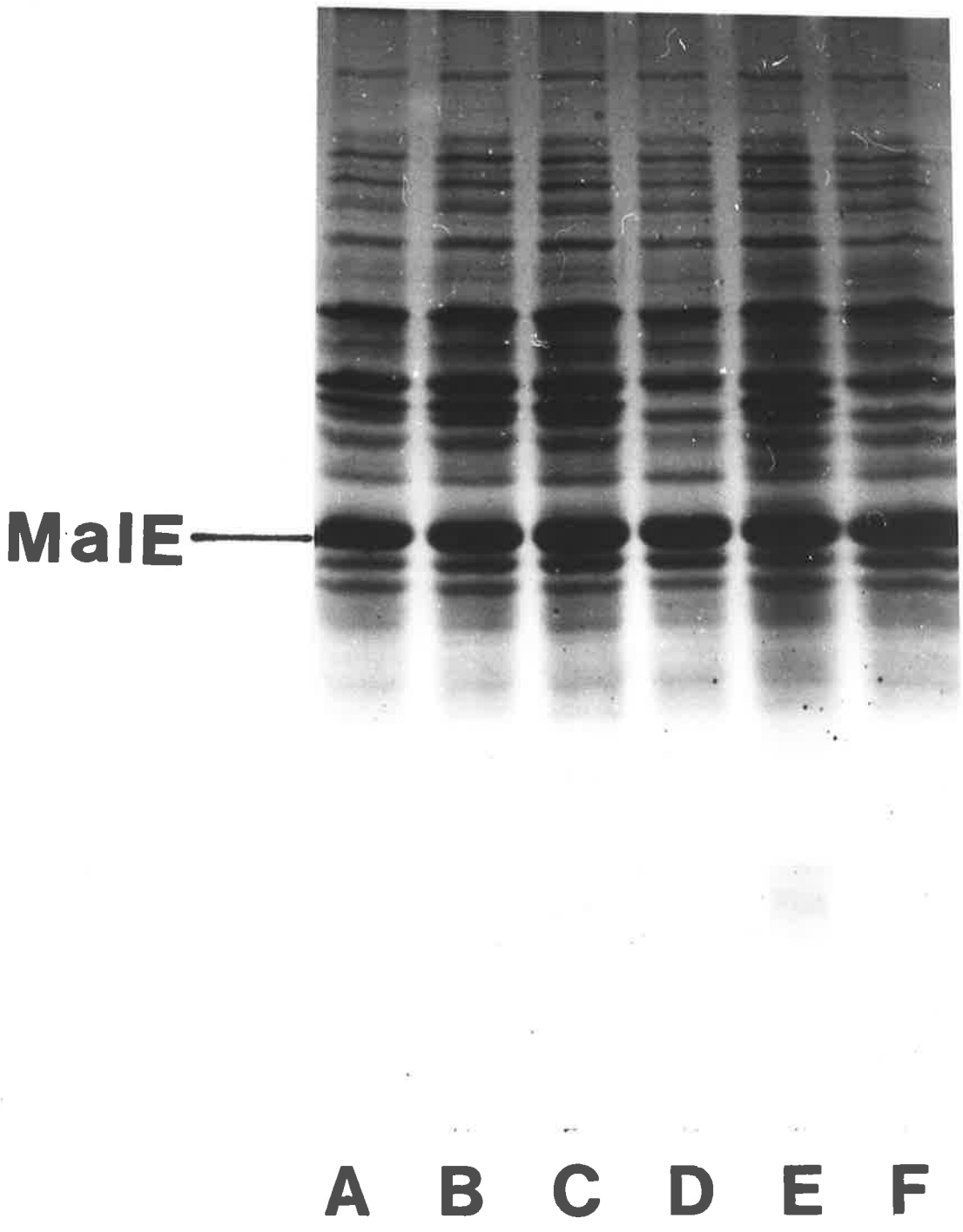

MalE
A B C D E F

Figure 4.6

Treatment of cells with EDTA to produce membrane fragments from cells treated with tris buffer.

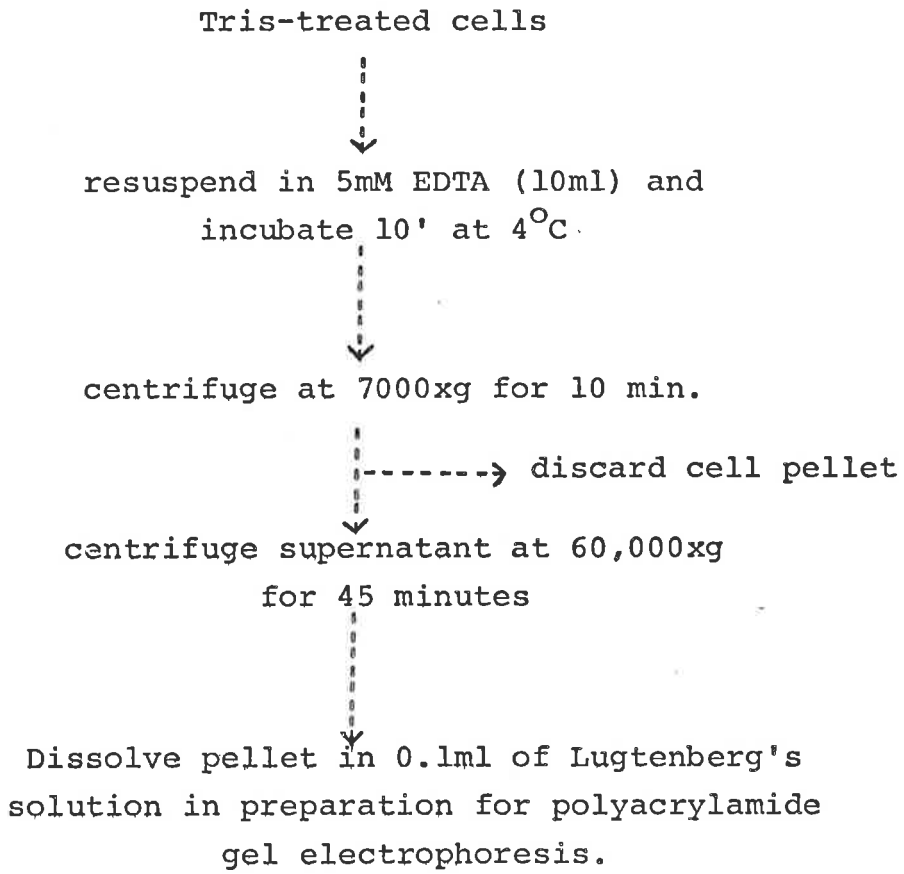


Figure 4.7

Polyacrylamide gel electrophoresis (Lugtenberg) of membrane fragments prepared after EDTA treatment of cells as described in figure 4.6. Equal (15µl) amounts loaded for each sample.

A : P2238

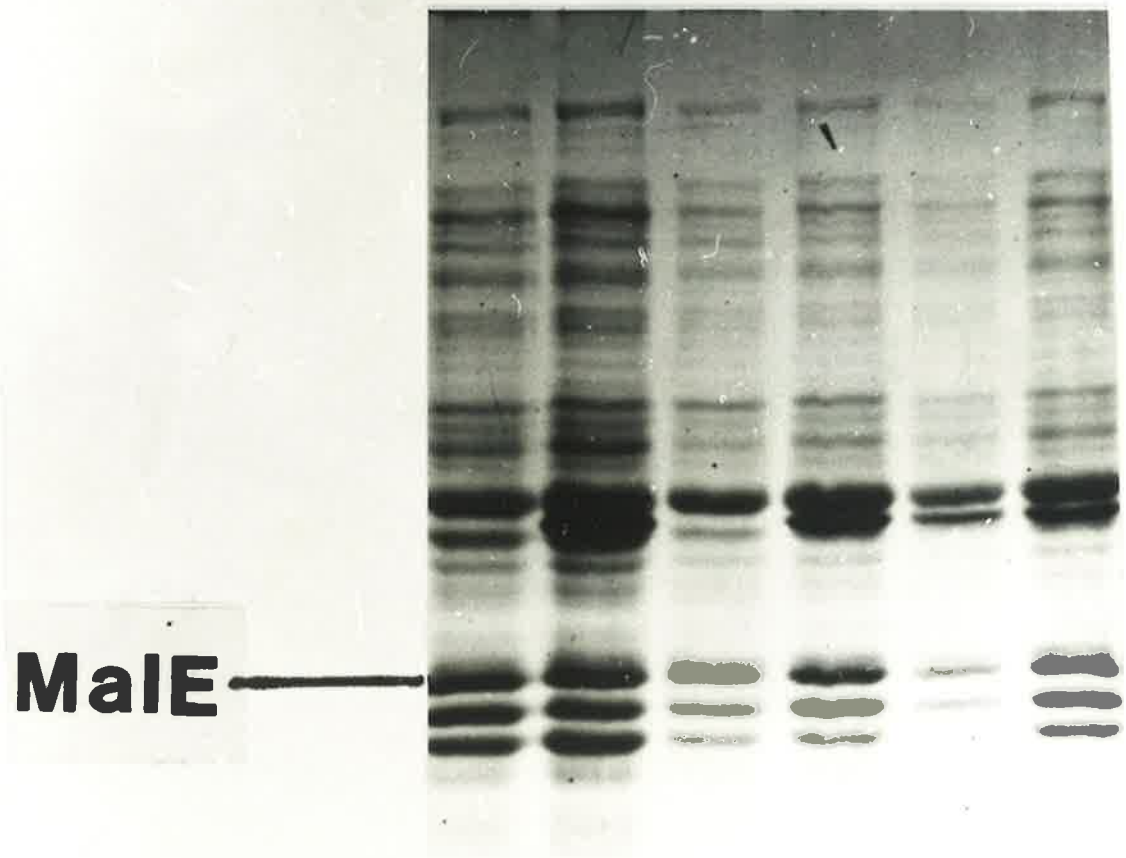
B : P2239

C : P2343

D : P2242

E : P2245

F : P2248



A B C D E F

Figure 4.8

Polyacrylamide gel electrophoresis in gels containing 4M urea after EDTA treatment of cells as described in figure 4.6. Equal (15µl) amounts loaded for each sample.

A : P2238

B : P2239

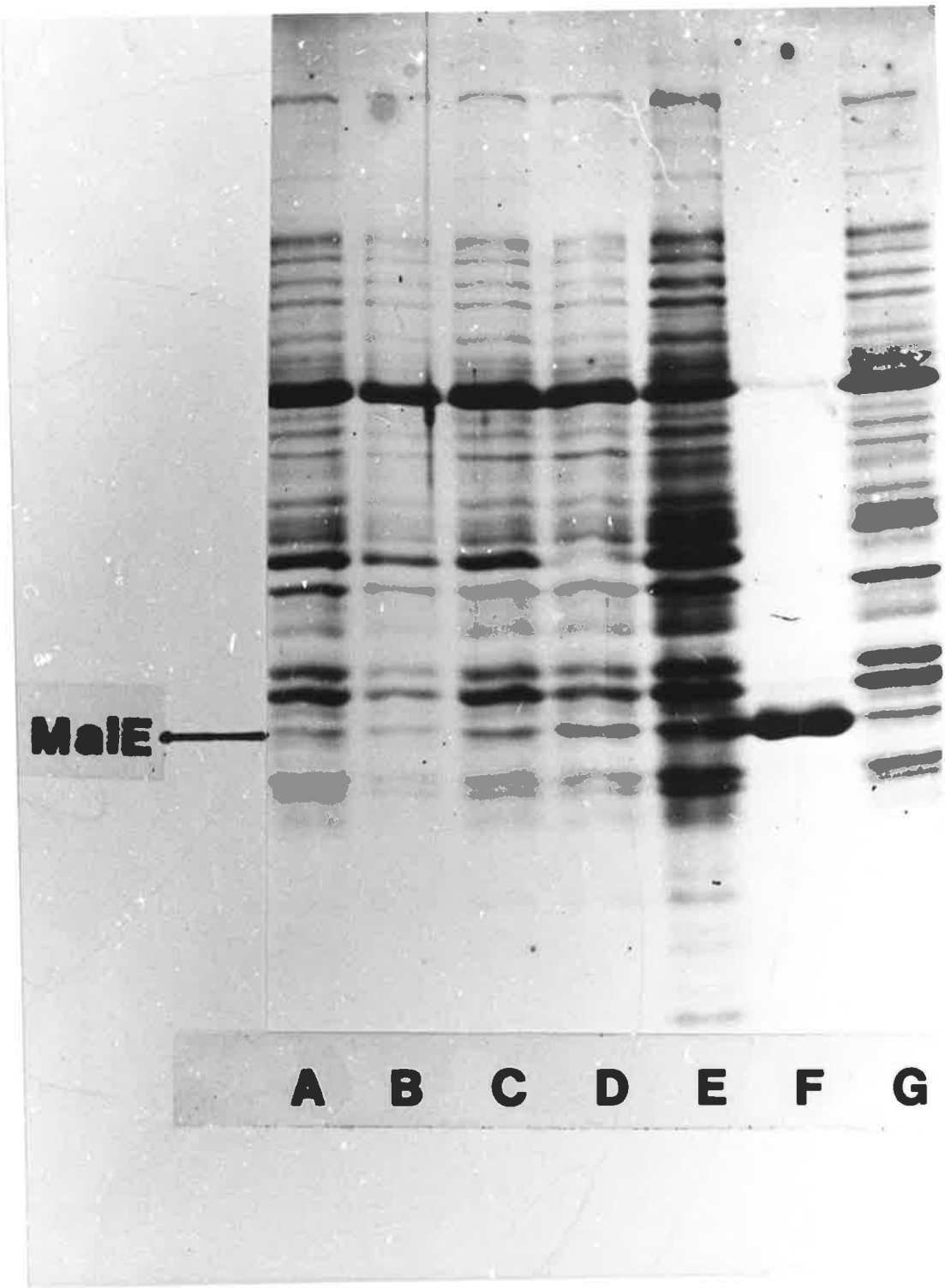
C : P2343

D : P2242

E : P2245

F : purified MalE protein

G : P2248


MalE
A
B
C
D
E
F
G

on 4M urea gels; the MalE protein separates substantially from the major outer membrane proteins and is readily discernable. It is apparent in all cases that the MalE protein is found with the EDTA released membrane fragments. It is not possible to conclude whether the relative abundance of the MalE protein bears any relationship to the presence of the LamB protein. It is noteworthy that tris-treatment of cells results in only about a release of 2% of the total MalE protein compared to that found with the EDTA membrane fragments.

4.6 SUMMARY AND CONCLUSIONS

It was not possible to demonstrate any direct association of the MalE protein with the LamB protein using the methods described. If such an interaction occurs it may be a very weak interaction and may be very difficult to demonstrate using these semi-*in vivo* methods. It is possible that the MalE protein may exist in two forms: one associated with the peptidoglycan and "free" in the periplasmic space. This appeared to be also possible for many other periplasmic proteins. It is also possible that lysozyme may result in a more efficient outer membrane disruption and therefore release of periplasmic proteins. The MalE protein does, however seem to be associated with outer membrane components. There is no detectable correlation between this association and the LamB protein.

CHAPTER 5

Tsx PROTEIN AS A PORE ANALAGOUS TO LamB PROTEIN

5.1 INTRODUCTION

It has been well documented that the Tsx protein promotes transport of nucleosides across the outer membrane (Hantke, 1976; Krieger-Brauer and Braun, 1980). The nucleoside uptake system and its associated outer membrane protein (Tsx protein) share some common features with the maltose/maltodextrin uptake system, which uses the LamB protein as a pore: (1) The dependence of nucleoside and maltose permeation pathways on the outer membrane protein only applies at low substrate concentrations. (2) There is no competitive inhibition of adsorption of specific bacteriophage by the substrate.

The common features shared by both maltose and nucleoside uptake systems, suggests that the Tsx protein may form an aqueous pore analogous to the LamB protein. This chapter tests the ability of the Tsx protein to promote the transport of molecules other than nucleosides across the outer membrane in *ompB* mutants. If the Tsx protein is analagous to the LamB protein, it would be expected that the Tsx protein would partially alleviate the *ompB* transport defect under conditions in which the outer membrane is limiting diffusion, in a similar fashion to the LamB protein.

5.2 BACTERIAL STRAINS

Two sets of bacterial strains were used in this

Table 5.1

Bacterial Strains

Strain	Relevant Genotype		Outer Membrane Proteins		
	ompB	*tsx*	OmpC	OmpF	tsx
P400	+	+	+	+	++
P407	+	*200*	+	+	-
P433	+	*201*	+	+	-
P1578	*105*	+	-	-	++
P1602	*105*	*203*	-	-	-
P1848	+	+	+	+	+
P1926	+	+	+	+	-
P1930	*106*	+	-	-	+
P2024	*106*	-	-	-	-

chapter: A set of strains derived from P400 which produces large amounts of Tsx protein (Manning, Pugsley and Reeves, 1977), and a set of strains derived from P1848 which produces "normal" amounts of Tsx protein. The relevant genetic background and outer membrane proteins are shown in table 5.1.

5.3 Polyacrylamide gel Electrophoresis

Figure 5.1 shows the proteins present in cell envelope preparations of the two groups of strains used in this chapter. Tracks A, D and F show the Tsx protein is produced as expected for *tsx*$^+$ strains. The *tsx* strains (tracks B, C and E) show no detectable Tsx protein. The *ompB105* strains, tracks D and E lack detectable levels of OmpC and OmpF protein. The P1848 derived strains only show Tsx protein present when the gel is overloaded. It is, however just possible to detect Tsx protein in the *tsx*$^+$ strains; tracks G and I. The *ompB106* derivatives; tracks I and J are devoid of detectable levels of OmpC and OmpF proteins.

5.4 Uptake of Labelled Nucleosides

With the exception of a brief study by Van Alphen *et al.*, (1978) the uptake of nucleosides has only been investigated in strains having normal amounts of OmpC and OmpF proteins. Figures 5.2 and 5.3 show the uptake of thymidine and adenosine by P400, P407, P433, P1578 and P1602. The rate of

Figure 5.1

Whole membrane preparations run on 11-20% gradient polyacrylamide gels showing relevant outer membrane protein.

A : P400

B : P407

C : P433

D : P1578

E : P1602

F : P400

G : P1848

H : P1926

I : P1930

J : P2024

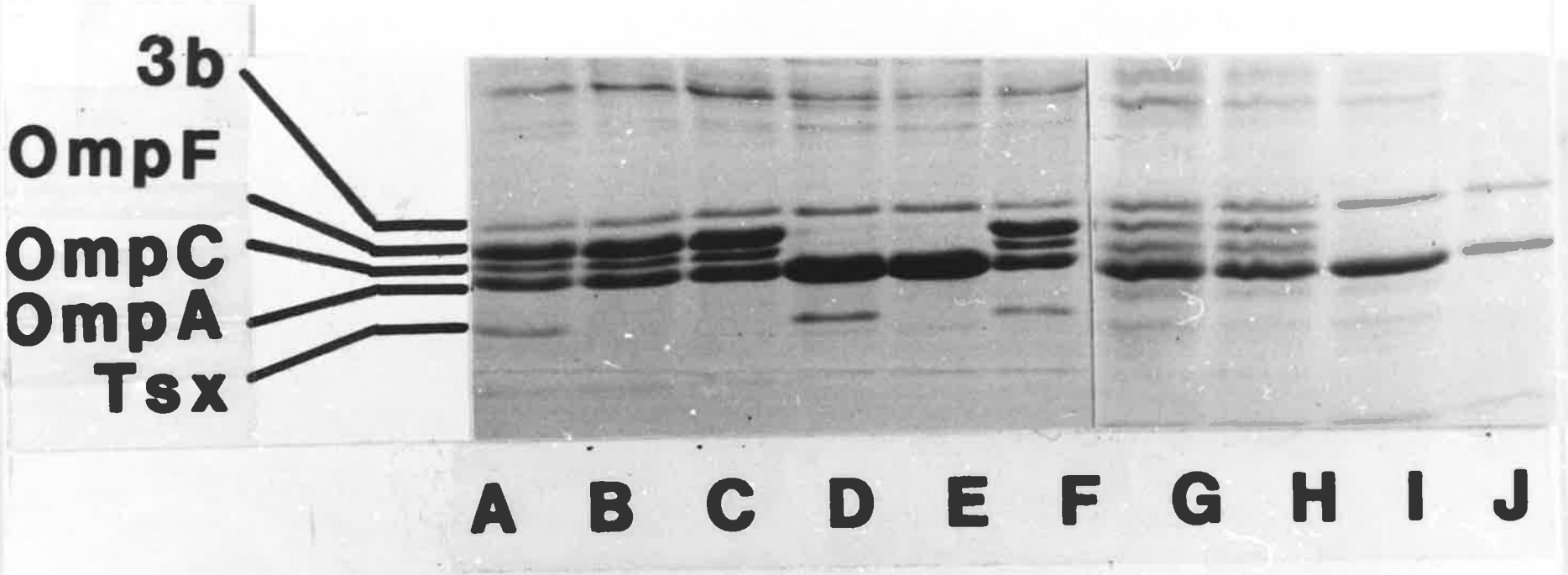

3b
OmpF
OmpC
OmpA
Tsx
A B C D E F G H I J

Figure 5.2

Uptake of (^{3}H) thymidine at 37^{o}C with a final concentration of 0.083μM in the uptake vessel.

Symbols: △ , P400; ▲ , P407; ○ , P433; ● , P1578, □ , P1602.

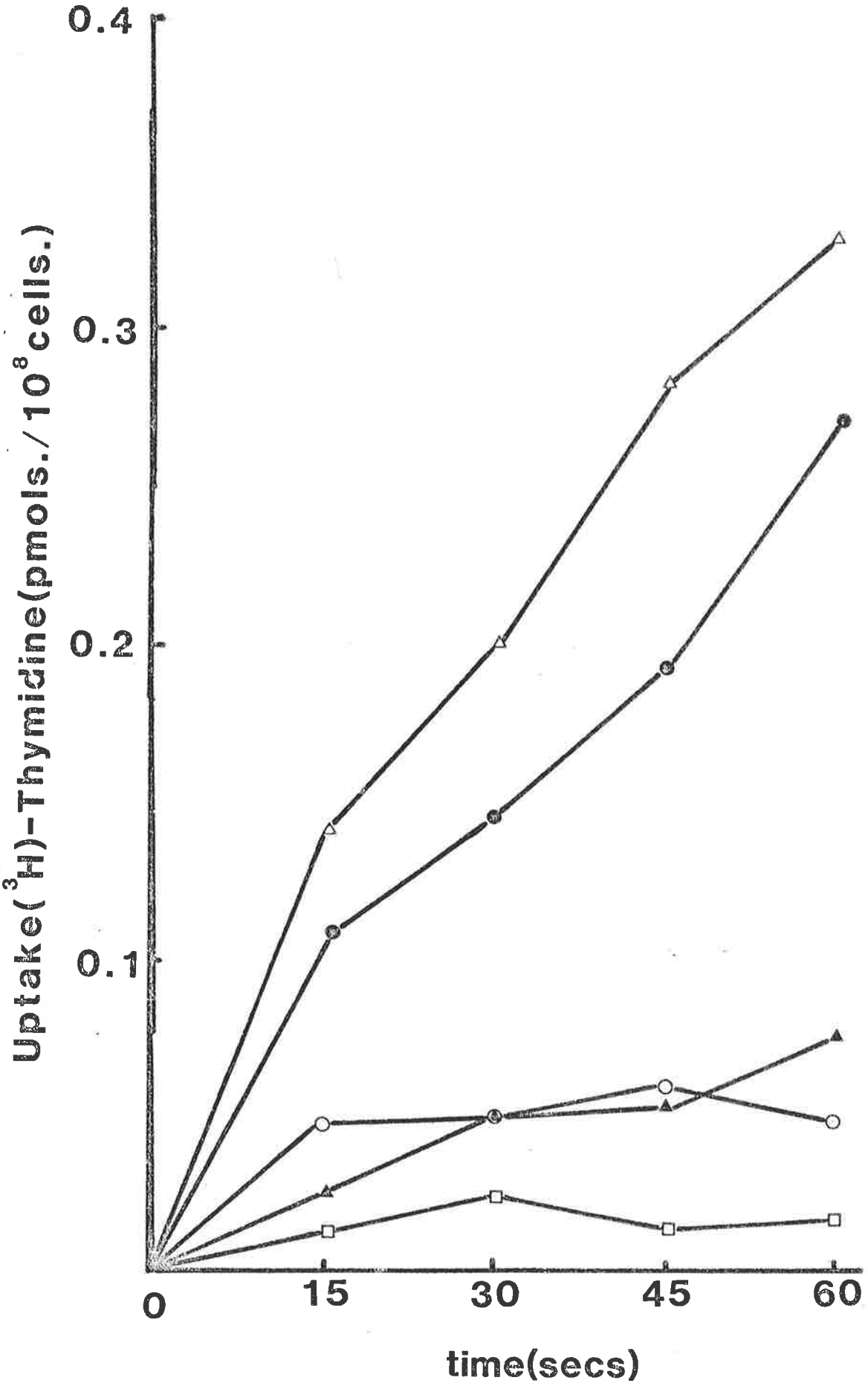

0.4
0.3
0.2
0.1
0
15
30
45
60
Uptake(^{3}H)-Thymidine(pmols./10^8cells.)
time(secs)

Figure 5.3

Uptake of (^{3}H) adenosine at 37^{o}C with a final concentration of 0.09μM in the uptake vessel.

Symbols: △ , P400; ▲ , P407; ○ , P433; ● , P1578; □ , P1602.

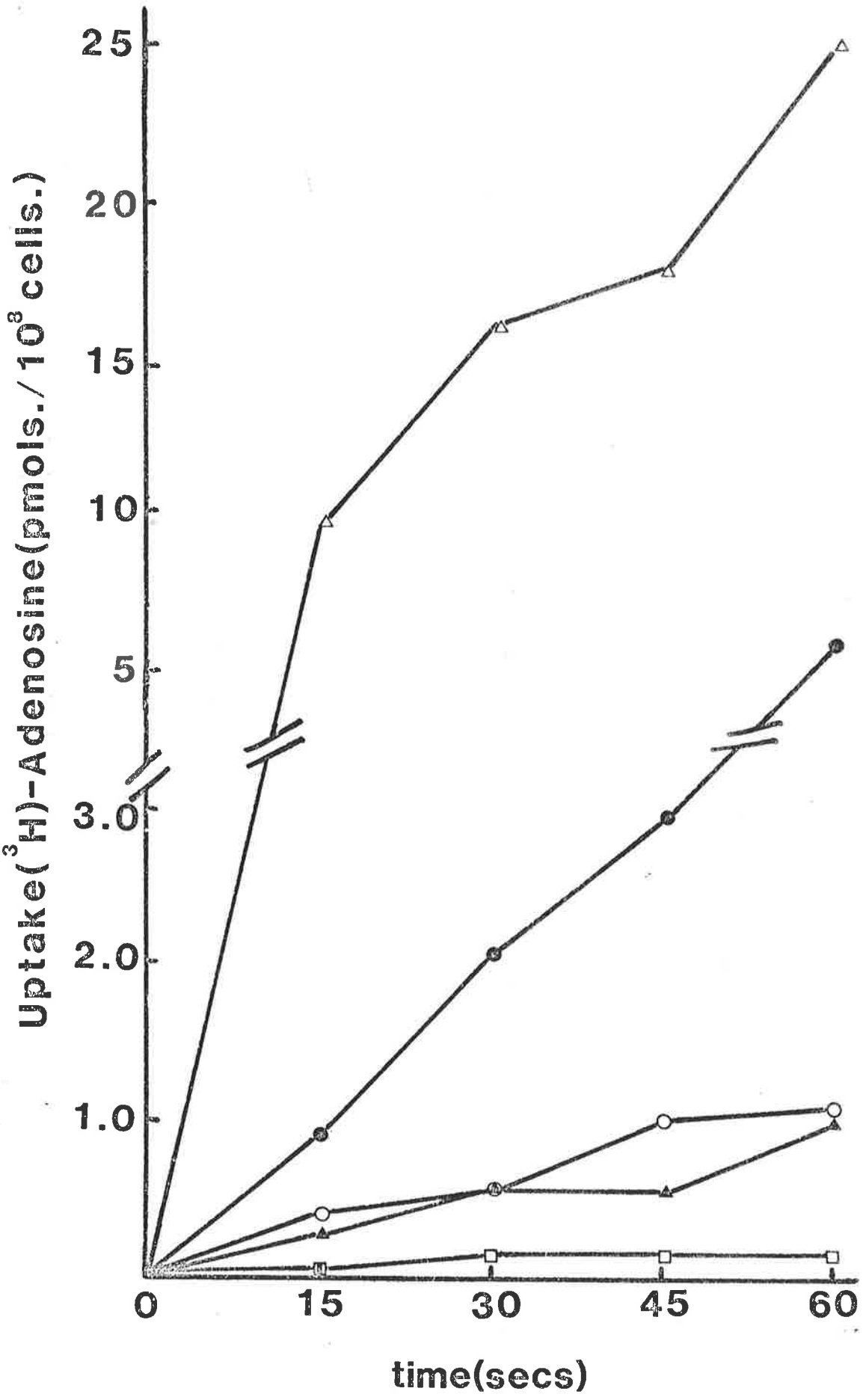

Uptake(^{3}H)-Adenosine(pmols./10^3 cells.)
25
20
15
10
5
3.0
2.0
1.0
0
15
30
45
60
time(secs)

Table 5.2 Rates of uptake of nucleosides in P1848 series of strains[a].

Strain	Relevant Genotype	Adenosine 0.09μM	Thymidine 0.08μM
P1848	wild-type	100(0.8)[b]	100(0.62)
P1926	*tsx*	12	29
P1930	*ompB*	127	91
P2024	*ompB tsx*	12	2

a. Calculated for first 45 sec. and given as a percentage of the uptake of wild-type.

b. Uptake rate in p.mols/10^8 cells/min.

nucleoside uptake is markedly reduced in *tsx* mutants, thus further confirming the work of Hantke (1976). The effect of the *ompB* mutation on both thymidine and adenosine uptake is significant, although the *tsx* mutation has the major effect in both cases. The *ompB tsx* double mutants are severely affected in their ability to take up these nucleosides. The set of strains derived from P1848 are similar in the uptake of nucleosides to the set of strains derived from P400. This result is summarized in table 5.2 The P400, and P1848 series of strains are behaving as predicted for nucleoside uptake, it is clear that OmpC and OmpF proteins promote nucleoside uptake as expected. The ability of Tsx protein to act as a non specific transport pore was now tested for molecules other than nucleosides.

5.5 UPTAKE OF SERINE, GLYCINE AND PHENYLALANINE

The uptake of (^{14}C)-serine by strains derived from P400 is presented in figure 5.4. It is clear that for serine uptake the Tsx protein can compensate for a lack of OmpC and OmpF proteins in P1578. The three *ompB*$^{+}$ strains P400, P407 and P433 show very similar uptakes as expected for strains retaining both OmpC and OmpF proteins. The *ompB* strain P1578, although lacking OmpC and OmpF proteins still has large amounts of Tsx protein in the outer membrane;

Figure 5.4

Uptake of (^{14}C) serine at 37^{o}C with a final concentration of 0.09µM in the uptake vessel.

Symbols: △ , P400; ▲ , P407, ○ , P433; ● , P1578; □ , P1602.

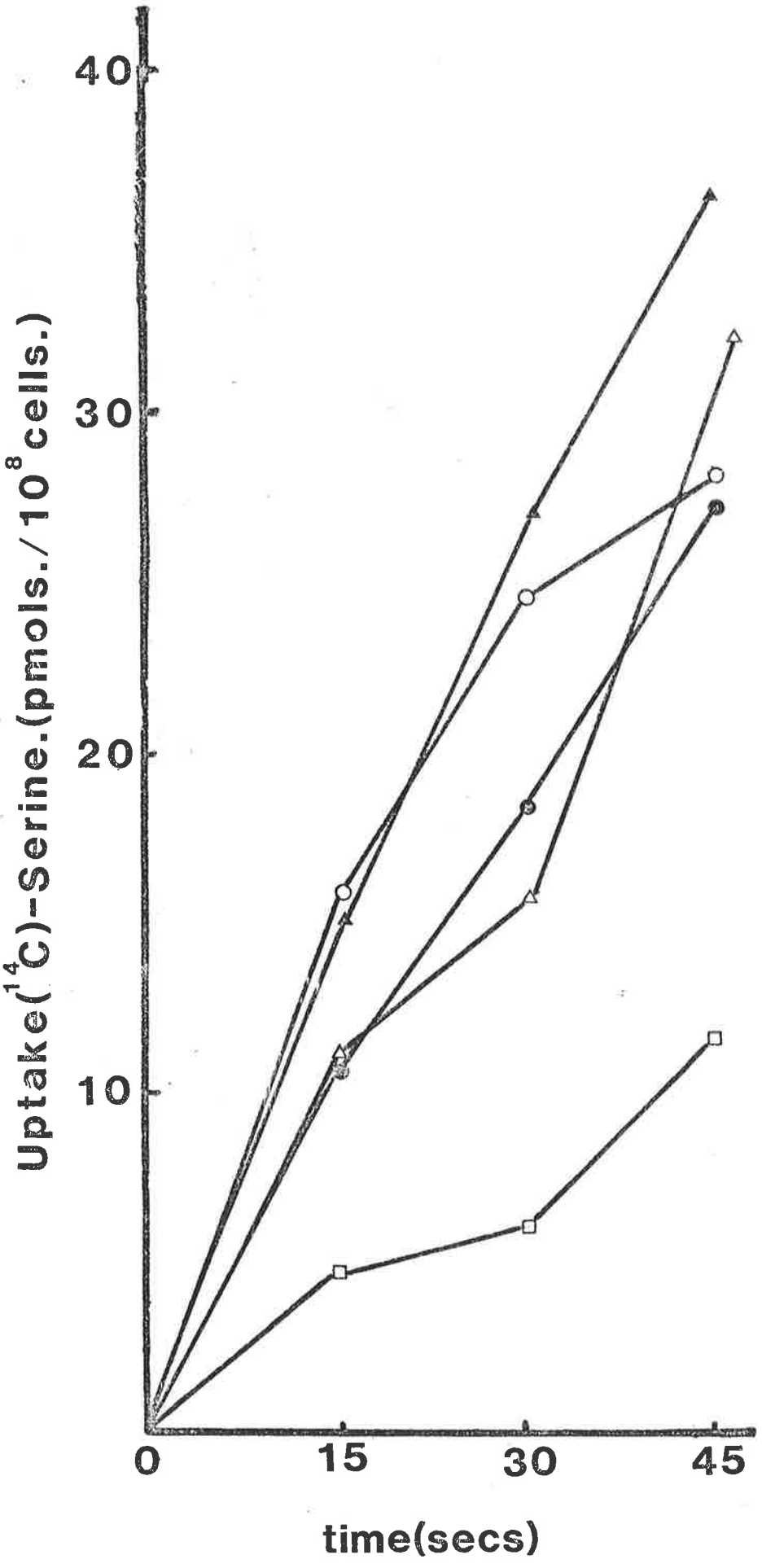

40
30
20
10
0
15
30
45
Uptake(^{14}C)-Serine.(pmols./10^8cells.)
time(secs)

Table 5.3 Rates of Uptake of labelled substrates[a.] for which Tsx protein enhances the uptake.

Strain	Relevant Genotype	Serine 0.9μM	Glycine 2.3μM	Phenylalanine 0.1μM
P400	wild type	100(7.6)[b.]	100(38)	100(1.4)
P407	*tsx*	120	74	132
P433	*tsx*	93	64	123
P1578	*ompB*	88	62	33
P1602	*ompB tsx*	35	41	15
P1848	wild type	100(120)	100(26)	
P1926	*tsx*	115	108	
P1930	*ompB*	68	115	
P2024	*ompB tsx*	23	79	

a. Calculated for first 45 sec. and given as percentage of uptake rate of appropriate wild type strain.

b. Uptake rate pmols/min/10^8 wild type cells.

the uptake rate of this strain is only slightly lower than those of P400, P407 or P433. The double mutant P1602 however appears to be considerably disadvantaged in its uptake ability, thus reflecting a substantial lack of outer membrane permeability. The Tsx protein can alleviate the effect of an *ompB* mutation, and one can conclude that serine crosses the outer membrane via the Tsx protein thus alleviating the *ompB* mutation. This ability of the Tsx protein is not confined to serine or the P400 genetic background; Table 5.3 summarizes the effect of Tsx protein on the uptake of glycine and phenylalamine. It can be seen that Tsx protein in an *ompB* mutant alleviates the uptake defect for both of these substrates. This effect is also apparent in the P1848 group of strains for serine and glycine uptake. It is noteworthy that the degree of alleviation is dependent upon the genetic background of the strain. The *tsx* mutations when present alone as in P407, P433 and P1926 quite often show an uptake rate equal to, if not exceeding that of the respective wild-type strains. It appears that the Tsx protein can promote the transport of at least three amino acids. However this effect is not true for all low molecular weight substrates.

5.5.1 Uptake of glucose and arginine

Figure 5.5 depicts the uptake of (^{14}C) glucose by P400, P407, P433, P1578 and P1602. In contrast to the result with serine the Tsx protein has no

Figure 5.5

Uptake of (^{14}C) glucose at 37^{o}C with a final concentration of 0.83μM in the uptake vessel.

Symbols: △ , P400; ▲ , P407; ○ , P433; ● , P1578; □ , P1602.

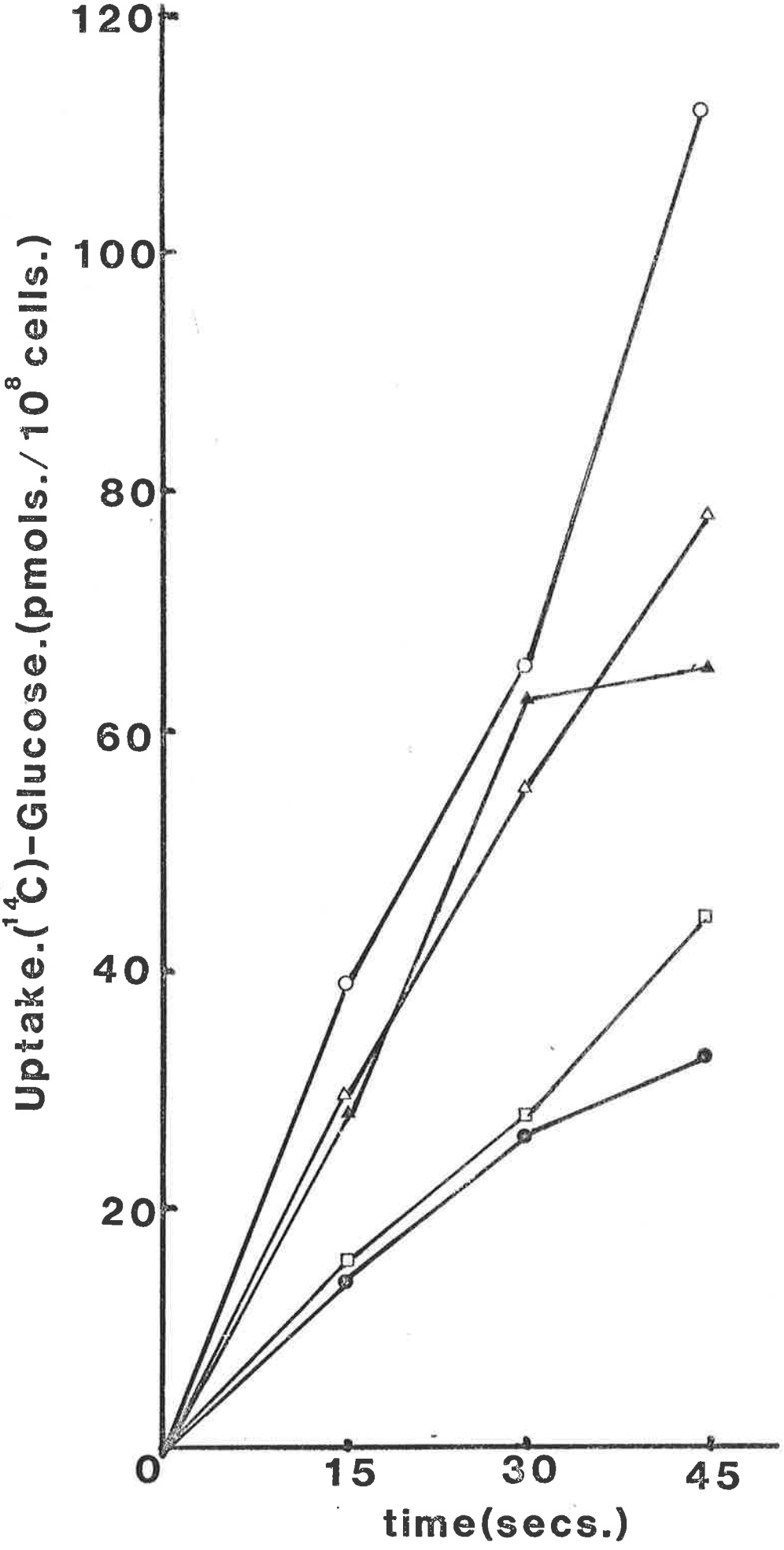
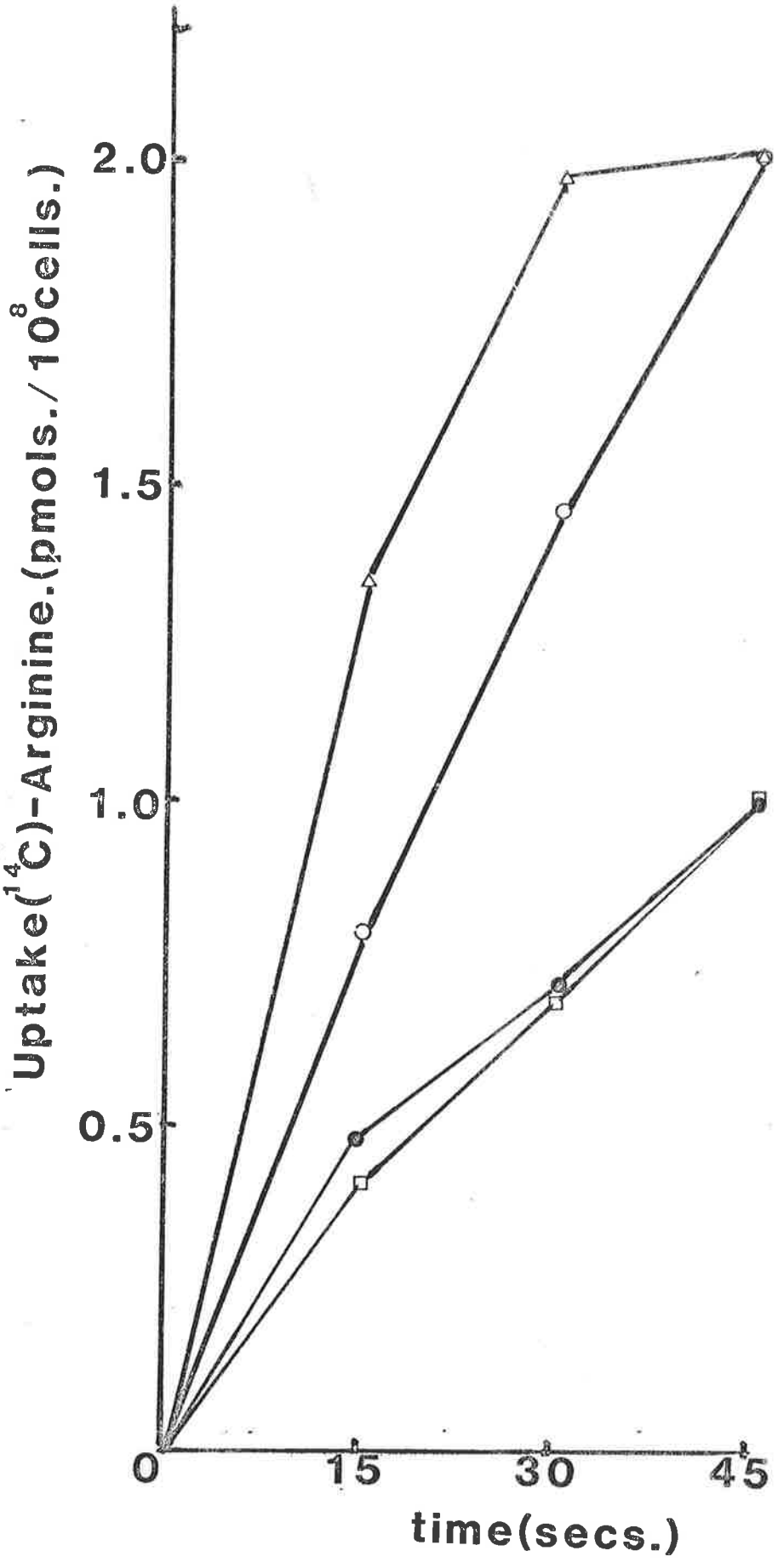

120
100
80
60
40
20
0
15
30
45
time(secs.)
Uptake.(^{14}C)-Glucose.(pmols./10^8 cells.)

Figure 5.6

Uptake of (^{14}C) arginine at 37^{o}C with a final concentration of 0.15μM in the uptake vessel.

Symbols: △ , P400; ▲ , P407; ○ , P433; ● , P1578; □ , P1602.


Uptake(^{14}C)-Arginine.(pmols./10^{8}cells.)
2.0
1.5
1.0
0.5
0
15
30
45
time(secs.)

detectable alleviating effect on the *ompB* mutation, since the uptake rates of P1578 and P1602 are almost identical. Figure 5.6 depicts the uptake of (^{14}C) arginine; the result is very similar to that obtained for glucose. There appears to be no significant uptake difference between P1578 and P1602. Similarly as for serine, glycine and phenylalanine, P407 and 433, often show uptake rates equal to, and exceeding wild-type for glucose and arginine. This may reflect increased amounts of OmpC and OmpF proteins in response to a lack of Tsx protein in the outer membrane.

5.5.2 Uptake of Glucosamine

Figure 5.7 depicts the uptake of (^{14}C) glucosamine. P400, P407, P433 and P1578 show almost identical uptake rates for this substrate; thus the *tsx* and *ompB* mutations have little effect on glucosamine uptake. P1602 shows a marginally improved uptake ability for glucosamine.

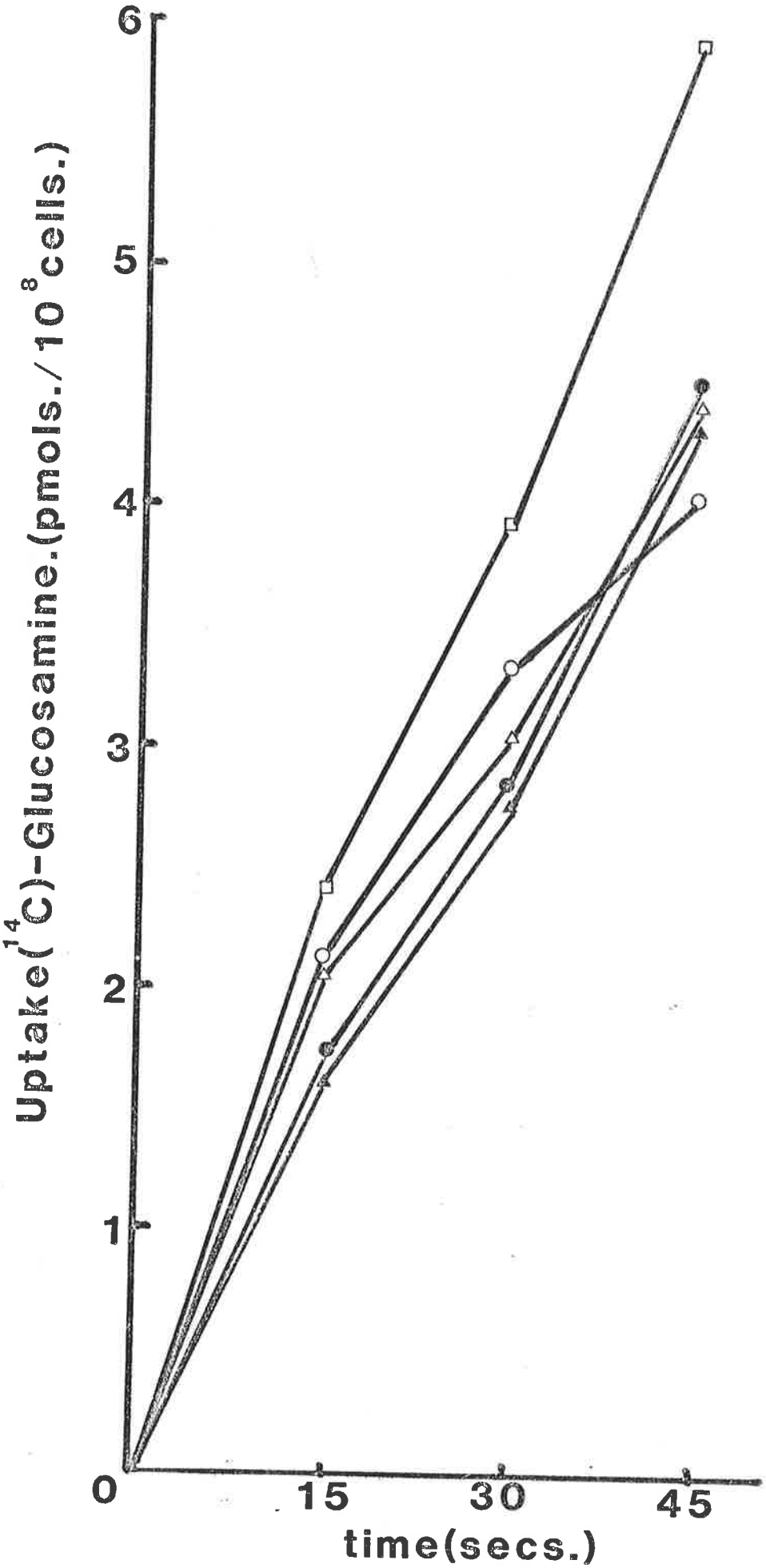
5.6 SUMMARY AND CONCLUSIONS

The Tsx protein is known to be involved in nucleoside permeation of the outer membrane. The extent of this function depends upon the nucleoside involved and the genetic background of the strain involved. The OmpC and OmpF proteins also appear to play a significant role in nucleoside permeation even at the low substrate concentrations employed

Figure 5.7

Uptake of (^{14}C) Glucosamine at 37^{o}C with a final concentration of 0.86μM in the uptake vessel.

Symbols: △ , P400; ▲ , P407; ○ , P433; ● , P1578; □ , P1602.


Uptake(^{14}C)-Glucosamine.(pmols./10^{8}cells.)
6
5
4
3
2
1
0
15
30
45
time(secs.)

(0.08-0.09μM), again the effect is dependent upon the nucleoside chosen and the strain.

The ability of the Tsx protein to facilitate the transport of molecules other than nucleosides was examined. The Tsx protein was shown to enhance the transport of serine, glycine and phenylalanine, while bearing no effect on either glucose or arginine transport. This effect appeared to vary depending on substrate and genetic background.

Glucosamine as we have seen previously in chapter 3 is not affected by the *ompB* mutation. The result here further confirms this and any effect of the Tsx protein is not apparent. This probably reflects the presence of another outer membrane transport system which masks the effect of either mutations.

CHAPTER 6

MUTATIONS AFFECTING THE PhoE PROTEIN

6.1 INTRODUCTION

It has been known for some time that pseudo-revertants of mutants deficient in OmpC and OmpF proteins produce a new membrane protein, which results in sensitivity to bacteriophage TC45 (Chai and Foulds, 1978). This new membrane protein has also been shown to produce pores similar to the OmpC and OmpF proteins (Lugtenberg *et al.*, 1978; Pugsley and Schnaitman, 1978 and van Alphen *et al.*, 1978).

Mutations resulting in the constituitive synthesis of the protein were mapped at two loci first designated *nmpA* and *nmpB* (Pugsley and Schnaitman, 1978; Foulds and Chai, 1978 and Lee *et al.*, 1979). During the initial stages of this work the observation was made (personal communication, Argast and Boos, 1980; Tomassen and Lugtenberg, 1980) that the new protein (and TC45 sensitivity) was co-regulated with the phosphate uptake system. The *nmpA* mutants were shown to map in the previously described *phoS*, *phoT* and *pst* genes and *nmpB* at *phoR*, all of which are involved in phosphate uptake and regulation. The structural gene for the new membrane protein was subsequently mapped at *phoE* (Tommassen and Lugtenberg, 1981), and the protein designated the PhoE protein.

Since the phosphate uptake system includes a binding protein which maps at *phoS* it is possible that the PhoS protein interacts with the PhoE

protein in a similar manner as the MalE protein with the LamB protein. A *phoS* mutant would therefore be expected to have enhanced uptake rates when compared to a *phoS*$^+$ in an *ompB* background. The experiments in this chapter compare the uptake abilities of both *phoS* and *phoT* strains. The *phoS* strain is a polar mutant on *phoT* (personal communication, Dr. H. Rosenberg) and so lacks both PhoS protein and the cytoplasmic PhoT protein.

6.2 STRAIN CONSTRUCTION

All strains used in this chapter were derived from either strain 101a (CGSC 5656) or C78 (CGSC 5651) which are listed in table 2.1 These strains were both found to be sensitive to bacteriophage TC45, but resistant to phage Tula. It was subsequently found that both strains possessed PhoE protein in whole membrane preparations but lacked the OmpF protein. It was already known that both strains were phage T2 resistant, and OmpF protein has been shown to be a receptor for phage T2 (Hantke, 1978). The derivatives of 101a and C78 used in this chapter are listed in table 6.1. The gene *ompB101* was transduced using phage P1 into both strains by the following procedure: Spontaneous *malA* mutants of either C78 or 101a were selected and then transduced with P1 phage grown on P530, selecting for simultaneous transduction of *malA*$^+$ and resistance to phage Tulb. These *omp101* strains were retained and subsequently used as parents for selection of spontaneous TC45 resistant mutants.

TABLE 6.1

Bacterial strains

Strain	Genotype	Cell envelope proteins			
		PhoE	OmpC	OmpF	PhoS
P2685	*ompB101* derivative of C78	+	-	-	-
P2686	*ompB101* derivative of 101a	+	-	-	+
P2687	indept. TC45^{R} mutant of P2685	r	-	-	-
P2688	indept. TC45^{R} mutant of P2685	r	-	-	-
P2689	indept. TC45^{R} mutant of P2686	r	-	-	+
P2690	indept. TC45^{R} mutant of P2686	r	-	-	+

r = reduced amounts

6.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

Figure 6.1 illustrates the whole membrane preparations of the strains run on polyacrylamide gels containing 4M urea, in order to differentiate PhoE protein from OmpC and OmpF.

It is apparent that C78 has more PhoE protein than 101a, but upon introduction of the *ompB* mutation, the amount of PhoE protein in the 101a derivative (P2686; track D) increases. The TC45 resistant strains (Tracks E, F, band H) have markedly reduced amounts of PhoE protein. This is more apparent for P2689 and P2690 (tracks E and F).

6.4 COMPARATIVE UPTAKE ABILITIES OF *phoS* AND *phoS*$^+$ STRAINS

The uptake abilities for a variety of substrates were tested for P2685 and P2686. The uptake were carried out in 10mM HEPES buffer pH7.5 and 5mM $MgCl_2$ rather than the phosphate based minimal medium used in previous uptake experiments.

6.4.1 Uptake of (^{14}C)-lactose, -glucose, -galactose, -mannitol and -glycerol

Figures 6.2, 6.3, 6.4, 6.5 and 6.6 illustrate the effects of the *phoS* mutation on the uptake abilities for the respective substrates. It can be seen that P2685 has no significant advantage over P2686 in uptake for any of these substrates. This is especially true when considering the apparently

FIGURE 6.1

Polyacrylamide gel electrophoresis of whole membrane preparations run on 11% acrylamide gels containing 4M urea.

A : C78

B : 101a

C : P2685

D : P2686

E : P2687

F : P2688

G : P2689

H : P2690

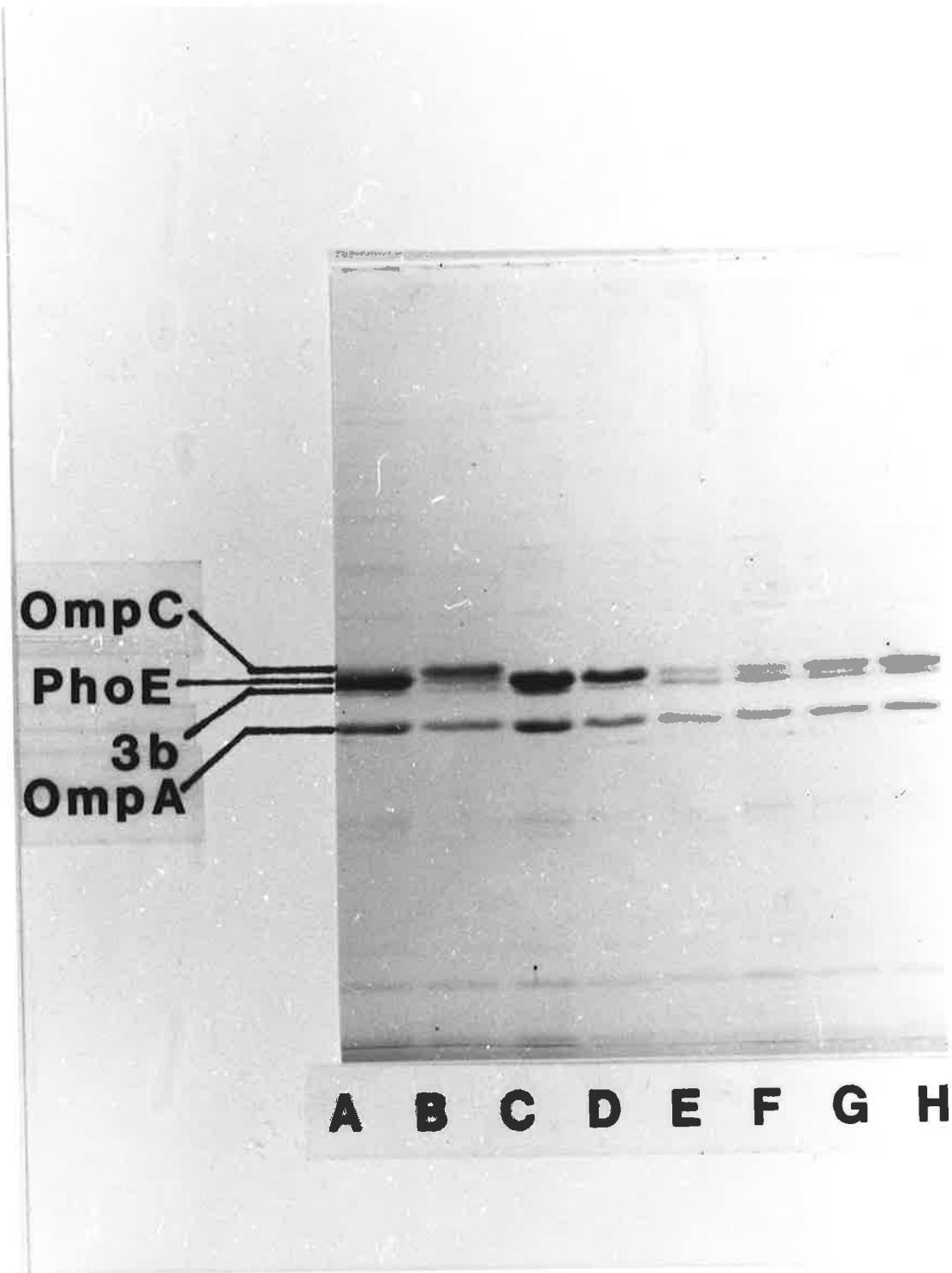

OmpC
PhoE
3b
OmpA
A B C D E F G H

FIGURE 6.2

Uptake of (^{14}C) lactose in 10mM HEPES and 6mM $MgCl_2$ pH7.5 with a final concentration of 1μM in the uptake vessel. Cells were induced with 10^{-2}M IPTG prior to uptake P2685, ● ; P2686, □ .

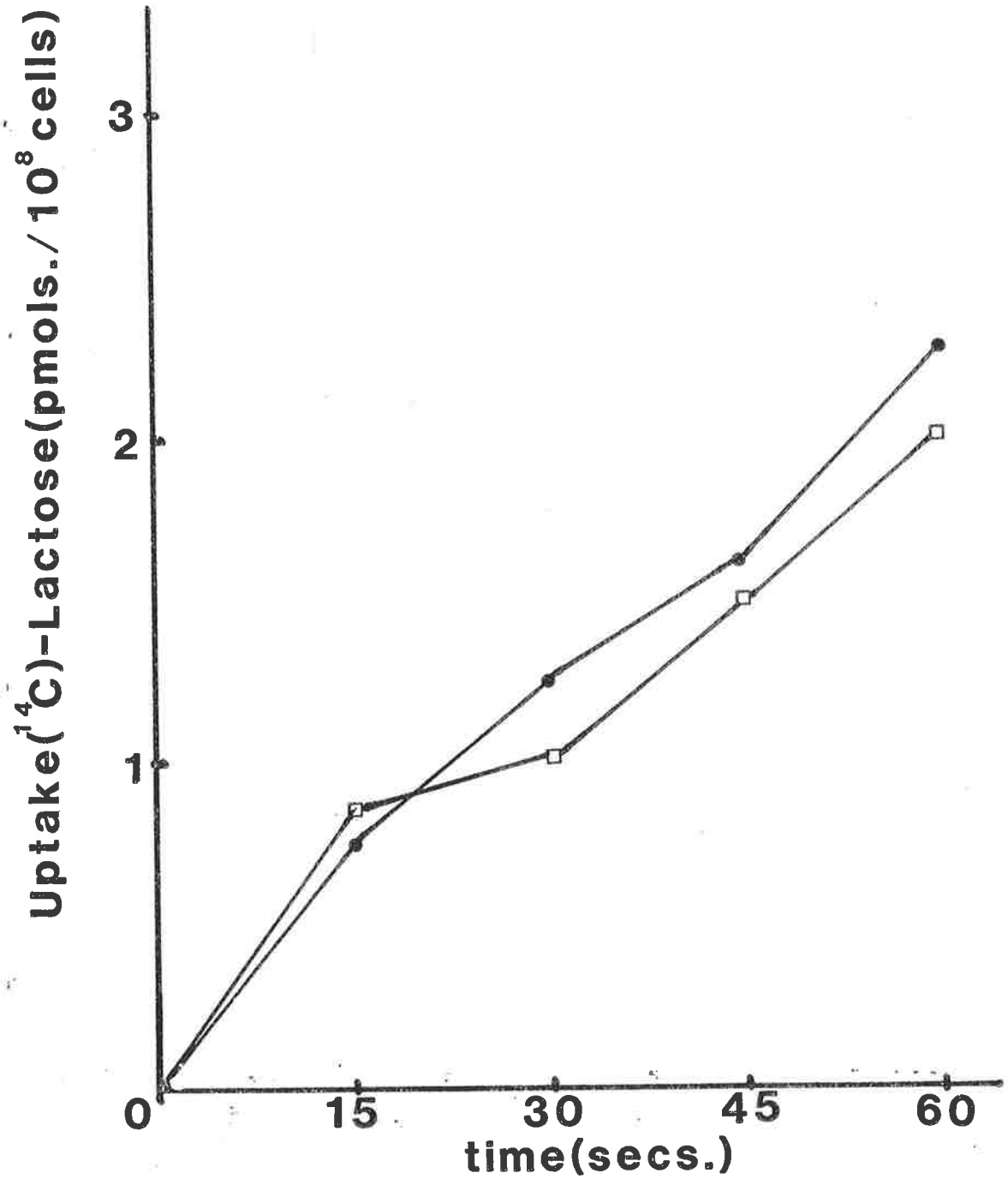

Uptake(^{14}C)-Lactose(pmols./10^8cells)
3
2
1
0
15
30
45
60
time(secs.)

FIGURE 6.3

Uptake of (^{14}C) glucose in 10mM HEPES and 5mM $MgCl_2$ pH7.5 with a final concentration of 0.83μM in the uptake vessel. P2685, ● ; P2686, □ .

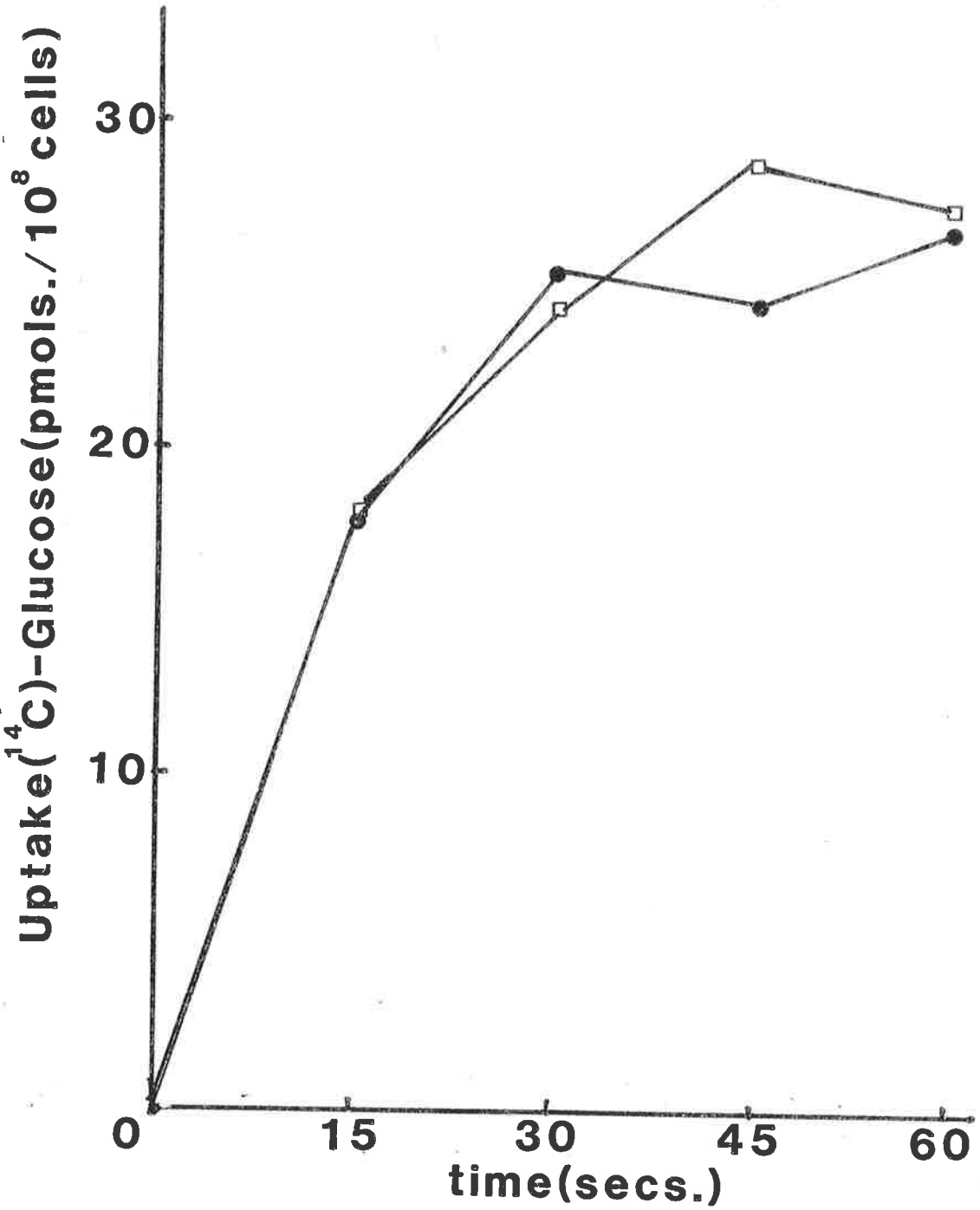

Uptake(^{14}C)-Glucose(pmols./10^8 cells)
30
20
10
0
15
30
45
60
time(secs.)

FIGURE 6.4

Uptake of (^{14}C) galactose in 10mM HEPES and 5mM $MgCl_2$ pH7.5 with a final concentration of 0.83μM in the uptake vessel. Cells were induced with 0.2% galactose prior to uptake. P2685, ● ; P2686, □ .

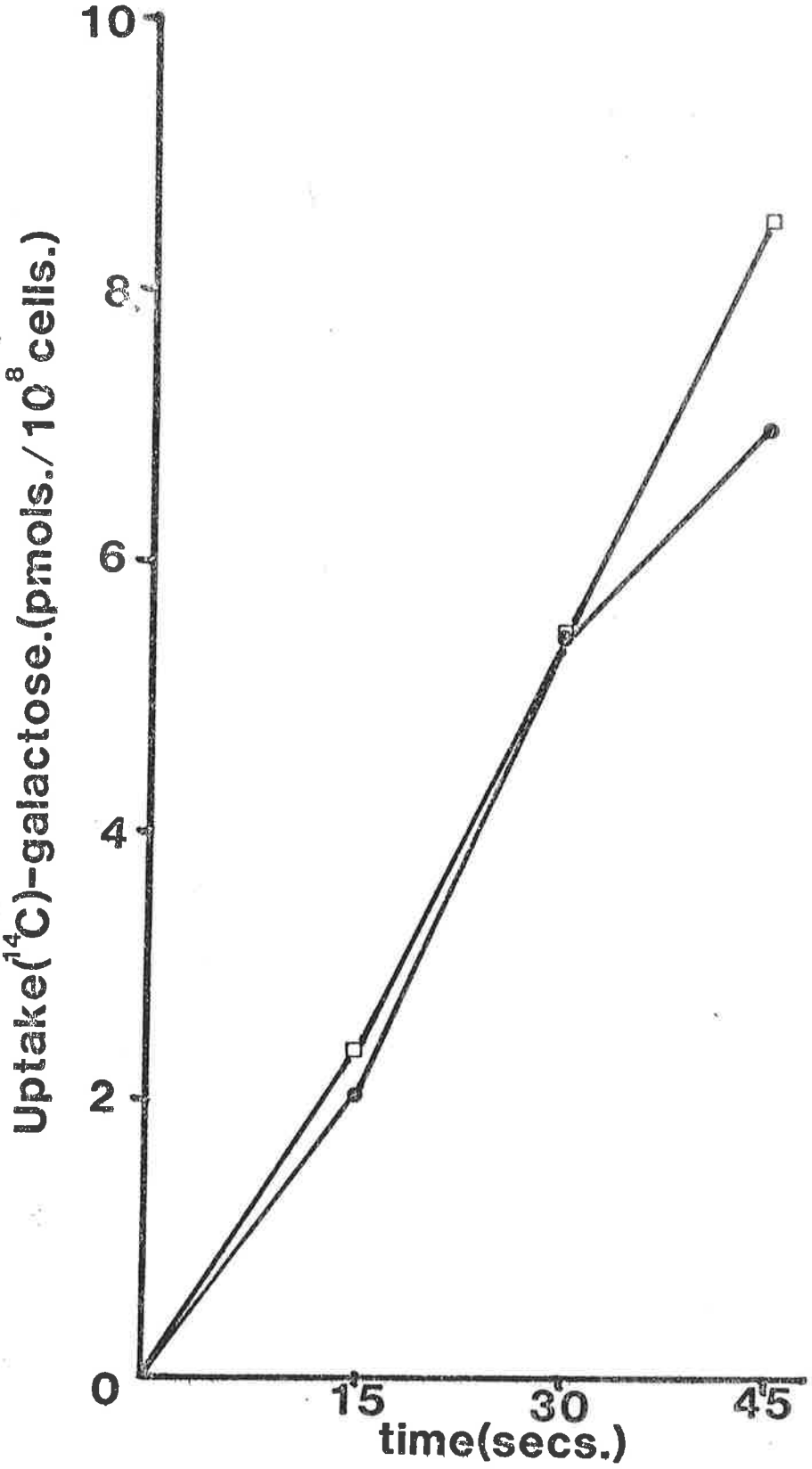

10
8
6
4
2
0
15
30
45
time(secs.)
Uptake(^{14}C)-galactose.(pmols./10^{8} cells.)

FIGURE 6.5

Uptake of (^{14}C) mannitol in 10mM HEPES and 5mM $MgCl_2$ pH7.5 with a final concentration of 0.175μM in the uptake vessel. Cells induced with 0.02% mannitol prior to uptake. P2685, ●; P2686, □.

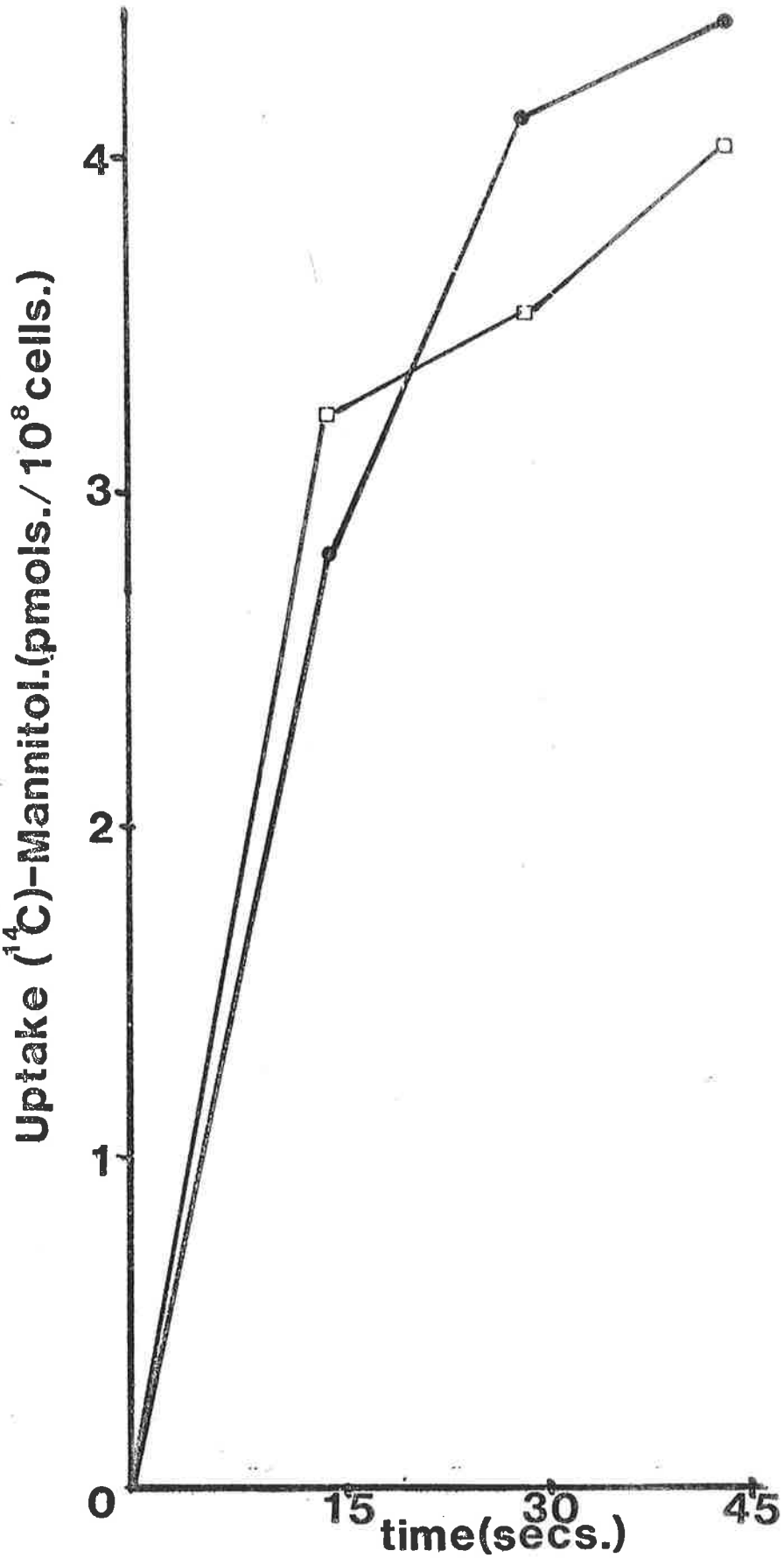
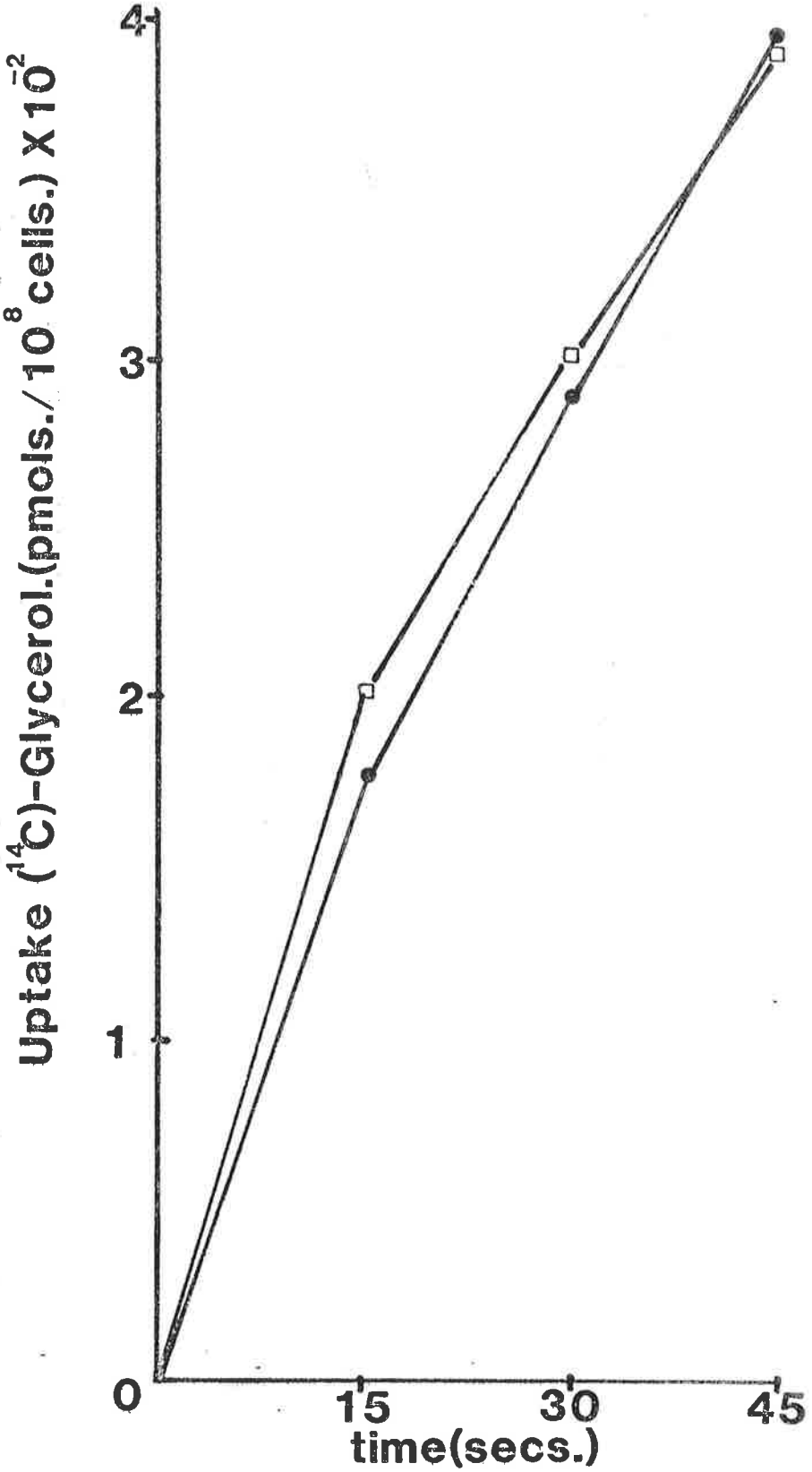

Uptake (^{14}C)-Mannitol.(pmols./10^8cells.)
0
1
2
3
4
15
30
45
time(secs.)

FIGURE 6.6

Uptake of (^{14}C) glycerol in 10mM HEPES and 5mM $MgCl_2$ pH7.5 with a final concentration of 0.63μM in the uptake vessel. P2685, ● ; P2686, □ .


Uptake (^{14}C)-Glycerol.(pmols./10^8 cells.) X 10^{-2}
4
3
2
1
0
15
30
45
time(secs.)

larger amounts of PhoE protein in the membrane of this strain as detected from electrophoresis of whole membrane preparations.

6.5 BACTERIOPHAGE AND COLICIN RESISTANCE PATTERNS

It was initially thought that since strains P2687, P2688, P2689 and P2690 still possessed some PhoE protein in the membrane and when resistance to bacteriophage TC45 was tested plaques with morphology similar to host range phage arose. In checking resistance of the strains to other bacteriophage it was found that the four strains tested were altered in their resistance patterns to phages C21 and U3, and therefore altered in their LPS structure (Rapin *et al.*, 1966; Rapin and Kalckar, 1971 and Watson and Paigen, 1972). They were also tested against colicins E2 and E2 which can kill the cells in the absence of OmpC and OmpF proteins when PhoE protein is present (Pugsley and Schnaitman, 1978). This data is presented in table 6.2 It was found that when resistance to bacteriophage TC45 was selected, some 50% of the TC45 resistant colonies became simultaneously altered in their resistance patterns to phage C21 and U3, indicating an LPS lesion.

6.6 DRUG AND DETERGENT SENSITIVITY

Table 6.3 depicts the sensitivity of the strains to various toxic agents and antibiotics. The TC45 resistant mutants have increased sensitivity to most

TABLE 6.2

Bacteriophage and colicin sensitivity.

	bacteriophage						colicin	
Strain	C21	U3	TC45	P1	T4	T7	E2	E3
P2685	R	S	S	S	S	S	S	S
P2686	R	S	S	S	S	S	S	S
P2687	S	R	R*	S	R	S	R	R
P2688	S	R	R*	S	R	S	R	R
P2689	S	R	R*	S	S	P	P	P
P2690	S	R	R*	S	S	P	P	P

* a few turbid plaques evident

R = resistant, P = partial resistance, S = sensitive

TABLE 6.3

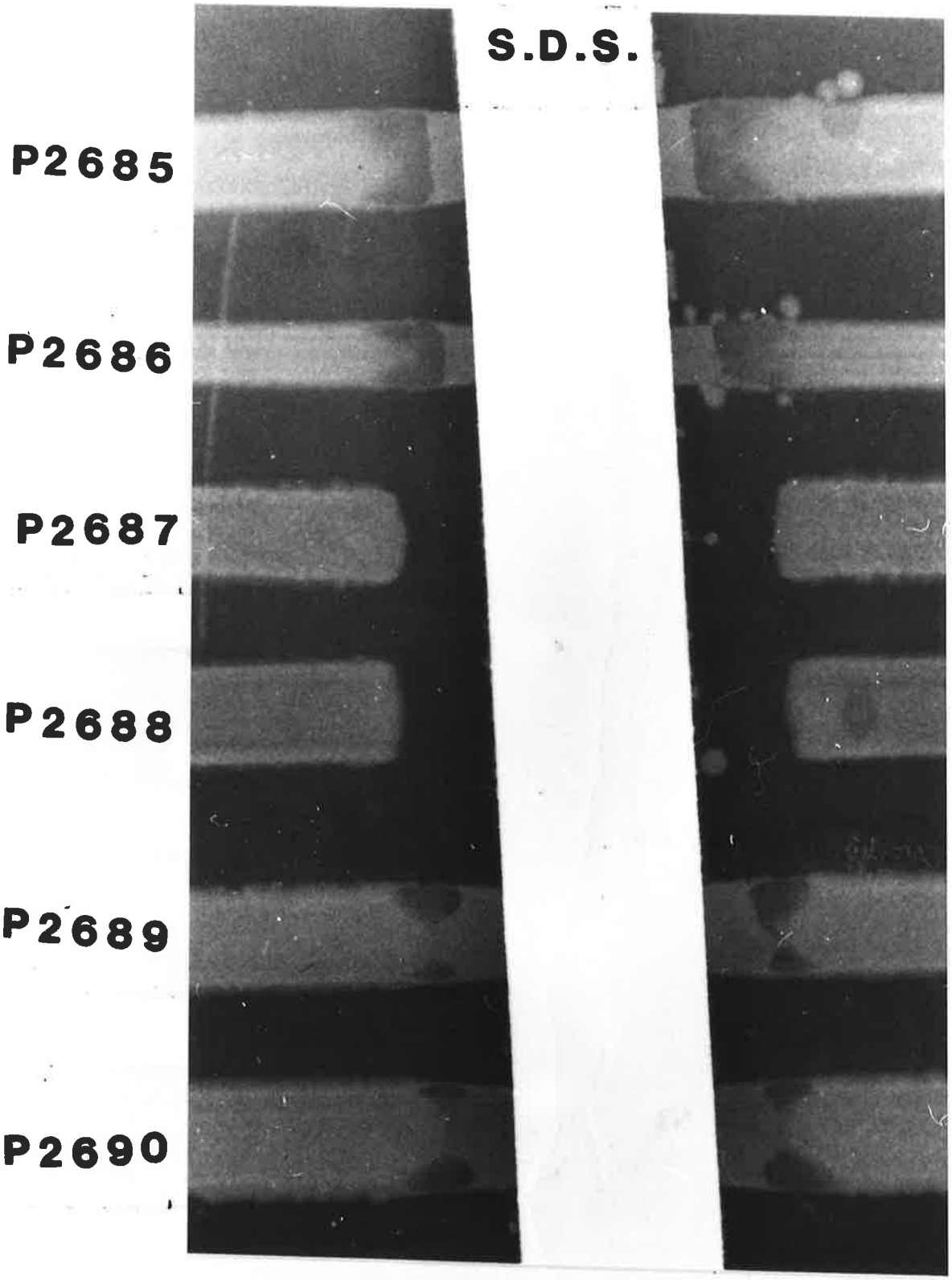
Drug and detergent sensitivity.

Agent	concn (mg/strip)	Inhibition zone width (mm)						
		phoS P2685	*phoT* P2686	*phoS* TC45^R P2687	P2688	*phoT* TC45^R P2689	P2690	Partition coeff[a]
Novobiocin	1.5	<10	<10	15	12	10	10	>20
Deoxycholate	50	15	15	19	19	16	16	1.09
Erythromycin	5	21	23	23	23	20	20	0.79
Nafcillin	1.0	28	28	34	33	30	30	0.31
Oxacillin	5	20	19	25	24	18	19	0.07
Polymyxin B	5	25	25	26	26	25	25	<0.05
Sodium dodecyl sulphate	50	<10	<10	19	19	10	10	0.02
Neomycin	5	25	25	30	29	25	25	0.01
Ampicillin	1.0	24	26	29	27	26	26	0.01

[a] Partition coefficients from Nikaido (1976), except sodium dodecyl sulphate and deoxycholate from Coleman and Leive (1979).

FIGURE 6.7

An example of the varying degrees of resistance of the strains to a filter paper strip containing 50mg of sodium dodecyl sulphate (S.D.S.).


S.D.S.
P2685
P2686
P2687
P2688
P2689
P2690

toxic agents. Strains P2687 and P2688 showing the most pronounced effect and this being most apparent with the more hydrophobic agents (by definition of Nikaido (1976) hydrophobic molecules have a partition coefficient in water/Octanol of >0.02). The increased sensitivity to hydrophobic antibiotics etc. is a property typical of strains affected in their LPS structure (Nikaido, 1979). Figure 6.7 illustrates an example of the different levels of sensitivity of the various strains to sodium dodecyl sulphate (SDS).

6.7 SUMMARY AND CONCLUSIONS

The absence of the phosphate binding protein (PhoS protein) does not appear to have any enhancing effect on the ability of the PhoE protein to act as a pore. During the course of this work it was thought that P2687, P2688, P2689 and P2690 were primarily altered in the production of PhoE protein. This was supported by cell envelope protein preparations and resistance to bacteriophage TC45 and the host range type plaques which appeared. P2687 and P2688 were very poor growers (data not shown) this being consistent with the pattern of growth in a strain which is low in porin. Radioactive uptake experiments supported this view, the decrease in uptake being less marked with P2689 and P2690 which is consistent with their lower complement of porin (data not shown). When testing these strains with bacteriophages C21 and

U3 it became apparent that the strains are LPS mutants; it also found that 50% of TC45 resistant mutants were also altered in C21 and U3 resistance patterns. These strains exhibited increased sensitivity to most of the hydrophobic agents tested. On the basis of phage resistance and sensitivity to antibiotics it appears there are two classes of LPS mutants; P2687 and P2688 being members of class 1 and P2689 and P2690 being members of class 2. These two classes probably represent LPS lesions of different degrees of severity.

CHAPTER 7

THE EFFECT OF *ompA* MUTATIONS ON TRANSPORT ACROSS THE OUTER MEMBRANE

7.1 INTRODUCTION

The *ompA* protein is generally thought not to be a pore forming protein. Recently, however, experimental data has appeared which suggests that OmpA protein does have an effect on the transport of some substrates.

The earliest such data is that of Manning *et al.*, (1977) who suggested that the *ompA* mutation results in a decrease in proline and glutamine uptake. Coulton and Braun (1979), were able to show that the *ompA* mutation reduced the *tonA* dependent uptake of iron in the form of ferrichrome. Krieger-Brauer and Braun (1980) while investigating the uptake of nucleosides by the Tsx protein found that thymidine and adenosine uptake was reduced 16-33% in *ompA* mutants. It is tempting to speculate that OmpA protein may physically interact with TonA and Tsx proteins. Another possibility arises: the absence of OmpA protein may affect outer membrane organization with a subsequent reduction in transport functions. In this chapter the effect of *ompA* mutations on the functioning of the OmpC and OmpF proteins is investigated. This idea can be extended by looking at the effects of *ompA* mutations on strains in which LamB protein is acting as a substitute major porin as described in chapter 3.

The initial uptake experiments were carried out on strains derived from P400. They are described in detail in table 3.1. Their relevant genotype and

TABLE 7.1

Relevant genotype and proteins carried by strains derived from P400.

		outer-membrane proteins			
Strain	relevant genotype	OmpA	OmpF	OmpC	Tsx
P400	wild-type	+	+	+	+
P407	*tsx*	+	+	+	-
P460	*ompA*	-	+	+	+
P1578	*ompB*	+	-	-	+
P1665	*ompA*	-	+	+	+
P1667	*ompA*	+[a.]	+	+	+
P1744	*ompA*	-	+	+	+[b.]

[a.] Altered protein *ompA* mutant. (Manning, Puspurs and Reeves, 1976).

[b.] Altered protein *tsx* mutant. (Manning and Reeves, 1978).

outer membrane protein pattern are described in table 7.1.

7.2 POLYACRYLAMIDE GEL ELECTROPHORESIS: P400 DERIVATIVES

The whole membrane preparations of the strains derived from P400 are shown in figure 7.1. It is noteworthy that P1667 is an altered protein mutant (Manning, Puspurs and Reeves, 1976), it has normal amounts of OmpA protein in membrane preparations, and is resistant to bacteriophage K3 and maps at *ompA*.

The other *ompA* mutants P460, P1665 and P1744 have no detectable OmpA protein in their membrane preparations. P1578, an *ompB* mutant and P407 a *tsx* mutant have a lack of detectable OmpC, OmpF and Tsx proteins respectively. P1744 is an altered Tsx protein mutant, which is resistant to phage T6, maps at *tsx* and has Tsx protein apparent in membrane preparations.

7.3 UPTAKE STUDIES: P400 DERIVATIVES

It can be seen from figures 7.2, 7.3 and 7.4 that *ompA* mutations which result in lack of OmpA protein also have reduced uptake rates for three substrates: phenylalanine, arginine and glucose respectively. This effect is most pronounced for arginine where uptake in *ompA* strains other than P1667 is comparable to P1578. In most cases the

Figure 7.1

Whole membrane preparations on 11-20% polyacrylamide gels. Equal (20µl) amounts loaded in each case.

A : P407

B : P1665

C : P1667

D : P1744

E : P400 (wild-type)

F : P460

G : P1578

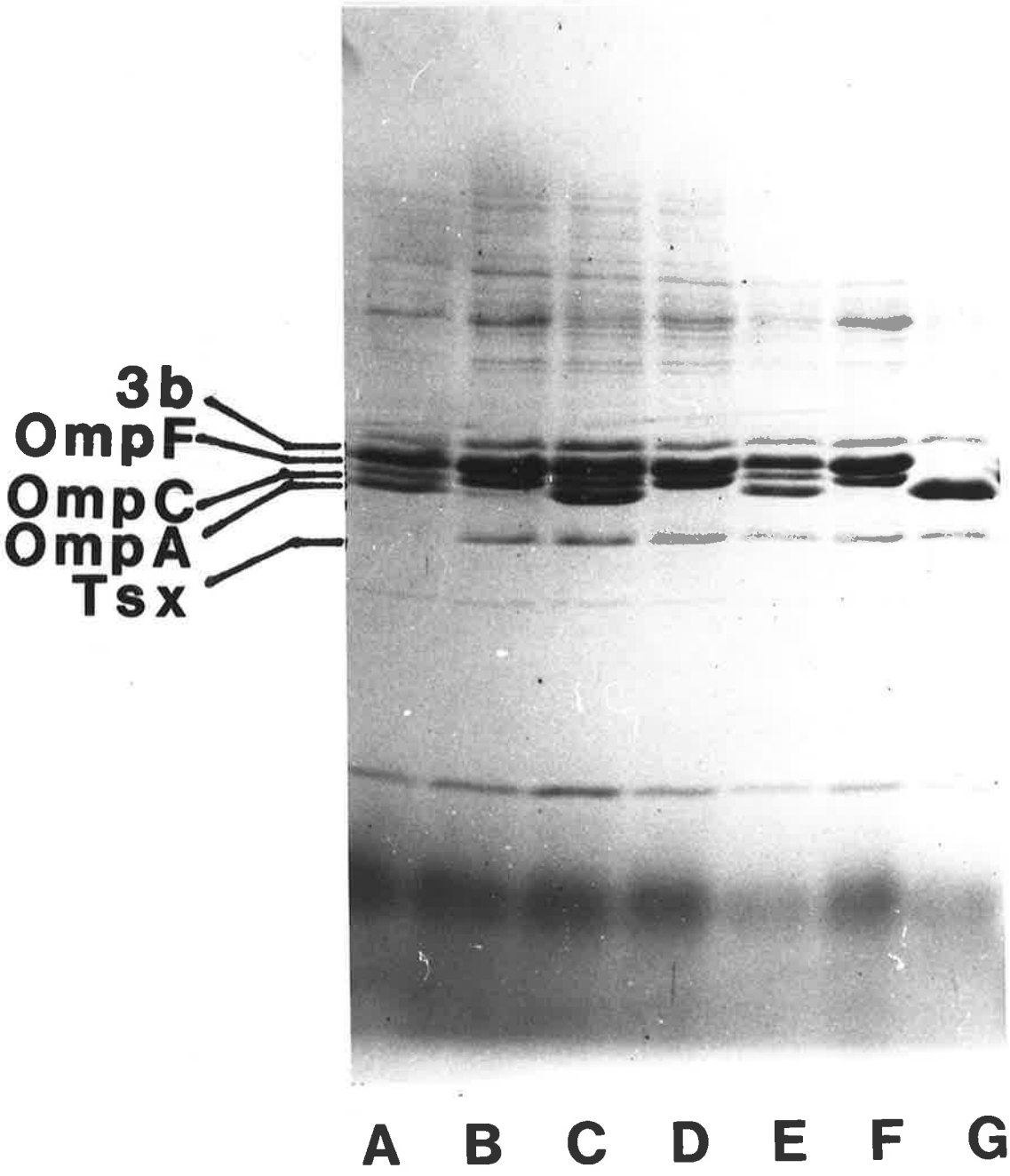

3b
OmpF
OmpC
OmpA
Tsx
A B C D E F G

FIGURE 7.2

Uptake of (^{3}H) phenylalanine in 10mM HEPES buffer and 5mM $MgCl_2$ pH7.5 at 37°C, and a final concentration of 0.107µM in the uptake vessel. P400, △ ; P407, ○ ; P460, ▲ ; P1578, ● ; P1665, + ; P1667, ■ ; P1744, □ .

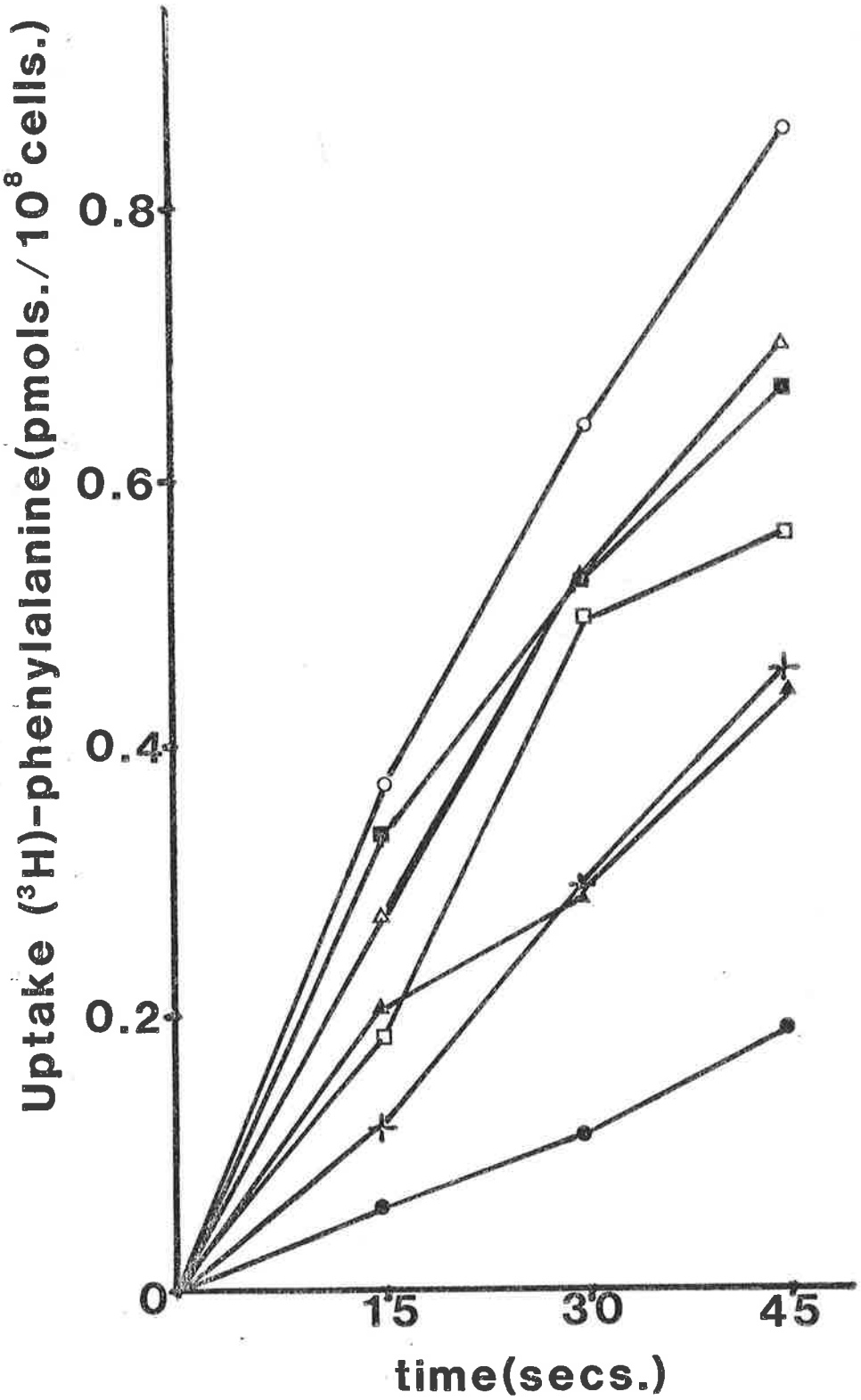

Uptake (^{3}H)-phenylalanine(pmols./10^{8}cells.)
0.8
0.6
0.4
0.2
0
15
30
45
time(secs.)

FIGURE 7.3

Uptake of (^{14}C) arginine in 10mM HEPES buffer and 5mM $MgCl_2$, pH7.5 at 37°C with a final concentration of 0.146µM in the uptake vessel. P400, △ ; P407, ○ ; P460, ▲ ; P1578, ● ; P1665, ┼ ; P1667, ■ ; P1744, □ .

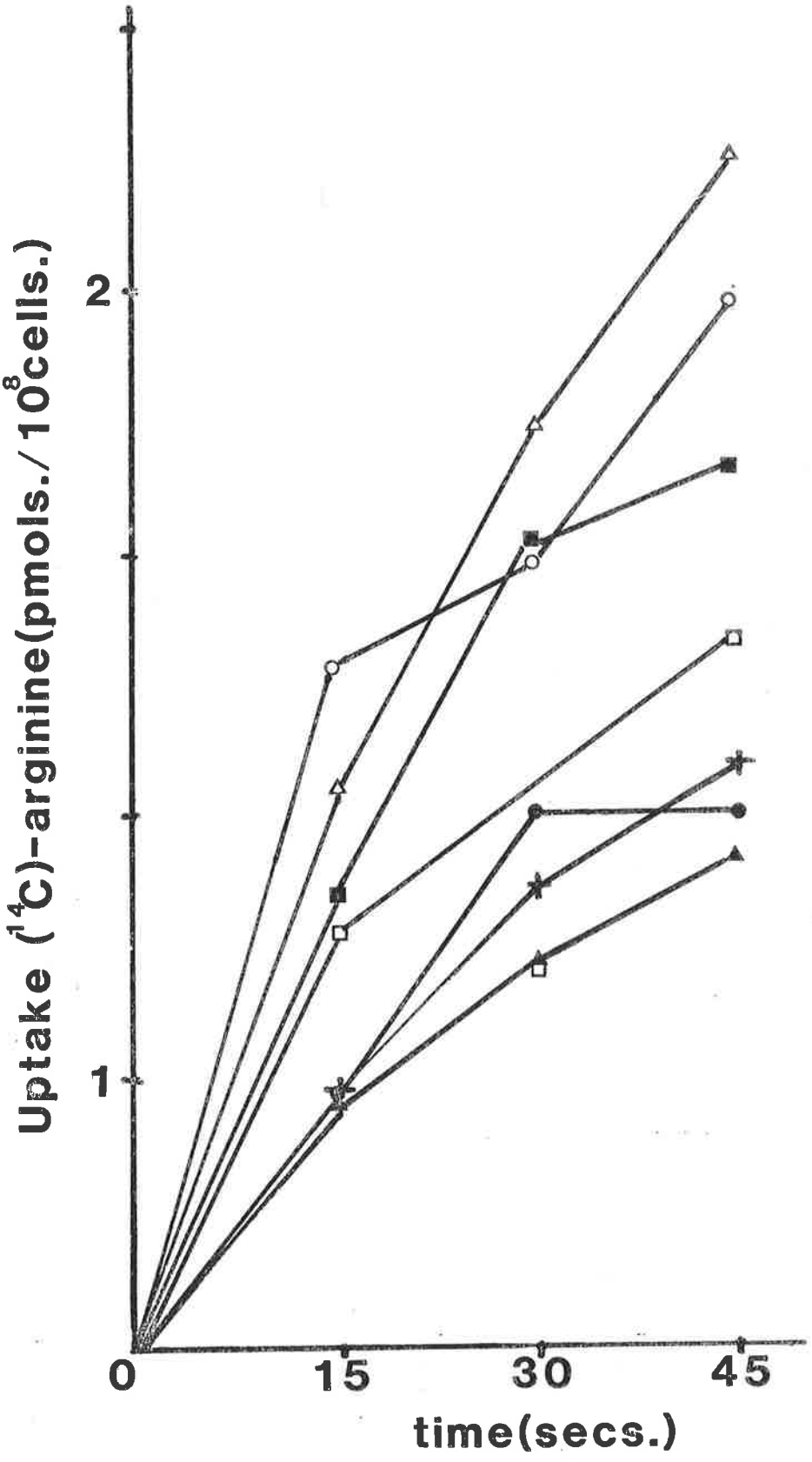
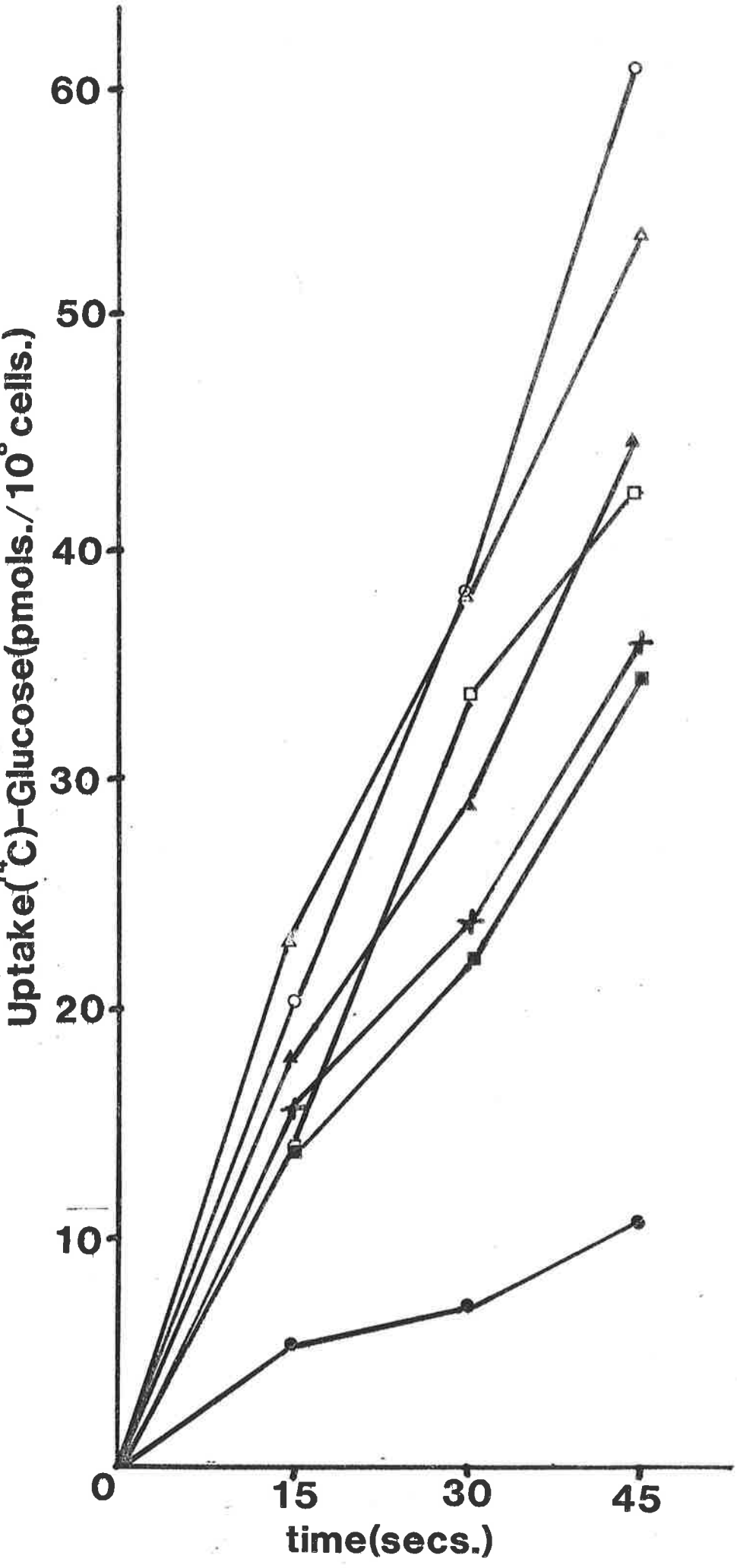

Uptake (^{14}C)-arginine(pmols./10^{8}cells.)
2
1
0
15
30
45
time(secs.)

FIGURE 7.4

Uptake of (^{14}C) glucose in 10mM HEPES buffer and 5mM $MgCl_2$, pH7.5 at 37°C, with a final concentration of 0.83µM in the uptake vessel. P400, △; P407, ○; P460, ▲; P1578, ●; P1665, +; P1667, ■; P1744, □.


60
50
40
30
20
10
0
15
30
45
time(secs.)
Uptake(^{14}C)-Glucose(pmols./10^{8} cells.)

uptake rate of the *ompA* strains, although reduced, is not reduced to the same level as in P1578. It is noteworthy that P1667 is able to transport these substrates at levels equivalent to P400.

7.3.1 The effect of *ompA* mutation on maltose transport

Figure 7.5 shows the effect of the *ompA* mutation on maltose transport in the P400 series of strains. In contrast to the effect of *ompA* on phenylalanine, arginine and glucose transport, maltose uptake is increased slightly rather than depressed.

7.4 THE EFFECT OF *ompA* ON TRANSPORT WHEN LamB PROTEIN IS ACTING AS A GENERAL PORE

The effect of *ompA* on the LamB protein when acting as a general pore as in an *ompB* mutant was examined.

7.4.1 Strain Construction

The strains P2343, P2342, P2341 and P2421 which are described in chapter 3 were used as the parent strains for the construction of *ompA* mutants. The LamB protein is produced in large amounts in all of these strains (except P2421) and can thus be considered a major outer membrane protein. These strains were treated with bacteriophage K3 to select for K3 resistant strains. The chosen K3 resistant strains were all further tested for resistance to bacteriophage K3*hr1*, a host range mutant of K3 (Manning, Puspurs and Reeves).

Figure 7.5

Uptake of (^{14}C) maltose in 10mM HEPES buffer and 5mM $MgCl_2$, pH7.5 at 37°C with a final concentration of 2.7µM in the uptake vessel. Cells were induced with 0.2% maltose prior to uptake. P400Δ.....Δ(not induced); P400,Δ ; P407,○ ; P460,▲ ; P1578,● ; P1667,■ ; P1744,□ .

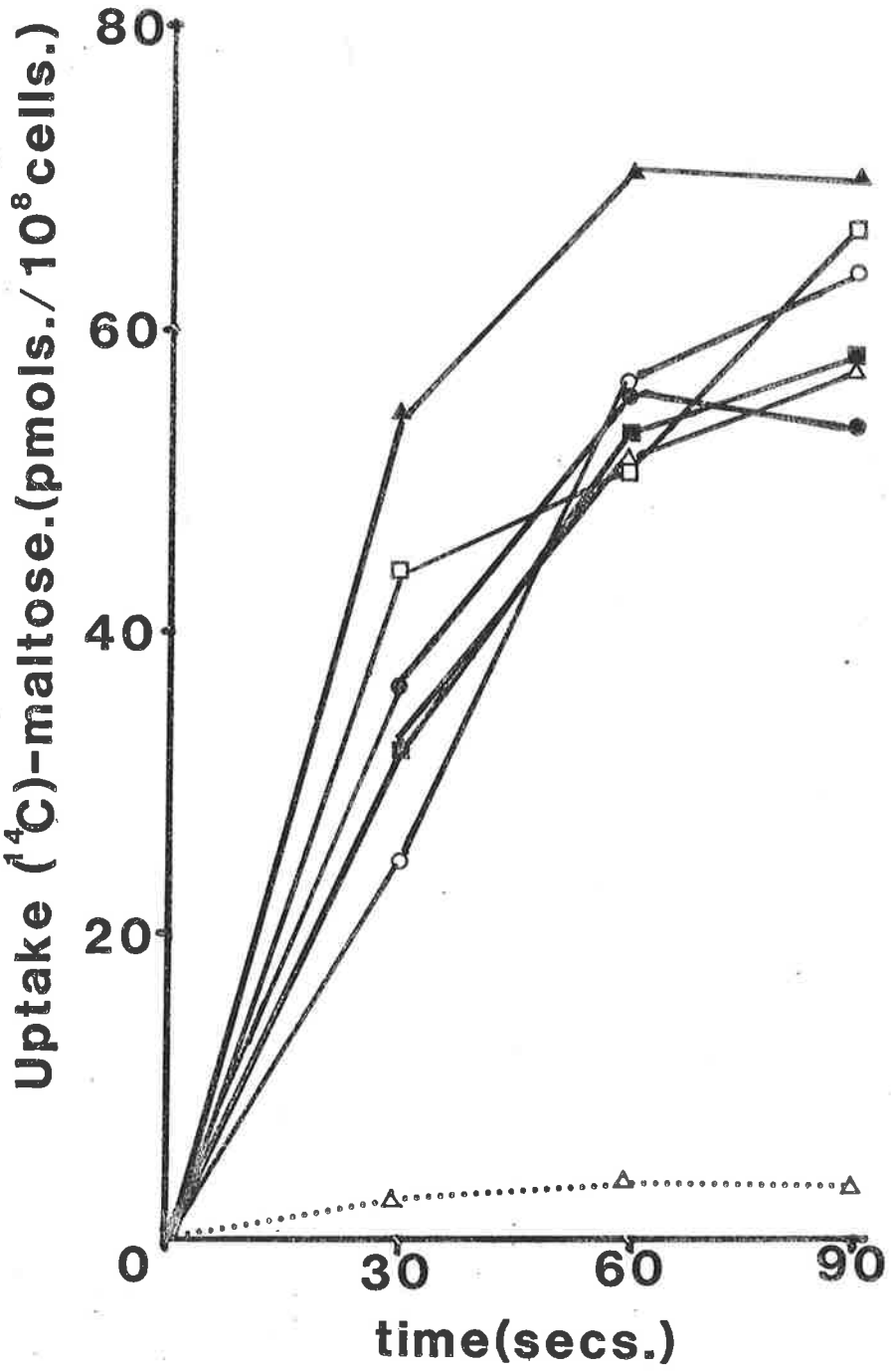

Uptake (^{14}C)-maltose.(pmols./10^8cells.)
80
60
40
20
0
30
60
90
time(secs.)

TABLE 7.2

Relevant genotype and outer membrane proteins of *ompA* strains derived in a *malT*^{c} background.

Strain	relevant genotype	membrane proteins			
		LamB	OmpC	OmpF	OmpA
P2684	*ompA738* derivative of P2343	+	+	+	-
P2691	*ompA739* derivative of P2342	+	-	-	-
P2692	*ompA740* derivative of P2341	+	-	-	-
P2693	*ompA741* derivative of P2421	-	-	-	-

Figure 7.6

Whole membrane preparations on 11-20% polyacrylamide gels. Equal (20µl) amounts loaded in each track.

A : P2343 parent of P2684

B : P2342 parent of P2691

C : P2341 parent of P2692

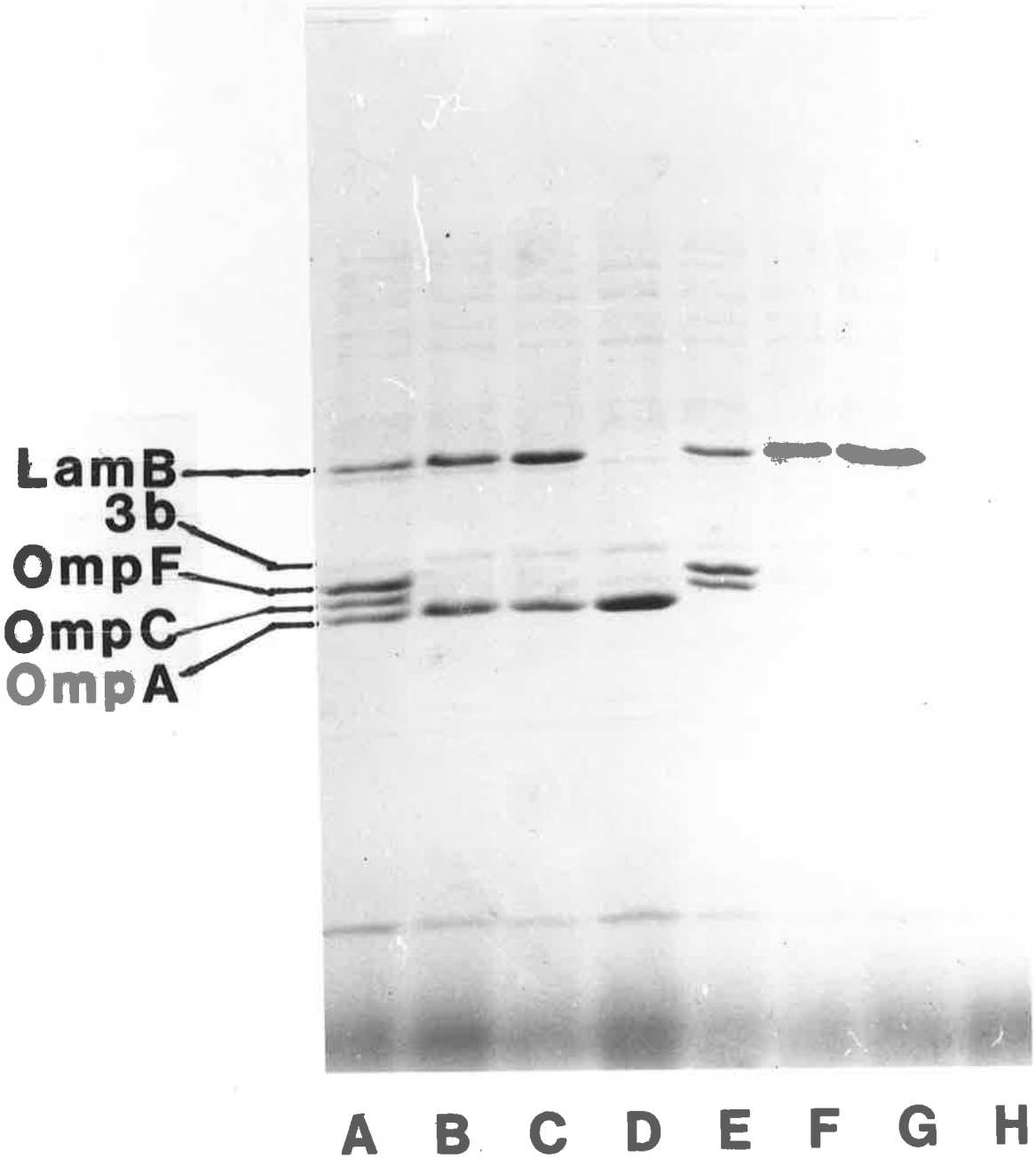
D : P2421 parent of P2693

E : P2684

F : P2691

G : P2692

H : P2693


LamB
3b
OmpF
OmpC
OmpA
A B C D E F G H

Strains resistant to K3*hr1* are probably totally devoid of OmpA protein. The lack of OmpA protein was verified on polyacrylamide gels: Whole membrane preparations of the four strains chosen, P2684, P2691, P2692 and P2693, are shown in figure 7.6. Their genetic and membrane characteristics are summarized in table 7.2.

7.4.2 The effect of *ompA* on LamB porin properties

Figure 7.7 illustrates the uptake of lactose for *ompA* derivatives in comparison to their parental strains. It is clear that all of the *ompA* derivatives except P2693 have enhanced uptake rates for lactose over their parent *ompA*$^+$ strains. This effect appears to be dependent upon the LamB protein, since P2691 shows the enhancing effect whereas P2693 which lacks both major porin (OmpC, OmpF proteins) and LamB shows no increase in uptake ability when compared to its parent strain P2421.

Figure 7.8 depicts the uptake of glucose; it is apparent that no significant difference in uptake ability exists between the *ompA* derivatives and their parental strains.

7.4.3 The effect of *ompA* on maltose transport

Figure 7.9 depicts the effect of *ompA* mutation on maltose transport in strains which are constituitive for maltose transport and lack the OmpC and OmpF proteins. The *malE* strains are not included since

FIGURE 7.7

Uptake of (^{14}C) lactose in 10mM HEPES buffer and 5mM $MgCl_2$, pH7.5 at 37°C, with a final concentration of 1.0μM in the uptake vessel. Cells were induced with 10^{-2}M IPTG prior to uptake. P2342, ●; P2343, ■; P2421, ▲; P2684, □; P2691, ○; P2692, +; P2693, △.

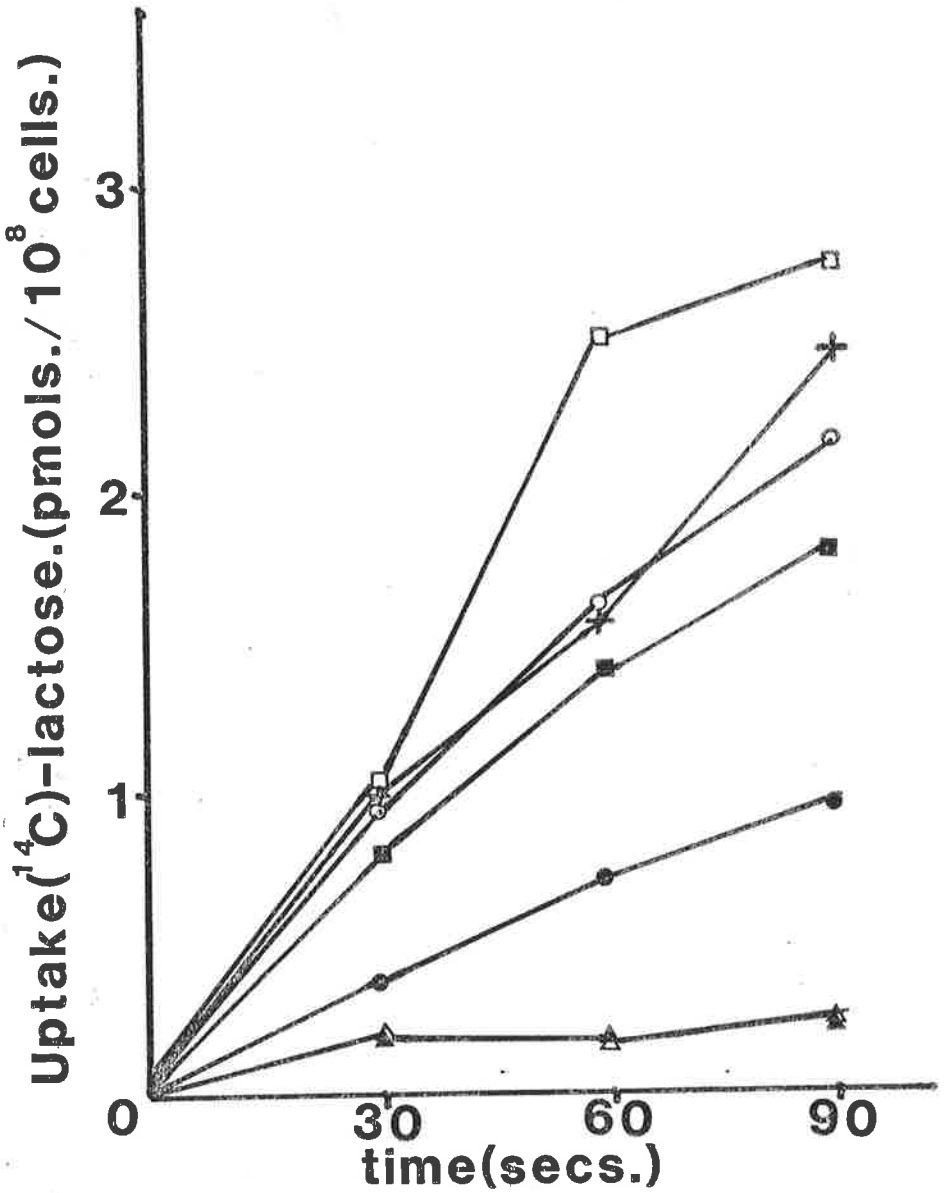

Uptake(^{14}C)-lactose.(pmols./10^{8} cells.)
3
2
1
0
30
60
90
time(secs.)

FIGURE 7.8

Uptake of (^{14}C) glucose in 10mM HEPES buffer and 5mM $MgCl_2$, pH7.5 at 37°C, with a final concentration of 0.83µM in the uptake vessel. P2343, ■ ; P2421, ▲ ; P2684, □ ; P2691, o ; P2692, + ; P2693, △ .

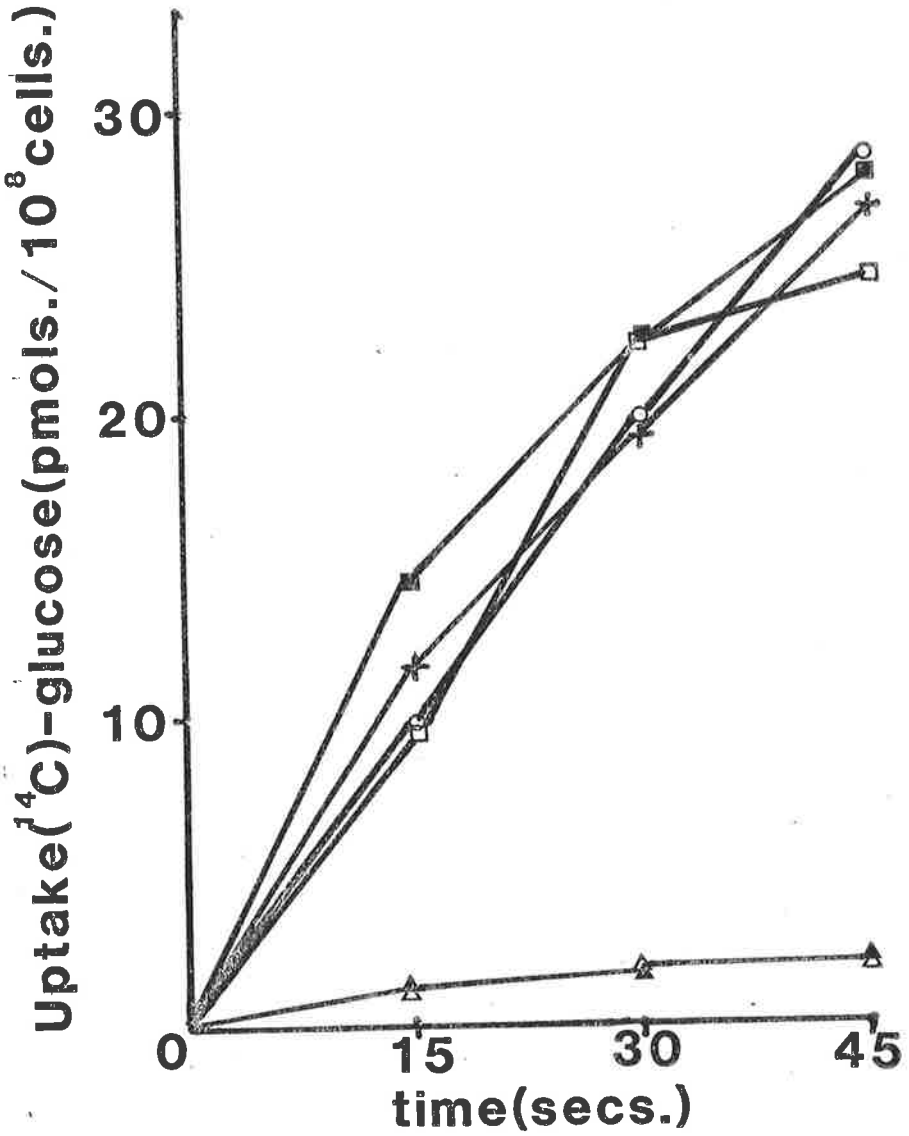
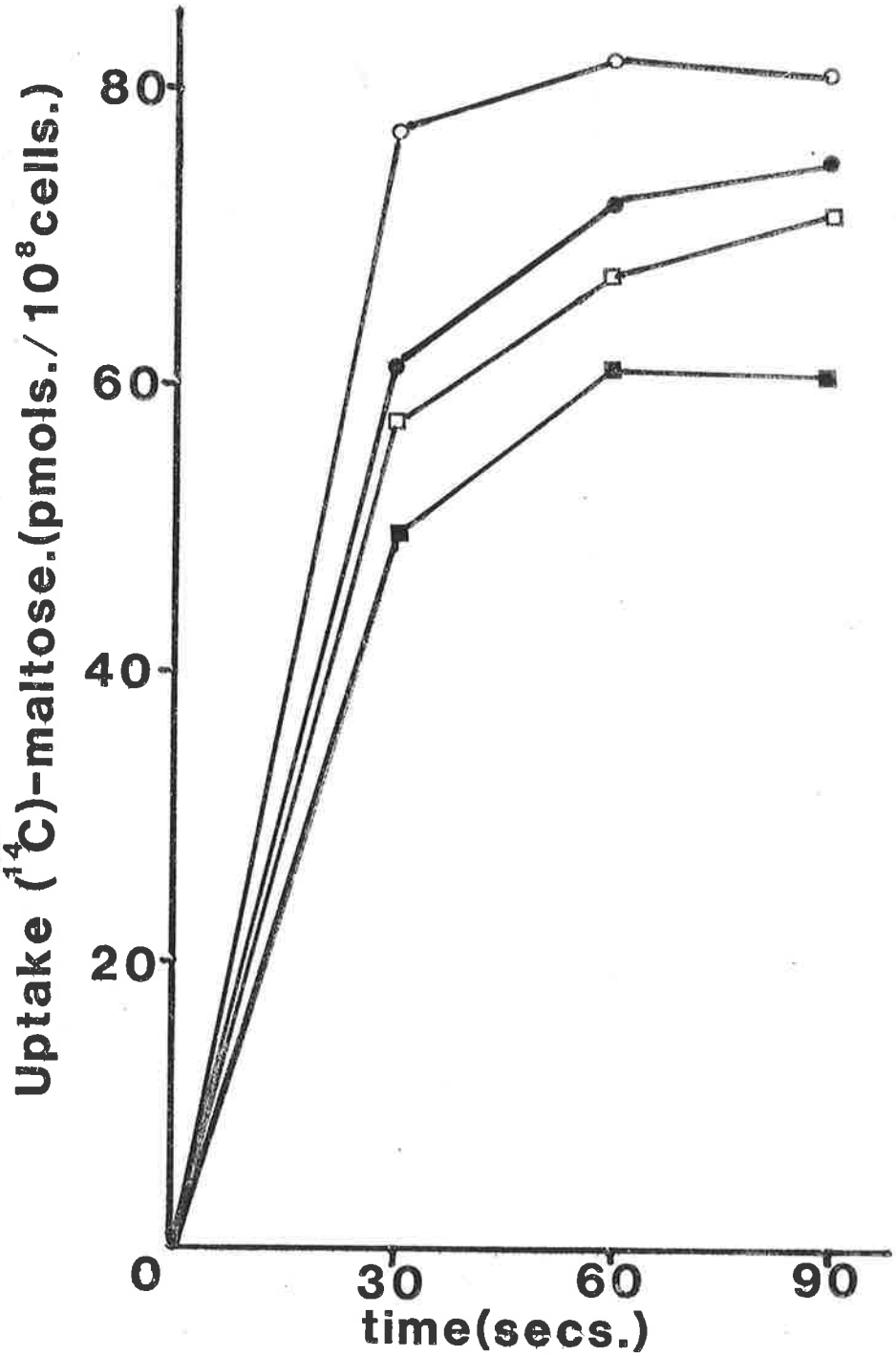

Uptake(^{14}C)-glucose(pmols./10^8cells.)
30
20
10
0
15
30
45
time(secs.)

FIGURE 7.9

Uptake of (^{14}C) maltose in 10mM HEPES buffer and 5mM $MgCl_2$, pH7.5 at 37°C, with a final concentration of 2.7µM in the uptake vessel. P2343, ■ ; P2342, ● ; P2684, □ ; P2691, ○ .


Uptake (^{14}C)-maltose.(pmols./10^8cells.)
80
60
40
20
0
30
60
90
time(secs.)

they are unable to transport maltose. It is apparent that *ompA* does not result in a general decrease in maltose transport, and indeed the *ompA* mutation results in somewhat enhanced levels of maltose uptake in P2684 and P2691 over their parental strains P2343 and P2342 respectively.

7.5 SUMMARY AND CONCLUSIONS

The *ompA* mutation results in general decline in the uptake of phenylalanine arginine and glucose in the P400 series of strains. The decline in the uptakes of these substrates is possibly due to an interaction of the *ompA* mutation with the OmpC and OmpF proteins. If the transport of maltose by its specific uptake system is examined no such decline is observed in either the P400 series of strains or those strains derived in a $malT^{c}$ background. When the ability of the LamB protein to act as a general pore is examined in *ompA* mutants it is found that for the two substrates tested, lactose and glucose; no decline in uptake is observed. Indeed a slight enhancement occurs. The *ompA* mutation would appear to affect the pore properties of the OmpC, OmpF and LamB proteins in such a way as to modify their ability to act as porins.

CHAPTER 8

DISCUSSION

8.1 INTRODUCTION

The major theme of this thesis has been the mechanism of specificity of the "specific" outer membrane transport systems of *E. coli*. The outer membrane possesses specific transport systems for a variety of substrates. These can be divided into two categories: Firstly there are those which involve a protein that strongly binds the substrate and for which bacteriophage and substrate competition for the common receptor can be demonstrated. Examples include the transport systems for iron complexes and vitamin B_{12}. The second class comprises those systems which are thought to act as more or less passive pores for the diffusion of the substrate molecules. These include the LamB protein which is a clearly documented example. The Tsx protein is a possibility since it shares features with the LamB protein which is discussed in chapter 5. The PhoE protein is known to be a porin and is part of the phosphate uptake system, and is probably a phosphate specific pore.

In this thesis the mechanism of specificity of the LamB protein is examined in detail. The Tsx and PhoE proteins are also examined and their similarity to the LamB system compared.

8.2 BINDING PROTEINS AND SPECIFICITY OF OUTER MEMBRANE PORES

It had been considered for some time that the

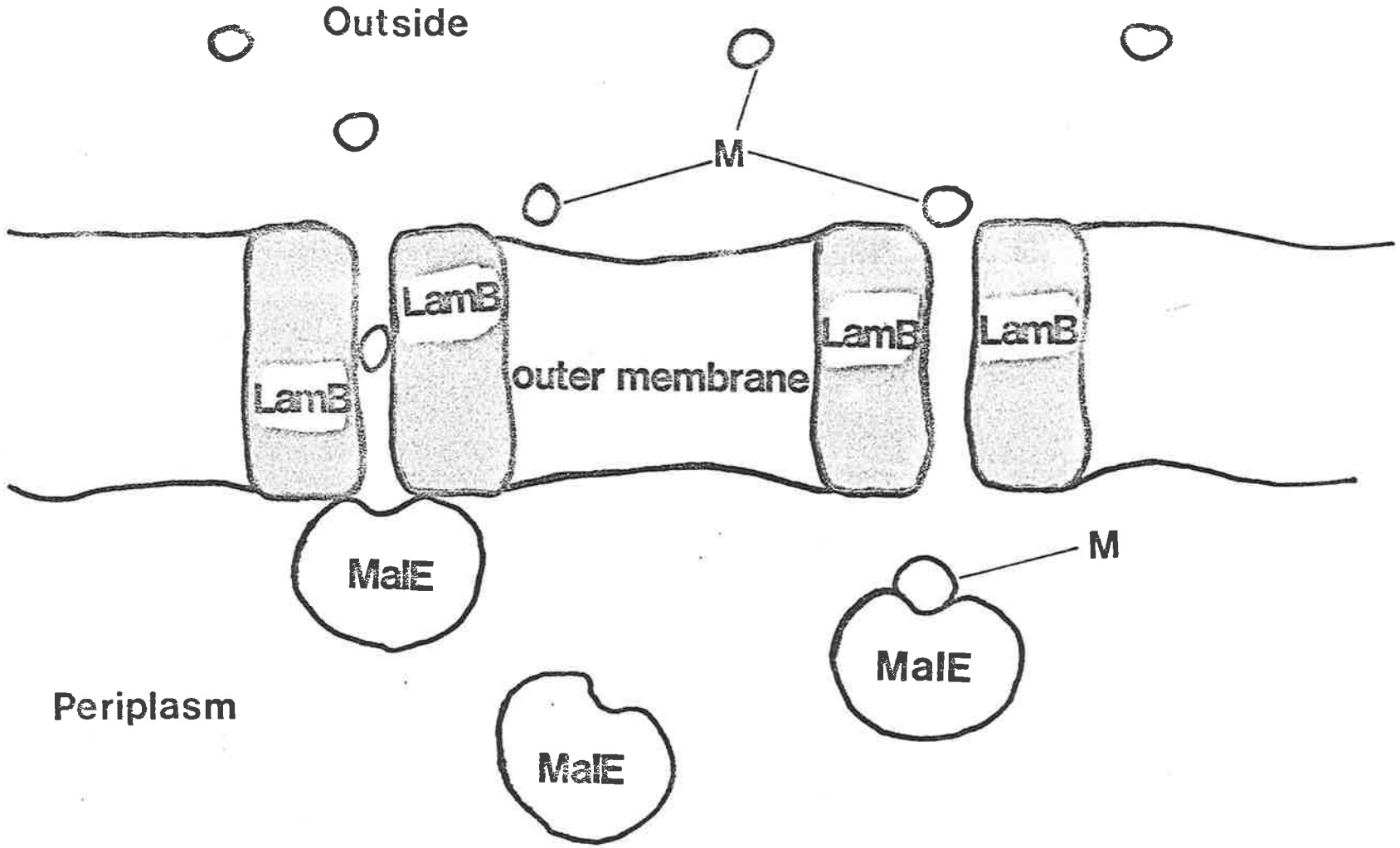
LamB protein has little or no affinity for maltose (Szmelcman *et al.*, 1976); however the maltose/ maltodextrin uptake system, which is described in detail in chapter 1 possesses a highly specific periplasmic maltose binding protein (MalE protein) which binds very strongly to its substrates. It is postulated in this thesis that association of this highly specific MalE protein with the outer membrane LamB protein, which is known to be a porin, confers specificity upon an otherwise non specific pore (see figure 8.1).

This hypothesis was tested in chapter 3. The rationale behind the experiments is that the association of the MalE protein with the LamB protein might impede the passage of molecules other than maltose and maltodextrins through the pore of the LamB protein, while the MalE protein is still available for through the pore binding of its substrate. In the absence of the MalE protein it would be expected that the LamB protein would be a non specific pore similar to the OmpC and OmpF proteins.

It was found that in an *ompB* mutant, when the LamB protein was substituting for the major porins, this alleviating effect of the LamB protein (Von Meyenberg and Nikaido, 1977) could be enhanced by the introduction of a *malE* mutation. This enhancing effect was, as expected, dependent upon the presence of the LamB protein, and could be demonstrated for a wide range of substrates; lactose, mannitol, adenosine,

Figure 8.1

Model for interaction between the LamB protein in the outer membrane and the maltose binding protein (MalE protein) in the periplasm. The MalE protein associates with the LamB pore protein, so as to "gate" the pore but dissociates after binding maltose/maltodextrin (M).


Outside
M
LamB
LamB
outer membrane
LamB
LamB
MalE
M
MalE
Periplasm
MalE

thymidine and some tri-peptides. The conclusion was that removal of the malE protein resulted in an opening or "un-gating" of the LamB pore, resulting in enhanced transport of these molecules.

The genetic evidence of Wandersman *et al.*, (1979), which appeared while this work was in progress, supports the idea of a MalE-LamB association. These workers were able to construct *malE* mutants which are still capable of transporting maltose, but unable to transport maltodextrins. These mutants thus express a Mal^+ Dex^- phenotype. It was concluded that the Dex^- phenotype is due to a mutation which results in a lack of MalE/LamB association. The isolated mutant MalE proteins still retain affinity for maltodextrins.

It was also recently shown by Ferenci and co-workers (Ferenci, 1980 and Ferenci *et al.*, 1980) that the LamB protein does in fact have an affinity for maltodextrins but a very much lower affinity for maltose. The binding capacity of the LamB protein increases with the molecular weight of the molecule. Luckey and Nikaido (1980) were able to demonstrate that the LamB protein is a pore with some specificity for maltose in an *in vitro* vesicle environment.

It is now possible to put forward an overall hypothesis for the mechanism of maltose/maltodextrin transport through the outer membrane. The LamB protein itself has an affinity for maltodextrins, and therefore such molecules would bind to the pore and undergo an

initial "selection" process; this would retain the molecule in the vicinity of the pore, thus facilitating entry of part of the molecule into the pore. The molecules after having passed into the body of the pore would then contact the MalE protein which presumably associates with the pore such that the active site is available to the maltodextrin molecule within the pore. The maltodextrin molecule would be held loosely by the LamB protein, but bind strongly to the MalE protein: it could then be "pulled" into the periplasmic space by diffusion of the MalE protein following its release from the MalE/LamB association by an allosteric conformational change by one or both proteins. The molecule is then available to the cytoplasmic membrane permease.

In addition to those molecules whose transport is enhanced by the *malE* mutation, there also exists a class of substrates which are not affected by the absence of the MalE protein. It might be that these molecules are small enough to "squeeze" through the LamB pore, even when gated by the MalE protein. The observed inability of the MalE protein to totally exclude lactose etc. or to detectably exclude molecules such as glycerol, glucose etc. could be explained by this "imperfect gate" hypothesis, alternatively one could postulate that a number of the LamB pores can be "un-gated" at any given time in a wild-type ($malE^+$) strain: this number of un-gated

pores may be sufficient to allow diffusion of these molecules at a great enough rate to mask any alleviating effect of the *malE* mutation.

8.3 DO OTHER "GATED" UPTAKE SYSTEMS EXIST?

The Tsx protein is involved in nucleoside permeation of the outer membrane. It has been shown that nucleoside permeation depends both upon the nucleoside involved and the genetic background of the strain employed (Hantke, 1976; Munch-Petersen *et al.*, 1979); Krieger-Brauer and Braun, 1980). The data in chapter 5 show that the major porin also plays a significant role in nucleoside permeation, and again this effect is dependent upon the substrate and the strain used.

Although the Tsx protein is known to facilitate the transport of nucleosides across the outer membrane, it was not known whether the Tsx protein acts as a porin, like the LamB protein, even though as we have seen previously the two systems share some similar properties.

Chapter 5 presents *in vivo* evidence suggesting that the Tsx protein is a porin, like LamB, and in addition is capable of mediating transport of molecules other than nucleosides. The ability of LamB protein to alleviate an *ompB* defect was first demonstrated by von Meyenberg and Nikaido (1977). Chapter 5 describes experiments similar to that conducted by these workers using strains in which the Tsx protein is a major outer

membrane protein. It was found that the presence of Tsx protein in the outer membrane alleviated the transport defects of an *ompB* mutant for serine, glycine and phenylalanine and for at least glycine and serine this effect could be demonstrated in two separate genetic backgrounds. This broad specificity suggests that like LamB, *tsx* encodes an open pore with some superimposed specificity. However, the Tsx protein had no effect upon transport of either arginine or glucose, and this again resembles LamB, which also shows preference for some substrates (Von Meyenberg and Nikaido, 1976; Luckey and Nikaido, 1980). The question remains however, what confers specificity upon the Tsx pore? The fact that the Tsx protein shows some selectivity in ability to transport nucleosides was already known (Hantke, 1976; Munch-Petersen, 1979 and Krieger-Brauer and Braun, 1980); a further dimension is now added in that Tsx protein affects the transport of molecules other than nucleosides and shows some selectivity in this process. The specificity of the LamB pore is conferred partly by the pore itself, and partly by the periplasmic binding (MalE) protein as seen from data from this thesis and that of other workers. It is not known whether the nucleoside uptake system possesses a binding protein, although a little circumstantial evidence exists to suggest that it does: Munch-Petersen *et al.*, 1979, were able to demonstrate that nucleoside transport could be impaired by osmotic

shock and this characteristic is usually associated with uptake systems which are dependent upon periplasmic binding proteins. If the nucleoside uptake system does involve a binding protein, then it is possible that the Tsx pores are "gated" in much the same way as the MalE protein "gates" the LamB pore, and if so then the substrates tested in chapter 5 must enter by one of the mechanisms discussed previously for the LamB protein.

It is possible that when the nucleoside uptake system is more thoroughly studied, removal of a binding protein (if it exists) may result in enhancement of the transport of molecules other than nucleosides. Alternatively, if no binding protein exists then specificity of the pore is conferred entirely by the internal properties of the Tsx protein itself.

Chapter 6 deals with another uptake system, involving phosphates. It is not known whether the PhoE protein, which is co-regulated with the phosphate uptake system, is a specific pore for phosphates, although it is highly likely that it is, considering that like the Tsx and LamB proteins, it is an outer membrane protein co-regulated as part of a specific uptake system. In this case the system is known to possess a periplasmic binding protein, the PhoS protein. This system offered the opportunity to examine the PhoE protein and its relationship, if any, with the PhoS protein in conferring specificity upon the pore. If the PhoE protein is analagous to the LamB protein,

it would be expected that in *ompB* mutants, a strain lacking the PhoS protein would transport a variety of substrates at an enhanced level when compared to an *ompB*, *phoS*$^+$ strain. Chapter 6 examines the uptake characteristics of the two strains, both *ompB* and producing the PhoE protein with the PhoS protein present or absent. It was apparent that for the substrates tested; lactose, glucose, galactose, mannitol and glycerol, the lack of PhoS protein does not detectably influence the alleviating effect of the PhoE protein on the *ompB* mutation. It is possible that the PhoS protein may exist as an imperfect "gate", if an association between PhoS and PhoE occurs.

8.4 ASSOCIATION OF THE MalE PROTEIN WITH CELL WALL COMPONENTS

Chapter 4 deals with an attempt to demonstrate a direct physical association of the MalE and the LamB proteins in a semi-*in vivo* system using whole cells which are subjected to osmotic shock under various conditions. During the course of these experiments, it was not possible to demonstrate a direct association of the MalE protein with LamB, although the data indicated that the LamB protein exists in two locations within the periplasm.

The peptidoglycan is, as discussed in chapter 1 a major structure within the periplasmic space and it has been shown that some outer membrane proteins,

including the LamB protein, are associated with the peptidoglycan (Enderman *et al.*, 1979); it is therefore possible to speculate that the binding proteins, which are thought to reside within the periplasm may be associated with this structure.

The first evidence for such an association is from Bewick and Lo (1979). If the binding proteins are attached to the peptidoglycan, disruption of the outer membrane by convential osmotic shock procedure would not necessarily dissociate these proteins efficiently; however treatment with lysozyme should result in release of proteins bound to peptidoglycan. The dicarboxylate binding protein is released in substantial amounts when subjected to standard osmotic shock; however, a further 60% is released upon treatment with lysozyme, suggesting at least 60% of the binding protein may be associated with the peptidoglycan.

Similar evidence for the existence of two populations of MalE protein, i.e. peptidoglycan associated and "free", is presented in chapter 4. The EDTA-osmotic shock treatment alone resulted in the release of only a limited number of detectable protein species, among these the MalE protein, however with addition of lysozyme to the EDTA-osmotic shock procedure a great deal of MalE and other proteins are released, presumably periplasmic since the pattern does not relate to either membrane or cytoplasm. The simple explanation is that MalE protein exists in two forms

as postulated. It is also possible to explain the results by maintaining that lysozyme treatment results in a more efficient disruption of the outer membrane and subsequent release of periplasmic proteins. Additional evidence that does not rely on the rather harsh treatment of EDTA osmotic shock also suggests that there are two populations of MalE protein. It is now quite well documented that treatment of cells with tris-buffer results in the release of many periplasmic proteins (Brass *et al.*, 1981; Irvin *et al.*, (a) 1981, and Irvin *et al.*, (b) 1981). The treatment of both *lamB* and *lamB*$^+$ cells with tris-buffer resulted in the release of a wide variety of proteins: the MalE protein appeared to be a major protein species released by this treatment, in both *lamB* and *lamB*$^+$ cells, as detected by polyacrylamide gel electrophoresis or specific anti-sera to MalE protein. Again there was no evidence to suggest a lesser release from cells carrying the LamB protein. The amount of MalE protein released by tris treatment is equivalent to about 2-5% of the total MalE protein: further treatment of these cells with EDTA results in the release of much larger amounts of MalE protein. It could be that we are observing the release of "free" rather than "bound" MalE protein, which seems to exist in larger amounts.

Brass *et al.*, (1981) on the basis of very similar experiments, speculate that MalE protein exists in two populations. However we find no effect

of the presence or absence of MalE protein: if the experiments indicate two populations of MalE proteins, it appears that they do not result from association or not with the LamB protein. Brass *et al.*, (1981) speculate that their results may indicate a surface location of MalE protein, however election microscope studies argue for a periplasmic location of the protein (Boos and Staehelin, 1981, personal communication). The idea of surface location of binding proteins has been discussed extensively by Lo (1979), who suggests the work of Medveczky and Rosenberg (1970) concerning reconstitution of phosphate transport represents the earliest evidence for this idea. Brass *et al.*, suggests that MalE protein if surface located probably associates with LPS. Bewick and Lo (1979) have evidence to suggest that the "free" form of the dicarboxylic binding protein is LPS associated. In LPS specifically labelled with (^{3}H) galactose in *galE* mutants (Elbein and Heath, 1965; Levy and Leive, 1968) the "free" form of the binding protein is found almost exclusively with the labelled LPS.

The question arises: if the MalE protein associates with the LamB protein as described in figure 8.1 and exists in two populations as postulated, why is it not possible to demonstrate this association physically, given the biochemical and genetic evidence in favour of the hypothesis? A number of reasons can be put forward to answer this question: If it is

assumed that it is only the free form of the MalE protein that associates with LamB, it is apparent from the data presented in this thesis, and by Brass *et al.*, that the free form of the MalE only represents around 10% or less of total MalE protein. It is possible not all of these proteins associate with LamB and that the association is very weak, and the minority of associated proteins may be impossible to detect using these methods. It may be that "free" MalE protein is LPS associated, this association may also be LamB dependent, but the treatments designed to liberate MalE protein also disrupt LPS. The treatment with EDTA may also so modify the periplasmic environment so that no direct association can be demonstrated. It may therefore be necessary to preserve the periplasmic environment as much as possible. It is possible to postulate that MalE protein exists in both "bound" and "free" forms. It is tempting to speculate that in addition to the association of MalE with LamB that the bound and un-bound forms of MalE protein may also interact during maltodextrin transport.

8.5 THE EFFECT OF OmpA PROTEIN ON PORE FORMERS

It has been discussed in the introduction that the OmpA protein does not act as a pore (Nakae, 1976; Bavoil *et al.*, 1977). It is known that lack of OmpA protein results in reduced uptake of *tonA* dependent uptake of iron as ferrichrome complex (Coulton and Braun, 1979). Mutants lacking major porins in this

system were not affected in the uptake of ferrichrome. It is possible that the OmpA protein physically interacts with the TonA protein. There is other evidence that the OmpA protein affects uptake systems. Manning *et al.*, (1977) present evidence that *ompA* mutants are reduced in the uptake of glutamine and proline. These results have been challenged, since the substrate concentrations employed are above the level for which the outer membrane acts as a permeability barrier. Krieger-Brauer and Braun (1980) have shown that *tsx* dependent uptake of thymidine and adenosine is reduced 16-33% in *ompA* mutants.

Chapter 7 presents evidence suggesting that OmpA protein does indeed interact with the major porins, the OmpC and OmpF proteins to cause a decline in the uptake ability of a number of substrates; phenylalanine, arginine and glucose. However, this effect is not as marked as that of an *ompB* mutant. It was also observed that an altered protein *ompA* mutant did not exhibit these transport effects to the same extent as strains lacking detectable OmpA protein. When the effect of *ompA* mutations was examined for the transport of maltose, rather than a decline a slight enhancement was observed.

This effect was also seen when examining the diffusion of lactose and glucose through the LamB pore when this protein is substituting for major porin in *ompB* mutants.

The evidence suggests that OmpA protein possibly physically interacts with TonA, Tsx, OmpC, OmpF and LamB proteins in such a way as to modify their pore forming abilities: in most cases the absence of the OmpA protein causes a decline in substrate transport, but in the case of LamB, there is no detectable effect or a slight increase occurs.

8.6 NON-PROTEIN INTERACTIONS WITH PhoE PROTEIN

Chapter 6 describes mutants which are resistant to bacteriophage TC45 which uses PhoE as part of its receptor. It has been shown for several bacteriophages, including TC45, which use major outer membrane proteins as receptors, that they have an *in vitro* requirement for LPS in addition to the specific protein (Foulds and Chai, 1978; Hantke *et al.*, 1978 etc.); however phage TC45 is unique in that it readily and directly selects LPS mutants. While several outer membrane proteins interact with LPS, it was not possible to select phage resistant LPS mutants using either T6 or K3 bacteriophage (Manning *et al.*, 1976; Manning and Reeves, 1978). The severity of the LPS lesion in the strains appears to be related to the amount of PhoE protein in the membrane. While this work was in progress Tommassen and Lugtenberg (personal communication) showed that three classes of mutants could be selected that are resistant to phage TC45; mapping at *phoB*, *phoE* and altered LPS mutants. The strains we have isolated appear to belong to this class

of LPS mutants, which result in a decrease in the amount of PhoE protein in the outer membrane.

8.7 CONCLUDING REMARKS

In this thesis it has been shown the Tsx protein affects the uptake of substrates unrelated to its specific substrates, and in this it resembles the LamB and PhoE proteins, and it appears that all three are open water filled pores in the outer membrane.

In the case of LamB it is shown in this thesis that its specificity as a pore derives in part from an association with the MalE binding protein. However in the case of the PhoE protein which is part of an uptake system involving a binding protein, no evidence could be found for an association of binding protein and the porin. It also seems likely that binding proteins, such as MalE protein exist in two forms; bound to the peptidoglyan and "free". The significance of this is not clear.

The OmpA protein, a non-porin, seems to modify the porin activity of both major porin and the LamB protein, possibly by protein-protein interaction within the membrane. The LPS has also been shown to affect the amount of the PhoE porin in the outer membrane.

APPENDIX

Material from this thesis has been accepted or submitted for publication in the following papers:

1. Heuzenroeder, M.W. and Reeves, P. 1980. Periplasmic maltose binding protein confers specificity on the outer membrane maltose pore of *Escherichia coli*. J. Bacteriol. 141: 431-435.

2. Heuzenroeder, M.W. and Reeves, P. 1981. The *tsx* protein of *Escherichia coli* can act as a pore for amino-acids. J. Bacteriol. (in press September 1981).

3. Heuzenroeder, M.W., Manning, P.A., and Reeves, P. 1981. Bacteriophage TC45 which uses PhoE protein as a receptor readily selects lipopolysaccharide mutants in *phoS* and *phoT* mutants of *Escherichia coli* K12. Submitted for publication.

BIBLIOGRAPHY

BIBLIOGRAPHY

Achtman, M., Schwuchow, S., Helmuth, R., Morelli, R., and Manning, P.A., 1978. Cell-cell interactions in conjugating *Escherichia coli: Con*⁻ mutants and stabilization of mating aggregates. Molec. Gen. Genetic. 164: 171-183.

Argast, M., and Boos, W. 1980. Co-regulation in *Escherichia coli* of a novel transport system for sn-glycerol-3-phosphate and the outer membrane protein Ic(e,E) with alkaline phosphatase and the phosphate binding protein. J. Bacteriol. 143: 142-150.

Bachmann, B.J., and Low, K.B. 1980. Linkage map of *Escherichia coli* K12, edition 6. Microbiol. Rev. 44: 1-56.

Bavoil, P., Nikaido, H., and von Meyenberg, K. 1977. Pleiotropic transport mutants of *Escherichia coli* lack porin a major outer membrane protein. Mol. Gen. Genet. 158: 23-33.

Bavoil, P., Hofnung, M., and Nikaido, H. 1980. Identification of a cytoplasmic membrane associated component of the maltose transport system of *Escherichia coli*. J. Biol. Chem. 255: 8366-8369.

Bassford, P.J. Bradbeer, C., Kadner, R.J. and Schnaitman, C.A. 1976. Transport of vitamin B12 in *tonB* mutants of *Escherichia coli*. J. Bacteriol. 128: 242-7.

Bassford, P.J., Diedrich, D.L., Schnaitman, C.A. and Reeves, P. 1977. Outer membrane proteins of *Escherichia coli VI*. Protein alteration in bacteriophage-resistant mutants. J. Bacteriol. 131: 608-622.

Beacham, I.R., Kahana, R., Levy, L., and Yagil, E. 1973. Mutants of *Escherichia coli* K12 'cryptic' or deficient in 5'-nucleotidase (uridine diphosphate sugar hydrolase) and 3'-nucleotidase (cyclic phosphodiesterase) activity. J. Bacteriol. 116: 957-964.

Beacham, I.R., Haas, D., and Yagil, E. 1977. Mutants of *Escherichia coli* 'cryptic' for certain periplasmic enzymes: evidence for an alteration of the outer membrane. J. Bacteriol. 129: 1034-1044.

Benz, R., Janko, K., and Läuger, P. 1979. Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. Biochim. Biophys. Acta. 551: 238-247.

Bewick, M.A., and Lo, T.C.Y. 1979. Dicarboxylic acid in *Escherichia coli* K12: Involvement of a binding protein in the translocation of dicarboxylic acids across the outer membrane of the cell envelope. Can. J. Biochem. 57: 653-661.

Birdsell, D.C. and Cota-Robles, E.H. 1967. Production and ultrastructure of lysozyme and EDTA-lysozyme spheroplasts of *Escherichia coli*. J. Bacteriol. 93: 427-437.

Boehler-Kohler, B.A., Boos, W., Dieterle, D., and Benz, R. 1979. Receptor for bacteriophage lambda of *Escherichia coli* forms larger pores in black lipid membranes than matrix protein (porin). J. Bacteriol. 138: 33-39.

Boman, H.G. and Monner, D.A. 1975. Characterization of lipopolysaccharide mutants from *Escherichia coli* K12 mutants. J. Bacteriol. 121: 455-464.

Bradbeer, C., Woodrow, M.L. and Khalifah, L.I. 1976. Transport of vitamin B12 in *Escherichia coli:* a common receptor system for vitamin B12 and bacteriophage BF23 on the outer membrane of the cell envelope. J. Bacteriol. 125: 1032-1039.

Bradbeer, C., and Woodrow, M.L., 1976. Transport of vitamin B12 in *Escherichia coli:* energy dependence. J. Bacteriol. 128: 99-104.

Brass, J.M., Boos, W., and Hengge, R. 1981. Reconstitution of maltose transport in *malB* mutants of *Escherichia coli* through calcium induced disruptions of the outer membrane. J. Bacteriol. (In press).

Braun, V. 1975. Covalent lipoprotein from the outer membrane of *Escherichia coli* K12. Biochim. Biophys. Acta. 415: 335-377.

Braun, V. 1978. Structure-Function relationships of gram-negative bacterial envelope. From Symposia of the Society for General Microbiology No. XXVIII. Relations between Structure and Function in the Prokaryotic cell.

Braun-Breton, C., and Hofnung, M. 1977. Genetic evidence for the existence of a region of the λ receptor involved in the maintenance of this protein in the outer membrane of *Escherichia coli* K12. FEMS. Microbiol. Lett. 1: 371-374.

Chai, T. and Foulds, J., 1978. Two bacteriophages which utilize a new *Escherichia coli* major outer membrane protein as part of the receptor. J. Bacteriol. 135: 164-170.

Chen, R., Schmidmayer, W., Krämer, C., Chen-Schmeisser, U., and Henning, V., 1980. Primary structure of major outer membrane protein II* (ompA protein) of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. 77: 4592-4596.

Coleman, W.G., and Leive, L., 1979. Two mutations which affect the barrier function of *Escherichia coli* K12 outer membrane. J. Bacteriol. 139: 899-910.

Costerton, J.W., Ingram, J.M. and Cheng, K.J., 1974. Structure and function of the cell envelope of gram negative bacteria. Bacteriol. Revs. 38: 87-110.

Coulton, J.W., and Braun, V., 1979. Protein II* influences ferrichrome-iron transport in *Escherichia coli* K12. J. Gen. Microbiol. 110: 211-220.

Cox, G.B., Gibson, F., Luke, R.K.J., Newton, N.A., O'Brien, I.G., and Rosenberg, H., 1970. Mutations affecting iron transport in *Escherichia coli*. J. Bacteriol. 104: 219-226.

Datta, D.B., Krämer, C., and Henning, V., 1976. Diploidy for a structural gene specifying a major outer membrane protein of the outer cell envelope membrane from *Escherichia coli* K12. J. Bacteriol. 128: 834-841.

Davies, J.K., 1974. Cross resistance to colicins. Ph.D. thesis. University of Adelaide.

Davies, J.K., and Reeves, P., 1975. Genetics of resistance to colicins in *Escherichia coli* K12: cross-resistance among colicins of group B. J. Bacteriol. 123: 97-101.

Davis, B.D., and Mingioli, E.S., 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. 60: 17-28.

Decad, G.M., and Nikaido, H., 1976. Outer membrane of gram negative bacteria XII. Molecular-sieving functions of the cell wall. J. Bacteriol. 128: 325-336.

De Felice, M., Guardiola, J., Lamberti, A., and Iaccarino, M., 1973. *Escherichia coli* K12 mutants altered in transport systems for oligo- and dipeptides. J. Bacteriol. 116: 751-756.

Di Masi, D.R., White, J.C., Schnaitman, C.A., and Bradbeer, C., 1973. Transport of vitamin B12 in *Escherichia coli*: common receptor for vitamin B12 and the E colicins on the outer membrane of the cell envelope. J. Bacteriol. 115: 506-513.

Earhart, C.F., Lundrigan, M., Pickett, C.L., and Pierce, J.R., 1979. *Escherichia coli* K12 mutants that lack major outer membrane protein *a*. FEMS Microbiol. Lett. 6: 277-280.

Elbein, A.D., and Heath, E.C., 1965. The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. The biochemical properties of a uridine diphosphate galactose 4-epimeraseless mutant. J. Biol. Chem. 240: 1919-1925.

Enderman, R. and Henning, U., 1979. Nearest neighbours of major proteins in the outer membrane of *Escherichia coli* K12. FEBS. Lett. 97: 339-342.

Enderman, R., Krämer, C., and Henning, U., 1978. Major outer membrane proteins of *Escherichia coli* K12: Evidence for protein II* being a transmembrane protein. FEBS. Lett. 86: 21-24.

Fairbanks, G., Steck, T.L. and Wallach, F.D.H., 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry. 10: 2606-2617.

Ferenci, T., 1980. Recognition of maltodextrins by *Escherichia coli*. Eur. J. Biochem. (In press).

Ferenci, T., Boos, W., Schwartz, M., and Szmelcman, S., 1977. Energy coupling of the transport system of *Escherichia coli* dependent upon maltose binding protein. Eur. J. Biochem. 75: 187-193.

Ferenci, T., and Klotz, U. 1978. Affinity chromatographic isolation of the periplasmic maltose binding protein of *Escherichia coli*. FEBS. Lett. 94: 213-217.

Ferenci, T., Schwentorat, M., Ullrich, S., and Vilmart, J., 1980. The lambda receptor in the outer membrane of *Escherichia coli* as a binding protein for maltodextrins and starch polysaccharides. J. Bacteriol. 142: 521-526.

Fiss, E.H., Hollifield, W.C., and Neilands, J.B. 1979. Absence of ferric interobactin receptor modification activity in mutants of *Escherichia coli* K12 lacking protein a. Biochim. Biophys. Res. Commun. 9: 29-34.

Foulds, J. 1972. Purification and partial characterization of a bacteriocin from *Serratia marcescens*. J. Bacteriol. 110: 1001-1009.

Foulds, J. 1974. Chromosomal location of the *tolG* locus for tolerance to bacteriocin JF246 in *Escherichia coli* K12. J. Bacteriol. 117: 1354-1355.

Foulds, J. 1976 *tolF* locus in *Escherichia coli:* chromosomal location and relationship to loci *cmlB* and *tolD*. J. Bacteriol. 128: 604-608.

Foulds, J. and Chai, T. 1978a. New major outer membrane protein found in an *Escherichia coli tolF* mutant resistant to bacteriophage Tulb. J. Bacteriol. 133: 1478-1483.

Foulds, J. and Chai, T. 1978b. Chromosomal location of a gene (*nmpA*) involved in the expression of a major outer membrane protein in *Escherichia coli*. J. Bacteriol. 136: 501-506.

Fredericq, P. 1949. Sur la resistance croisee entre colicine E et bacteriophage II. Compt. rend. soc. biol. 143: 1011-1013.

Fredericq, P. 1951. Recherches sur l'origine des mutants de *Escherichia coli* V produisant la colicine M. Compt. rend. soc. biol. 145: 930-933.

Fredericq, P., and Smarda, J. 1970. Complexité du facteur colicinogène B. Ann. Inst. Pasteur (Paris) 118: 767-774.

Galanos, C., Luderitz, O., and Westphal, O., 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. 9: 245-249.

Gayda, R.C., Avni, H., Berg, P.E., and Markovitz, A. 1979. Outer membrane protein *a* and other polypeptides regulate capsular polysaccharide synthesis in *Escherichia coli* K12. Molec. Gen. Genet. 175: 325-332.

Gayda, R.C., and Markovitz, A., 1978. A cloned DNA fragment specifying major outer membrane protein a in *Escherichia coli* K12. J. Bacteriol. 136: 369-380.

Guterman, S.K., 1973. Colicin B: mode of action and inhibition by enterochelin. J. Bacteriol. 114: 1217-1224.

Hall, M.N., and Silhavy, T.J., 1979. Transcriptional regulation of *Escherichia coli* major outer membrane protein 1b. J. Bacteriol. 140: 342-350.

Hancock, R.E.W., 1974. Resistance to coliphages. Ph.D. thesis, University of Adelaide.

Hancock, R.E.W., Hantke, K., and Braun, V., 1976. Iron transport in *Escherichia coli* K12: involvement of the colicin B receptor and a citrate inducible protein. J. Bacteriol. 125: 409-415.

Hancock, R.E.W., Hantke, K., and Braun, V., 1977. Iron transport in *Escherichia coli* K12 2,3 dihydroxybenzoate-promoted iron uptake. Arch. Microbiol. 114: 231-239.

Hancock, R.E.W., and Braun, V., 1976. Nature of the energy requirement for the irreversible adsorption of bacteriophages T1 and Ø80 to *Escherichia coli*. J. Bacteriol. 125: 409-415.

Hancock, R.E.W., and Reeves, P., 1976. Lipopoly-saccharide deficient, bacteriophage resistant mutants of *Escherichia coli* K12. J. Bacteriol. 127: 98-108.

Hantke, K. 1976. Phage T6-colicin K receptor and nucleoside transport in *Escherichia coli*. FEBS. Lett. 70: 109-112.

Hantke, K. 1976. Major outer membrane proteins of *Escherichia coli* K12 serve as receptors for phages T2 (Protein 1a) and 434 (Protein 1b). Molec. Gen. Genet. 164: 131-135.

Hantke, K., and Braun, V. 1975. Membrane receptor dependent iron transport in *Escherichia coli*. FEBS. Lett. 49: 301-304.

Hasegawa, Y., Yamada, H., and Mizushima, S. 1976. Interactions of outer membrane proteins 0-8 and 0-9 with thepeptidoglycan sacculus of *Escherichia coli* K12. J. Biochem. 80: 1401-1409.

Havekes, L., Tommassen, J., Hoekstra, W., and Lugtenberg, B. 1977. Isolation and characterization of *Escherichia coli* K12 F⁻ mutants defective in conjugation with an I-type donor. J. Bacteriol. 129: 1-8.

Henning, U., and Haller, I. 1975. Mutants of *Escherichia coli* K12 lacking all major proteins of the outer cell envelope membrane. FEBS. Lett. 55: 161-164.

Henning, U., Hindenmach, I., and Haller, I. 1976. The major proteins of *Escherichia coli* outer cell envelope membrane: evidence for the structural gene of protein II*. FEBS. Lett. 61: 46-48.

Henning, U., Schmidmayer, W., and Hindennach, I. 1977. Major proteins of the outer cell envelope membrane of *Escherichia coli* K12: multiple species of protein I. Mol. Gen. Gent. 154: 293-298.

Hirota, Y., Suzuki, H., Nishimura, Y., and Yasuda, S. 1977. On the process of cellular division in *Escherichia coli*: A mutant of *Escherichia coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. U.S.A. 74: 1417-1420.

Hogg, R.W., and Hermodsen, M.A. 1977. Amino-acid sequence of the L-arabinose binding protein from *Escherichia coli* B/r. J. Biol. Chem. 252: 5135-5141.

Hofnung, M. 1974. Divergent operons and genetic structure of the maltose B region in *Escherichia coli*. Genetics. 76: 169-184.

Hofnung, M., Hatfield, D., and Schwartz, M. 1974. *malB* region in *Escherichia coli* K12: characterization of new mutations. J. Bacteriol. 117: 40-47.

Hofnung, M., Jezierska, A., and Braun-Breton, C. 1976. *lamB* mutations in *Escherichia coli* K12. Growth of lambda host range mutants and effects of nonsense suppressors. Mol. Gen. Genet. 145: 207-213.

Hofnung, M., Schwartz, M., and Hatfield, D. 1971. Complementation studies in the maltose A region of *Escherichia coli* K12 genetic map. J. Mol. Biol. 61: 681-694.

Hollifield, W.C., and Neilands, J.B. 1978. Ferric enterobactin transport system in *Escherichia coli* K12. Extraction, assay, and specificity of the outer membrane receptor. Biochemistry 17: 1922-1928.

Ichihara, S., and Mizushima, S., 1978. Identification of membrane protein responsible for the binding of the Fe-enterochelin complex to *Escherichia coli* cells. J. Biochem. 83: 137-140.

Ichihara, S., and Mizushima, S., 1978. Characterization of major outer membrane proteins O8 and O9 of *Escherichia coli* K12. Evidence that the structural genes for the two proteins are different. J. Biochem. (Japan) 83; 1095-1100.

Inouye, H., and Beckwith, J. 1977. Synthesis and processing of an *Escherichia coli* alkaline phosphatase precursor *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 71: 2396-2400.

Inouye, M., Shaw, J., and Shen, C. 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. 246: 8154-8159.

Irvin, R.T., MacAlister, T.J. and Costerton, J.W. 1981. Tris(hydroxymethyl) aminomethane Buffer Modification of *Escherichia coli* outer membrane permeability. J. Bacteriol. 145: 1397-1403.

Irvin, R.T., MacAlister, T.J., Chan, R., and Costerton, J.W. 1981. Citrate-Tris (hydroxymethyl) aminomethane-Mediated of Outer Membrane Sections from the Cell Envelope of a deep-rough (Heptose-Deficient Lipopoly-saccharide) strain of *Escherichia coli* 08. J. Bacteriol. 145: 1386-1396.

Ito, K., Sato, T., and Yura, T. 1977. Synthesis and assembly of membrane proteins of *Escherichia coli*. Cell 11: 551-559.

Kamio, Y., and Nikaido, H. 1976. Outer membrane of *Salmonella typhimurium:* accessibility of phospholipid head groups to phospholipase C and cyanogen bromide activated dextran in the external medium. Biochemistry. 15: 2561-70.

Kellerman, O., and Szmelcman, S. 1974. Active transport of maltose in *Escherichia coli* K12: Involvement of a periplasmic maltose binding protein. Eur. J. Biochem. 47: 139-149.

Kleckner, N., Roth, J., and Botstein, D. 1977. Genetic engineering *in vitro* using translocatable drug resistance elements. J. Mol. Biol. 116: 125-129.

Krieger-Brauer, H.J., and Braun, V. 1980. Functions Related to the Receptor Protein Specified by the *tsx* Gene of *Escherichia coli*. Arch. Microbiol. 124: 233-242.

Langman, L., Young, I.G., Frost, G.E., Rosenberg, H., and Gibson, F. 1972. Enterochelin system of iron transport in *Escherichia coli*.mutations affecting ferric-enterochelin esterase. J. Bacteriol. 112: 1142-1149.

Langridge, R., Shinagawa, H., and Pardee, A.B. 1970. Sulphate binding protein from *Salmonella typhimirium*: Physical Properties. Science 169: 59-61.

Lee, R.L., Schnaitman, C.A., and Pugsley, A.P. 1979. Chemical heterogeneity of the major outer membrane pore proteins of *Escherichia coli*. J. Bacteriol. 138: 861-870.

Levy, S.B., and Leive, L. 1968. An Equilibrium between two fractions of lipopolysaccharide in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 61: 1435-1439.

Lo, T.C.Y. 1979. The molecular mechanisms of substrate transport in gram-negative bacteria. Can. J. Biochem. 57: 289-301.

Luckey, M., and Nikaido, H. 1980. Specificity of the diffusion channels produced by the λ phage receptor protein of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 77: 167-171.

Luckey, M., Wayne, R., and Neilands, J.B. 1975. *In vitro* competition between ferrichrome and phage for the outer membrane T5 receptor complex of *Escherichia coli*. Biochem. Biophys. Res. Commun. 64: 687-693.

Lüderitz, O., Westphal, O., Staub, A.M., and Nikaido, H. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides. In Microbiol. Toxins IV: 145-233.

Lugtenberg, E.J.J., and Peters, R. 1976. Distribution of lipids in gytoplasmic and outer membranes of *Escherichia coli* K12. Biochim. Biophys. Acta. 441: 38-47.

Lugtenberg, B.J., Meijers, J., Peters, R., van der Hoek, P., and van Alphen, L. 1975. Electrophoretic resolution of the 'Major outer membrane protein' of *Escherichia coli* K12 into four bands. FEBS Letts. 58: 254-258.

Lugtenberg, B., Peters, R., Bernheimer, H., and Berendsen, W. 1976. Influence of cultural conditions and mutants on the composition of the outer membrane proteins of *Escherichia coli*. Mol. Gen. Genet. 147: 251-262.

Lugtenberg, B., van Boxtel, R., Verhoeff, C., and van Alphen, W. 1978. Pore protein e of the outer membrane of *Escherichia coli* K12. FEBS. Lett. 96: 99-105.

Manning, P.A. 1977. Characterization of conjugation defective mutants of *Escherichia coli* K12. Ph.D. thesis. University of Adelaide.

Manning, P.A., and Reeves, P. 1975. Recipient ability of bacteriophage resistant mutants of *Escherichia coli* K12. J. Bacteriol. 124: 575-577.

Manning, P.A., and Reeves, P. 1976(b). Outer membrane of *Escherichia coli* K12: *tsx* mutants (resistant to bacteriophage T6 and colicin K) lack an outer membrane protein. Biochem. Biophys. Res. Commun. 71: 446-471.

Manning, P.A., Pugsley, A.P., and Reeves, P. 1977(a). Defective growth functions in mutants of *Escherichia coli* K12 lacking a major outer membrane protein. J. Mol. Biol. 116: 285-300.

Manning, P.A. and Reeves, P. 1977. Outer membrane protein 3b of *Escherichia coli*: effect of growth temperature on the amount of the protein and further characterization on acrylamide gels. FEMS. Microbiol. Lett. 1: 275-278.

Manning, P.A., and Reeves, P. 1978. Outer membrane of *Escherichia coli* K12: isolation of a common receptor for bacteriophage T6 and colicin K. Molec. Gen. Genet. 158: 279-286.

Manning, P.A., Puspurs, A., and Reeves, P. 1976. Outer membrane of *Escherichia coli* K12: isolation of mutants with altered protein 3A by using host range mutants of bacteriophage K3. J. Bacteriol. 127: 1080-1084.

McKeown, M., Kahn, M., and Hanawalt, P. 1976. Thymidine uptake and utilization in *Escherichia coli*: a new gene controlling nucleoside transport. J. Bacteriol. 126: 814-822.

Medveczky, N., and Rosenberg, H. 1970. The phosphate binding protein of *Escherichia coli*. Biochim. Biophys. Acta. 211: 158-168.

Miller, J.H. 1972. "Experiments in Molecular Genetics:. Cold Spring Harbour Laboratories.

Mizushima, S. and Yamada, H. 1975. Isolation and characterization of two outer membrane preparations from *Escherichia coli*. Biochim. Biophys. Acta. 375: 44-53.

Movva, N.R., Nakamura, K., and Inouye, M. 1980. Regulatory region of the gene for the *ompA* protein, a major outer membrane protein of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 77: 3845-3849.

Mülhradt, P.F., and Golecki, J.R. 1975. Asymmetrical distribution and artifactual distribution of lipopolysaccharide on the bacterial cell surface. Eur. J. Biochem. 35: 471-481.

Mühlradt, P., Menzel, J., Golecki, J.R., and Speth, V. 1974. Lateral mobility and surface density of lipopolysaccharide in the outer membrane of *Salmonella typhimirium*. Eur. J. Biochem. 43: 533-9.

Munch-Petersen, A., Mygind, B., Nicolaisen, A., and Pihl, N.J., 1979. Nucleoside transport in cells and membrane resicles from *Escherichia coli* K12. J. Biol. Chem. 254: 3730-3737.

Mutoh, N., Nagasawa, T., and Mizushima, S. 1981. Specialized transducing bacteriophage Lambda carrying the structural gene for a major outer membrane matrix protein of *Escherichia coli* K12. J. Bacteriol. 145: 1085-1090.

Nakamura, K., Pirtle, R.M., and Inouye, M. 1979. Homology of the gene coding for outer membrane lipoprotein within various gram negative bacteria. J. Bacteriol. 137: 595-604.

Nakae, T. 1976a. Identification of the major outer membrane protein of *Escherichia coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochim. Biophys. Res. Commun. 71: 877-884.

Nakae, T. 1976b. Outer membrane of *Salmonella*. Isolation of protein complex that produces transmembrane channels. J. Biol. Chem. 251: 2176-2178.

Nakae, T. 1979. A porin activity of purified λ receptor protein from *Escherichia coli* in reconstituted membrane vesicles. Biochem. Biophys. Res. Commun. 71: 874-877.

Nikaido, H., Bavoil, P. and Hirota, Y. 1977. Outer membranes of gram negative bacteria *XV*. Transmembrane diffusion rates in lipoprotein deficient mutants of *Escherichia coli*. J. Bacteriol. 132: 1045-1047.

Osborn, M.J., Gander, J.E., Parisi, E., and Carson, J. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimirium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. 247: 3962-3972.

Ouchterlony, O. 1962. Diffusion-in-gel methods for Immunological analysis in Progress in Allergy. (Ed. Kallôs, P., and Waksman, B.H.) 6: 30.

Overbeeke, N., and Lugtenberg, B. 1980. Expression of outer membrane protein e of *Escherichia coli* K12 by phosphate limitation. FEBS. Lett. 112: 229-232.

Oxender, D.L., and Quay, S.C. 1976. Methods of Membrane Biology (Korn, E.D., ed.) pp. 183-242, Plenum Press, New York.

Pagès, J.M., Piovant, M., Varenne, S., and Lazdunski, C. 1978. Mechanistic aspects of the transfer of nascent periplasmic proteins across the cytoplasmic membrane in *Escherichia coli*. Eur. J. Biochem. 86: 589-602.

Picken, R.N., and I.R. Beacham. 1977. Bacteriophage resistant mutants of *Escherichia coli* K12. Localization of receptors within the lipopolysaccharide. J. Gen. Microbiol. 102: 305-318.

Pollack, J.R., and Neilands, J.B. 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. 38: 989-993.

Prehm, P., Stirm, S., Jann, B., Jann, K., and Boman, H.G. 1976. Cell wall lipopolysaccharides of ampicillin resistant mutants of *Escherichia coli* K12. Eur. J. Biochem. 66: 369-377.

Pugsley, A.P., and Reeves, P. 1976. Iron uptake in colicin B resistant mutants of *Escherichia coli*. J. Bacteriol. 126: 1052-1062.

Pugsley, A.P., and Reeves, P. 1977. The role of colicin receptors in the uptake of ferrienterochelin by *Escherichia coli* K12. Biochem. Biophys. Res. Commun. 74: 903-911.

Pugsley, A.P., Lee, D.R., and Schnaitman, C.A. 1980. Genes affecting the major outer membrane proteins of *Escherichia coli* K12: mutations at *nmpA* and *nmpB*. Mol. Gen. Genet. 117: 681-690.

Pugsley, A.P., and Schnaitman, C.A. 1978. Identification of three genes controlling production of new outer membrane pore proteins in *Escherichia coli* K12. J. Bacteriol. 135: 1118-1129.

Quiocho, F.A., Meador, W.E., and Pflugrath, J.W. 1979. Preliminary crystallographic data of receptors for transport and chemotaxis in *Escherichia coli:* D-Galactose and Maltose binding proteins. J. Mol. Biol. 133: 181-184.

Raibaud, O., Clément, J., and Hofnung, M. 1979. Structure of the *malB* region in *Escherichia coli* K12. III Correlation of the genetic map with the restriction map. Molec. Gen. Genet. 174: 261-267.

Raibaud, O., Roa, M., Braun-Breton, C., and Schwartz, M. 1979a. Structure of the *malB* region of *Escherichia coli* K12. I. Genetic map of the *malK-lamB* operon. Molec. Gen. Genet. 174: 241-248.

Randall-Hazelbauer, L., and Schwartz, M. 1973. Isolation of bacteriophage lambda receptor from *Escherichia coli*. J. Bacteriol. 116: 1436-1446.

Rapin, A.M.C., Kalckar, H.M., and Alberico, L. 1966. The metabolic basis for the masking of receptor sites on *Escherichia coli* K12 for C21, lipopolysaccharide core specific phage. Arch. Biochem. Biophys. 128: 95-105.

Rapin, A.M.C., and H.M. Kalckar, 1971. The relation of bacteriophage attachment to lipopoly-saccharide structure. Microbial Toxins IV (eds. Weinbaum, G., Kadis, S., and Ajl, S.J.), 267-307. Academic Press, N.Y. and London.

Reeves, P. 1979. Bacterial Outer Membranes, Biogenesis and functions. (Inouye, M., ed.) pp.253-291. J. Wiley, New York.

Reynolds, B.L., and Pruul, H. 1971. Protective role of smooth lipopolysaccharide in the serum bacteriocidal reaction. Infect. Immun. 4: 764-771.

Roa, M. 1979. Interaction of bacteriophage K10 with its receptor, the *lamB* protein of *Escherichia coli*. J. Bacteriol. 140: 680-686.

Rosenbusch, J.P. 1974. Characterization of the major envelope protein from *Escherichia coli*. J. Biol. Chem. 249: 8019-8029.

Rosen, B.P., and Heppel, L.A. Bacterial Membranes and Walls (Leive, L., ed.). pp.209-239. Marcel Dekker Inc. New York.

Sabet, S.F., and Schnaitman, C.A. 1971. Localization and solubilization of colicin receptors. J. Bacteriol. 108: 422-429.

Sabet, S.F., and Schnaitman, C.A. 1973. Purification and properties of the colicin E_3 receptor of *Escherichia coli*. J. Biol. Chem. 248: 1797-1806.

Sarma, V. 1978. Genetic and functional aspects of the outer membrane proteins of *Escherichia coli* K12. Ph.D. thesis. University of Adelaide.

Sarma, V., and Reeves, P. 1977. Genetic locus *(ompB)* affecting a major outer membrane protein in *Escherichia coli* K12. J. Bacteriol. 132: 23-27.

Sato, T., and Yura, T. 1979. Chromosomal location and expression of the structural gene for major outer membrane protein 1a of *Escherichia coli* K12 and of the homologous gene of *Salmonella typhimirium*. J. Bacteriol. 139: 468-477.

Scandella, C.J., and Kornberg, A. 1971. A membrane bound phospholipase A1 purified from *Escherichia coli*. Biochemistry 10: 4447-4456.

Schacterle, G.R., and Pollack, R.L. 1973. A simplified method for the quantitative assay of small amounts of protein in biological material. Anal. Biochem. 51: 654-655.

Schnaitman, C.A. 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. 104: 890-891.

Schnaitman, C.A. 1974. Outer membrane proteins of *Escherichia coli*. III. Evidence that the major protein of *Escherichia coli* OIII outer membrane consists of four distinct polypeptides. J. Bacteriol. 118: 442-453.

Schwartz, M. 1967. Sur l'existence chez *Escherichia coli* d'une régulation commune à la biosynthèse des récepteurs du bactériophage λ et au mètabolisme du maltose. Ann. Inst. Pasteur. (Paris) 113: 685-704.

Schwartz, M., Kellerman, O., Szmelcman, S., and Hazelbauer, G. 1976. Further studies on the binding of maltose to maltose binding protein of *Escherichia coli*. Eur. J. Biochem. 71: 167-170.

Schweizer, M., and Henning, V. 1977. Action of a major outer membrane cell envelope protein in conjugation of *Escherichia coli* K12. J. Bacteriol. 129: 1651-1652.

Silhavy, T.J., Brickman, E., Bassford, P.J., Casadaban, M.J., Shuman, H.A., Schwartz, V., Guarente, L., Schwartz, M., and Beckwith, J.R. 1979. Structure of the *malB* region in *Escherichia coli* K12 II. Genetic map of the *malE,F,G* operon. Molec. Gen. Genet. 174: 249-259.

Silhavy, T.J., Casadaban, M.J., Shuman, H.A., and Beckwith, J.R. 1976. Conversion of β-galactosidase to a membrane bound state by gene fusion. Proc. Natl. Acad. Sci. U.S.A. 74: 5411-5415.

Silhavy, T.J., Shuman, H.A., Beckwith, J., and Schwartz, M. 1977. Use of gene fusions to study outer membrane protein localization in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 74: 5411-5415.

Skurray, R.A., Hancock, R.E.W., and Reeves, P. 1974. *Con*⁻ mutants: a class of mutants in *Escherichia coli* K12 lacking a major cell wall protein and defective in conjugation and adsorption of bacteriophage. J. Bacteriol. 119: 726-735.

Sonntag, I., Schwarz, H., Hirota, Y., and Henning, V. 1978. Cell envelope and shape of *Escherichia coli*: Multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. 136: 280-285.

Stock, J.B., Rauch, B., and Roseman, S. 1977. Periplasmic space in *Salmonella typimirium* and *Escherichia coli*. J. Biol. Chem. 252: 7850-7861.

Suzuki, H., Nishimura, Y., Yasuda, S., Nishimura, A., Yamada, M., and Hirota, Y. 1978. Murein-lipoprotein of *Escherichia coli*: A protein involved in the stabilization of bacterial cell envelope. Mol. Gen. Genet. 167: 1-9.

Szmelcman, S., Schwartz, M., Silhavy, T.J., and Boos, W. 1976. Maltose transport in *Escherichia coli* K12: A comparison of transport kinetics in wild-type and λ resistant mutants with the dissociation constants of the maltose binding protein as measured by fluorescence quenching. Eur. J. Biochem. 65: 13-19.

Tamaki, S., and Matsuhashi, M. 1973. Increase in sensitivity to antibiotics and lysozyme on deletion of lipopolysaccharide in *Escherichia coli* strains. J. Bacteriol. 114: 453-454.

Thirion, J.P., and Hofnung, M. 1972. On some genetic aspects of phage λ resistance in *Escherichia coli* K12. Genetics. 71: 207-216.

Tokunaga, M., Tokunaga, H., and Nakae, T. 1979. The outer membrane permeability of gram negative bacteria. Determination of permeability rate in reconstituted vesicle membranes. FEBS. Lett. 106: 85-88.

Tommassen, J., and Lugtenberg, B. 1980. Outer membrane protein e of *Escherichia coli* K12 is co-regulated with alkaline phosphatase. J. Bacteriol. 143: 151-157.

Tommassen, J., and Lugtenberg, B. 1981. Localization of *phoE*, the structural gene for outer membrane protein e in *Escherichia coli* K12. J. Bacteriol. (Submitted for publication).

Van Alphen, L., Havekes, L., and Lugtenberg, B. 1977. Major outer membrane protein d of *Escherichia coli* K12. Purification and *in vitro* activity of bacteriophage K3 and f-pilus mediated conjugation. FEBS. Lett. 75: 285-290.

Van Alphen, L., Lugtenberg, B., Boxtel, R. van, and Verhoef, C. 1976. Architecture of the outer membrane of *Escherichia coli* K12 I. Action of phospholipases A2 and C on wild-type strains and outer membrane mutants. Biochim. Biophys. Acta. 466: 257-268.

Van Alphen, W., van Selm, N., and Lugtenberg, B. 1978. Pores in the outer membrane of *Escherichia coli* K12. Involvement of proteins b and e in the functioning of pores for nucleotides. Mol. Gen. Genet. 159: 75-83.

Verhoef, C., de Graaff, P.J., and Lugtenberg, E.J.J. 1977. Mapping of a gene for a major outer membrane protein of *Escherichia coli* K12 with the aid of a newly isolated bacteriophage. Mol. Gen. Genet. 150: 103-106.

Verkleij, A., Van Alphen, L., Bijvelt, J., and Lugtenberg, B. 1977. Architecture of the outer membrane of *Escherichia coli* K12 II. Freeze fracture morphology of wild-type and mutant strains. Biochim. Biophys. Acta. 466: 269-282.

Von Meyenberg, K. 1971. Transport limited growth rates in a mutant of *Escherichia coli*. J. Bacteriol. 107: 878-888.

Von Meyenberg, K., and Nikaido, H. 1977. Outer membrane of gram negative bacteria XVII. Specificity of transport process catalysed by the λ receptor in *Escherichia coli*. Biochem. Biophys. Res. Commun. 78: 1100-1107.

Wandersman, C., Moreno, F., and Schwartz, M. 1980. Pleiotropic mutations rendering *Escherichia coli* K12 resistant to bacteriophage TP1. J. Bacteriol. 143: 1374-1383.

Wandersman, C., and Schwartz, M. 1978. Protein la and the lamB protein can replace each other in the constitution of an active receptor for the same coliphage. Proc. Natl. Acad. Sci. U.S.A. 75: 5636-5639.

Wandersman, C., Schwartz, M. and Ferenci, T. 1979. *Escherichia coli* mutants impaired in maltodextrin transport. J. Bacteriol. 140: 1-13.

Wanner, B.L., Sarthy, A., and Beckwith, J. 1979. *Escherichia coli* Pleiotropic mutant that reduces amounts of several periplasmic and outer membrane proteins. J. Bacteriol. 140: 229-239.

Watson, G., and Paigen, K. 1972. Intracellular transfer by phage receptor site lipolysaccharide. Nature (New. Biol.) 239: 120-122.

Wayne, R., Frick, K., and Neilands, J.B. 1976. Siderophore protection against colicins M, B, V and Ia in *Escherichia coli*. J. Bacteriol. 126: 7-12.

Wayne, R., and Neilands, J.B. 1975. Evidence for a common site for ferrichrome compounds and bacteriophage Ø80 in the cell envelope of *Escherichia coli*. J. Bacteriol. 121: 497-503.

White, J.C., Di Girolamo, P.M., Fu, M.L., Preston, Y.A., and Bradbeer, C. 1973. Transport of vitamin B_{12} in *Escherichia coli*: location and properties of the initial B_{12}-binding site. J. Biol. Chem. 248: 3978-3986.

Wilkinson, R.G., Gemski, P., and Stocker, B.A.D. 1972. Non smooth mutants of *Salmonella typhimurium*: Differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. 70: 527-554.

Woodrow, G.C., Langman, L., Young, I.G., and Gibson, F. 1978. Mutations affecting the citrate dependent iron uptake system in *Escherichia coli*. J. Bacteriol. 133: 1524-1526.

Wookey, P., and Rosenberg, H. 1978. Involvement of inner and outer membrane components in the transport of iron and in colicin B action in *Escherichia coli*. J. Bacteriol. 133: 661-666.

Wu, H.C. 1972. Isolation and characterization of an *Escherichia coli* mutant with alterations in the outer membrane proteins of the cell envelope. Biochim. Biophys. Acta. 290: 274-289.

Wu, H.C., How, C., Lin, J.J.C., and Yem, D.W. 1977. Biochemical characterization of a mutant lipoprotein of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 74: 1388-1392.

Wu, H.C., and Osborn, M.J. 1980. Proteins of the outer-membrane of gram negative bacteria. Ann. Rev. Microbiol. 34: 369-422.

Yem, D.W., and Wu, H.C. 1978. Physiological characterization of an *Escherichia coli* mutant altered in the structure of murein lipoprotein. J. Bacteriol. 133: 1419-1426.

Young, I.G., Cox, G.B., and Gibson, F. 1967. 2,3-dihydroxybenzoate as a bacterial growth factor and its route of biosynthesis. Biochim. Biophys. Acta. 141: 319-331.