To my parents, in memory of Stephen

The University of Aston in Birmingham

Studies on porins of bacteria

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The effect of various plasmolysing agents on the resistance of *Pseudomonas aeruginosa* and *Escherichia coli* to rifampicin and bacitracin was investigated. It was found that the resistance of cells treated with 0.22 M NaCl + 0.01 M $MgCl_2$ was unaffected, whereas 0.3 M NaCl or 0.5 M sucrose increased the sensitivity of the organisms to the antibiotics.

It was determined that isomaltitol (OS 346) was identified by the maltose uptake system in *E. coli* after growth of the organism in nutrient broth. Isomaltotriitol (OS 508) and isomaltotetritol (OS 670) were not recognised. After growth of the organism in chemically defined media (CDM) in the absence of maltose, OS 346 was actively removed from the environment but at a reduced level.

An *in vivo* double label assay for determining the size of the outer membrane pores in Gram-negative bacteria was designed. The assay was based on a comparison of the uptake into the periplasmic space of ^{3}H labelled molecules from an isomaltitol series with that of ^{14}C sucrose. The exclusion limit of *E. coli* pores was found to be approximately 700 d mw, confirming results obtained by other workers. The pores in *P. aeruginosa* were found to have an exclusion limit of approximately 360 d mw or less, in contrast to previous data.

The effect of various growth environments on pore size and outer membrane protein composition was investigated. It was found that alterations in the growth environment of either organism did not significantly affect the outer membrane pore size. The nutritional richness of the growth media (CDM as compared with nutrient broth) affected the protein composition of the *P. aeruginosa* outer membrane, whereas the *E. coli* outer membrane proteins were unaffected by variation in the organism's CDM growth environment.

The finding that *P. aeruginosa* has outer membrane pores with an exclusion limit less than that of *E. coli* is significant in that *P. aeruginosa* is unusually resistant to many antibiotics in comparison with other Gram-negative bacteria. This high resistance could be explained by the very low exclusion limit of the outer membrane pores.

Key words: *Escherichia coli*; *Pseudomonas aeruginosa*; double label assay; porins; pore size.

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Abbreviations

^{14}C	$^{14}_{6}C$, carbon fourteen
cm	centimetre
d	daltons
dpm	disintegrations per minute
^{o}C	degrees centigrade
E	dpm ^{14}C in efflux material ÷ dpm ^{3}H in initial solution
EDTA	ethylenediaminetetraacetic acid
F	(I - E)
g	relative centrifugal force
gm	gramme
^{3}H	$^{3}_{1}H$, tritium
I	dpm ^{14}C in initial solution ÷ dpm ^{3}H in initial solution
Kd	kilodaltons
l	litres
ln	logarithms to the base e (natural logarithms)
log	logarithms to the base 10
LPS	lipopolysaccharide
M	Moles per litre
m	metre
mCi	millicurie
ml	millilitre
mmol	millimole
mOsm	milliOsmoles
min	minute
mw	molecular weight
μ	growth rate
μCi	micro Curie
μCi/μmol	micro Curies per micromole, specific activity

µl	microlitre
µmol	micromole
NE260	NE260 micellar scintillant
nm	nanometre
OD_{470}	optical density read at 470 nm wavelength
OS 346	isomaltotitol
OS 508	isomaltotriitol
OS 670	isomaltotetritol
OS 833	isomaltopentitol
OS 995	isomaltohexitol
OS 1157	isomaltoheptitol
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
phosphate buffer	0.1 M sodium phosphate buffer pH 7.2
plasmolysing agent	0.22 M NaCl plus 0.01 M $MgCl_2$ final concentrations
s	seconds
SDS	sodium dodecyl sulphate
v/v	volume by volume

Origin and scope of the work

Gram-negative bacteria have a cell envelope composed of two membranes, the cytoplasmic membrane similar to that found in many other cells, and the outer membrane (Costerton, Ingram and Cheng, 1974). The latter structure has been shown to be partly responsible for the general high resistance of Gram-negative organisms against compounds which are comparatively efficacious with Gram-positive organisms (Nikaido and Nakae, 1979). Amongst the Gram-negative organisms of medical interest is *P. aeruginosa*, which is considerably more resistant to antibiotics and dyes than *E. coli* and many other Gram-negative bacteria.

The outer membrane primarily functions as a permeability barrier, preventing molecules from penetrating to the periplasmic space (Payne and Gilvarg, 1968; Nikaido, 1976; Nikaido, 1979). However, it was found that some major outer membrane proteins functioned as water filled pores enabling small, hydrophilic molecules to cross the outer membrane (Nakae, 1975, 1976; Decad and Nikaido, 1976; Hancock and Nikaido, 1979). These proteins are generally termed porins and include OmpF and OmpC in *E. coli* K12, OmpF, D and C in *Salmonella typhimurium* and protein F in *P. aeruginosa*. Using various methods, generally *in vitro* assays involving membrane vesicles, the pores of *E. coli* and *S. typhimurium* were found to have an exclusion limit of 600-700 d mw. However, *in vitro* assays with *P. aeruginosa* showed the pores as having an exclusion limit of approximately 6000 d mw.

Although the data obtained for the *E. coli* pores fitted in well with previous knowledge of the organism, including its resistance to antibiotics (Nikaido, 1976), the results with *P. aeruginosa* were unexpected and did not easily support data related to the permeability

of the outer membrane with respect to antibiotics. Many antibiotics which are able to cross the outer membrane of *E. coli* via the pores should also be able to penetrate the outer membrane of *P. aeruginosa* via the pores, if these are ten times larger; but in practice the organism is more resistant to such compounds than *E. coli*. Various attempts to explain the discrepancy have been made, all assuming that the pores in *P. aeruginosa* are large (Benz and Hancock, 1981; Angus et al., 1982).

However, the data obtained for *P. aeruginosa* are from *in vitro* assays, and could therefore be artefacts. Consequentially, an *in vivo* investigation of the pore size of *P. aeruginosa* and *E. coli* was necessary, and this need lead to the double label assay used in this thesis. The potential scope of such an *in vivo* assay is considerable, as it can be used to examine bacteria grown under various conditions, or treated in different ways.

In order to design such an assay it was necessary to determine whether its various components would either affect the cells, or themselves be altered. Consequently, this thesis is written so as to present the various stages of investigation in a logical progression, each section of experimental work being self-explanatory, but linked with previous sections.

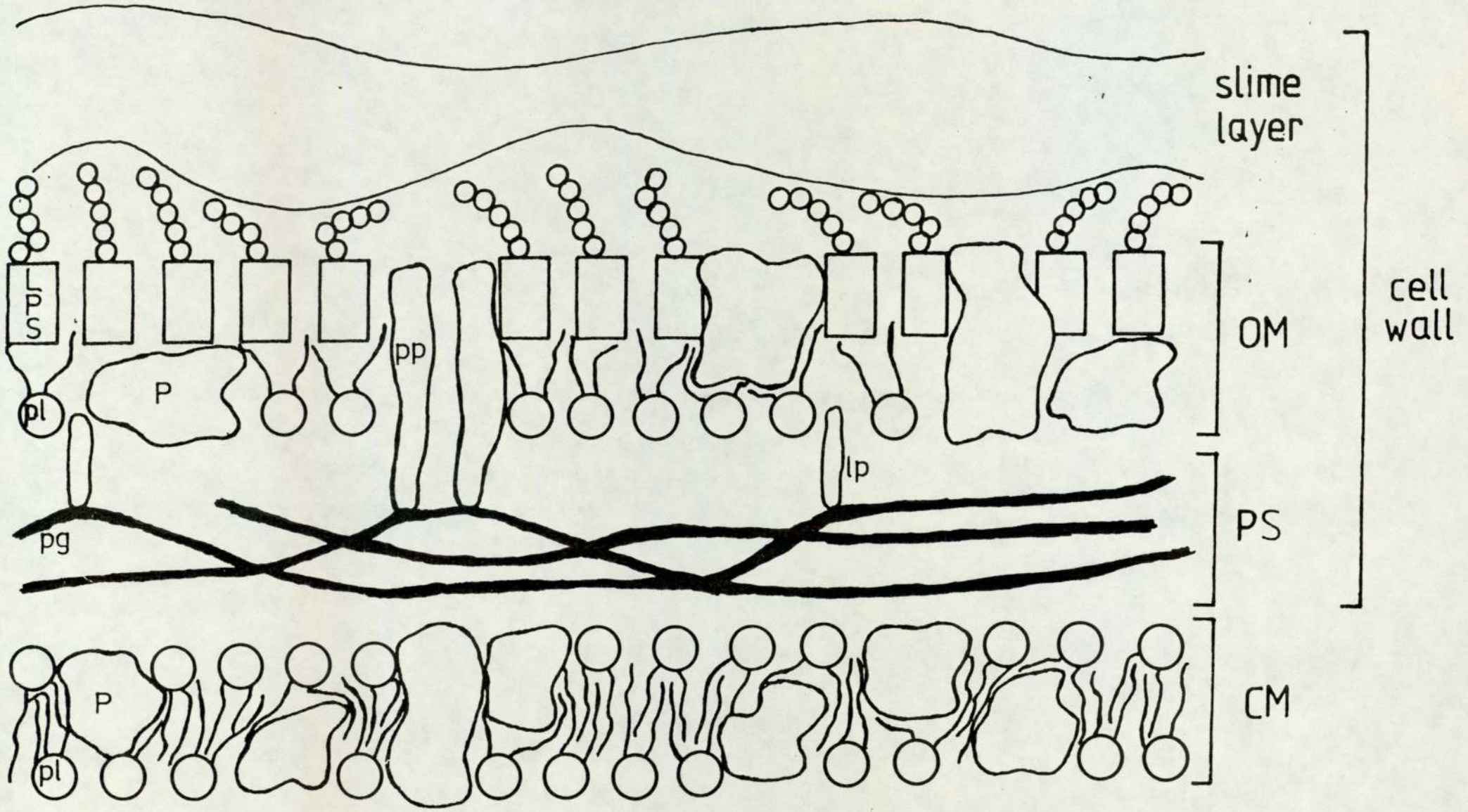
1. INTRODUCTION

1. Introduction

The integrity of any cell, prokaryotic or eukaryotic, is dependent on the presence of a stable barrier between the environment and the molecules which comprise the cytoplasm. In addition, this boundary provides simultaneously a potential barrier to the assimilation of vital nutrients and metabolites, which must of necessity be overcome, and a protection against lethal compounds which clearly has to be maintained. In nearly all cells, this structure is present in the form of a cytoplasmic or plasma membrane, a lipid bilayer containing many proteins. However, several species of bacteria, collectively known as Gram-negative bacteria, increase the protection afforded by the cytoplasmic membrane with a further "outer" membrane, outside the cytoplasmic membrane, the two being separated by the periplasmic space, which contains a layer of peptidoglycan, a polymer based on N-acetyl muramic acid (Figure 1.1).

These are together called the cell envelope (Costerton, Ingram and Cheng, 1974). The integrity of this structure is partially dependent on the links between the outer membrane and both the peptidoglycan and the cytoplasmic membrane. The former are the result of the binding of various outer membrane proteins to the peptidoglycan. The latter links, between the two membranes, occur at the Bayer's points of adhesion, of which there are some 200-400 per cell in E. coli (Bayer, 1968a, b).

The cell envelope in Gram-negative bacteria is therefore the interface between the cytoplasm and environment, through which molecules have to pass to enter the cell. It has been shown that the peptidoglycan does not constitute any permeability barrier (Nakae and Nikaido, 1975) and such properties are therefore associated with the two membranes.


slime
layer
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The nature of the two membranes has therefore been extensively investigated with a view to determining how molecules do enter the cell. It has been observed that all the active transport systems known of in Gram-negative bacteria are present in the cytoplasmic membrane, accompanied by systems for oxidative phosphorylation and biosynthesis of certain macromolecules (DiRienzo, Nakamura and Inouye, 1978) including some of those finally located in the outer membrane (Inouye, 1979). Whereas the outer membrane contains bacteriophage (Bradbeer, Woodrom and Khalijah, 1976) and colicin (Konisky, 1979) receptors, is involved in conjugation (Manning and Achtman, 1979) and septation, has some specific uptake systems (as opposed to active transport), e.g. for iron (Nikaido and Nakae, 1979) and vitamin B12 (Bradbeer et al., 1976) and acts as a diffusion barrier against various molecules (Robbie and Wilson, 1969; Leive, 1974) including antibiotics and other antibacterial compounds.

The outer membrane is dissimilar from the inner, cytoplasmic membrane in both function and structure. As a consequence, the outer membrane has been, in recent years, extensively investigated to determine how it affects the bacterium's ability to obtain any essential nutrients, whilst protecting the cell from lethal agents, and if either process fails, why. The properties and function of this diffusion barrier can only be considered in the light of an adequate understanding of the somewhat novel structure of the Gram-negative bacterial outer membrane.

1.1 Composition and structure of the outer membrane of Gram-negative bacteria

The outer membrane of Gram-negative bacteria can be separated from the cytoplasmic membrane using methods dependent on the greater density of the outer membrane, or on the ability of some detergents to solubilize the cytoplasmic membrane but not the outer membrane (DiRienzo et al., 1978). This in turn has led to the clarification of the three major structural components present in the isolated outer membrane. These are phospholipid, lipopolysaccharide and protein.

1.1.1 Phospholipids

Phospholipids can be extracted directly from whole cells of Gram-negative organisms, using methods adapted from Bligh and Dyer (1959) based on the lipid solubilising properties of chloroform and methanol. However, unless the two membranes are first separated, the phospholipids from both are extracted, and it is not possible to distinguish between those from the cytoplasmic membrane and the phospholipids from the outer membrane. In the few cases where the outer membrane and cytoplasmic membrane have been separated, and the phospholipids then extracted, it has been found that there is considerably less phospholipid present in the outer membrane than in the cytoplasmic membrane. Amongst the Enterobacteriaceae, the qualitative phospholipid composition of the two membranes is very similar, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and very small amounts of cardiolipin being present (Osborn, Gander and Parisi, 1972 ; Cronan, 1979). However, it has been shown that the outer membranes of wild type Salmonella typhimurium and

Escherichia coli are enriched with PE when compared with the respective inner membranes (Cronan, 1979). In Pseudomonas aeruginosa PAO1, the phospholipid and fatty acid composition of both membranes have been found to be identical (P. Lambert, personal communication).

Attempts to express the relative proportion of the outer membrane which is comprised of phospholipid or any other component are made difficult by the variation in the amount of LPS present. Therefore it is more meaningful to express the relative amounts in terms of the numbers of molecules present. In S. typhimurium, approximately 1.5×10^6 molecules of phospholipid were measured per μm^2 section of the outer membrane (Smit, Kamio and Nikaido, 1975) which in turn represents 2 μm^2 of the membrane bilayer. This amount of phospholipid was concluded as being inadequate to cover one side of a lipid bilayer (which is the usual structure of the inner membrane) (Smit et al., 1975; Kamio and Nikaido, 1976). As a result of this, and other observations, there has been considerable debate as to whether the phospholipid is organised in the outer membrane as a single layer on the inner side of a bilayer, or as parts of a lipid bilayer. Certainly, freeze fracture studies (Bayer, Koplow and Goldfine, 1975; Smit et al., 1975; Verkleij, van Alphen, Bijvelt and Lugtenberg, 1977; Schweizer, Schwarz, Sonntag and Henning, 1976) have shown that the outer membrane does not have a typical lipid bilayer structure. Cronan (1979) suggests that the phospholipid in wild type outer membranes is organised in a lipid bilayer which only occurs in small patches. This is in particular supported by Jones and Osborn (1977a, b) who incorporated exogenous lipids into intact cells and then showed that the incorporation exhibited the properties expected of a direct fusion of two phosphilipd bilayers. In contrast with this, Kamio and

Nikaido (1976) found that in smooth and only slightly rough (Rc) strains of S. typhimurium there were no PE head groups exposed on the outer surface of the outer membrane. Using cyanogen bromide activated dextran to label such phospholipid head groups, Rd and Re strains, with less complete LPS and therefore reduced amounts of protein in the outer membrane, were shown to have PE exposed on the outer surface of the outer membrane. This suggested that, in those strains with wild type or only slightly altered LPS, the phospholipid was either only present in the inner leaflet of the outer membrane, or otherwise the proteins in the outer membrane covered the phospholipid head groups. In either situation, the reduction in the amount of protein present in the outer membrane of Rd and Re mutants would expose the phospholipid head groups. Because of the conflicting evidence, a definitive solution as to the arrangement of phospholipid in the outer membrane of Gram-negative bacteria has not yet been obtained.

1.1.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is unique to the outer membrane of Gram-negative bacteria. It is an amphipathic molecule comprised of a hydrophilic, polysaccharide section and a hydrophobic portion, known as lipid A. The polysaccharide is generally regarded as having two parts, the central "R-core", which links the lipid A to the outer "O-antigen". The R-core almost always contains an 8-carbon sugar acid, 2 keto-3 deoxyoctonic acid, known as KDO, although it has been shown that this sugar does not occur in LPS found in Psuedomonas cepacia (Mariello, Heymann and Adair, 1979). KDO, because it is unique to LPS, is used as a means of assaying for

the presence of the outer membrane component in such organisms as E. coli, S. typhimurium and P. aeruginosa. The O-antigens, which vary extensively in composition between strains and, in rough and deep rough mutants, in length (Nikaido, 1976) are formed of repeating units of oligosaccharide.

The whole molecule is aligned at right angles to the membrane so that the hydrophilic portion protrudes away from the cell into the environment, whereas the hydrophobic Lipid A is located in the centre of the outer membrane. Further, it is now believed that the LPS is located entirely in the outer surface of the outer membrane, giving the membrane an asymmetric structure (Muhlradt and Golecki, 1975; Funahara and Nikaido, 1980). It has been calculated that there are approximately 5.8×10^5 molecules of LPS monomers present per μm^2 section of the outer membrane of S. typhimurium (Smit et al., 1975) (a figure of 3.4×10^6 has been suggested for E. coli K12 (Lugtenberg, 1981)) and that this would in total cover about 0.7 μm^2 of a 2 μm^2 area of outer membrane (Nikaido and Nakae, 1979).

1.1.3 Protein

In comparison with the cytoplasmic membrane, there are few protein species located in the outer membrane of Gram-negative bacteria. Therefore, after isolation of the outer membranes, the separation and identification of the various types of protein, using polyacrylamide gel electrophoresis (Lugtenberg, Meijers, Peters, van der Hock and van Alphen, 1975) is easily carried out. As a consequence, rapid progress has been made in identifying the various outer membrane proteins, and for many the function is at least partly known.

1.1.3.1 Proteins found in the outer membrane of *E. coli* and *S. typhimurium*

There have been shown to be several "major" outer membrane proteins, i.e. those which are predominant in the wild type organism. This can be misleading as under varying environmental conditions the "major" proteins may be less important than some more usually regarded as "minor" proteins. In *E. coli* K12 the major outer membrane proteins include the matrix proteins, OmpA protein and lipoprotein (see Table 1.1 for nomenclature).

Initially, the matrix protein was identified in strains of *E. coli* and *S. typhimurium* as a dominant outer membrane protein of molecular weight in the region of 35-36 K daltons, which was bound in some manner to the peptidoglycan (Rosenbusch, 1974). These proteins were also identified as being the components required to confer permeability to vesicles comprised of outer membranes (Nakae, 1976a, b) and they were as a result called porins. However, these are in fact not necessarily single protein species in either organism. *E. coli* K12 was found to have two major structural outer membrane proteins, both matrix proteins, called variously 1a, TolF, b, O-9, Ia, OmpF, and 1b, c, O-8, Ib, OmpC (Table 1.1). The unified nomenclature (Osborn and Wu, 1980; Lugtenberg, 1981) suggests that the proteins should be known by the name given to the gene coding for each molecule, leading to the choice of OmpF and OmpC as the names for the two proteins. In *E. coli* B strains, only one porin protein, equivalent to the OmpF protein in *E. coli* K12 strains, has been identified (Rosenbusch, 1974).

S. typhimurium has three porin proteins, which were initially known by their apparent molecular weights (Nakae, 1976a). But the genes coding for these proteins have been given the same names

Table 1.1

Constitutively synthesised proteins present in the outer membranes of *E. coli* and *S. typhimurium*.

Protein	Alternative symbols		Apparent mw (d)	Gene	Function
	E. coli	*S. typhimurium*			
OmpA	TolG, II*, d, O-10, 3a	33 K	33,159	*ompA* (*tolG*, *con*, *tut*)	Involved in conjugation, influences cell shape
OmpC	Ib, c, O-8, 1b	36 K	36,000	*ompC* (*par*, *meoA*)	general hydrophilic pore
OmpB	-[a]	34 K	37,205	*ompD*	as OmpC
OmpF	TolF, Ia, b, O-9, 1a	35 K	37,205	*ompF* (*tolF*, *colB*, *coa*, *cry*)	as OmpC
Lipoprotein			7,200	*lpp* (*mlpA*, *lpm*, *lpo*)	attachment of peptidoglycan to outer membrane. Influences cell shape
Phage T6 receptor			26,000	*tsx*	receptor for phage T6. pore for nucleosides.

a. No OmpD protein has been observed in *E. coli*

as in E. coli, because of the considerable similarity of the gene products and functions. The proposed nomenclature for the major outer membrane proteins which are porins is OmpC (previously 36 K), OmpD (34 K) and OmpF (35 K) (see Table 1.1).

In both organisms a further gene is necessary for the expression of the omp genes. This, the ompB gene, does not have a structural protein product, and is clearly a regulatory gene (Sato and Yura, 1979). Using various genetic techniques, Hall and Silhavy (1979) showed that ompB regulated the expression of the ompC gene. More recently, they have extended this work (Hall and Silhavy, 1981a) and shown that the ompF gene expression is also controlled by ompB.

The ompB region has been shown to be highly complex, consisting of at least two genes, envZ (also called tpo (Wandersman, Moreno and Schwarz, 1980))and perA (Wanner, Sarthy and Beckwith, 1979)) and ompR (Hall and Silhavy, 1981b; Taylor, Hall, Enquist and Silhavy, 1981). Although the situation is not yet clear, Hall and Silhavy (1981b) propose that the ompR gene codes for a bifunctional protein capable of regulating the expression of ompC and ompF in a positive manner. The envZ gene product is suggested as being an "envelope" protein (which section thereof is not mentioned) capable of sensing the cell's external environment, in particular the medium osmolarity. However, as is pointed out, a complete, correct model must be more complex, in order to explain porin regulation.

Previous to the work showing ompB as the regulatory gene and ompC and ompF as the structural genes in E. coli, it was thought that the two outer membrane proteins were derived from ompB, and were subject to post-translational control by the products of the ompF and ompC genes (Bassford, Diedrich, Schnaitman and Reeves, 1977). This was because both membrane polypeptides are structurally

similar, and have similar functions. This was misleading, and it has been shown since that the two structural proteins differ extensively in their amino-acid composition (Ichihara and Mizushima, 1978). It has been suggested that the two genes developed by duplication of an ancestral genome, followed by independent mutation of the two systems.

The ease with which outer membrane proteins can be isolated has led to a gradual increase in knowledge of their structure and organisation. The matrix proteins have in general been found to have molecular weights in the region of 35,000 d (Table 1.1). Rosenbusch (1974) showed that in E. coli B, the OmpF porin was a single polypeptide containing 336 amino acids and apparently lacking in any non-protein moiety. None of the porins purified so far are particularly hydrophobic in their amino-acid composition (Rosenbusch, 1974; Garten and Henning, 1974) and they are known to carry a negative charge (Schmitges and Henning, 1976). It has also been found that both OmpC and OmpF proteins have very high contents of β structure (Rosenbusch, 1974; Nakamura and Mizushima, 1976) which is in contrast to many other "intrinsic" membrane proteins (cytoplasmic and outer membranes) which have high α-helix contents. These outer membrane proteins show considerable alteration in mobility on sodium dodecyl sulphate (SDS) polyacrylamide gels after heating of the proteins in SDS. Nakamura and Mizushima (1976) showed that this was due to gross conformational changes in the protein which altered the β structure to α-helix.

The modification of the protein after heating in SDS is characteristic of porins. So too is their association with the underlying peptidoglycan. This was demonstrated by treatment of the cell envelope at temperatures below 70°C, which solubilized

all the outer membrane components other than the lipoprotein and the peptidoglycan associated proteins (the porins). The lipoprotein is associated with the peptidoglycan through covalent bonds whereas the matrix protein association with the peptidoglycan is non-covalent (Rosenbusch, 1974).

The OmpA protein, with approximately 10^5 molecules per cell (Lugtenberg, 1981) was found to have an apparent molecular weight of 25,000 d when separated from other outer membrane proteins on SDS polyacrylamide gels, but, like the matrix proteins, was found to be modified if heated in SDS prior to the electrophoresis (Schnaitman, 1973). The OmpA protein was also rich in β structure and, as with the porin proteins, heating the protein in SDS converted this to α-helix, which gross conformational change almost certainly was responsible for the altered behaviour on polyacrylamide gels (Nakamura and Mizushima, 1976). However, the OmpA protein is not bound in any way to the underlying peptidoglycan.

The structural gene for this protein in *E. coli* is *ompA*, which was previously known as *tolG* and *con* (Table 1.1). In *S. typhimurium* a very similar protein has been identified, previously known as the 33K protein.

The lipoprotein (or Braun's lipoprotein) (structural gene *lpp*) found in *E. coli* and species of *Salmonella* and *Serratia*, is a small, well characterised protein, which is frequently linked to the underlying peptidoglycan via the amino group of its C-terminal lysine residue. At its N-terminal end, the cysteine carries an amide-linked fatty acid as well as a diglyceride moiety (Braun, 1975). Also in contrast to the heat modifiable proteins, the lipoprotein is rich in α-helical regions (Braun, 1975).

The lipoprotein is present in very large numbers (7×10^5 molecules per cell), possibly more than any other outer membrane proteins in E. coli (Nikaido and Nakae, 1979). Approximately two thirds of the total amount is present in a free form, the remaining third being covalently bound to the peptidoglycan (Inouye, Shaw and Shen, 1972). Both free and bound forms of similar lipoproteins have been located in Salmonella and Serratia species, but in Proteus mirabilis, only the bound form of such a lipoprotein has been detected (Braun, Rehn and Wolff, 1970; Halegoua, Hiroshima and Inouye, 1974) and this is only present in relatively small quantities (Gmeiner, Kroll and Martin, 1978).

Although the major outer membrane proteins are generally regarded as being the matrix proteins, OmpA and the lipoprotein, there are other proteins which can be of great significance in various circumstances. These include the lamB protein and the new outer membrane proteins.

Synthesis of the lamB gene product, which is the receptor for the bacteriophage , is induced when E. coli is grown in the presence of maltose. It then becomes a major outer membrane protein comparable in abundance with the matrix proteins (Braun and Krieger-Brauer, 1977). In addition to inducing the synthesis of the λ receptor protein, growth of E. coli on maltose induces expression of all the mal operon, which includes a periplasmic maltose binding protein. The lamB protein has an apparent molecular weight of 47,000 d and resembles porins in that it is associated with the underlying peptidoglycan (Braun and Krieger-Brauer, 1977). A similar protein has recently been identified in S. typhimurium (Palva, 1978; Palva, 1979) as a 44,000 d molecule induced by growth

Table 1.2

Inducible proteins present in the outer membrane of *E. coli*

Protein	Induced by	Alternative designation	Apparent mw (daltons)	Gene	Function
PhoE	phosphate limitation	e, NmpA, NmpB, Ic, E	40,000	*phoE*	general pore for hydrophilic compounds
Lambda[a] receptor	presence of maltose	*lamB* protein, maltose pore	47,000	*lamB*	phage λ receptor. Specific pore for maltose and maltodextrins.
BtuB protein	Vitamin B_{12} limitation		60,000	*btuB* (*bfe*)	uptake of vitamin B_{12}. phage BF23 receptor.
Cir protein	Fe^{3+} limitation		74,000	*cir*	uptake of complexed Fe^{3+}?
TonA protein	Fe^{3+} limitation		78,000	*tonA*	uptake of ferrichrome
Fec protein	Fe^{3+} limitation	Cit protein	80,500	*fec*, *cit*	uptake of Fe^{3+}-citrate
FepA protein	Fe^{3+} limitation		81,000	*fep*, *feuB*	uptake of Fe^{3+}-enterochelin
83K protein	Fe^{3+} limitation		83,000	-	uptake of complexed Fe^{3+}?
Protein 2	Lysogeny of *E. coli* DNA with phage PA-2		38,000	prophage PA-2	replaces OmpC (the PA-2 receptor) with a new non-receptor, general hydrophilic pore protein.

a. An equivalent to the lambda receptor has been identified in *S. typhimurium*.

of the organism on maltose. The two proteins have been shown to be immunologically related, but the function of the polypeptide located in the outer membrane of S. typhimurium has not yet been identified.

It has been shown that in organisms with mutations suppressing the expression of the structural genes ompF and ompC, the phenotypic effect of the mutation can be mitigated by the presence of outer membrane proteins with functions similar to the porin proteins. These proteins were termed new membrane proteins, presumably because they had not been observed previously, nor were they synthesised constitutively and therefore usually present in the outer membrane. In addition, the new membrane proteins were electrophoretically distinct from the other outer membrane proteins. At least three genetic loci associated with these polypeptides were identified, nmpA (Foulds and Chai, 1978a; Pugsley and Schnaitman, 1978b), nmpB (Pugsley and Schnaitman, 1978b) and nmpC (Pugsley and Schnaitman, 1978b). It is probable that protein Ic (Henning, Schmidmayr and Hindennach, 1977) and protein e (Lugtenberg van Boxtel, Verhoef and van Alphen, 1978; van Alphen, Lugtenberg, van Boxtel, Hack, Verhoef and Havekes, 1979; van Alphen, van Selm and Lugtenberg, 1978) are the same as protein NmpA and protein E (Foulds and Chai, 1978a, b). Although it was initially believed that the NmpA and OmpF proteins were of similar structure (Henning et al., 1977) it was shown that the two polypeptides were dissimilar in several ways including apparent molecular weight and peptide profiles (Lee, Schnaitman and Pugsley 1979). Immunological cross reactivity had also produced discrepancies between the two proteins (Chai and Foulds 1979; Pugsley and Schnaitman, 1978b), although there is some immunological relationship between OmpC, OmpF and NmpA (Overbeeke, Scharrenberg and Lugtenberg, 1980).

There is at present confusion as to the function of the genes so far isolated which are involved in the production of new membrane proteins. It was found that nmpB and nmpA mutants were identical under several criteria (Pugsley and Schnaitman, 1978b) other than the electrophoretic mobility of the new membrane protein produced. But the two genetic loci, nmpA and nmpB, were clearly distinct (Pugsley and Schnaitman, 1978b). Overbeeke and Lugtenberg (1980a) showed however, that expression of protein e (possibly NmpA) was induced by growth of E. coli in phosphate deficient media. Further, they found that protein e has only one structural gene, phoe, which maps at a separate location from either nmpA or nmpB (Tommassen and Lugtenberg, 1981). The synthesis of this PhoE protein is controlled by the same regulatory mechanism as that which controls the synthesis of alkaline phosphatase and nmpA was found to be identical to one of phoS, phoT or pst (all involved in the uptake of inorganic phosphate) whereas nmpB was identical with phoR, a regulatory gene of the pho region (Tommassen and Lugtenberg, 1980). Although there are still problems, it would appear probable that the nmpA and nmpB genes are regulatory for the phoe gene product, which is a new outer membrane protein, PhoE (formerly e, NmpA, NmpB, Ic, E).

Lee et al. (1979) showed that protein NmpC and protein 2 (an outer membrane protein produced in E. coli after lysogeny with phage PA-2 (Pugsley and Schnaitman, 1978a)) were very similar, comparisons of proteolytic peptide maps showing only one different peptide, and there being slight differences in the isoelectric focusing profiles of the two molecules. Both proteins were also shown to be subject to catabolite repression (Lee et al., 1979; Pugsley and Schnaitman, 1978a).

The work carried out on the new membrane proteins used mutant strains lacking expression of the ompC and ompF loci. However, if the nmp mutations are imposed onto a wild type genetic background (i.e. if strains with a normal, wild type expression of ompC and ompF are used to obtain nmp mutations) the new membrane proteins are produced (Pugsley and Schnaitman, 1978b). There is a related reduction in the amount of protein OmpF and OmpC produced in such mutants. Further, strains lacking OmpF and OmpC produce more of the new membrane proteins than those in which ompC and ompF are expressed. It would therefore appear that the expression of the new outer membrane proteins is regulated in a manner similar to the other porin proteins. As an aspect of this, the envZ (perA) region of ompB has recently been implicated in regulating the expression of alkaline phosphotase (Lundrigan and Earhart, 1981).

As yet, no new outer membrane protins have been identified in S. typhimurium or other species of enteric bacteria.

Further outer membrane proteins found in E. coli and other enteric bacteria include those associated with the uptake of specific components such as iron, or vitamin B12 (see Table 1.2). It is known that the feuB gene product is involved with Fe^{3+}-enterochelin (Hancock, Hantke and Braun, 1976) and the tonA gene product with (Fe^{3+}) ferrichrome (Hantke and Braun, 1975). Ferric citrate is presumed to be taken up with some involvement of the cit gene product, which is induced by growth of E. coli on citrate (Hancock et al., 1976).

These proteins, and others thought to be involved in Fe^{3+} transport (Braun, Hancock, Hantke and Hartmann, 1976), are all located in the outer membrane. So too is the protein associated

with vitamin B12 uptake (Bradbeer et al., 1976). There have also been identified several proteins to which no function has, as yet, been ascribed. These include a 15000 d molecule known as protein III. Until recently the phage T6 receptor would also have belonged in this group, but more recently this protein, the tsx gene product, of molecular weight 26,000 d, has been found to have a specific pore function (Hantke, 1976; McKeown, Kahn, and Hanwalt, 1976).

Unlike the cytoplasmic membrane, enzymes are not generally located in the outer membrane. So far, only two outer membrane proteins have been identified in E. coli as being enzymes, phospholipase A (Scandella and Kornberg, 1971; Nishijima, Nakaike, Tamon and Nojima, 1977) and a protease of some kind (Nikaido and Nakae, 1979; Leytus, Bowles, Konisky and Mangel, 1981).

1.1.3.2 Proteins of the outer membrane of Pseudomonas aeruginosa

Although Stinnett and Eagon (1973) investigated the protein composition of the outer membrane of P. aeruginosa in the early seventies, more work was carried out on the enteric bacteria, and it is only recently that interest in the outer membrane protein composition of P. aeruginosa has really increased (Mizuno and Kageyama, 1978, 1979a, b; Hancock and Carey, 1979). This initial slow start was probably the result of the greater difficulty of separating the outer and cytoplasmic membranes of P. aeruginosa, caused by the hypersensitivity of the organism to EDTA (Roberts, Gray and Wilkinson, 1970; Matsushita, Adachi, Shnagawa and Ameyama, 1978) which was frequently used in the preparation of enterobacteriaceae outer membranes. Additionally, the amount of knowledge already accumulated about E. coli, in particular information about the organism's genetics, made it an obvious candidate for study, whereas P. aeruginosa was of less interest and importance.

As a result of the more recent investigations of the outer membrane of P. aeruginosa, several proteins have been identified (Stinnett and Eagon, 1973; Booth and Curtis, 1977; Hancock and Nikaido, 1978; Matsushita et al., 1978; Mizuno and Kageyama, 1978). These were initially given different systems of nomenclature by the various groups, but that of Mizuno and Kageyama (1978, 1979b) has been used in general since Hancock and Carey (1979) identified the various proteins found by the different groups (Table 1.3).

Thus it is now known that of the at least eight major outer membrane proteins identified (Hancock and Carey, 1979), five are heat modifiable, three are non-covalently bound to the underlying peptidoglycan and three are to some degree induced by glucose when P. aeruginosa is grown with this compound as the carbon source.

Hancock and Carey (1979) showed that proteins D1, D2, F, G and H1 were heat modifiable, as was demonstrated by the different patterns of migration of the altered proteins on SDS-polyacrylamide gels. Proteins D1, D2, G and H1 gave results similar to those obtained with the OmpA protein of E. coli K12 (Schnaitman, 1973) in that heating of the proteins between 70^{o}-100^{o}C in SDS caused a decrease of the mobility of the proteins (i.e. an increase in the apparent molecular weight) on SDS-polyacrylamide gels. Also, if LPS were added to the heat modified forms of D1, D2, G and H1, the modification was reversed to the same extent as with similar treatment of heat modified OmpA protein. This led to the conclusion that these four polypeptides belonged to the same class of heat modifiable proteins (Hancock and Carey, 1979).

Protein F has been identified as being the porin protein (Hancock and Nikaido, 1978). Like the OmpF and OmpC proteins, it has a molecular weight of around 35-37,000 d. It is also heat

Table 1.3

Some of the major proteins found in the outer membrane of *P. aeruginosa*

Protein	Alternative designations	Apparent mw (daltons)	Properties
D1	D, I	46000	Induced by glucose, forms a glucose specific pore.
D2	D, I	45500	Induced by glucose, possibly involved in glucose uptake
E	II	44000	As protein D2
F	porin, III, A	35000-37000	general, non-specific pore
G	IV	25000	
H1	H, V, B	21000	Induced by growth in Mg^{2+} deficient media
H2	H, V, B	20500	Novel lipoprotein
I	lipoprotein	8000	Lipoprotein analagous to Braun lipoprotein in *E. coli*

modifiable (Mizuno and Kageyama, 1978; Hancock and Carey, 1979) but does not belong to the same group of heat modifiable proteins as do D1, D2, G and H, for protein F requires long periods of boiling in SDS to effect the change from the unmodified to the heat modified form (Hancock and Carey, 1979). It has been shown that this protein is rich in β structure (Mizuno and Kageyama, 1979b) as are the matrix proteins of E. coli and S. typhimurium, which may explain the mechanism by which the heat modification occurs (Nakamura and Mizushima, 1976). (This may also be true of the other class of P. aeruginosa heat modifiable proteins, but there is as yet no evidence about the presence or otherwise of β structure within these molecules.) Also in keeping with its properties as the porin protein of P. aeruginosa, protein F has been found to be non-covalently bound to the underlying peptidoglycan (Mizuno and Kageyama, 1979b; Hancock, Irvin, Costerton and Carey, 1981). It is not precisely clear how the protein and peptidoglycan are linked, but Hancock et al. (1981) suggest that the association is complex, involving more than one kind of bond.

In addition to protein F being bound to the underlying peptidoglycan, it has also been found that protein I and protein H2 are associated with the peptidoglycan (Hancock et al., 1981). Initially, it was not shown that proteins H1 and H2 were distinct, Mizuno and Kageyama (1979b) finding that proteins F and H were associated with the peptidoglycan. Protein H was then identified as a novel lipoprotein, with a counterpart in E. coli of molecular weight 21000 d (Mizuno, 1979). These two novel lipoproteins have been shown to have similar amino acid compositions, but equally have no apparent correlation with the common Braun's lipoprotein.

It is probable that the lipoprotein identified initially as protein H is in fact protein H2, as this (unlike protein H1) was peptidoglycan associated (Hancock et al., 1981).

Protein I in P. aeruginosa exists in both the free (Mizuno and Kageyama, 1979a) and bound form, attached to the peptidoglycan (Mizuno and Kageyama, 1979a; Hancock et al., 1981). It has also been found to be rich in α-helical structure (Mizuno and Kageyama, 1979a) as is the Braun's lipoprotein in E. coli, and it is clear that protein I is analogous to the Braun's lipoprotein of E. coli (Mizuno and Kageyama, 1979a).

Proteins D1 and D2 are of similar molecular weight, and perhaps because of this have not always been identified as separate polypeptides (Mizuno and Kageyama, 1979b, Hancock and Carey, 1979). Both are strongly induced in cells grown in media containing glucose as the sole carbon source (Hancock and Carey, 1979). Protein D1 is not observed in cells grown under nutritionally rich conditions, whereas D2 is present at low levels. Protein E, although not heat modifiable (unlike D1 and D2), is also slightly induced by growth of the organism on glucose, although it is generally present in the outer membrane irrespective of the growth conditions.

Protein H1 was probably not identified in the earlier work done on the outer membrane of P. aeruginosa (Mizuno and Kageyama, 1978, 1979b) because it only becomes an important major outer membrane protein when the organism is grown in Mg^{2+} deficient media. A linear, reciprocal relationship has been found between the Mg^{2+} levels in the cell envelope and the amount of protein H1 present (Nicas and Hancock, 1980).

As yet, there has been little or no work done on the occurrence of "new membrane proteins" in P. aeruginosa, such as occur in E. coli.

This is perhaps a reflection on the more recent development of the knowledge of P. aeruginosa outer membrane proteins, and also of the lesser interest in the genetics of the organism.

As in E. coli, there are several high molecular weight outer membrane proteins which are induced when P. aeruginosa is grown in iron deficient conditions. These proteins have been reported as having molecular weights of between 70,000 and 80,000 d (Mizuno and Kageyama, 1978; Meyer, Mock and Abdallah, 1979; Ohkawa, Shiga and Kageyama, 1980), and some appear in concentrations similar to those of the major outer membrane proteins (Ohkawa et al., 1980).

Two enzymes have been found associated with the outer membrane of P. aeruginosa, phospholipase A and DD carboxypeptidase (Booth and Curtis, 1977). However, no information is available as to how the enzymes are actually related to the outer membrane.

1.1.4 Supramolecular structure and organisation of the outer membrane

When the outer membrane of Gram-negative organisms is considered as a whole, it has been found that in S. typhimurium there are approximately 1.5×10^6 molecules of phospholipid per 1 μm^2 of outer membrane, and 1.9×10^5 molecules of LPS in the same area (Smit et al., 1975). This latter figure represents the number of LPS molecules, which were each assumed to contain three monomeric units. Although Smit et al. (1975) suggested that the phospholipid covered 80% of the surface of the membrane and LPS 40% (expressed as a percentage of the total membrane area covered, and therefore the coverage of both surfaces of the membrane gives a value of 200%), it has more recently been suggested that the phospholipid and LPS cover 90% and 70% respectively of the total area of the

wild type outer membrane (Nikaido and Nakae, 1979). It is known that in the wild type organisms all the LPS is to be found located on the outer surface of the outer membrane (Muhlradt and Golecki, 1975; Funahara and Nikaido, 1980) and the phospholipid is present on the inner surface (Kamio and Nikaido, 1976). This asymmetry may well be maintained by the underlying peptidoglycan (Muhlradt and Golecki, 1975).

The asymmetry of the outer membrane is considerably reduced in mutants with defective LPS (in which the O chain is reduced) known as "rough" and "deep rough" mutants (the wild type is known as "smooth"). In these mutants much of the protein normally incorporated into the outer membrane is not (Ames, Spudick and Nikaido, 1974; Koplow and Goldfine, 1974), possibly because the defective LPS is unable to interact with the proteins. There is a related increase in the amount of phospholipid present in the outer membrane, and it exceeds that required to make up the inner surface of the outer membrane (Smit et al., 1975). Thus in such mutants there is phospholipid as well as LPS present in the outer surface of the outer membrane (Kamio and Nikaido, 1976). A similar effect can be achieved with mutants producing reduced quantities of outer membrane proteins.

It is important to remember that E. coli K12 (a strain of which is used in this thesis) and E. coli B, the strains of E. coli most frequently used in investigations of the outer membrane proteins of the organism, are both rough mutants, and not wild type in terms of LPS biosynthesis.

A probable 40-75% of the surface area of the outer membrane of the wild type Gram-negative bacterial cell is covered by protein. It has been shown that there are approximately 6×10^5 molecules of

matrix protein per cell (Steven, ten Heggeler, Muller, Kistler and Rosenbusch, 1977) and 10^5 molecules of OmpA protein (Lugtenberg, 1981). The location of the outer membrane proteins is not entirely understood, but in S. typhimurium many of the protein species, including all proteins identified as porins, have been located in the outer half of the outer membrane, exposed on the cell surface (Kamio and Nikaido, 1977). The only major protein which does not appear to feature on the outside of the cell is the Braun's lipoprotein in E. coli and S. typhimurium, which is probably only exposed should there be structural faults in the cell membrane (Nikaido and Nakae, 1979).

Begg and Donachie (1977) proposed a model for the growth of the cell surface of E. coli which involved insertion of newly made porin at the poles of the cell. This in turn would have led to a concentration of porin molecules in these two areas. However, both Begg (1978) and Smit and Nikaido (1978) have found that the matrix protein is distributed evenly throughout the outer membrane. This fits in well with the work of Steven et al. (1977) who have reported that the matrix protein of E. coli is arranged in a periodic monolayer covering at least 60% of the surface of the peptidoglycan. They showed that although the proteins were firmly attached to the peptidoglycan, the periodic structure was maintained in its absence, and was therefore based primarily on strong protein-protein interactions. The arrangement appeared to be a hexagonal lattice, the repeat being 7.7. nm. Each unit probably contained 3 molecules of matrix protein, each with an indentation, which, it was suggested, was occupied by lipoprotein (Steven et al., 1977). The main arguments for this were that the matrix protein covered most of

the cell surface, not allowing room for the very abundant lipoprotein unless it was in some way slotted in amongst the matrix protein arrangement. The lipoprotein was believed to be linked to the porin proteins in some way (De Martini and Inouye, 1978) and in rough strains was exposed on the cell surface (Braun, 1975) which supported this theory.

However, using freeze fracturing electron microscopy techniques, van Alphen, Verkleij, Leunissen-Bijvelt and Lugtenberg (1978) observed that the various porin proteins complexed with the LPS rather than the lipoprotein. It does seem more likely that the porin molecules are associated with the LPS (Yu and Mizushima, 1977) and in P. aeruginosa LPS-protein complexes have been clearly identified (Rogers, Gilleland and Eagon, 1969; Gilleland, Stinnett, Roth and Eagon, 1973; Stinnett, Gilleland and Eagon, 1973).

A very important feature of the matrix protein is the evidence that they exist as trimers (Steven et al., 1977; Ichihara and Mizushima, 1979; Palva and Randall, 1978; Reithemeier and Bragg, 1977; Tokunuga, Tokunuga, Okajima and Nakae, 1979), as well as dimers (Reithemeier and Bragg, 1977). The trimers, which in E. coli have been shown to be heterogeneous and homogeneous combinations of OmpF and OmpC proteins (Ichihara and Mizushima, 1979), are the biologically active form of the porin proteins. It has also been shown that the trimers are sufficiently long to span the thickness of the outer membrane (Tokunuga et al., 1979b).

The OmpA protein in E. coli has been shown to cross link readily with the Braun lipoprotein (Palva and Randall, 1976), and it has been suggested that this protein exists in complexes containing several copies of both itself and the lipoprotein, associated with the LPS.

An important feature of the integrity of the outer membrane is divalent cations. Some of the proteins present in large numbers exposed on the outer surface of the outer membrane are strongly acidic (Schmitges and Henning, 1976), and the hydrophilic portion of the LPS is polyanionic. There would therefore be powerful electrostatic repulsion between these compounds, probably providing the major destabilising force in the outer membrane. Divalent cations are known to be necessary for the integrity of the outer membrane, as EDTA treatment of E. coli (Leive, 1965) and P. aeruginosa (Rogers et al., 1969; Roberts et al., 1970) releases predominantly LPS and LPS-protein complexes from the two organisms respectively. These divalent cations probably act by neutralising and bridging the anionic groups. However, their effect is not complete in that under a variety of growth conditions, the phenomenon of "blebs" has been observed (de Petris, 1967; Smit et al., 1975). This may be caused by the strong electrostatic repulsion at the outer surface between membrane components, and it has been suggested that the Braun lipoprotein serves as an anchor, pulling the outer membrane down to the underlying peptidoglycan layer (Nikaido and Nakae, 1979). E. coli mutants lacking the Braun lipoprotein produce very large "blebs" in the outer membrane which can be reduced by adding Mg^{2+} to the medium (Suzuki, Nishimura, Yasuda, Nishimura, Yamada and Hirota, 1978; Fung, MacAlister and Rothfield, 1978).

Mutants lacking both the lipoprotein and the OmpA protein, have a drastically altered outer membrane (Sonntag, Schwarz, Hirota and Henning, 1978). Digestion of the peptidoglycan with lysozyme also causes extensive reorganisation of the outer membrane components (Shands, 1966; Muhlradt and Gotecki, 1975). It would appear that E. coli and S. typhimurium maintain their highly asymmetric outer

membrane organisation, at least in part, depending on the lipoprotein-peptidoglycan system and divalent cations. P. aeruginosa has relatively more anionic groups present in its outer membrane (Gray and Wilkinson, 1965) and usually requires high concentrations of Mg^{2+} to stabilise the outer membrane and maintain its integrity.

1.2 Properties of the outer membrane of Gram-negative bacteria

The outer membrane of Gram-negative bacteria has several properties which fall into two main categories. There are those properties associated with the fact that the outer membrane is the external limit of the cell (i.e. related to the surface of the organism). The remaining properties are a result of the barrier functions of the outer membrane, dividing environment from cell.

1.2.1 Surface properties of the outer membrane

The outer membrane of *E. coli*, in particular the outer membrane proteins, is associated with conjugation. The OmpA protein in *E. coli* has been found to be necessary for conjugation (Manning and Achtman, 1979). Other proteins may also be involved, but as yet little is known about the interaction between outer membrane components and the changes in the cell envelope required to enable the transfer of DNA to occur.

In terms of the role of the surface of the outer membrane, the most studied function is that of several outer membrane proteins which act as receptor sites for colicins and bacteriophages. The most noteable examples of these include the λ phage receptor, the colicin E and phage BF23 receptor, the colicin K and phage T6 receptor, which are respectively the *lamB* gene product, the protein involved in the specific transport of vitamin B12, and the *tsx* gene product (Konisky, 1979).

Amongst the major outer membrane proteins, OmpA has been shown to be the receptor for phage TuII* (Manning, Puspurs and Reeves, 1976), and although both the OmpA protein and LPS are involved, there is evidence that the actual protein moiety acts as the receptor (Datta, Kramer and Henning, 1976; Datta, Arden and Henning,

1977). The matrix proteins OmpC and OmpF are receptors for phage TuIa and TuIb in E. coli (Datta et al., 1977) and both OmpC and OmpF act independently as receptors for various phages such as PA-2 (OmpC) and TP1 (OmpF).

In S. typhimurium, at least two major outer membrane proteins are believed to be phage receptors. These are polypeptides with apparent molecular weights of 34 Kd and 26 Kd (Konisky, 1979).

The outer membrane is involved in the pathogenicity of the organisms, as the O chains of the LPS are known to have considerable antigen properties. The importance of these is somewhat diminished by the presence of the "enterobacterial common antigen", in at least the Enterobacteriaceae, which is present outside the outer surface of the outer membrane, but anchored to it (Nikaido and Nakae, 1979).

Some outer membrane components may be partially responsible for maintaining the morphology of the cell. Sonntag et al. (1978) found that mutants of E. coli lacking both lipoprotein and protein A became spherical and required increased concentrations of electrolytes (in particular the divalent cations, Ca^{2+} and Mg^{2+}) for optimal growth. However, Henning and Haller (1975) showed that mutants of E. coli K12 lacking the matrix and OmpA proteins showed no morphological difference from the parent strains.

For a more detailed coverage of the surface properties of Gram-negative organisms, see "Bacterial Outer Membranes: Structure and Function" (Ed. Inouye, 1979).

1.2.2 Barrier properties of the outer membrane

The outer membrane forms a semi-permeable barrier around the Gram-negative cell, protecting it from the environment and yet

permitting some compounds, both hydrophilic and hydrophobic, to cross the membrane. The barrier role of the outer membrane is more easily understood and better considered in the light of the manner in which molecules are normally able to cross into the cells through the outer membrane.

1.2.2.1 Diffusion of hydrophobic substances

Investigation of the permeation of hydrophobic compounds through the outer membrane was initiated by the observation that the presence of LPS in the outer membrane was particularly involved in the drug resistance of E. coli (Tamaki, Sato and Matsuhashi, 1971). Removal of much of the LPS with EDTA resulted in considerable increases in susceptibility of the organism to benzyl penicillin (Hamilton-Miller, 1965), actinomycin (Leive, 1965), polymyxin and detergents (Muschel and Gustafson, 1968) which were almost certainly due not to changes in transport systems but to an increase in the passive permeability of the outer membrane (Leive, 1968).

Gustafsson, Nordstrom and Normark (1973) showed that the rate of "uptake" of gentian violet by E. coli was dependent on the nature of the LPS present, that is, the length of the O chain attached to the LPS core, and hence the amount of hydrophobic material present in the outer membrane. This in turn led to the investigations of Nikaido (1976) who showed that permeation of hydrophobic compounds through the outer membrane is the rate limiting step in their penetration into the cell, and that in wild type S. typhimurium and rough mutants (Ra and Rc) the permeation constants were very low whereas in deep rough mutants they were much higher. The work also investigated the efficacy of various antibiotics against the wild type S. typhimurium and deep rough mutants (Nikaido, 1976). This data suggested that in the presence of wild type LPS the

hydrophobic compounds were less able to penetrate the organisms than the deep rough mutants where there was relatively less LPS and the outer membrane has a less asymmetric arrangement. However, most of the hydrophilic compounds tested were unaffected by the change in the amount present in the outer membrane. The molecular weight of the hydrophobic compounds did not appear to be relevant.

Together, these data suggest that the nature of the LPS is very important in determining whether hydrophobic compounds can diffuse across the outer membrane. Where the LPS is defective, the hydrophobic molecules are able to penetrate the outer membrane more easily than in those organisms with wild type LPS.

The mechanism of diffusion of these hydrophobic molecules through the outer membrane was concluded as being similar to that proposed by Stein (1967). Here it was suggested that the permeant compounds initially dissolve in the hydrophobic interior of the membrane, diffuse through the hydrocarbon layer and then cross the membrane by partitioning into the aqueous phase on the other side of the membrane. Nikaido (1976) investigated various aspects of this as applied to S. typhimurium and found that among compounds of a similar size, the more hydrophobic compounds diffused more quickly, the diffusion rate being temperature dependent. Further, compounds of fairly large size were apparently able to penetrate the outer membrane by this mechanism, with no clear-cut size restriction. These properties, in particular the high temperature coefficient, are known to be features of the diffusion process using the dissolution of compounds into the membrane interior (Nikaido, 1976). It was therefore concluded that hydrophobic compounds could certainly penetrate deep rough mutants by a similar mechanism.

However it is in fact true that the wild type E. coli and S. typhimurium outer membranes are exceptionally impermeable to hydrophobic compounds when compared with other biological membranes and artificial lipid bilayers (Nikaido, 1979). Although the presence of LPS does theoretically enable hydrophobic compounds to diffuse across the outer membrane, it has been found that for many of the compounds to which wild type strains (i.e., smooth) of E. coli and S. typhimurium are sensitive, such as neomycin, cycloserine, ampicillin and cephalothin, alterations in the LPS have little effect on the efficacy of the antibiotics. These molecules tend to be hydrophilic, and it is presumed that they penetrate the outer membrane using routes other than the LPS. The hydrophobic compounds, such as actinomycin D, erythromycin, novobiocin, rifamycin SV, crystal violet and other dyes, and detergents such as bile salts and sodium dodecyl sulphate, which penetrate the cells as described above, are very much less effective against E. coli and S. typhimurium wild type strains than against Gram-positive organisms, and it is only against deep rough mutants, with very much less LPS, that these compounds become active.

This distinction between the efficacy of hydrophobic and hydrophilic compounds against E. coli and S. typhimurium is undoubtedly a result of the different routes for crossing the outer membrane available to different substances (Nikaido, 1979). Interestingly, P. aeruginosa is unusually resistant to all antibiotics and other compounds used against Gram-negative bacteria. This is particularly significant with respect to hydrophilic compounds, as the relationship between the organism and hydrophobic compounds is probably similar to that of E. coli and S. typhimurium.

1.2.2.2 Passage of specific substances across the outer membrane

In addition to the non-specific routes by which hydrophobic (and hydrophilic) molecules cross the outer membrane into the periplasmic space, there are various specific pathways, already mentioned. These include the maltose uptake system, the vitamin B12 system and those concerned with the accumulation of iron. The first of these is in the nature of a pore through the outer membrane (Ferenci and Boos, 1980; Luckey and Nikaido, 1980a, b). It is also possible that a similar system, but involving the specific uptake of glucose rather than maltose, exists in P. aeruginosa, involving protein D1 (Hancock and Carey, 1980).

The systems for the uptake of iron utilise enterochelin, ferrichrome and Fe^{3+} citrate in E. coli and S. typhimurium. In P. aeruginosa, although there are various proteins known to be induced by growth of the organism in iron deficient media, it is not definite that these are involved in the uptake of iron. However, it would seem likely. Iron, because it has a very low solubility when in the ferric form, is not easily obtained by bacteria.

As iron is required in several metabolic processes, bacteria have evolved these systems for acquiring and accumulating iron, as to rely solely on diffusion through the existing channels would be inadequate. The mechanism for the specific transport of vitamin B12 belongs in the category of systems designed to aid the transmembrane diffusion of compounds which would otherwise be unable to penetrate into the cells. The need for the maltose uptake system, and even more surprisingly, a glucose uptake system in P. aeruginosa is difficult to understand, as no real attempts have as yet been made to explain their existence.

1.2.2.3 Diffusion of hydrophilic compounds across the outer membrane

As has been mentioned, it became apparent that the outer membranes of Gram-negative bacteria such as *E. coli*, *S. typhimurium* and *P. aeruginosa* contain pathways by which hydrophilic compounds can cross the membrane. The study of this area has become one of the major activities in membrane bacteriology in recent years, and very considerable advances in knowledge of these pathways have been made (DiRienzo et al., 1978; Nikaido, 1979; Nikaido and Nakae, 1979; Osborn and Wu, 1980). The nature and function of these pathways is discussed in detail in the following section.

1.3 Gram-negative bacterial outer membranes: diffusion channels for hydrophilic compounds

Gilvarg and Katchalski (1965) on the basis of studies with peptides of graded sizes, first noted that although the outer membrane of E. coli was permeable to hydrophilic compounds, there was however some exclusion factor which determined which molecules could pass through the outer membrane. This limit was due either to the size, or the component parts of the permeant molecule (Payne and Gilvarg, 1968a). Both these aspects were investigated, leading to the conclusion that the exclusion limit was associated with the size of the diffusive molecule (Payne and Gilvarg, 1968b).

The outer membrane of E. coli was described as resembling a molecular sieve, with hydrophilic molecules up to a certain size being able to enter the cell through "pores" in the outer membrane (Payne and Gilvarg, 1968b). The various components of the outer membrane were therefore candidates for forming the structures enabling the passage of such hydrophilic molecules into the periplasmic space. (The underlying peptidoglycan was shown not to be required (Nakae and Nikaido, 1975)). Vesicles made of phospholipid and LPS did not show permeability to small, hydrophilic molecules comparable to that of the entire outer membrane (Nikaido and Nakae, 1973), and it was concluded that protein was the component causing the increased membrane permeability (Nakae and Nikaido, 1975). This protein was then identified as being a specific, single protein, given the general title of porin (Nakae, 1976b), and further identified as being the matrix protein.

Knowledge and a somewhat limited understanding of the porin proteins and the pores which they form, have since developed in several directions. These include the structure of the pores and

the manner in which they operate, their size exclusion limit, and their specificity.

1.3.1 Structure and mode of function of the pores

Membrane vesicle reconstitution studies showed that proteins were the source of the permeability of the outer membrane. Vesicles composed of various combinations of the outer membrane components were formed and their permeability measured (Nikaido and Nakae, 1973; Nakae and Nikaido, 1975; Nakae, 1976a, b). This was shown for both *E. coli* and *S. typhimurium* in the mid seventies, and, more recently, for *P. aeruginosa* (Hancock and Nikaido, 1978; Hancock, Decad and Nikaido, 1979). In each case it was shown that the matrix or porin protein was the polypeptide instrumental in enabling various molecules to diffuse across the membrane.

These matrix proteins are organised in trimers (Steven et al., 1977; Ichihara and Mizushima, 1979; Nakae, Ishii and Tokunuga, 1979) forming "pores" through the outer membrane, protruding on the outer surface of the cell, and anchored at the inner surface to the peptidoglycan. There is debate as to whether each monomer of porin contains a channel, or whether a channel can only be formed after association of three porin molecules forming a trimer (Nikaido, 1979).

Perhaps the most important general observations about the channels formed by the porin proteins were the restrictions as to which molecules could pass through them. It appeared that those compounds able to permeate through the pores had to be hydrophilic (Decad and Nikaido, 1976). Otherwise, the matrix protein pores were non-specific, not enabling any molecules preferentially to cross the outer membrane (Payne and Gilvarg, 1968b; Decad and Nikaido, 1976; Nikaido, 1976). This non-specificity goes so far

as to enable antibiotics and other bactericidal compounds to diffuse through the outer membrane (Nikaido, 1976).

Methods for determining which compounds can diffuse across the outer membrane fall into two groups. Some workers tested whether a certain protein was capable of facilitating diffusion through the outer membrane (e.g. Foulds and Chai, 1978; Pugsley and Schnaitman, 1978a, b), or whether a mutant lacking in such outer membranes had been isolated (e.g. Manning, Pugsley and Reeves, 1977). These methods in general investigate the uptake of labelled substrates used in the metabolism of the organism (Manning et al., 1977; Pugsley and Schnaitman, 1978a, b). In addition, in these methods E. coli was most commonly utilised as the test organism.

The second general approach, used with several Gram-negative organisms, was to determine the actual exclusion limit of the pores.

1.3.2 The exclusion limit of the outer membrane pores

The first assay for determining which compounds could cross the outer membrane was a double label assay developed to measure the "saccharide permeable space" in a cell (Decad, Nakae and Nikaido, 1974; Decad and Nikaido, 1976). It was found that ^{3}H labelled oligosaccharides up to approximately 600 d in molecular weight could penetrate into S. typhimurium, E. coli and Proteus morganii. It was less easy to interpret the results obtained for P. aeruginosa, but it was concluded that the organism's outer membrane had a similar exclusion limit to that of E. coli (Decad and Nikaido, 1976; Hancock and Nikaido, 1978; Hancock et al., 1979). Nevertheless, the data could be interpreted as showing that the organism had very large pores.

The use of whole cells was (and is) open to various technical problems. Only certain solutes could be used for measuring permeation through the outer membrane, and it was possible that some of these molecules could cross the outer membrane by pathways other than the pores. In particular, using whole cells, which have been plasmolysed includes the risk of "leaky" outer membranes. In order to obtain an adequate periplasmic volume, Decad and Nikaido (1976) plasmolysed the cells, which would have imposed considerable stress on the outer membrane, and possibly led to artificial results, suggesting the presence of large pores. P. aeruginosa in particular is known to have a fragile outer membrane, and it is possible that the unusual results obtained with the double label assay (Decad and Nikaido, 1976) were the result of plasmolysing the cells and consequentially damaging the outer membrane.

To overcome the inherent risks of plasmolysing the cells, and the restriction on the available choice of permeant compounds, various techniques were developed utilising membrane vesicles composed of phospholipid, LPS and porin molecules extracted from the appropriate organism. These vesicles were then used in a system similar to the double label assay, and with S. typhimurium vesicles, very similar results were obtained (Nakae and Nikaido, 1975).

In another method membrane vesicles have been prepared in the presenc of radiolabelled compounds of various sizes (Nakae, 1975; Nakae, 1976a, b; Hancock and Nikaido, 1978). These are then removed from the radioactive solution, resuspended in buffer and compounds capable of moving through the outer membrane vesicle pores are identified by measuring the radioactivity of the (previously non-radioactive) buffer. The exclusion limit of the pores could thus be determined. This method is considerably more satisfactory than

the double label assay for membrane vesicles as it is both easier to operate and to interpret.

Determining which molecules could or could not cross the outer membrane vesicles was not the only method used for investigating the permeability properties of Gram-negative organisms. Several workers have made black lipid films containing a specific porin protein, and have then measured the conductance caused by the formation of the ion permeable channels through the film. This has enabled the diameter of the pore to be determined, and hence, which compounds are likely to be able to diffuse through them (Benz, Janko and Laugher, 1979; Boehler-Kohler, Boos, Dieherle and Benz, 1979; Benz, Ishii and Nakae, 1980; Benz and Hancock, 1981).

All the above methods are limited in that they do not determine the rate at which compounds are able to penetrate through the outer membrane to the periplasmic space. As a result, two further systems employing membrane vesicles have been developed. Tokunuga, Tokunuga and Nakae (1979) prepared membrane vesicles in the presence of α-glucosidase or α-galactosidase. The vesicles were resuspended in buffer to which was added maltose or maltotriose (α-glucosidase) or raffinose (α-galactosidase) and the rate of liberation of glucose and galactose determined. The rate of degradation of the substrate was then calculated, and hence the rate of penetration of the compounds into the vesicle.

This method is dependent on the use of molecules for which suitable enzymes are available, and is therefore somewhat restricted. However, Luckey and Nikaido (1980a, b) developed a system based on a technique frequently used for measuring the permeability of non-protein containing liposomes. Liposomes containing the porin protein to be investigated were prepared and suspended in an isotonic

solution containing the sugar, of known size, the permeation of which was to be investigated. The rate of swelling of the vesicle, as the sugar penetrated through the pores, was determined, and this represented the rate of permeation of the sugar into the liposome. This method is excellent in that it directly and simply measures the rate at which molecules can pass through the pores, using a system based on well established techniques. A disadvantage about it is that no LPS can be present in liposomes as this molecule is too inflexible to allow the liposomes to expand.

The various techniques have been used to obtain data about the exclusion limit of the outer membrane pores of Gram-negative organisms including E. coli, S. typhimurium, Neisseria gonorrhoea and P. aeruginosa, and to investigate the rate of diffusion of various sugars into E. coli and S. typhimurium. Where the exclusion limit of the outer membrane has been defined, it has generally been found that molecules up to approximately 600 d molecular weight (or possibly a little larger) can cross membrane vesicles containing porin protein from E. coli and S. typhimurium. Using the technique developed by Hancock and Nikaido (1978) it was found that such vesicles containing outer membrane fragments from N. gonorrhoea had an apparent exclusion limit of approximately 870 d, suggesting that the pores in this organism were slightly larger than those of E. coli (Heasley, 1980). P. aeruginosa was found to have very much larger pores, permitting the passage through outer membrane vesicles of molecules of at least 6000 d molecular weight. (The result was generally given as 6000 ± 3000 d.) (Hancock and Nikaido, 1978; Hancock et al., 1979). This was in contrast to the conclusions obtained from the only studies carried out with whole cells of P. aeruginosa (Decad and Nikaido, 1976).

In support of the evidence indicating that P. aeruginosa has very large pores through the outer membrane, work with black lipid films containing protein F showed that the porin channel had a diameter of 2.2 nm (Benz and Hancock, 1981) in contrast to a channel diameter of between 0.8 and 1.4 nm for porins obtained from E. coli and S. typhimurium (Benz et al., 1979; Bochter-Kohler et al., 1979; Benz et al., 1980; Benz and Hancock, 1981).

The studies of the rate of diffusion of molecules through membrane vesicles containing outer membrane proteins has been limited to organisms with small pores, presumably because of the difficulty of obtaining suitable, large compounds of a clearly defined molecular weight. Using the rate of degradation of a substrate (Tokunuga et al., 1979b) it was found that, irrespective of the outer membrane porin used in the vesicle, maltose diffused through the pores more quickly than maltotriose or raffinose, which two sugars diffused at similar rates. Measuring the rate of swelling of liposomes demonstrated that monosaccharides were able to diffuse through OmpF protein pores more quickly than disaccharides (Nikaido, Luckey and Rosenberg, 1980). This in itself is not surprising, and information as to the relative rates of diffusion of larger compounds was not supplied, reducing the usefulness of the work.

Although early whole cell experiments were restricted and problematic, they have a major advantage over any system employing membrane vesicles. In the latter, an artificial, non-viable, copy of the outer membrane of the parent organism has been made, in some cases not even using bacterial phospholipids (Tokunuga et al., 1979b). As a result there is no guarantee that the vesicles represent the whole cell outer membrane, or that they behave as such. Without the

support of the peptidoglycan and cytoplasmic membrane, particularly the points of adhesion, the membrane vesicles may be organised, and behave differently from the cell outer membranes.

However, in a recent study carried out by Nikaido and Rosenberg (1981) using a technique based on the rate of growth of porin deficient mutants of E. coli on various carbon sources of different sizes, it was concluded that E. coli does have small pores (0.6 nm in diameter) and that very small compounds, such as glycerol, diffused into the periplasmic space more quickly than glucose, to which the outer membrane was more permeable than to sucrose. Thus it seemed reasonable to assume that data obtained with membrane vesicles did resemble the truth, and it is therefore at present believed that E. coli and S. typhimurium outer membranes have exclusion limits of approximately 600 daltons, and P. aeruginosa of 6000 ± 3000 d.

1.3.3 The specificity of the outer membrane pores

Much of the work carried out investigating the passive diffusion of compounds through the protein channels in the outer membrane assumed that the pores were non-specific, composed of the matrix (porin) protein, or their equivalent (i.e. OmpF, OmpC, OmpD, protein F). However, various discrepancies were observed, for example, it was noted that ompA mutants of E. coli grew less well than the wild type strains in various nutritional environments (Manning et al., 1977). In particular, these mutants were defective in their ability to acquire amino acids from external sources. As a result, it was proposed that the OmpA protein functioned as a pore (Manning et al., 1977) possibly facilitating the diffusion of amino-acids.

This apparent ability of the OmpA protein to form pores capable of facilitating diffusion of specific compounds is not unique.

Van Alphen et al. (1978) showed that the OmpF protein facilitated permeation of various low molecular weight nucleotides, and also adenosine. None of the other normally occurring outer membrane proteins (e.g. OmpC, OmpA, lamB gene protein) seemed to be involved. However, in mutants in which the nmp genes were expressed it was shown that the PhoE protein (see section 1.1.3.1) could also facilitate the uptake of nucleosides, but did not totally mimic OmpF as adenosine diffusion was not aided (van Alphen et al., 1978).

Lutkenhaus (1977) and Manning et al. (1977) observed that both OmpF and OmpC functioned as pores but that probably there were additional pores, as the loss of both OmpC and OmpF functions did not appear to affect nutrient uptake by laboratory mutants under normal growth conditions. (Under conditions in which very low concentrations of the necessary nutrients were present, the outer membrane protein mutants were at a disadvantage.) It was also noted (Lutkenhaus, 1977) that there could be some separate site through which thymidine permeated the outer membrane. This possibly linked in with the nucleotide and adenosine diffusion facilitating function of the OmpF pore (van Alphen et al., 1978) or was due to the apparent link between the tsx gene product, which is the page T6 receptor protein, and the facilitated diffusion of nucleosides (Hantke, 1976; McKeown et al., 1976; van Alphen et al., 1978). The latter is more likely as the tsx gene product has been clearly shown as functioning as a specific pore facilitating the diffusion of nucleosides, including thymidine (Hantke, 1976; McKeown et al., 1976). Recently it has been demonstrated that the tsx gene product can also act as a specific diffusion pathway for amino acids, including serine, glycine and phenylalanine, but not for arginine (Heuzenroeder and Reeves, 1981).

Although the new outer membrane protein, PhoE, is induced by growth of E. coli in conditions of inorganic phosphate depletion (Overbeeke and Lugtenberg, 1980; Tommassen and Lugtenberg, 1980; Nesmeyanova, Tsfaman and Kulaev, 1981) it would appear that the PhoE protein pore is not specific in facilitating transport of this nutrient (Overbeeke and Lugtenberg, 1980). Rather, these pores seem to be active in facilitating the diffusion of negatively charged compounds (up to a certain size) through the outer membrane of E. coli (Overbeeke and Lugtenberg, 1980; Tommassen and Lugtenberg, 1980).

This contrasts with the possible cationic selectivity of the matrix proteins in E. coli (Benz, Janko and Laugher, 1979). Here it was shown that at neutral pH, pores comprised of matrix proteins in black lipid films were four times more permeable for alkali ions than for chloride ions.

In addition to the observations of pores specific for such compounds as nucleotides, nucleosides and amino-acids, in which the diffusion through that pore is in some manner more favourable for one compound that for another, very specific uptake systems involving periplasmic binding proteins have been observed. In E. coli and P. aeruginosa these have been extensively characterised.

The λ receptor protein in E. coli also acts as a pore, permitting the diffusion of small, hydrophobic molecules, but undoubtedly facilitating the diffusion of maltose and higher maltodextrins (Boehler-Kohler et al., 1979; Ferenci and Boos, 1980; Ferenci, Schwentarat, Ullrich and Vilmart, 1980). Associated with this pore, the genetic locus for which, lamB, is part of the maltose operon, is the periplasmic maltose binding protein. The precise nature of the interaction between the two is as yet unknown, but is has been

suggested that the maltose binding protein is sited at the bottom of the λ pore. The synthesis of both gene products is induced by the presence of maltose in the growth environment, and under normal, maltose-free conditions, the mal operon is not expressed. A protein similar to the λ pore, also induced by growth of the organism on maltose, has been identified in S. typhimurium (Palva, 1978, 1979) but whether this is actually part of a maltose uptake system has not yet been shown.

In P. aeruginosa a similar system has been observed, but induced by, and involved in, the uptake of glucose. Two inducible pathways for the uptake of glucose by P. aeruginosa have been observed, one, with a high affinity of glucose (low Km value of 8 μM for glucose) induced by growth of the organism on glucose, but not gluconate, glycerol, succinate or citrate (Midgley and Dawes, 1973; Dawes, Midgley and Whiting, 1976). The other system, with a low affinity for glucose (Km = 1-2 mM) is induced by growth on glucose, gluconate and glycerol but is repressed by succinate and citrate (Midgley and Dawes, 1973; Dawes et al., 1976).

Stinson, Cohen and Merrick (1977) found a glucose binding protein in the periplasmic space of P. aeruginosa induced by growth of the organism on glucose. In addition, the outer membrane proteins D1, D2 and E are induced by growth of the organism on glucose (Hancock and Carey, 1979). Protein D1 is specifically induced by growth on glucose, and non-metabolisable analogues but not by a variety of other carbon sources, including gluconate, glycerol, citrate and succinate (Hancock and Carey, 1980). Their results indicated that under conditions in which the high affinity glucose uptake system was induced (including the periplasmic binding protein) the D1 protein was also present, whereas there was no correlation

between the presence of D1 and the induction of the low affinity glucose uptake system. Thus D1 would appear to be co-regulated with the high affinity glucose uptake system. Further, protein D1 has been shown to form pores permeable to sucrose and glucose, and possibly the trisaccharide raffinose (Hancock and Carey, 1980). The results in general imply that the outer membrane protein, D1, and the periplasmic binding protein form a high affinity glucose uptake system in P. aeruginosa similar to that found in E. coli for the uptake of maltose.

Despite the wide range of data, it is not entirely clear whether the matrix protein (or their equivalent) pores so far investigated are specific for certain compounds or not. Nikaido et al. (1980) argue that, despite evidence to the contrary (van Alphen et al., 1978) those pores formed by proteins OmpF and OmpC were non-specific other than the limitation of size. This is primarily because the overall rate of a compound such as a nucleoside is determined by the V_{max} and Km of the active transport process, as well as the permeability of the outer membrane. In studies such as those of van Alphen et al. (1978) the rate limiting step cannot be identified, and therefore drawing conclusions as to the specificity of the outer membrane pore is invalid. This conclusion (of Nikaido et al., 1980) clashes in particular with the findings of Benz et al. (1979) that the matrix proteins of E. coli K12 form pores which facilitate the diffusion of cations, at least when present in black lipid films. Little work has been done on the specificity of the pores in P. aeruginosa other than the determination of any relationship between charge and diffusion (Benz and Hancock, 1981), where again, the protein F pores were found to be more permeable to positively charged compounds.

When discussing the non-specificity of OmpF and OmpC protein pores, Nikaido et al. (1980) suggested that there were no differences between the two pore types and their response to the hydrophobicity or charge of the permeant compounds. But this did not rule out the possibility that both pore types facilitate diffusion of cationic compounds, due to the presence of negative charges on the inside of the pore (which is possible as porins are anionic).

The specificity of other pores, such as that formed by the PhoE protein, has not been questioned. However it may well be true in these cases, too, that the conclusions are invalid, due to the difficulties in ascertaining which step (outer membrane permeation or cytoplasmic permeation) is rate limiting.

The debate about the specificity or otherwise of the matrix protein pores has extended to the maltose pore. Although this pore is known to be specific for maltose in some manner, it was suggested that this specificity was in fact conferred by the periplasmic maltose binding protein, possibly located at the base of the λ pore (Heuzenroeder and Reeves, 1980). Using ompB mutants of E. coli which were therefore porin deficient, it was shown that uptake of many substrates was decreased. However, in the absence of the maltose binding protein, the maltose pore appeared to restore permeation of some of these substrates. Also, reconstituted membrane vesicles containing the λ receptor protein appeared to be permeable to various sugars structurally unrelated to maltose (Nakae, 1979). However, Nikaido et al. (1980) believed this work in general to give inadequate data. As a result, the rate of diffusion of various substances through liposomes containing the lamB gene protein was determined. It was found (Luckey and Nikaido, 1980a; Nikaido et al., 1980) that the pore did discriminate between maltose and other

sugars such as sucrose, cellobiose; maltose diffusing into the liposomes considerably faster than the other compounds. This was interpreted as meaning that the lamB protein pore contained a site or sites which did loosely interact with solutes (Luckey and Nikaido, 1980a, b; Nikaido et al., 1980) and that therefore the pore was specific for maltose and related compounds such as maltodextrins.

Another interesting aspect of the debate about porin specificity was introduced when Bewick and Lo (1979, 1980) showed that E. coli dicarboxylate binding protein was not only located in the periplasmic space but also attached to the outer surface of the outer membrane. They demonstrated that this dicarboxylate binding protein played an essential role in the translocation of dicarboxylic acids across the outer membrane (Bewick and Lo, 1979), and that of the integral outer membrane proteins, only OmpF and OmpC were involved in outer membrane dicarboxylate diffusion. When functional dicarboxylate binding protein was absent, the translocation of succinate through the matrix protein channels was very much less efficient, and appeared to be non-specific (Lo and Bewick, 1981). It was proposed that the dicarboxylate binding protein acted either as a gate or plug to the porin channel, or as a substrate recognition component. Although Lo and Bewick (1981) were unable to identify which, if either, of these systems was used, their results suggested that the binding protein could be acting by recognising the substrate.

1.3.4 Mode of operation of the outer membrane pores reconsidered

The multiplicity of the types of pore can now be appreciated. There are primarily non-specific pores with a low molecular weight exclusion limit (approximately 600 d), which may facilitate the diffusion of cationic compounds, found in E. coli and S. typhimurium,

composed of the matrix proteins. These organisms also have similar pores, of probably similar exclusion limits, which do appear to have some specificity of function, such as the PhoE protein pores. There is also present the λ phage receptor pore, which has been shown to be specific for maltose, in E. coli, and possibly an equivalent in S. typhimurium. In P. aeruginosa there is the protein F pore, with a large exclusion limit of approximately 6000 d. There exists a glucose uptake system with pores large enough to accommodate sucrose, comprised of protein D1. And there is the possibility that binding proteins, such as the dicarboxylate binding protein, exist on the outer surface of the outer membrane, conferring specificity on otherwise non-specific pores.

It would seem unlikely that in vivo all the pores appropriate to an organism are present in the same physiological state all the time. It has been suggested (Schindler and Rosenbusch, 1978) that pores formed by E. coli matrix protein could be "opened" or "closed", depending on the potential across the membrane (which has been shown to exist due to the presence of a high level of anion within the periplasmic space (Stock et al., 1977), and it appeared that when there was a high negative potential across the membranes used, the pores were inactivated (or closed). Nikaido et al. (1980) reported that pores formed in E. coli with OmpF protein were approximately ten times more active than OmpC pores to any given solute. As the difference in the number of porin molecules per cell of the two types is not that great, it was thought possible that most of the OmpF channels were "open", whereas many of those formed of OmpC were "closed" under the conditions imposed by their assays (Nikaido et al., 1980).

Benz and Hancock (1981) observed that the activity of purified porin F from P. aeruginosa was approximately one hundred times lower than the equivalent porins obtained from E. coli and S. typhimurium. It was suggested that in P. aeruginosa a large number of the porin channels were closed or inactivated in some way, although not through a voltage gate such as proposed for E. coli (Schindler and Rosenbusch, 1978; Nikaido et al., 1980). The most probable solution was that only a few of the protein F pores were actually functional, which was not the case with E. coli or S. typhimurium pores (Benz and Hancock, 1981).

Recently, Nikaido and Rosenberg (1981), on the basis of the rate of uptake of various carbon sources by porin deficient mutants of E. coli, concluded that there could be no voltage controlled "opening" and "closing" of the pores in the E. coli outer membrane. This was because their results suggested that it would be necessary for a strain of E. coli wild type of porins to have all the pores open all the time.

An additional problem when considering the operation of the outer membrane pores is that they have been described as being "water filled" (Decad and Nikaido, 1976). It is difficult to associate the concept of a channel which is simply filled with water, and the same channel being specific for a given compound.

There are many reported discrepancies concerning the control of the pores in the outer membrane of E. coli, S. typhimurium and P. aeruginosa, and such areas as the "open" and "closed" concepts, and the possibility of outer membrane outer surface binding proteins are of particular importance. It will be necessary for much further work to be carried out to identify the mechanisms by which the pores operate.

1.4 The role of pores and other outer membrane proteins in the resistance of Gram-negative bacteria to antibacterial agents

Nikaido (1976) when discussing the transmembrane diffusion of various substances, found that there were some antibiotics to which the sensitivity of S. typhimurium was not affected, when wild type and deep rough strains were compared. These compounds were mostly hydrophilic, with a molecular weight below 650 d, including penicillin G, ampicillin, cephalothin, carbenicillin, chloramphenicol and tetracycline, and it was suggested that these compounds were crossing the outer membrane through the aqueous pores. Zimmermann and Roslett (1977) supported this as, although they found that the outer membrane acted as a permeability barrier for both penicillins and cephalosporins, they observed that the diffusion of these compounds which did occur fitted a pattern appropriate to passage through an aqueous pore.

Mutant strains of S. typhimurium deficient in porin proteins were found to be less permeable than the wild type to cephaloridine (Nikaido, Song, Shaltiel and Nurminen, 1977) and E. coli OmpF mutants were shown to be more resistant to various antibiotics including tetracycline, chloramphenicol and cepthalothin (Foulds and Chai, 1978b). Sensitivity of the latter mutants was restored in ompF nmpA mutants, in which the new pore-forming membrane protein PhoE was expressed. It was also shown (van Alphen, van Boxtel, van Selm and Lugtenberg, 1978) that E. coli K12 outer membranes composed of protein OmpF allowed the passage, acrosss the outer membrane of cephaloridine and ampicillin. Although it was not clear, OmpC pores possibly performed the same function.

However, this work depended on finding outer membrane protein mutants of E. coli and S. typhimurium and demonstrating that they

were resistant to various antibiotics. Recently, Harder, Nikaido and Matsuhashi (1981) found that various carbenicillin resistant mutants of *E. coli* K12 had reduced levels of the OmpF protein, and that similarly resistant mutants of *E. coli* B/r also had reduced levels of porin. More exactly, the *E. coli* B/r mutants were shown to be moderately resistant to all the β lactams investigated. In the *E. coli* K12 *ompF* mutants, the situation was more complex as the organism compensated for the absence of OmpF by overproducing OmpC. It has been shown (Nikaido et al., 1980) that OmpC is ten times less efficient than OmpF as a pore, and therefore the channels formed from this overproduced outer membrane component would enable permeation of the antibiotics at a reduced efficiency. However, the *E. coli* K12 strains were observed to be resistant to some of the β lactams (as opposed to somewhat less sensitive to all the antibiotics investigated). This was probably due to the variation amongst the antibiotics in the individual rates of permeation through the outer membrane, particularly associated with the negative charges on dibasic β lactams. One of the *E. coli* K12 *ompF* mutants was in fact found to be very resistant to both dibasic and very hydrophobic β lactam antibiotics (Harder et al., 1981).

Some tetracycline and chloramphenicol resistant mutants of *E. coli* lack the OmpF protein (Foulds and Chai, 1978b) and this combined with data obtained about the carbenicillin resistance of the organism suggests strongly that the pores found in *E. coli* and *S. typhimurium* play an important role in antibiotic interaction with the cells. The presence of the pores would in fact seem to confer some disadvantage on the organisms in their attempt to survive, which must be an argument in favour of there being some opening and closing mechanism for the channels.

This should be even more true of P. aeruginosa, where the pores through the outer membrane are reported to be of such considerable size. However, as already mentioned, Benz and Hancock (1981) have suggested that for much of the time a very high proportion of the protein F pores are closed in some manner. Further, specific outer membrane proteins, other than protein F, have been implicated in the resistance of P. aeruginosa to antibiotics. For example, P. aeruginosa grown in Mg^{2+} deficient media become resistant to EDTA and polymyxin B (Brown and Melling, 1969). This could be reversed by transferring the cells to Mg^{2+} sufficient media for several generations. Mutants resistant to polymyxin and EDTA can also be isolated (Brown and Watkins, 1970; Nicas and Hancock, 1981). It was shown (Nicas and Hancock, 1981) that both the mutants and strains grown with insufficient Mg^{2+} had increased levels of the outer membrane protein H1 as compared with the wild type or revertants growing in Mg^{2+} adequate media respectively. It was also determined that in all cases the concentration of Mg^{2+} present associated with the outer membrane varied inversely with the amount of protein H1. The concentration of H1 in the membrane also seemed to be directly related to the resistance of the organisms to aminoglycosides. A possible function of protein H1 was that it replaced Mg^{2+} at sites on the LPS which were otherwise available to attack by cationic antibiotics and EDTA.

Other outer membrane proteins have been implicated in P. aeruginosa hypersensitivity to carbenicillin. Irvin, Govan, Fyfe and Costerton (1981) found that for a pair of mucoid strains of P. aeruginosa, one of which was resistant, and the other hypersensitive to carbenicillin, the hypersensitive strains had two additional outer membrane proteins. These, of molecular weight 32 K and 25 K d

were termed N1 and N2 respectively. Neither were heat or 2 mercapto-ethanol modifiable and further information as to their function was not presented.

So far, increased resistance to antibiotics of P. aeruginosa has not been associated with the absence of the outer membrane porin protein. This may be due to the lack of experience of workers with protein F deficient mutants of the organism. But it would seem more likely that it stems from the very high resistance of P. aeruginosa to many antibiotics and dyes. This resistance is much greater than that of other enterobacteriaceae to the same compounds. Furthermore, it is in contradiction of the findings that P. aeruginosa has pores in its outer membrane of possibly ten times greater exclusion limit than those in enteric bacteria. It would therefore be expected that P. aeruginosa would be sensitive to a wide range of compounds, including those of molecular weight greater than 650 d (Nikaido, 1976) and possibly to more hydrophobic reagents (Nikaido, 1976; Harder et al., 1981) or those carrying a greater charge (Harder et al., 1981).

The role of the outer membrane pores in bacterial resistance would appear to be confusing. In P. aeruginosa, where the large pores would be expected to confer a great disadvantage on the organism, they do not appear to; whereas these enteric bacteria such as E. coli and S. typhimurium, in the wild type form, when pores are present, seem to be at a disadvantage as compared with mutants expressing reduced amounts of any porin. At present, there seems no easy resolution of these problems, although it is clear that in the case of enteric bacteria, where these are disease causing it would be valuable to be able to maintain the organisms in the wild type state. Unfortunately for such medical considerations, it has been suggested that E. coli

and other enteric bacteria are the exception rather than the rule, and that most Gram-negative, non-enteric bacterial outer membranes will more closely resemble the outer membrane of P. aeruginosa (Nikaido, 1979; Benz and Hancock, 1981).

1.5 The relationship between the environment and the outer membrane of Gram-negative bacteria

The outer membrane, like much of the bacterial cell, is extensively influenced by the prevailing environmental conditions (Brown, 1975). Lack of nutrients, e.g. Mg^{2+} (Kenward, Brown and Fryer, 1979) affects the chemical composition of the cell envelope. Lipids and phospholipids are particularly affected by the growth temperatures of the organism (Cronan and Gelmann, 1975), and it has been shown that there is an inverse relationship between the amount of unsaturated fatty acids present in the membrane lipids, and the temperature at which the organisms are growing (Raetz, 1978). This is almost certainly associated with the need for the bacteria to maintain, and therefore regulate, membrane fluidity. This is linked with the outer membrane proteins, for it has been found that the lipid fluidity and physical state of the membrane affected the synthesis and assembly of the outer membrane proteins (DiRienzo and Inouye, 1979).

The influence of the environment on the outer membrane proteins has been investigated with particular reference to the porin proteins. Lugtenberg, Peters, Bernheimer and Berendsen (1976) found that both the composition of the growth medium and the temperature at which the organism was grown, affected the amounts of the various outer membrane proteins found in *E. coli* K12. The ratio of OmpF to OmpC proteins was greatly influenced by the growth media, and although no clear pattern was observed, it seemed that in nutritionally rich media there was far more OmpC and less OmpF protein present in the outer membrane than in cells grown in glucose minimal media. In the latter, the amount of OmpF protein present tended to be 1.5-4 times as great as that of OmpC. Although the amounts of the two

porins varied, the total amount of matrix protein altered far less within any one strain, suggesting that the system regulating the porin concentration was geared to maintaining an approximately constant amount of the porin proteins present in the outer membrane. The effect of growth temperature on the outer membrane protein composition was less dramatic, but it was found that at higher temperatures there was an increase in the concentration of the OmpC protein, and a related decrease in OmpF. Again, the more significant finding was the maintenance of the status quo in terms of the concentration of porin present in the outer membrane (Lugtenberg et al., 1976).

Another important environmental factor found to affect dramatically the porin composition of the E. coli outer membrane was the osmolarity of the growth medium (van Alphen and Lugtenberg, 1977; Kawaji, Mizuno and Mizushima, 1979). van Alphen and Lugtenberg (1977) showed that by supplementing the growth media with high concentrations of NaCl, KCl or sucrose, the amount of OmpF protein present in the outer membrane decreased dramatically, whereas the OmpC porin concentration increased by a roughly equal amount. Similar results were obtained by Kawaji et al. (1979) when the growth medium of E. coli K12 was supplemented with high concentrations of sugars and low molecular weight dextrans. In these conditions, expression of ompC was induced whereas that of ompF was suppressed, leading to a reduction in the concentration of the OmpF protein in the outer membrane, and an increase in OmpC. It was also found that the size of the compound used to influence the osmolarity of the growth environment influenced the extent to which the synthesis of the two outer membrane proteins was switched. Isomaltose, isomaltotriose

and other sugars of up to trisaccharide size were observed to be inefficient at effecting an alteration in the ratio of the two outer membrane proteins, a high concentration being required to cause the change; whereas molecules of 1100 d molecular weight or greater were all highly efficient, the concentration of the compounds required to effect the switch being very much lower. A 700 d molecular weight dextran was found to be intermediate in its ability to change the porin composition of the outer membrane. It was concluded (Kawaji et al., 1979) that those molecules which were unable to pass through the outer membrane pores (exclusion limit 600-700 daltons) were best able to alter the synthesis of porins from OmpF to OmpC. This was probably due to the recognition in some way, by the outer membrane - peptidoglycan layer, of the difference in the osmotic pressure exerted on either side of this layer (see Stock et al., 1977 for discussion of osmotic pressure in Gram-negative bacteria).

Kawaji et al. (1979) suggested that the concentration of the two major outer membrane protein could be affected by environment osmolarity as a response to the differing specificity of the pores formed of OmpF and OmpC proteins. Alternatively, they suggested that the rigidity of the outer membrane - peptidoglycan layer could be different, depending on which outer membrane protein was present, and the change in the concentration of the proteins might be related to this possible difference. However, no ideas supported by data as to why these changes should occur have as yet been presented.

The major conclusion which can be drawn from all the work carried out on the effect of the environment on outer membrane proteins is that E. coli controls the synthesis of the two porins,

and probably other outer membrane proteins such that the overall concentration of the molecules in the outer membrane remains approximately constant. This has been supported by work on the surface density of the major outer membrane proteins in S. typhimurium under varying growth conditions and growth rates (Aldea, Herrero, Esteve and Guerrero, 1980). Here it was shown that, despite considerable variation in growth rate (between 2.4 and 0.31 doublings h^{-1}) the surface density of major outer membrane proteins only varied between 0.9×10^5 and 1.2×10^5 molecules per μm^2, whereas the total surface area of the cell halved between these extremes of the doubling times. Variation in the growth media also had little effect on the surface density of the major outer membrane proteins. It had previously been suggested (Lugtenberg et al., 1976; Boyd and Holland, 1979) that the actual mechanism regulating incorporation of outer membrane proteins into the envelope was determined by the area of cell surface available for occupation by these proteins. These data (Aldea et al., 1980) was taken as strongly supporting that theory.

In addition to such alterations of overall outer membrane protein composition caused by changes in the growth environment, there are the inducible proteins already discussed. These include the lamB gene protein in E. coli and its equivalent in S. typhimurium, proteins D1, D2 and E in P. aeruginosa, and the iron binding proteins in all these organisms. Also some of these proteins which have been shown to be associated with resistance of an organism may be inducible, such as H1 in P. aeruginosa. It would seem likely that there are other inducible proteins, and certainly many other ways in which the growth environment affects the outer membrane of Gram-negative organisms.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Organisms

Escherichia coli K12 W3110, a kind gift of Dr. R. M. M. Klemperer, Department of Pharmacy, University of Aston, and Pseudomonas aeruginosa NCTC 6750, originally obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, were used in these studies. Both organisms were maintained on nutrient agar slopes stored at $4^{o}C$, fresh slope cultures being prepared from multiple colonies at approximately three month intervals.

Nutrient agar plate cultures of each bacterium were also maintained, fresh plates being prepared from a sample every fortnight, and checked for homogeneity. Samples, comprised of many colonies, from these plates were used to inoculate media.

2.1.2 Media

Nutrient agar (NA) Oxoid nutrient agar CM3.

Nutrient broth (NB) Oxoid nutrient broth CM1.

Both these media were obtained from Oxoid Ltd., London SE1 9HF, and were prepared according to the manufacturer's instructions.

Chemically defined medium (CDM) for E. coli: the basic CDM used for routine liquid culture experiments with E. coli is described in Table 2.1. Modifications to the media to provide conditions of nutrient limitation are described in 3.1.2.

CDM used for P. aeruginosa: the basic CDM used for routine liquid culture studies with P. aeruginosa is described in Table 2.2. Modifications to the media to obtain conditions of nutrient limitation are described in 3.1.2.

Table 2.1

Chemically Defined Medium used for E. coli K12 W3110 R- after Klemperer, Ismail and Brown, 1979)

Nutrient	Concentration added (mM)
Glucose	27.0
$MgSO_4.7H_2O$	0.2
KCl	0.3
$NH_4 H_2 PO_4/(NH_4)_2HPO_4$ [a]	25.0
$(NH_4)_2SO_4$ [b]	0.2

a pH 7.2

b added only during studies on Mg^{2+} limited growth

Table 2.2

Chemically Defined Medium used for P. aeruginosa 6750

Nutrient	Concentration added (mM)
KCl	3.00
NaCl	3.00
$(NH_4)_2SO_4$	12.00
$Me_7SO_4.7H_2O$	3.20
$FeSO_4.7H_2O$	0.02
$KH_2PO_4,3H_2O$	1.40
MOPS[a]	50.00
Glucose	20.00

a adjusted to pH 7.4 with NaOH

All media were sterilised by autoclaving for 20 minutes at 115°C. For *E. coli* CDM, glucose, $MgSO_4$ and KCl were autoclaved separately, and for *P. aeruginosa* CDM glucose, K_2HPO_4 and $FeSO_4$ were autoclaved separately. These components were added to the rest of the media (phosphate last) immediately prior to use.

2.1.3 Chemicals

All chemicals were of AnalaR grade, supplied by BDH, Poole, Dorset, or equivalent standard, other than those used in growth limitation studies with iron, where Aristar grade chemicals were used.

Bacitracin (molecular weight (MW) 1411 daltons) was obtained from Sigma Chemicals, London, rifampicin (MW 823 d) was obtained from CIBA Laboratories, Horsham, Sussex, and tetracycline was obtained from Lederle, Cyanamide of Great Britain, London. All were made up in aqueous solution and used immediately.

Dextran T10 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The average molecular weight, and analysis of the dextran molecular weight distribution were provided.

Radiochemicals (^3H n-hexadecane, ^{14}C n-hexadecane, KB$^3H|H_3|$ 616 m Ci/mmol, D$|U^{14}$-C|glucose 3.0 m Ci/m mol, $|U\text{-}^{14}C|$ sucrose 477 m Ci/m mol, $|^3H|$ inulin 5.6 C/m mol, $|6,6'(n)\text{-}^3H|$ sucrose 9.8 C/m mol) were supplied by Amersham International Ltd, Amersham, Buckinghamshire. Prior to use they were diluted, where necessary, in 3% aqueous ethanol.

NE260 micellar scintillant (NE260) was obtained from New England Nuclear, Sighthill, Edinburgh, UK.

2.1.4 General apparatus

Spectrophotometers: optical density measurements for growth curves and lysis experiments, and absorbance of the coloured product of the cysteine-sulphuric acid assay were made using a Unicam SP600 supplied by Pye-Unicam Instruments Ltd, York Street, Cambridge.

Measurements of pH were made using the Pye model 290 pH meter, obtained from Pye-Unicam Instruments Ltd.

Batch cultures were grown in a Mickle reciprocating shaker bath, Camlab Ltd, Nuffield Road, Cambridge, or an orbital shaking incubator, Gallenkamp, Crawley, Sussux, UK. Magnetic stirrers for use with 2l batch cultures were obtained from Gallenkamp.

Cells from 2l cultures were harvested using an MSE Highspeed 18 refrigerated centrifuge, supplied by Measuring and Scientific Equipment Co Ltd, Manor Royal, Crawley, Sussex, or an International refrigerated centrifuge, model B20, manufactured by the International Equipment Company, Second Avenue, Needham Heights, Massachusetts, USA. Other cells were harvested in the above or in an MSE Super Minor bench centrifuge.

Microscopical examination of cultures were carried out using a "Wild" model B20 binocular phase contrast microscope, obtained from Micro Instruments (Oxford) Ltd, Clarendon Street, Oxford.

Millipore membrane filtration apparatus was obtained from Millipore UK Ltd, Heron House, Wembley, Middlesex.

2.1.5 Treatment of glassware

Glassware for all growth of the organisms was washed by total immersion in 5% (v/v) extran 300 (BDH, Poole, Dorset) overnight at room temperature. The glassware was rinsed once in

distilled water, immersed in 1% (v/v) hydrochloric acid and then rinsed six times in distilled water and three times in double distilled water. The glassware was air dried at 60^{o}C, closed with aluminium foil and sterilised by heating at 160^{o}C for 3 hours.

2.2 Methods

2.2.1 Measurement of turbidity

The wavelength for measuring the turbidity of bacterial cultures in suspension was determined as described by Kenward (1975). It was found that the use of 470 nm to measure optical density (OD) of suspensions of both *E. coli* and *P. aeruginosa* was suitable, and that the relationship between optical density and cell concentration obeyed the Beer-Lambert Law provided an OD_{470} of 0.3 was not exceeded. Therefore, this wavelength was selected for obtaining all measurements of optical density of the bacterial suspensions unless otherwise stated.

To ensure that the measurements of turbidity remained within the limits of the relationship between the optical density and cell concentration, those bacterial suspensions with an OD_{470} greater than 0.3 were diluted either x5 or x10 into prewarmed 0.1 M sodium phosphate buffer pH 7.2, or prewarmed CDM minus glucose.

2.2.2 Liquid culture studies

E. coli was grown at 37°C in chemically defined medium (CDM) pH 7.2 (Table 2.1), all components in excess unless otherwise stated, or in nutrient broth, in a shaking water bath, 120 rpm, in 25 ml volumes in 100 ml flasks or in an orbital shaking incubator, 140 rpm, in portions of 200 or 500 ml, in 1 litre conical flasks.

P. aeruginosa was grown at 37°C in CDM pH 7.4 (Table 2.2), all components in excess unless otherwise stated, or in nutrient broth in a shaking water bath or an orbital incubator as described above.

2.2.2.1 Batch cultures of E. coli and P. aeruginosa in 2 litre volumes

Five litre conical flasks containing 2 litres of medium as described previously were used. These were inoculated directly from a 200 ml culture of the same medium grown for 12 hours at 37°C as described above, in the case of cultures to be grown in nutrient broth or complete CDM. For media to provide nutrient limited conditions of growth, a 200 ml complete CDM culture was grown as described above, centrifuged at 5500 g, washed once in medium minus the limiting nutrient, and resuspended in the same. After inoculation the initial OD_{470} of the 2 litre cultures was approximately 0.001.

Flasks containing inoculated media were incubated either in an orbital incubator (140 rpm, 37°C) or in a warm room maintained at 37°C, each stirred with a magnetic follower. The speed of the followers was adjusted so that the vortex generated extended down to the follower. As a result, numerous small air bubbles were produced in the medium thus providing a large surface area for gaseous exchange between the atmosphere and the culture.

Where the cells were required to have grown to mid-log phase, the cultures were harvested when they had attained an OD_{470} of approximately 0.6. Those cells required to have grown to stationary phase, or to a condition of nutrient depletion, were harvested approximately four hours after the cessation of exponential growth.

In cases where tetracycline was used to limit the growth of the cells, E. coli was grown in complete CDM 1 to an OD_{470} of approximately 0.2. 1 ml tetracycline (1 mg ml^{-1}) was added and the culture allowed to grow for a further two generations, to an OD_{470} of approximately 0.8.

2.2.3 Viable counts

Cells were grown as described above (2.2.1). Using suspensions with an OD_{470} of approximately 1.0 (equivalent to about 10^9 cells ml^{-1}) tenfold dilutions were made in 0.1 M sodium phosphate buffer pH 7.2, unless otherwise stated, to give approximately 10^3 cells ml^{-1}.

Six 0.1 ml samples of the final dilution were spread on overdried nutrient agar (control) plates, and in experiments investigating the effect of plasmolysing the cells, three portions were spread on overdried nutrient agar plates containing appropriate antibiotic concentrations. At the end of the experiment, a further six control plates were set up. This was to determine any loss of viability of the suspensions during the experiment.

Plates were incubated for 18 hours at $37^{o}C$ and the number of colonies present on each counted.

2.2.4 Total counts

Total counts of cultures were determined using haemocytometer counting chambers, having a chamber depth of 0.1 mm and "Improved Neubauer" rulings.

1 ml of a suspension to be counted was added to 1 ml 4% (v/v) formaldehyde. Further dilutions were made if necessary, such that when counting, there were approximately 8-12 cells per small square of the grid. The dilution was allowed to run under the coverslip, which had previously been pressed down over the counting chamber until Newton's rings were visible, so that there were no air-bubbles in the chamber, nor did material overflow into the troughs beside the counting grid. The chamber was allowed to stand for 20 minutes prior to counting. It was then viewed under phase contrast with a

x40 objective and a x15 binocular eyepiece. Bacteria in 80 small squares were counted, and the total count determined.

2.2.5 Scintillation counting

Scintillation counting was carried out using a Packard Tri Carb 2660 Liquid Scintillation System (Packard TriCarb Instrument Corporation Inc., Downers Grove, Illinois, USA). Quench curves were programmed using ^{3}H n-hexadecane and ^{14}C n-hexadecane standards in NE260, following the manufacturer's instructions. Thus all data obtained were adjusted for the efficiency of the scintillant, and were expressed as disintegrations per minute (dpm).

2.2.6 Preparation of ^{3}H labelled oligosaccharides

2.2.6.1 Manufacture

To obtain an homologous series of oligosaccharides with molecular weights between 300 and 1500 daltons, a 20% (w/v) solution of Dextran T10 in 0.1 M sodium phosphate buffer, pH 6.0, was made up. To 20 ml of this solution were added 4 ml dextranase solution (10 units ml^{-1} in 0.1 M sodium phosphate buffer pH 6.0). The solution was mixed and incubated in a shaking water bath for 2 hours at 37°C, 120 rpm. The mixture was then heated at 100°C for 10 minutes to denature the enzyme, and stored at -20°C until required.

To label the oligosaccharides, 0.2 ml 10 M NaOH was added to 6 ml of the hydrolysed dextran solution, to increase the pH to at least pH 10. To this were added 25 mCi $KB|^{3}H|H_{3}$, the solution was mixed and incubated at room temperature for 9 hours. 60 mg potassium borohydride (unlabelled) were then added and, after mixing, the solution was allowed to stand at room temperature for a further 16 hours. To stop the reduction, 0.3 ml glacial acetic acid was

added, the solution mixed thoroughly and allowed to stand until any effervescence had ceased before use. Where necessary, the solution was stored at $4^{o}C$.

To obtain unlabelled, reduced oligosaccharides, the above procedure was carried out, omitting the addition of $KB|^{3}H|H_{3}$ and the first, 9 hour, incubation period.

2.2.6.2 Separation of reduced oligosaccharides

The reduced oligosaccharides, labelled or unlabelled, were separated using a custom-built gel filtration column of Bio-Gel P2 - 400 mesh (Bio-Rad, California, USA) 150 cm long, 18 mm diameter, jacketed and heated at $65^{o}C$, and eluted continuously with deaerated distilled water at a rate of 0.8 ml $minute^{-1}$ using a Super Mini Pump (Model NIC) (supplied by Michael Smith Engineering, Woking, Surrey, UK) (John, Trenel and Dellweg, 1969). Samples were loaded on to the top of the gel through the rubber diaphragm in a GLC injection head (Pye-Unicam, Cambridge) which was connected to the top of the column by a flanged teflon headpiece. The flange on the teflon connection was clipped to a corresponding glass flange on the top of the column, providing a pressure-tight seal.

The column was calibrated against a similar column run by Dr. P. Sommers, Department of Organic Chemistry, University of Birmingham, using a sample of unlabelled, hydrolysed dextran and one of unlabelled, reduced, hydrolysed dextran, both samples containing added glucose. This calibration enabled immediate and certain identification of the compounds obtained in the fractions collected from the column.

To separate the various oligosaccharides, 6 ml of the hydrolysed, reduced, dextran solution were mixed with 1 ml glucose solution (30 mg ml^{-1}) and, for the separation of labelled sugars, 0.05 ml

^{14}C glucose solution (2.02 μ Ci ml^{-1}). 0.5 ml of this solution was then loaded on to the top of the gel. Fractions of 3 ml volume were collected using a Redirac 2112 fraction collecter (LKB Instruments Ltd., Croydon, Surrey, UK) and the samples were analysed for the presence of carbohydrate and, if appropriate, radioactivity.

2.2.6.3 Cysteine-sulphuric acid assay for the presence of carbohydrate

The presence of carbohydrate was determined using the method of Hatt (1970). Solutions containing 10, 20, 25, 40 and 50 $\mu g\ ml^{-1}$ dextran T10 dissolved in filtered, deionised distilled water were made up. 0.5 ml of each standard was placed in chemically clean, dry, stoppered tubes and 2.5 ml cysteine-sulphuric acid reagent (0.7 $g\ L^{-1}$ cysteine in 86% (v/v) sulphuric acid) were added, with mixing. The tubes were heated in a boiling water bath for 3 minutes, cooled to room temperature and the absorbance, at 414 nm, of the solution was measured. A calibration curve of optical density (absorbance) against concentration of dextran was plotted.

The fractions collected from the column were diluted to a suitable concentration by mixing 0.05 ml of each fraction with 0.45 ml filtered, deionised distilled water in chemically clean, stoppered tubes. 2.5 ml cysteine-sulphuric acid reagent were added to each tube and the assay continued as above. The optical density appropriate to each fraction was plotted against fraction number.

2.2.6.4 Radiochemical assay

0.1 ml of each fraction was mixed with 5 ml NE260 in suitable scintillation vials, which were counted for one minute. The dpm present in each vial were plotted against fraction number.

2.2.6.5 Identification and storage of the labelled oligosaccharides

Using the plots of the amount of sugar or radiolabel against fraction number, those fractions containing clearly separated,

Table 2.3

^{3}H labelled oligosaccharides manufactured

Name[a, b, c]	Molecular weight (daltons)	Final concentration (μM)	Approximate specific activity ($\mu Ci/\mu mol^{-1}$)
isomaltitol	346	120.0	10.0
isomaltotriitol	508	120.0	10.0
isomaltotetritol	670	120.0	10.0
isomaltopentitol	833	120.0	10.0
isomaltohexitol	995	120.0	10.0
isomaltoheptitol	1157	120.0	10.0

a. For correct chemical nomenclature, see Appendix 1.

b. The method of identification is described in the text.

c. In the text, these will normally be referred to as OS (oligosaccharide) followed by the molecular weight, e.g. OS 346, OS 833.

labelled oligosaccharides were identified (Table 2.3). To each of these fractions was added 0.1 ml absolute alcohol and those fractions containing molecules of the same size were pooled. The individual oligosaccharides were identified by comparing the graphs showing sugar or radiolabel concentration against fraction number with the similar plots obtained from the column at Birmingham University. As glucose is not obtained when Dextran T10 is hydrolysed, the peaks representing glucose could be used to align the calibration plot with those obtained from separating the oligosaccharides.

Reduced, unlabelled sugars were pooled and identified in the same manner, and stored at -20°C until required.

The concentration of material present in each oligosaccharide solution was determined using the cysteine-sulphuric acid assay. Each solution of radiolabelled sugars was diluted to a concentration of 120 µM in 3% ethanol. The dpm ml^{-1} present in the solutions were determined by mixing 0.1 ml of each solution with 5 ml NE260, in triplicate, and counting these for 10 minutes. The average dpm ml^{-1} was calculated for each solution, and also the specific activity (µCiµ mol^{-1}). Where necessary, the specific activity of the labelled oligosaccharide solutions was adjusted using the appropriate, reduced, unlabelled oligosaccharide so that there were approximately 10 µ Ci µ mol^{-1}, whilst retaining the molarity of the oligosaccharide solutions at 120 µM.

The radiolabelled solutions were sterilised by filtration through 0.22 µm membrane filters, previously washed in three changes of boiling water to remove wetting agents (Brown, Farwell and Rosenbluth, 1969). The standardised oligosaccharide solution were stored at 4°C until required.

2.2.6.6 Purity of the labelled oligosaccharide solutions

To ensure that the various oligosaccharide solutions were not contaminated with other sugars, 0.3 ml of the oligosaccharide solution was mixed with 0.05 ml glucose solution (30 mg ml^{-1}) and and 0.01 ml ^{14}C glucose (2.02 μ Ci ml^{-1}). This solution was loaded on to the top of the gel and 3 ml fractions collected as before.

The fractions were analysed as described above. Any oligosaccharide solutions containing a 5% impurity, or greater were discarded.

To ascertain whether there was any decomposition of the oligosaccharides, the above procedure was repeated at two to three week intervals. The redetermined specific activity of the substance was compared with that in the original solution, and if decomposition exceeded 10%, the solution was discarded.

2.2.7 Sonication of cells

Cells were sonicated over ice after suspension in water or 0.1 M sodium phosphate buffer pH 7.2 using a Soniprobe Automatic Type 7532A, (Dawe Instruments Ltd., Western Avenue, London, UK). Any remaining whole cells were removed by centrifugation for 10 minutes at 5000 g, 4°C.

2.2.8 Extraction of outer membrane proteins

Outer membrane proteins were extracted from whole cells of E. coli and P. aeruginosa using 2% sodium lauryl sarcosine (sarkosyl) after the method of Filip, Fletcher, Wulff and Earhart (1973). After washing twice with water, the proteins were resuspended in 0.5 ml water and stored at -20°C until required.

2.2.9 Analysis of outer membrane proteins

The protein composition of the outer membrane of E. coli and P. aeruginosa grown under various conditions was analysed using sodium dodecyl sulphate (SDS) - 12% polyacrylamide gel electrophoresis, after the method of Lugtenberg et al. (1975). The outer membrane proteins were prepared for the gel electrophoresis with a sarkosyl extraction (2.2.8).

3. EXPERIMENTAL

3.1 Nutrient limitation studies

3.1.1 Introduction

An accepted method of studying the effect of the environment on bacteria is to limit the growth of the organisms in batch culture by reducing the amount of a specific nutrient added to the media (Klemperer, Ismail and Brown, 1979). To study the effect of the environment on the size of the outer membrane pores, it was decided to restrict the growth of E. coli and P. aeruginosa with various limiting factors, such as some of the compounds required for the growth of the organisms, and with E. coli also to investigate the effect of adding tetracycline to the growth medium (Brown and Garrett, 1964).

Media designed specifically for the purpose of limiting growth with a variety of compounds have been determined for E. coli W3110 R- (Klemperer et al., 1979) and P. aeruginosa 6750 (Ombaka, 1980). However, in order to ensure that suitable growth limiting concentrations of nutrients and tetracycline were used, studies were carried out in which the amount of a specific compound was varied, and the consequent growth of the bacteria determined.

3.1.2 Method

3.1.2.1 E. coli: carbon limitation studies

E. coli was grown overnight in a 25 ml volume of complete CDM (2.1.2, Table 2.1). Cells were harvested by centrifugation (5500 g, 10 minutes, RT), washed twice in CDM minus glucose and resuspended in the same to an OD_{470} of 0.5. 1 ml of this suspension was used to inoculate 25 ml portions of CDM containing different

concentrations of glucose, as follows:- zero, 0.0005 M, 0.001 M, 0.002 M, 0.003 M, 0.005 M, 0.010 M, 0.020 M, 0.027 M. All concentrations were present in triplicate. The initial OD_{470} was read and the flasks incubated as described (2.2.2). Further readings were taken every 30 minutes, diluting the culture in 0.1 M sodium phosphate buffer where appropriate (i.e. so that the reading did not exceed 0.3) until the cell populations were in stationary phase.

The log of the OD reading was plotted against time for the various cell populations, and the optical density reached at the point of cessation of logarithmic growth determined for each culture. These were plotted against the concentration of glucose appropriate to each reading. The growth rate (μ) of the organism in each medium was also determined.

3.1.2.2 E. coli: magnesium limitation studies

E. coli was grown overnight as described above, harvested, washed twice in CDM minus Mg^{2+} and resuspended in the same to an OD_{470} of 0.5. 1 ml portions were used to inoculate 25 ml portions of CDM containing different concentrations of $Mg\ SO_4$, as follows: zero, 0.01 mM, 0.03 mM, 0.05 mM, 0.10 mM, 0.20 mM. All concentrations were present in triplicate. The experiment was carried out as described above.

3.1.2.3 P. aeruginosa: carbon limitation studies

P. aeruginosa was grown overnight in a 25 ml volume of complete CDM (2.1.2, Table 2.2). Cells were harvested by centrifugation (5500 g, 10 minutes, RT) washed twice in CDM minus glucose and resuspended in the same to an OD_{470} of 0.5. 1 ml of this suspension was used to inoculate 25 ml portions of CDM containing different

concentrations of glucose as follows: zero, 0.001 M, 0.002 M, 0.004 M, 0.006 M, 0.008 M, 0.01 M. All concentrations were present in triplicate. The initial OD_{470} was read and the experiment carried out as described above.

3.1.2.4 P. aeruginosa: iron limitation studies

P. aeruginosa was grown to mid-log phase (OD_{470} approximately 0.5) in a 25 ml volume of complete CDM as before. A further 25 ml volume of CDM minus iron was inoculated from this first culture and allowed to grow overnight. Cells were harvested as above, washed twice in CDM minus iron and resuspended in the same to an OD_{470} of 0.5. 1 ml of this suspension was used to inoculate 25 ml portions of CDM containing various concentrations of iron (added as $FeSO_4.7H_2O$) as follows: zero, 4.4×10^{-8} M, 8.9×10^{-8} M, 4.4×10^{-7} M, 8.9×10^{-7} M, 8.9×10^{-6} M. All concentrations were present in triplicate. The experiment was carried out as before.

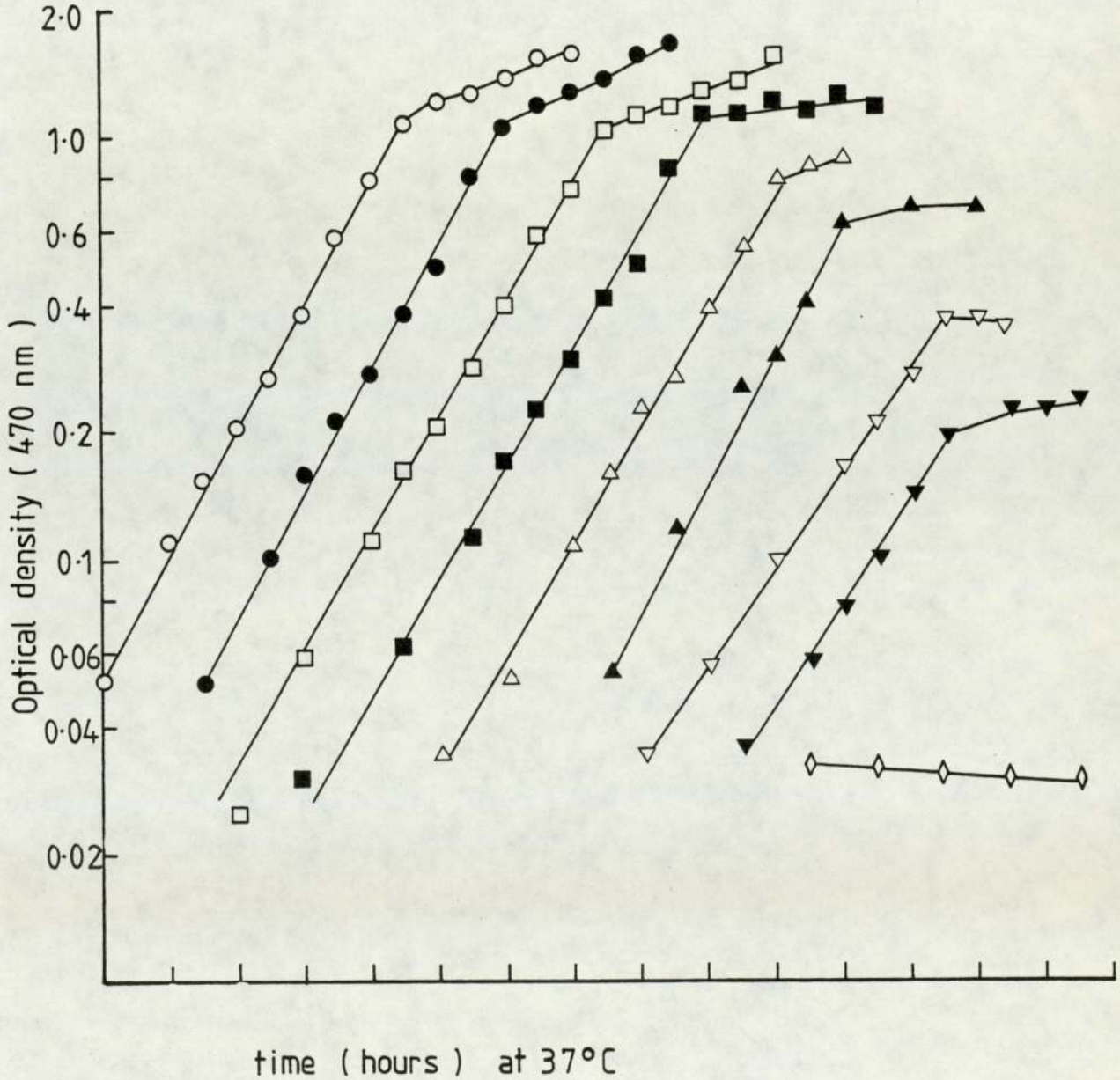
3.1.2.5 E. coli: tetracycline limitation studies

E. coli was grown up overnight in a 25 ml volume of CDM (2.1.2, Table 2.1), harvested, washed twice in complete CDM and resuspended in the same to an OD_{470} of 0.5. 1 ml portions of this suspension were used to inoculate 25 ml portions of complete CDM containing varying concentrations of tetracycline as follows: zero, 0.06 $\mu g\ ml^{-1}$, 0.125 $\mu g\ ml^{-1}$, 0.25 $\mu g\ ml^{-1}$, 0.5 $\mu g\ ml^{-1}$, 1.0 $\mu g\ ml^{-1}$ 2.0 $\mu g\ ml^{-1}$, 8.0 $\mu g\ ml^{-1}$. All concentrations were present in triplicate. The experiment was carried out as described above.

3.1.3 Results

3.1.3.1 E. coli: carbon limitation studies

Figure 3.1 shows the growth of E. coli W3110 in CDM containing various concentrations of glucose. Where the media contain 0.5, 1.0


2·0
1·0
0·6
0·4
0·2
0·1
0·06
0·04
0·02
Optical density (470 nm)
time (hours) at 37°C

and 2.0 mM glucose, there was an abrupt cessation of logarithmic (exponential) growth, at an increasingly high OD, whereas with higher glucose concentrations there is a more gradual change from exponential growth to stationary phase. The growth rates (μ) (Table 3.1) for the populations growing with low concentrations of glucose, in particular 0.5 and 1.0 mM, are slower than those obtained for cultures containing 2.0 mM glucose or more.

Figure 3.2 expresses the relationship between the concentration of glucose present in the media and the OD_{470} reached when the cell population ceases to grow exponentially. It can be seen that in this media the quantity of glucose present is the limiting factor with concentrations less than 3 mM. Addition of amounts of glucose greater than this does not enable further exponential growth.

3.1.3.2 E. coli: magnesium limitation studies

Figure 3.3 shows the growth of E. coli W3110 in CDM containing various concentrations of Mg^{2+}. In media containing zero, 0.001, 0.003 and 0.005 mM Mg^{2+} the OD reached before cessation of logarithmic growth is increasingly high; whereas those cell populations grown in the presence of greater concentrations of Mg^{2+} do not reach a higher OD before the cells cease to grow exponentially than E. coli growing in the presence of only 0.005 mM Mg^{2+}. The growth rates also reflect the change in the quantity of Mg^{2+} in the media (Table 3.2), μ increasing with increasing concentration of Mg^{2+} in cultures containing up to 0.1 mM of the divalent cation. Thereafter, μ remained approximately constant irrespective of the concentration of the ion.

The linear relationship between the point of cessation of exponential growth and the concentration of added Mg^{2+} is shown in

Table 3.1

Calculated growth rate (μ) of E. coli W3110 R- grown in CDM containing graded concentrations of added glucose[a].

Concentration of glucose (M)	(h^{-1})
0.0005	0.54
0.001	0.49
0.002	0.69
0.003	0.65
0.005	0.65
0.010	0.64
0.020	0.67
0.027	0.68

Table 3.2

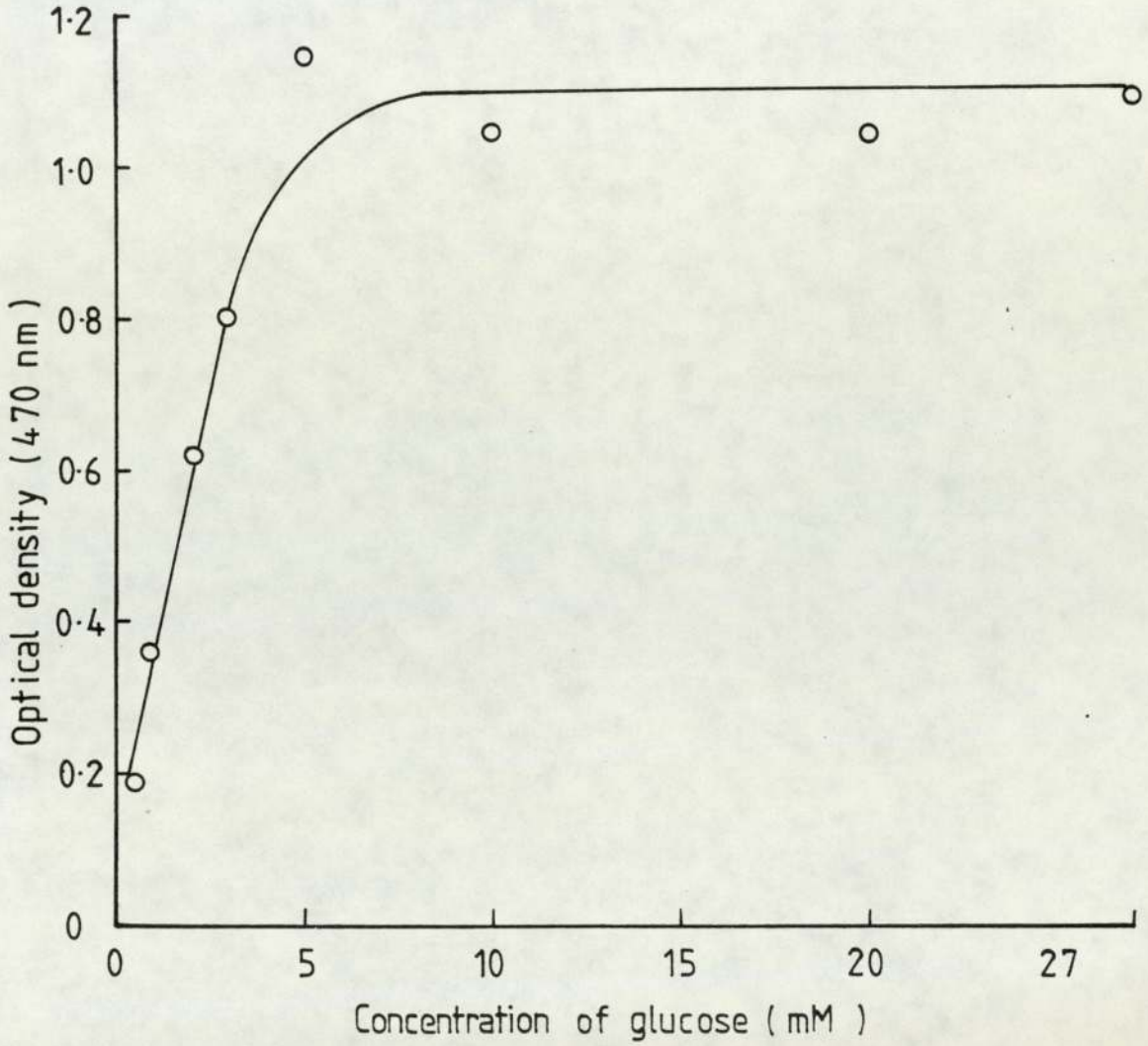
Calculated growth rate (μ) of E. coli W3110 R- grown in CDM containing graded concentrations of added Mg^{2+}[a].

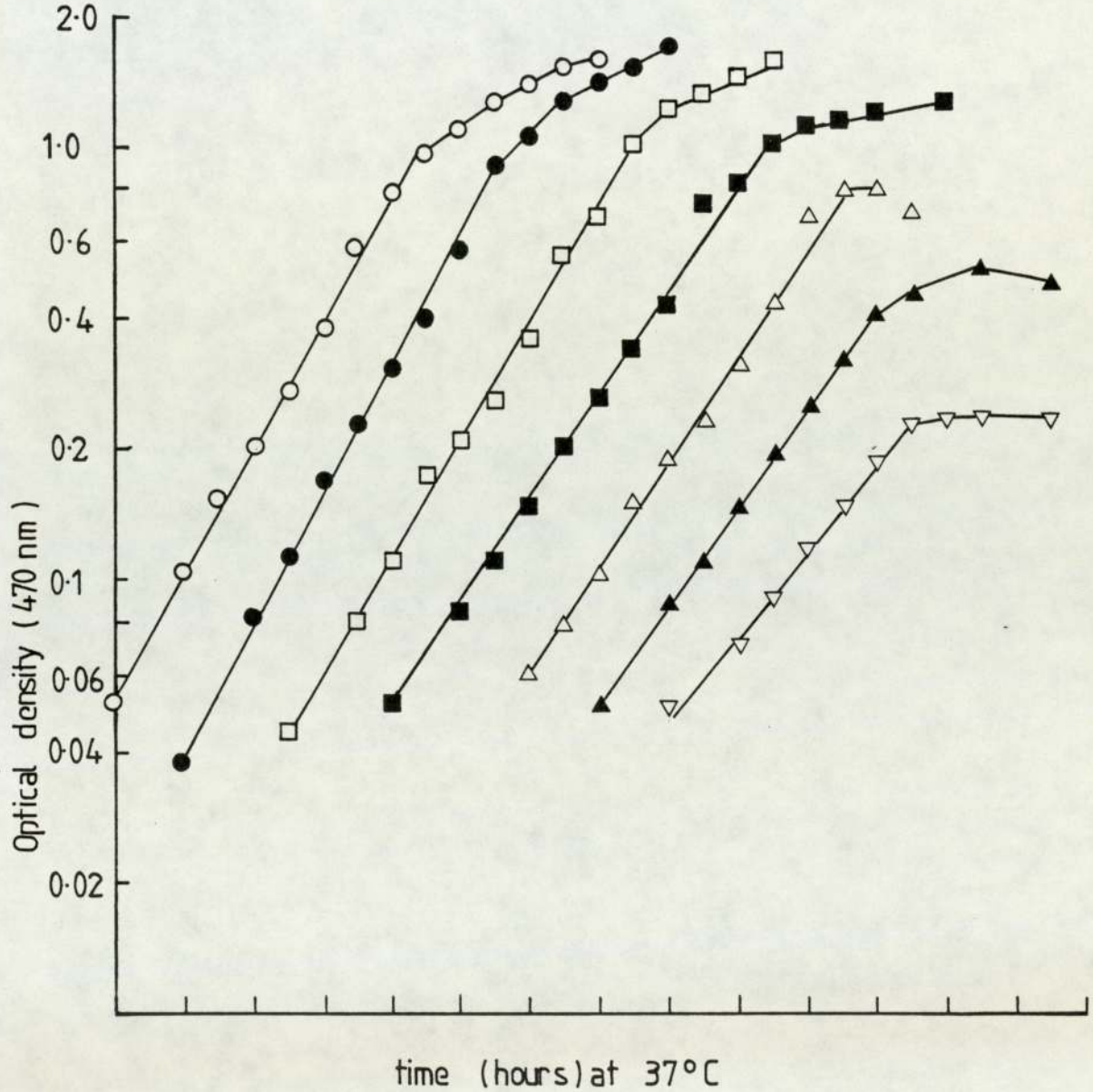
Concentration of Mg^{2+} (mM)	(h^{-1})
0	0.46
0.01	0.51
0.03	0.57
0.05	0.57
0.10	0.66
0.15	0.69
0.20	0.68

a. The growth rate was calculated by determining the mean generation time (MGT) of the organism during logarithmic phase growth in each medium, and using this to calculate the growth rate, μ where

$$\mu = \frac{\log_e 2}{MGT}$$

Figure 3.2: Optical density at the point of cessation of logarithmic growth against concentration of added glucose for *E. coli* W3110 R-.


1·2
1·0
0·8
0·6
0·4
0·2
0
Optical density (470 nm)
0
5
10
15
20
27
Concentration of glucose (mM)


2·0
1·0
0·6
0·4
0·2
0·1
0·06
0·04
0·02
Optical density (470 nm)
time (hours) at 37°C

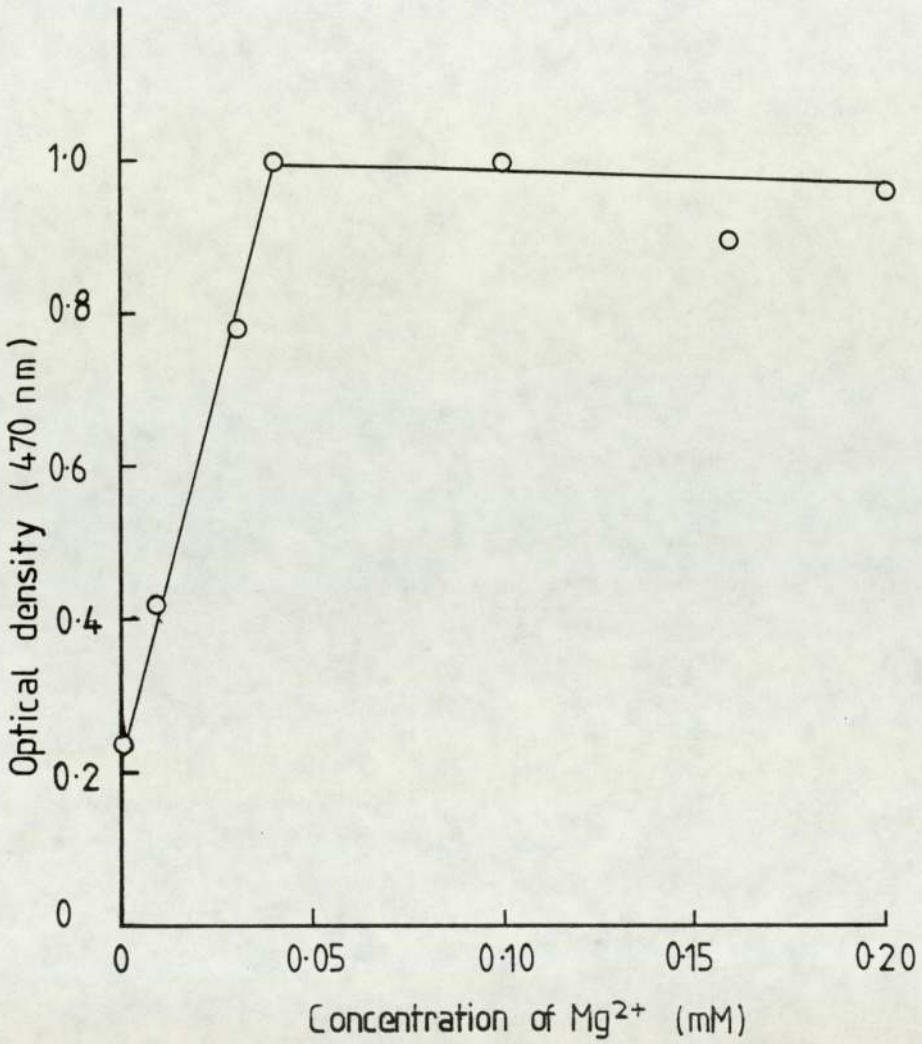

Optical density (470 nm)
1·0
0·8
0·6
0·4
0·2
0
0
0·05
0·10
0·15
0·20
Concentration of Mg2+ (mM)

Figure 3.4. This continued until 0.035 mM Mg^{2+} was added, when further addition of the cation did not affect the logarithmic growth of the organism. As a result, it was decided to add 0.015 mM Mg^{2+} to the CDM in experiments where the growth of E. coli was to be limited by the concentration of Mg^{2+} present.

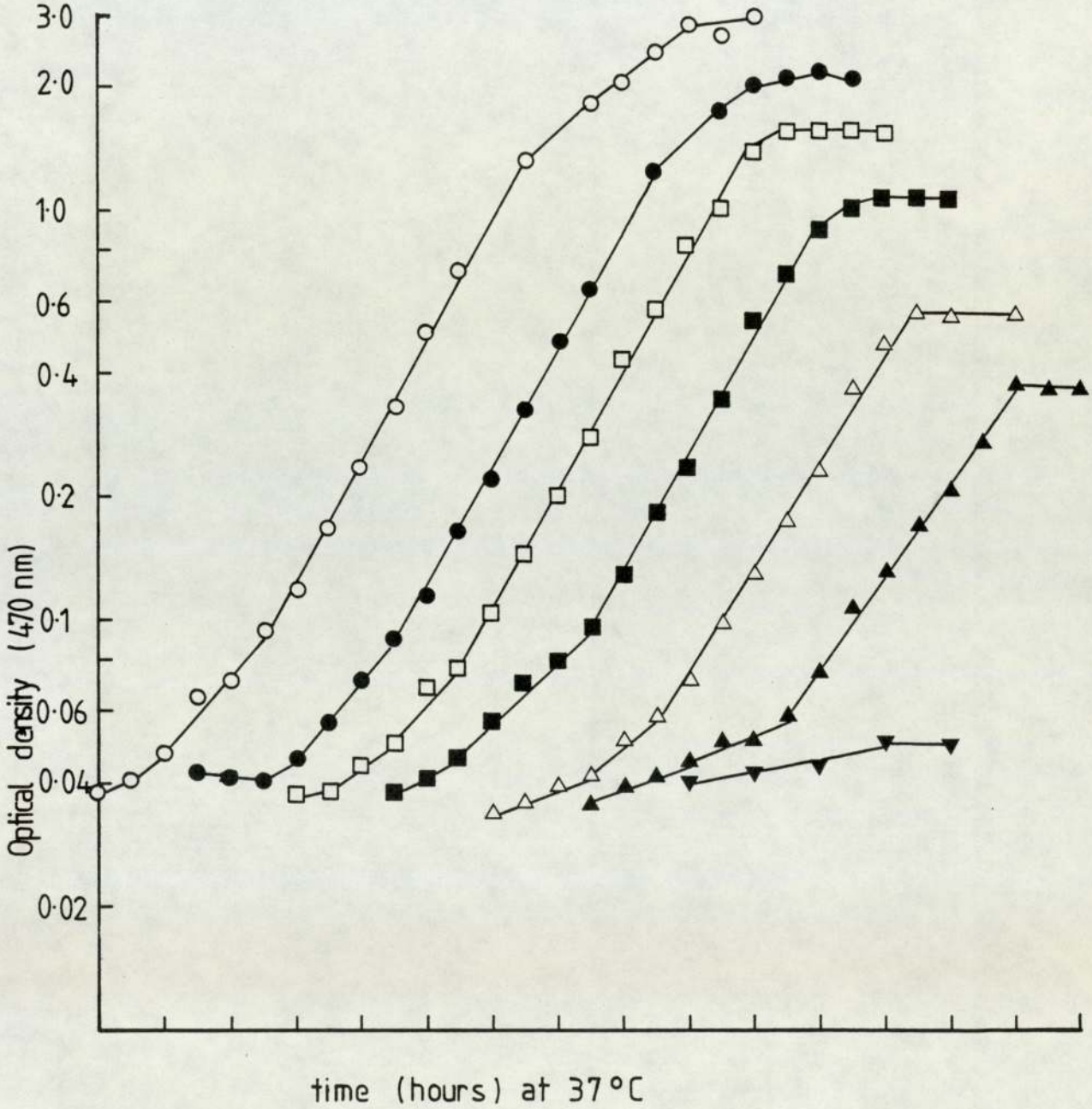
3.1.3.3 P. aeruginosa: carbon limitation studies

Figure 3.5 shows the growth of P. aeruginosa 6750 in CDM containing various concentrations of glucose. Those populations grown with 0.001 M, 0.002 M, 0.004 M and 0.006 M glucose show a very abrupt cessation of exponential growth, whereas with higher glucose concentrations the cells enter stationary phase more gradually. The growth rates obtained from the different populations are slower for those cultures growing with lower concentrations of glucose, most notably 0.001 M and 0.002 M (Table 3.3).

Figure 3.6 shows that the point at which exponential growth ceases is, within limits, proportional to the concentration of glucose. It can be seen that in this CDM the amount of glucose present is a limiting factor at concentrations less than 0.006 M. Addition of further glucose does not enable exponential growth to continue to a higher OD.

3.1.3.4 P. aeruginosa: iron limitation studies

The growth of P. aeruginosa 6750 in the presence of various concentrations of iron is shown in Figure 3.7. Those cell populations grown without iron, or with 4.4×10^{-8} M, 8.9×10^{-8} M or 4.4×10^{-7} M added iron reach increasingly high optical densities before cessation of logarithmic growth. The patterns of growth of P. aeruginosa in media containing 4.4×10^{-7} M, 8.96×10^{-7} M or 8.96×10^{-6} M iron are all similar. The growth rates (Table 3.4) of the cultures decreased with decreasing iron concentration.


3·0
2·0
1·0
0·6
0·4
0·2
0·1
0·06
0·04
0·02
Optical density (470 nm)
time (hours) at 37°C

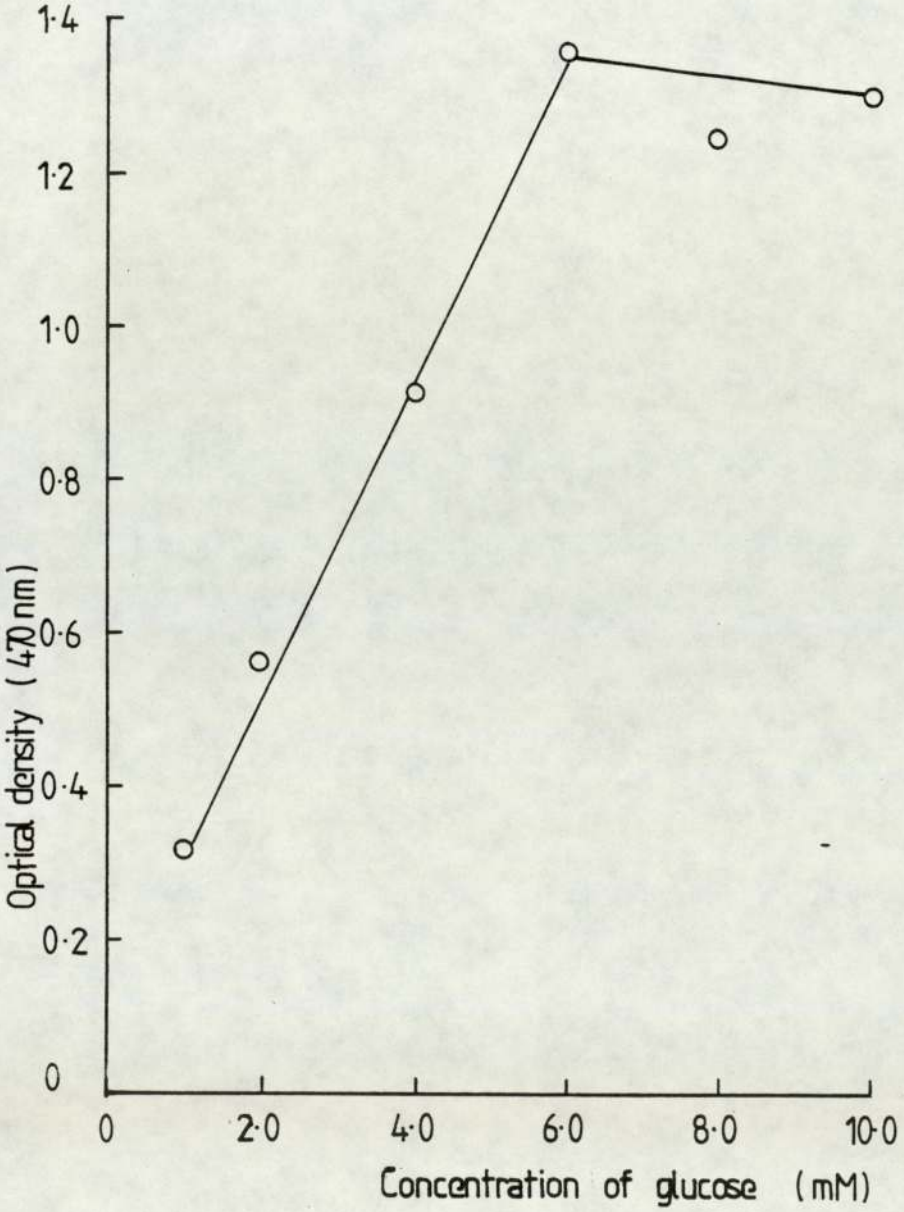

1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Optical density (470 nm)
0
2·0
4·0
6·0
8·0
10·0
Concentration of glucose (mM)

Table 3.3

Calculated growth rate (μ) of P. aeruginosa 6750 grown in CDM containing graded concentrations of added glucose[a].

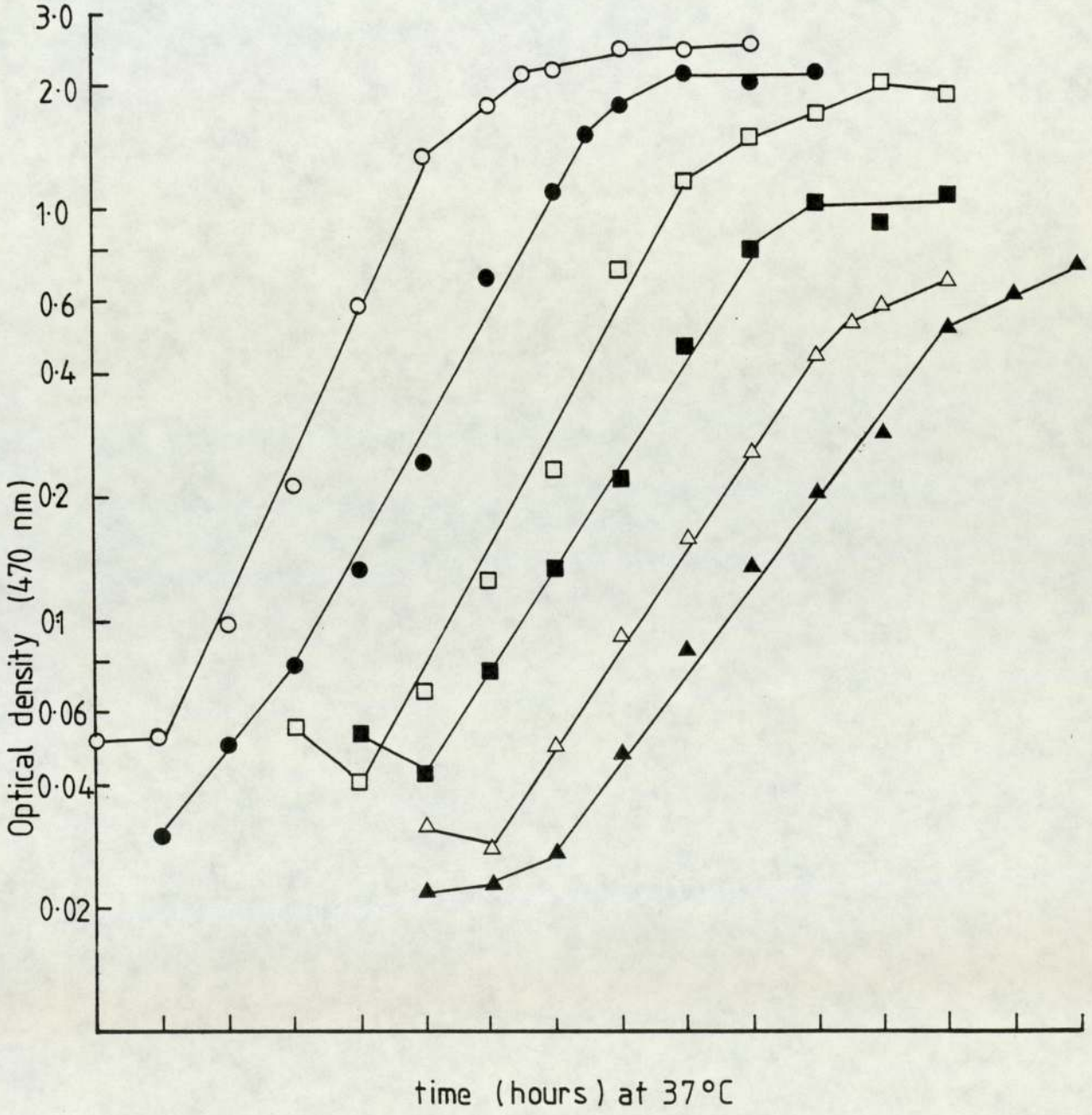
Concentration of glucose (M)	(h^{-1})
0.001	0.51
0.002	0.60
0.004	0.65
0.006	0.67
0.008	0.66
0.010	0.66

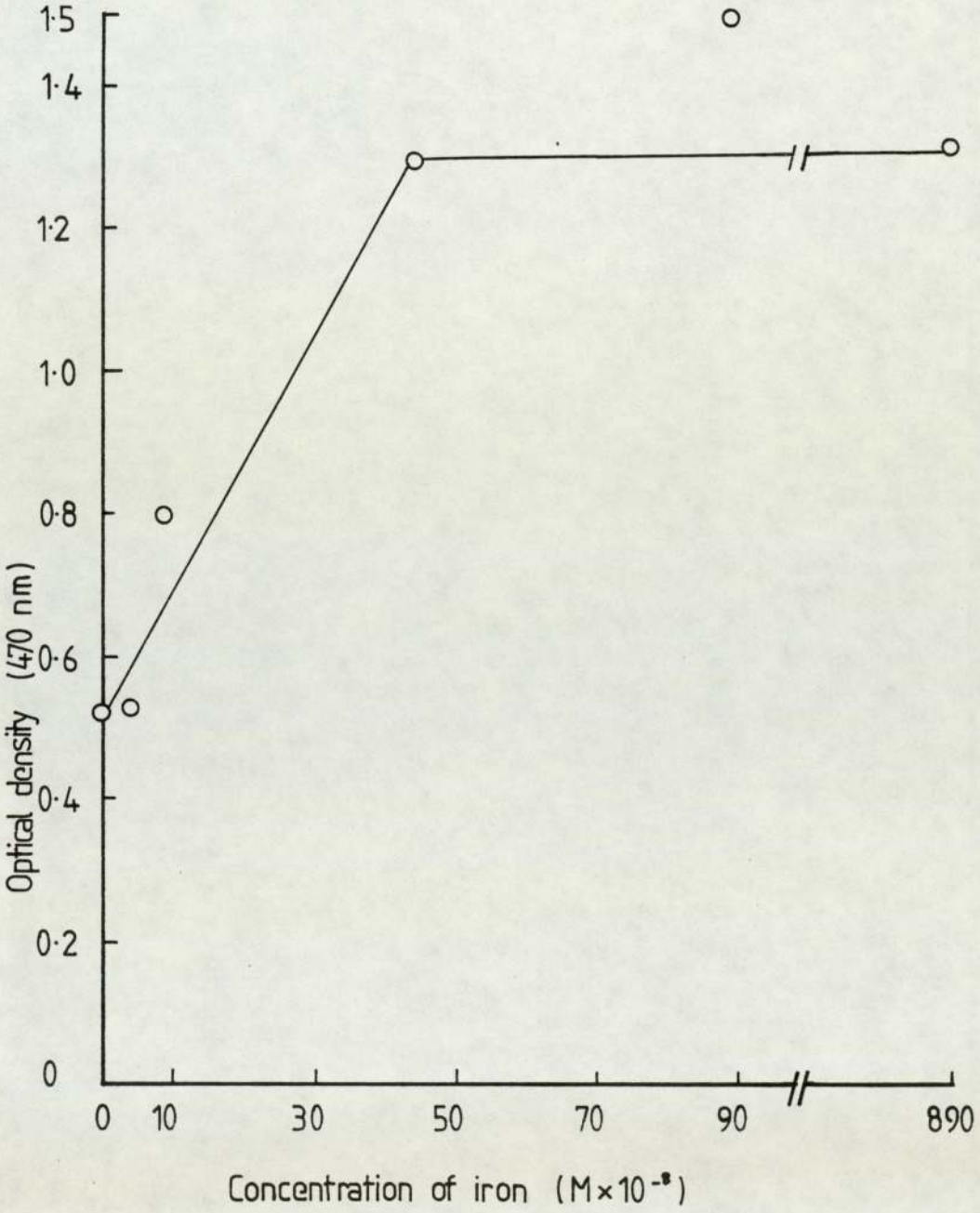
Table 3.4

Calculated growth rate (μ) of P. aeruginosa 6750 grown in CDM containing graded concentrations of added iron [a].

Concentration of iron (M)	(h^{-1})
0	0.48
4.4×10^{-8}	0.53
8.9×10^{-8}	0.59
4.4×10^{-7}	0.63
8.9×10^{-7}	0.69
8.9×10^{-6}	0.75

a. For calculation of μ, see Tables 3.1 and 3.2.


3·0
2·0
1·0
0·6
0·4
0·2
0·1
0·06
0·04
0·02
Optical density (470 nm)
time (hours) at 37°C


1·5
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Optical density (470 nm)
0
10
30
50
70
90
890
Concentration of iron (M×10⁻⁸)

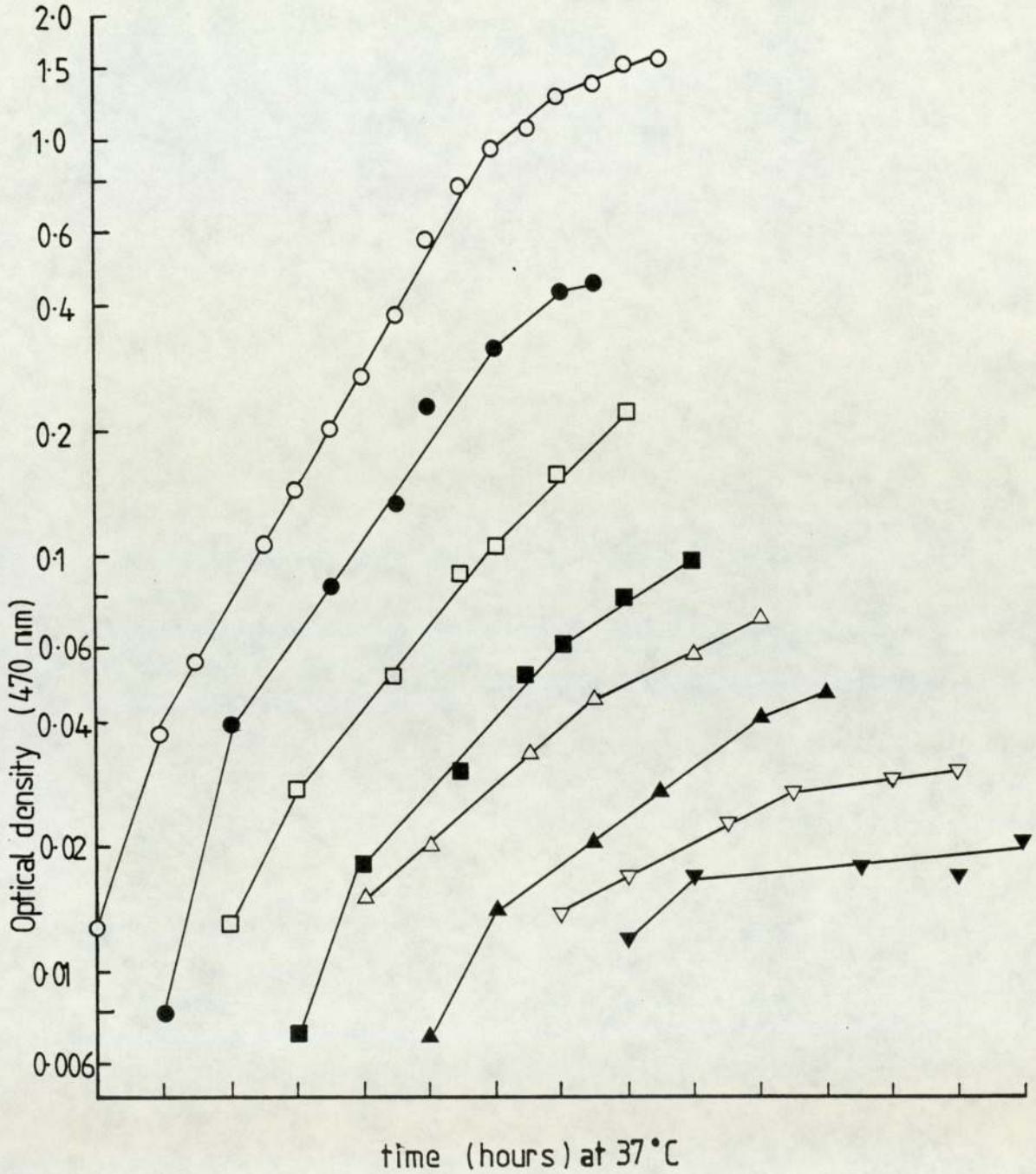

2·0
1·5
1·0
0·6
0·4
0·2
0·1
0·06
0·04
0·02
0·01
0·006
Optical density (470 nm)
time (hours) at 37 °C

Table 3.5

Calculated growth rate (μ) of E. coli W3110 grown in CDM containing graded concentrations of tetracycline[a].

Concentration of tetracycline (μg ml^{-1})	(h^{-1})
0	0.66
0.06	0.53
0.125	0.46
0.25	0.41
0.50	0.32
1.00	0.28
2.00	0.19

a. for calculation of μ, see Tables 3.1 and 3.2.

The OD at which exponential growth ceases is proportional to the initial concentration of added iron up to 4.4×10^{-7} M (Fig. 3.8). Thereafter, any further addition of iron does not enable the media to support logarithmic growth to a noticeably greater degree. It was therefore decided to add 4.4×10^{-8} M iron to the CDM used in experiments where the growth of P. aeruginosa was to be limited by the concentration of iron added.

3.1.3.5 E. coli: tetracycline limitation studies

The effect on the growth of E. coli of the addition of various concentrations of tetracycline to complete CDM is shown in Figure 3.9. It can be seen that the addition of increasing concentrations of the antibiotic reduces both the rate of growth and the duration for which the growth rate is maintained. The growth rates (Table 3.5) are depressed considerably by concentrations of tetracycline greater than 0.5 $\mu g\ ml^{-1}$.

For experiments in which the rate of growth of E. coli was to be reduced using tetracycline, it was decided to add 0.5 $\mu g\ ml^{-1}$ of the antibiotic. This would give a growth rate approximately half that normally obtained for E. coli growing in complete CDM.

3.1.4 Discussion

The two media used for the growth of E. coli W3110 and P. aeruginosa 6750 were based respectively on those devised by Klemperer et al. (1979) and Ombaka (1980). However, that used for the growth of E. coli was buffered with an ammonium phosphate buffer as opposed to MOPS. This may have caused the discrepancies in the OD of cessation of exponential growth when E. coli was limited with either glucose or magnesium, as compared with previous results, in which higher ODs were obtained (Klemperer et al., 1979).

However, the growth rates obtained were similar. A possible explanation for this discrepancy, other than the alteration in the media composition is that the cultures were more efficiently aerated in the previous experiments, although this is unlikely as the same experimental procedure was used.

The maximum growth rates of E. coli grown in the various limiting conditions are all very similar, being approximately 0.66-0.68 h^{-1}. However, whilst the concentrations of glucose and magnesium have to be reduced considerably for the growth rate to be affected, the addition of very low levels of tetracycline appeared to have an immediate effect on the growth rate of the organism (Brown and Garrett, 1964).

With P. aeruginosa 6750 the results for both carbon and iron limited growth of the organism are almost identical with those obtained by Ombaka (1980), both in the relationship between the initial concentration of limiting nutrient and the OD_{470} at the cessation of logarithmic growth, and the growth rates.

To obtain log phase E. coli W3110 or P. aeruginosa 6750, the bacteria were harvested when the cultures had attained an OD_{470} of 0.6. It can be seen that in these media, at such a point in the batch culture growth of the organism, both are growing exponentially. The assumption that such cells will represent log (and fast growing) cells is therefore correct.

Therefore these media, with the adjustments described in the results (3.1.3) were used in all experiments where it was required to grow E. coli W3110 or P. aeruginosa 6750 in chemically defined conditions.

3.2 An investigation of the effects of plasmolysing agents on E. coli and P. aeruginosa

3.2.1 Introduction

The volume of the periplasmic space of Gram-negative bacteria has been variously reported as a small percentage of the total cell volume (Decad and Nikaido, 1976) or a relatively large fraction (Stock et al., 1977). The sensitivity of the method described in this thesis for determining the size of the outer membrane pores is affected by the periplasmic space, a small volume reducing the sensitivity considerably. Therefore, because of the discrepancy between the reported determinations of the periplasmic volume, it was necessary to carry out experiments to investigate the effects of various plasmolysing agents on the bacteria used. Furthermore, plasmolysing agents can be destructive of the integrity of the outer membrane (Bayer, 1968; Decad and Nikaido, 1976) leading to artificial results which could indicate that the membrane is highly permeable to many compounds, which may be a false observation. It was possible that the degree of damage done to cells during plasmolysis could be reduced by using a suitable plasmolysing agent. In order to determine this, and to identify a suitable relatively non-destructive agent, the effect of two drugs, rifampicin (mw 823 d) and bacitracin (mw 1411 d), on the viability of E. coli and P. aeruginosa plasmolysed with different compounds was investigated.

3.2.2 Method

All the plasmolysing agents used were made up to the concentration four times that finally required. Final concentrations in the experiments were NaCl 0.3 M; sucrose 0.5 M; NaCl 0.22 M + $MgCl_2$ 0.01 M;

sucrose 0.44 M + $MgCl_2$ 0.01 M.

Drug agar was made by adding 10 ml of antibiotic solution and sterile distilled water in appropriate ratios to nutrient agar, kept at 45°C. Final drug concentrations used were as described in Table 3.6.

P. aeruginosa and E. coli were grown up in 200 ml volumes of their respective CDM (see 2.2.1) to an OD_{470} of approximately 0.5 (i.e. mid-log phase). Cells were harvested by centrifugation (5500 g, 10 minutes, RT) and resuspended in 0.1 M sodium phosphate buffer pH 7.2 to an OD_{470} of approximately 4.0. A total count of the suspension was obtained (2.2.4.1).

1 ml of this suspension was added to a tube containing 3 ml 0.1 M sodium phosphate buffer pH 7.2, mixed with a whirlimixer, and allowed to incubate at room temperature for 5 minutes. Viable counts were determined on nutrient and drug agar (2.2.2).

Further 1 ml portions of the suspension were added to tubes containing 2 ml 0.1 M sodium phosphate buffer pH 7.2 and 1 ml plasmolysing agent, mixed and allowed to incubate at room temperature as described above.

The appropriate plasmolysing agents, made up to the final concentrations in phosphate buffer were used to dilute the suspensions, and colony counts made on nutrient and drug agar.

The number of cells from each suspension surviving on the various concentrations of drug agar were expressed as a percentage of the control count (on nutrient agar). These percentages were plotted against drug concentration.

Table 3.6

Concentrations of Rifampicin and Bacitracin used in experiments with *P. aeruginosa* and *E. coli*.

P. aeruginosa

Rifampicin µg ml^{-1}	20	10	5	2.5	1.25	0.625	0.313
Bacitracin IU ml^{-1}	400	200	100	50	25	12.5	6.25

E. coli

Rifampicin µg ml^{-1}	2.5	1.25	0.625	0.313	0.157	0.079
Bacitracin IU ml^{-1}	25	12.5	6.25	3.13	1.57	0.79

3.2.3 Results

3.2.3.1 P. aeruginosa

Figures 3.10 and 3.11 show the effect of various concentrations of rifampicin and bacitracin on the viability of P. aeruginosa. It can be seen that where the organism is incubated in the presence of either NaCl or sucrose as plasmolysing agent, and then grown on nutrient agar containing rifampicin (Fig. 3.10) there is a dramatic reduction in the viability of the cells. The addition of $MgCl_2$ to the two plasmolysing agents decreases this effect, and the combination of 0.22 M NaCl and 0.01 M $MgCl_2$ appears to enable more cells to survive on the drug agar than do those from the unplasmolysed control suspension. The significance of the difference between the control cells and those incubated with sucrose and $MgCl_2$ for each antibiotic concentration was determined using a t-test, and the values of t were generally found to be significant (Table 3.7).

Where P. aeruginosa is grown on nutrient agar containing bacitracin (Fig. 3.11), the effect of the various plasmolysing agents is less dramatic. Again, those cells incubated in the presence of NaCl plus $MgCl_2$ as the plasmolysing agent appear to have a somewhat greater percentage survival than the control. The degree of difference in viability of the remaining cell suspensions as compared with the control is less clear. However, P. aeruginosa incubated with NaCl has a significantly reduced level of viability as compared with the control (Table 3.7) when incubated on all the antibiotic concentrations other than 25 IU ml^{-1}, and therefore the difference between the control and cells plasmolysed with 0.5 M sucrose is also significant. Interestingly, there is a significant difference between the survival of unplasmolysed P. aeruginosa and

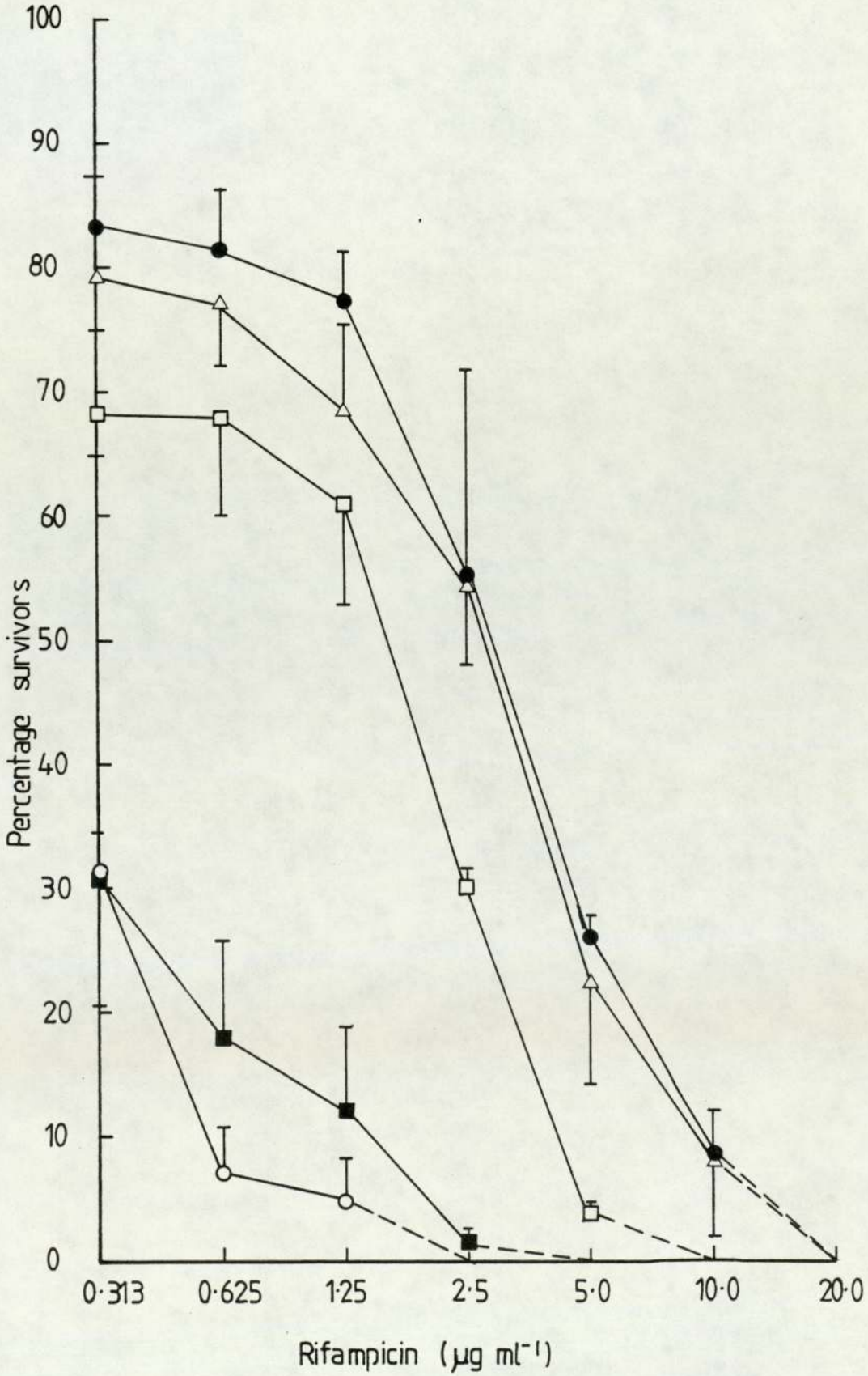

100
90
80
70
60
50
40
30
20
10
0
Percentage survivors
0·313
0·625
1·25
2·5
5·0
10·0
20·0
Rifampicin (μg ml⁻¹)

Table 3.7

t-tests to determine the significance of the difference in viability between plasmolysed and unplasmolysed P. aeruginosa grown on nutrient agar containing rifampicin or bacitracin

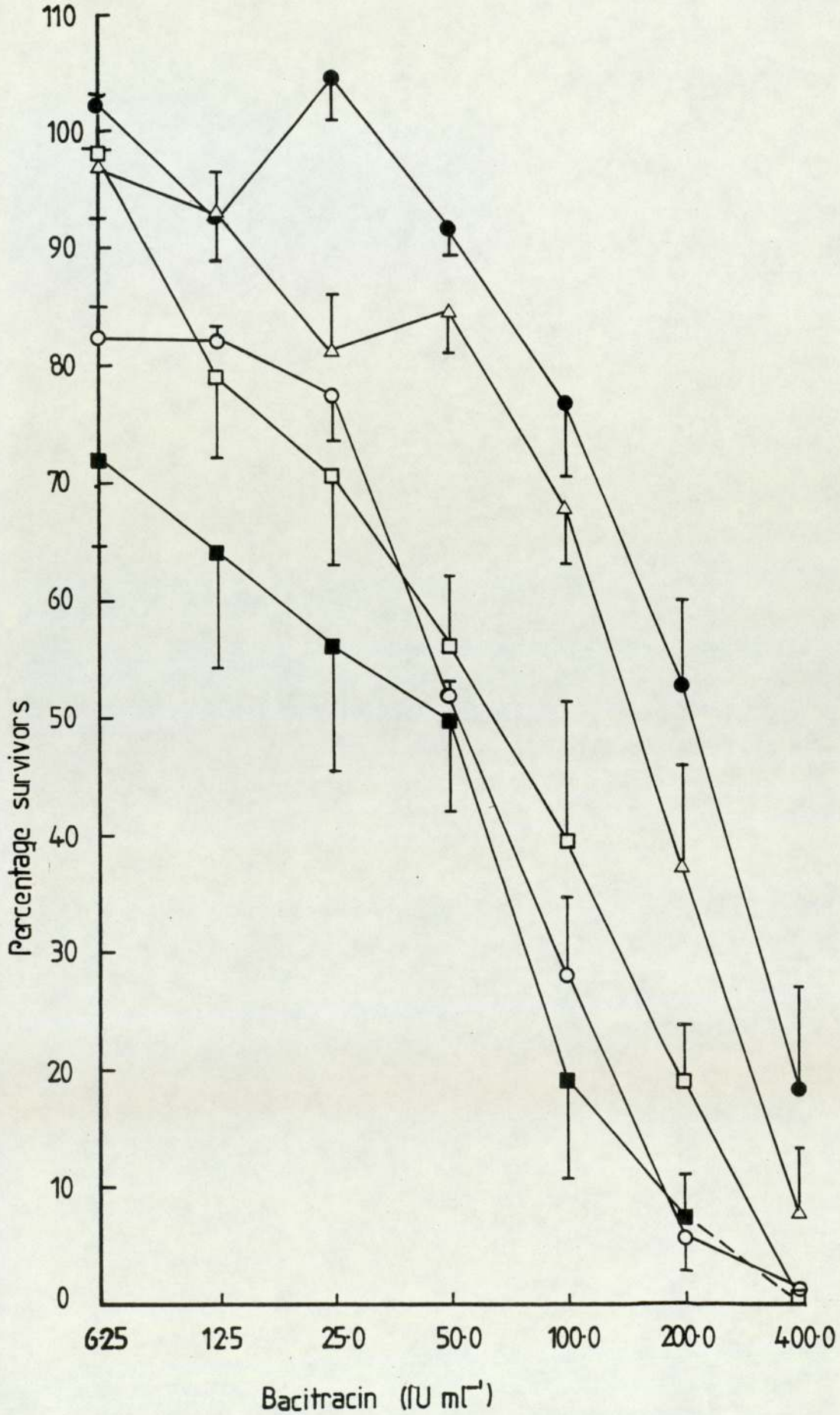
Concentration of rifampicin ($\mu g\ ml^{-1}$)	Calculated value of t for cells plasmolysed with sucrose + $MgCl_2$	Concentration of bacitracin ($IU\ ml^{-1}$)	Calculated value of t for[a] cells plasmolysed with Sucrose + $MgCl_2$	NaCl
20	0	400	1.227	1.938
10	1.855	200	2.018	6.051
5	3.302	100	4.071	9.932
2.5	5.491	50	3.889	16.021
1.25	1.315	25	1.726	1.050
0.625	2.046	12.5	3.080	5.218
0.313	3.272	6.25	1.119	9.071
$t(p = 0.05)$[b]	1.833	$t(p = 0.05)$[b]	1.943	1.860

a. t is calculated using the formula

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{(n_1 - 1) + (n_2 - 1)} \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

For determination of the significance of values of t, a one-tailed test was used.

b. Values of F calculated for the data obtained for each concentration of antibiotic will be found in Appendix 2.


110
100
90
80
70
60
50
40
30
20
10
0
Percentage survivors
6·25
12·5
25·0
50·0
100·0
200·0
400·0
Bacitracin (IU ml⁻¹)

those incubated with sucrose plus $MgCl_2$ when the cells are grown on four of the seven concentrations of bacitracin used (Table 3.7). As there is no significant difference between the viability of the control cells and those plasmolysed with sucrose plus $MgCl_2$ growing on both the maximum and minimum concentrations of the antibiotic it is likely that, overall, the incubation of the organism in 0.44 M sucrose plus 0.01 M $MgCl_2$ significantly reduces the resistance of P. aeruginosa to bacitracin.

3.2.3.2 E. coli

Figures 3.12 and 3.13 show the viability of E. coli when grown on nutrient agar plates containing various concentrations of rifampicin and bacitracin, after incubation of the cells with different plasmolysing agents. Incubation with either sucrose plus $MgCl_2$ or NaCl plus $MgCl_2$ does not appear to reduce the viability of the organism as compared with the unplasmolysed control when the cells are grown on agar containing rifampicin (Fig. 3.12). However, the values of t ($p = 0.05$) (Table 3.8) calculated for the data obtained when the cells are incubated with sucrose as plasmolysing agent as compared with those obtained for the unplasmolysed cells are significant for every concentration of antibiotic other than the highest (2.5 $\mu g\ ml^{-1}$). These differences will therefore also be significant for cells plasmolysed with 0.3 M NaCl when compared with the control, except where the organism is grown on the lowest concentration of rifampicin (0.079 $\mu g\ ml^{-1}$).

Overall, there is little difference in the viability of E. coli when grown on different concentrations of bacitracin after incubation with the various reagents (Fig. 3.13). This is supported by the values of F calculated for the data appropriate to each

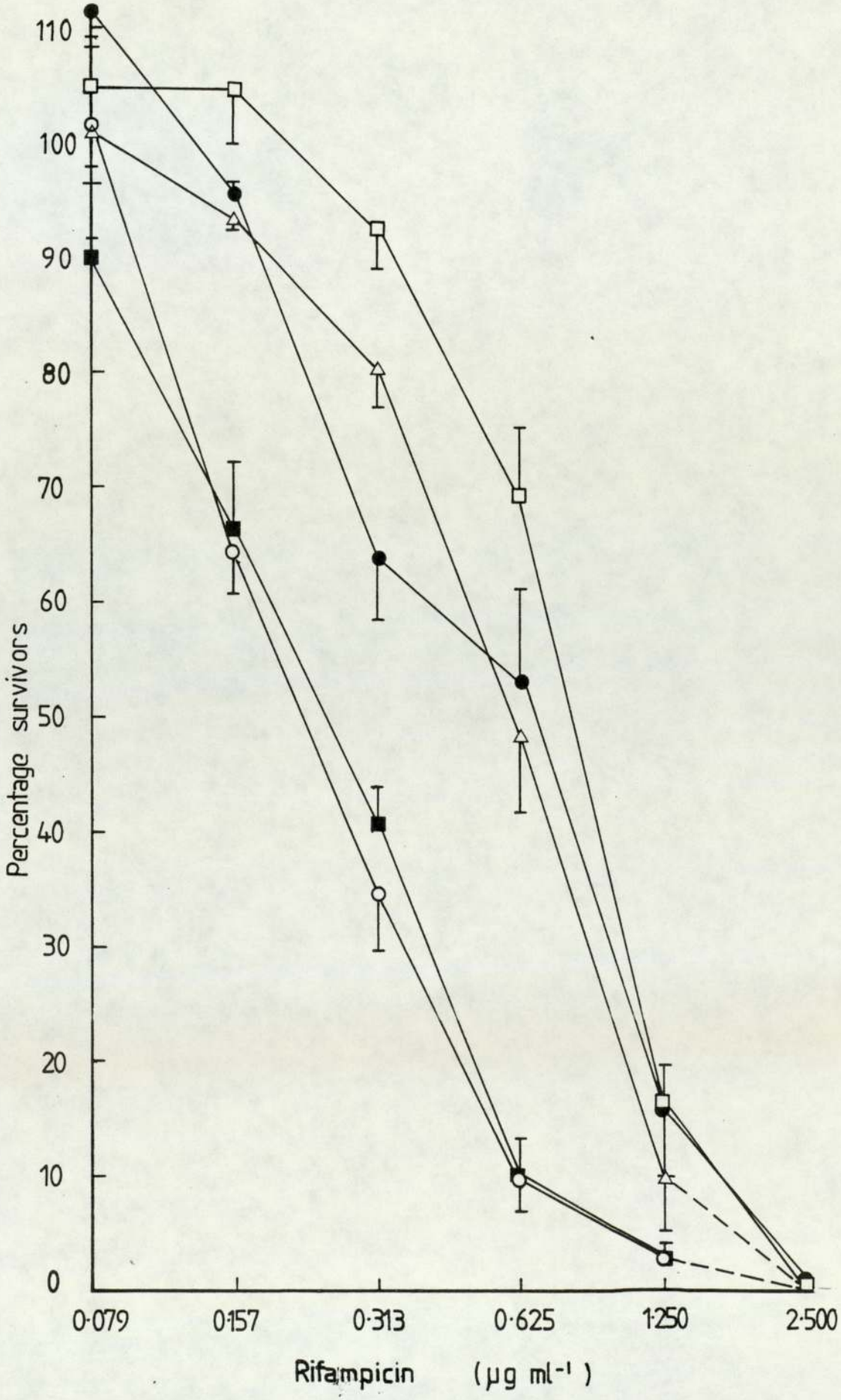

110
100
90
80
70
60
50
40
30
20
10
0
Percentage survivors
0·079
0·157
0·313
0·625
1·250
2·500
Rifampicin (μg ml⁻¹)

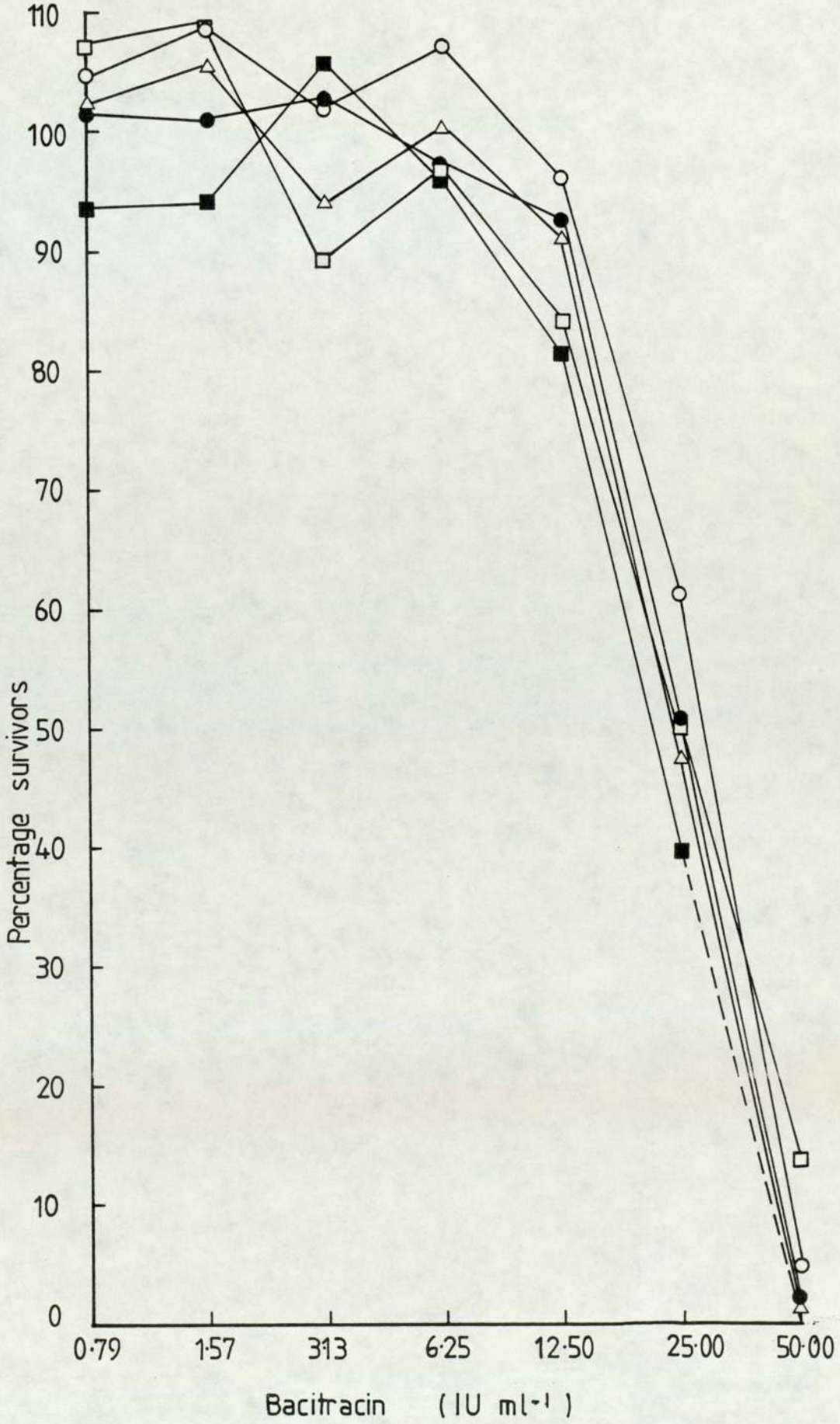
Table 3.8

t-tests to determine the significance of the difference in viability between plasmolysed and unplasmolysed E. coli grown on nutrient agar containing rifampicin.

Concentration of rifampicin (μg ml^{-1})	Calculated value[a] of t for cells plasmolysed with sucrose
2.5	0
1.25	2.159
0.625	7.580
0.313	12.344
0.157	6.585
0.079	5.002
$\bar{t}(p = 0.05)$[b]	2.132

a. The formula for calculating t will be found at Table 3.7. For determination of the significance of t a one-tailed test was used.

b. Values of F calculated for the data obtained for each concentration of the antibiotic will be found in Appendix 2.


110
100
90
80
70
60
50
40
30
20
10
0
Percentage survivors
0·79
1·57
3·13
6·25
12·50
25·00
50·00
Bacitracin (IU ml⁻¹)

concentration of antibiotic, which are only significant ($p = 0.05$) when the cells are grown on 50 IU ml^{-1}, 3.13 IU ml^{-1} and 1.57 IU ml^{-1} (see Appendix 2). In the latter two cases, this is likely to be the result of extreme variation, rather than any significant influence of the incubation system.

3.2.4 Discussion

Plasmolysing Gram-negative bacteria primarily affects the cytoplasm, causing it to reduce in volume and contract, pulling away from the outer membrane. The cytoplasmic membrane remains associated with the cytoplasm, and its structure is probably little affected by the stringencies of plasmolysis. However the outer membrane is undoubtedly disturbed, in part because it loses much of the structural support provided by the cytoplasmic membrane. It is therefore possible that on plasmolysis, the pores in the outer membrane of *E. coli*, or other Gram-negative bacteria, would be enlarged, due to the stress of the situation. Equally, the integrity of the outer membrane could be so affected as to allow it to break in regions other than the pores, enabling the passage of molecules to the periplasmic space to occur.

To investigate whether this could be occurring during the plasmolysis of the bacteria, the ability of plasmolysed *E. coli* and *P. aeruginosa* to grown on nutrient agar containing bacitracin (mw 1411 d) or rifampicin (mw 823 d) was investigated. These antibiotics were selected because of their molecular weights (both being greater than the determined exclusion limit of *E. coli*). Also, rifamycin SV, a member of the same group of compounds as rifampicin, probably penetrates the outer membrane via the hydrophobic membrane diffusion pathway (Nikaido, 1976) and, should the efficacy

of the antibiotic against an organism increase it, implies a distortion of parts of the outer membrane other than the pores.

Both molecules are considerably smaller than the determined size exclusion limit of the pores in P. aeruginosa (Hancock and Nikaido, 1978), and it would be expected that the antibiotics should pass freely through the outer membrane pores. However, it will be noted that P. aeruginosa 6750 is approximately 10 times as resistant as E. coli to both antibiotics, which is in keeping with the unusually high resistance of the organism to many compounds. Because the evidence suggests that the outer membrane of P. aeruginosa does exclude the molecules, viability of the organism on agar containing either antibiotic is a suitable indicator of the damage incurred by the outer membrane during plasmolysis.

Both sucrose and NaCl are regularly used as plasmolysing agents, and were chosen for this reason. The concentrations of both were based on those used by Decad and Nikaido (1976). $MgCl_2$ was added in low concentrations to each in an attempt to determine whether the presence of additional divalent cations would maintain the integrity of the membranes, despite the demanding plasmolysing conditions. The amount of sodium chloride or sucrose used in the plasmolysing agent was adjusted in either case to maintain the approximate osmolarity of the reagent.

Overall, the data suggests that P. aeruginosa is more susceptible to damage during plasmolysis than E. coli, which is in accord with the effect of physical stress on P. aeruginosa, such as cold shock (Kenward, Brown, Hesslewood and Dillon, 1977; Kenward, Brown and Fryer, 1979). In fact, plasmolysis hardly alters the resistance of E. coli to bacitracin, but the effect of rifampicin

on both organisms after plasmolysis is marked, suggesting that the compound's smaller size enables it to cross the outer membrane more easily.

Plasmolysing either P. aeruginosa or E. coli does affect their viability when grown on nutrient agar containing bacitracin or rifampicin. When either sucrose or NaCl is used to plasmolyse the bacteria, it would appear that the organisms become more vulnerable to both antibiotics, particularly rifampicin, the outer membrane having been damaged in some way. This is particularly true of P. aeruginosa. But the addition of $MgCl_2$ to either plasmolysing agent enables the organisms to respond in a similar manner as the unplasmolysed cells to the presence of the antibiotics. In some cases, the presence of $MgCl_2$ even appears to increase the resistance of the organism, particularly when with NaCl. This is likely to be a result of the properties of Mg^{2+} with respect to the outer membrane components, and it is likely that the presence of divalent cations compensates for some of the stress which would have damaged the outer membrane, primarily by holding the anionic outer membrane proteins and LPS together. The slight increases in resistance of cells plasmolysed with NaCl and $MgCl_2$ together, or E. coli with sucrose and $MgCl_2$ are probably a result of the outer membrane being held together more tightly than in unplasmolysed cells.

Although no plasmolysing agent can be ideal, as by their very nature they change the bacteria from one state to another which is less natural, it would appear that, considering all the factors, 0.22 M NaCl plus 0.01 M $MgCl_2$ would be the most suitable plasmolysing agent. There is a situation in which this reagent will be inappropriate, where E. coli grown to magnesium limitation is to be investigated. Addition of Mg^{2+}, for whatever reason, and at

whatever stage of an experiment, could affect the membrane architecture of such bacteria. Thus, when magnesium limited *E. coli* is used, 0.3 M NaCl is used as the plasmolysing agent. As the *E. coli* outer membrane is not as susceptible as that of *P. aeruginosa* to damage as a result of plasmolysis, using this reagent will not unduly affect the results obtained in any experiment, although it is a factor which may have to be considered.

3.3 Studies on the uptake of isomaltitol and other compounds by E. coli

3.3.1 Introduction

E. coli, unlike P. aeruginosa can grow using maltose as the only carbon source (Hofnung and Schwarz, 1972; Ferenci, 1980). The operon coding for the various structural proteins involved in the uptake and catabolism of maltose is not expressed constitutively, and it is under both catabolite repression (when, for example, glucose is the carbon source of the cell) and positive control (Hofnung and Schwarz, 1972). This means that for the maltose operon to be expressed maltose has to be present in the environment of the bacteria.

Isomaltitol (OS 346) resembles maltose, the two differences being that maltose is comprised of two closed glucose rings linked by an α1-4 bond whereas isomaltitol is made up of one closed glucose ring and one reduced glucose chain linked by an α1-6 link. It is likely that the two compounds are adequately similar for isomaltitol to be taken up by the periplasmic maltose binding protein and used as a carbon source by E. coli. It was necessary to investigate this possibility, thus determining whether it was feasible to use OS 346 in the investigations of the size of the pores in the outer membrane of E. coli.

As the uptake of maltose, and hence, potentially, of OS 346 is enzymatic, it would be considerably affected by temperature. Thus it was decided to investigate the uptake of OS 346, isomaltotriitol (OS 508) and isomaltotetritol (OS 670) in the presence of various reagents as affected by temperature. This was done by measuring the loss of labelled material from the supernatant after various periods of incubation of the cells and labelled oligosaccharides at different temperatures.

3.3.2 Method

E. coli was grown in 2 litre volumes of nutrient broth as described (2.2.2.1) unless otherwise stated to an OD_{470} nm of 0.8 and harvested (10 minutes, RT, 8000 g), washed once in 0.1 M sodium phosphate buffer pH 7.2 and resuspended to 40% v/v in the same.

For experiments carried out at room temperature, into 1.5 ml plastic centrifuge tubes (Eppendorf, Hamburg, Germany) were placed 0.25 ml cell suspension, 0.125 ml labelled oligosaccharide and 0.125 ml of either plasmolysing agent plus maltose (0.22 M NaCl, 0.01 M $MgCl_2$, 0.02 M maltose, final concentrations), plasmolysing agent (0.22 M NaCl, 0.01 M $MgCl_2$, final concentrations), maltose alone (0.02 M maltose, final concentration) or water (described collectively as the reagents). This was done in at least triplicate for each of the reagents, and thus for each of the oligosaccharides in groups of at least twelve. Each group of twelve was incubated for a specific period of time, between 1 and 20 minutes, centrifuged in an Eppendorf bench centrifuge (Model 5412) for one minute (9980 g, RT) and the supernatant removed. 0.2 ml of the supernatant were then added to 5 ml NE260 and counted for 10 minutes.

The total amount of radioactivity added to each portion of cell suspension was determined by mixing three 0.125 ml portions of those solutions of radiolabelled oligosaccharides used with 5 ml NE260, and counting these for 10 minutes. All data were expressed as disintegrations per minute (dpm).

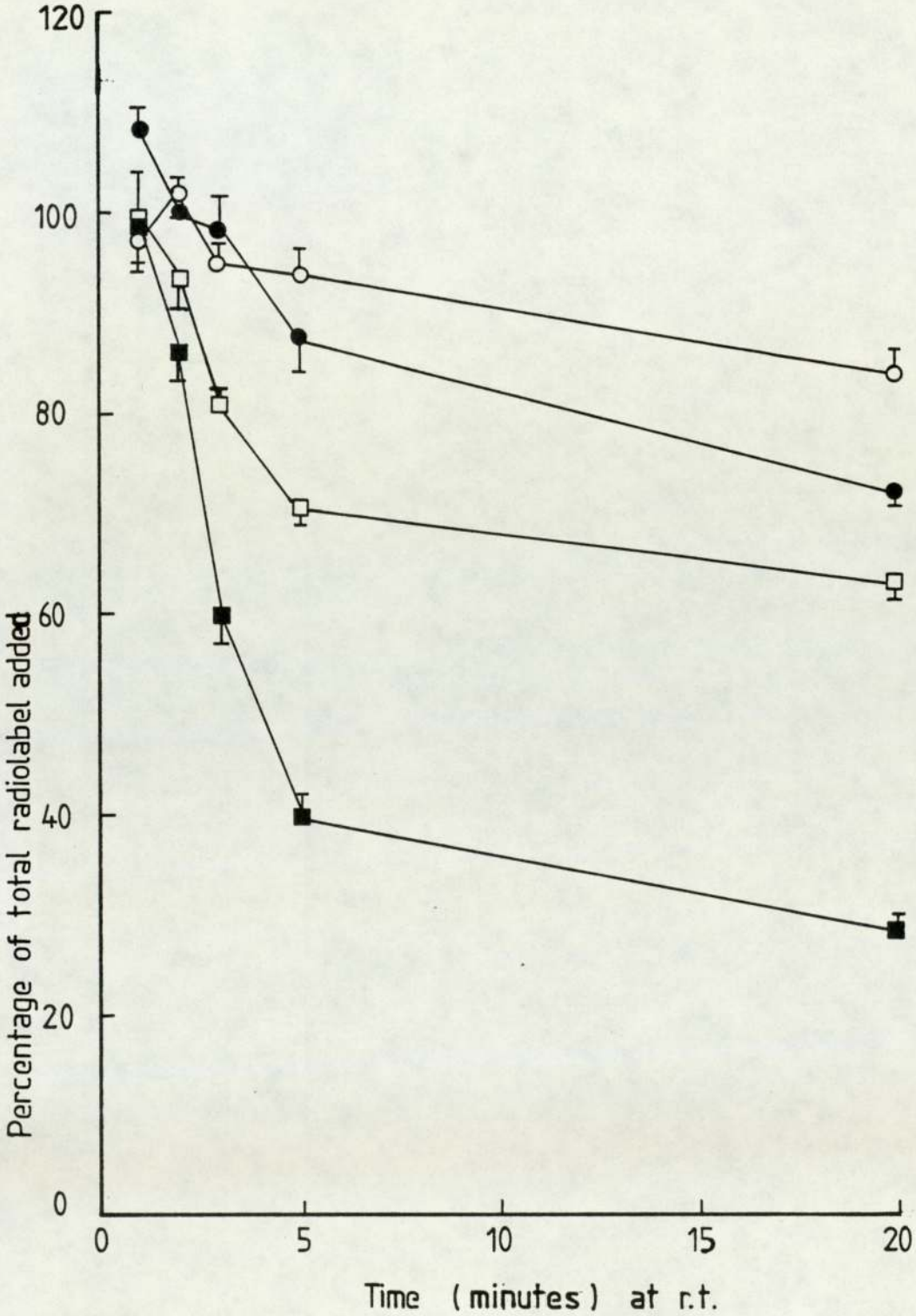
Where experiments were carried out at different temperatures, all materials were at the chosen temperature prior to use. Tubes were set up as described above and then incubated in a waterbath at the appropriate temperature, or on ice. The experiment was then carried out as before.

3.3.3 Calculation of results

The mean and standard deviation of the results for each time point with each combination of reagents and labelled oligosaccharide were determined. This represented the amount of radioactivity remaining in 0.2 ml, or 40% of the total volume used in each centrifuge tube. This was adjusted so that the data represented the amount of radioactive material in 0.5 ml, or 100% of the total working volume. These adjusted data were then expressed as a percentage of the total amount of radioactive material originally added to the centrifuge tube, and the resulting figures for the various combinations of reagents and labelled oligosaccharides were plotted against time.

3.3.4 Results

Figures 3.14, 3.15 and 3.16 show the loss of OS 346, OS 508 and OS 670 from the supernatant when the radiolabelled sugars were incubated with nutrient broth grown *E. coli* at room temperature. It is clear that the concentrations of the two larger molecules in the supernatant remain fairly constant throughout the experiment, and that the presence of various reagents, such as 0.22 M NaCl, 0.01 M $MgCl_2$ (plasmolysing agent), or 0.02 M maltose has no significant influence on any potential uptake of the compounds into the cells. It would appear from these results that neither compound is actively removed from the environment by the organism. This is confirmed when the removal of OS 508 and OS 670 from the environment by *E. coli* was investigated at 37°C (Figs. 3.17, 3.18). During the course of the experiments, there is little change in the concentration of the oligosaccharides in the environment, and there is no loss of either from the supernatant into the cells, irrespective of the reagent present with the cells and radiolabelled sugars.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Percentage of total radiolabel added
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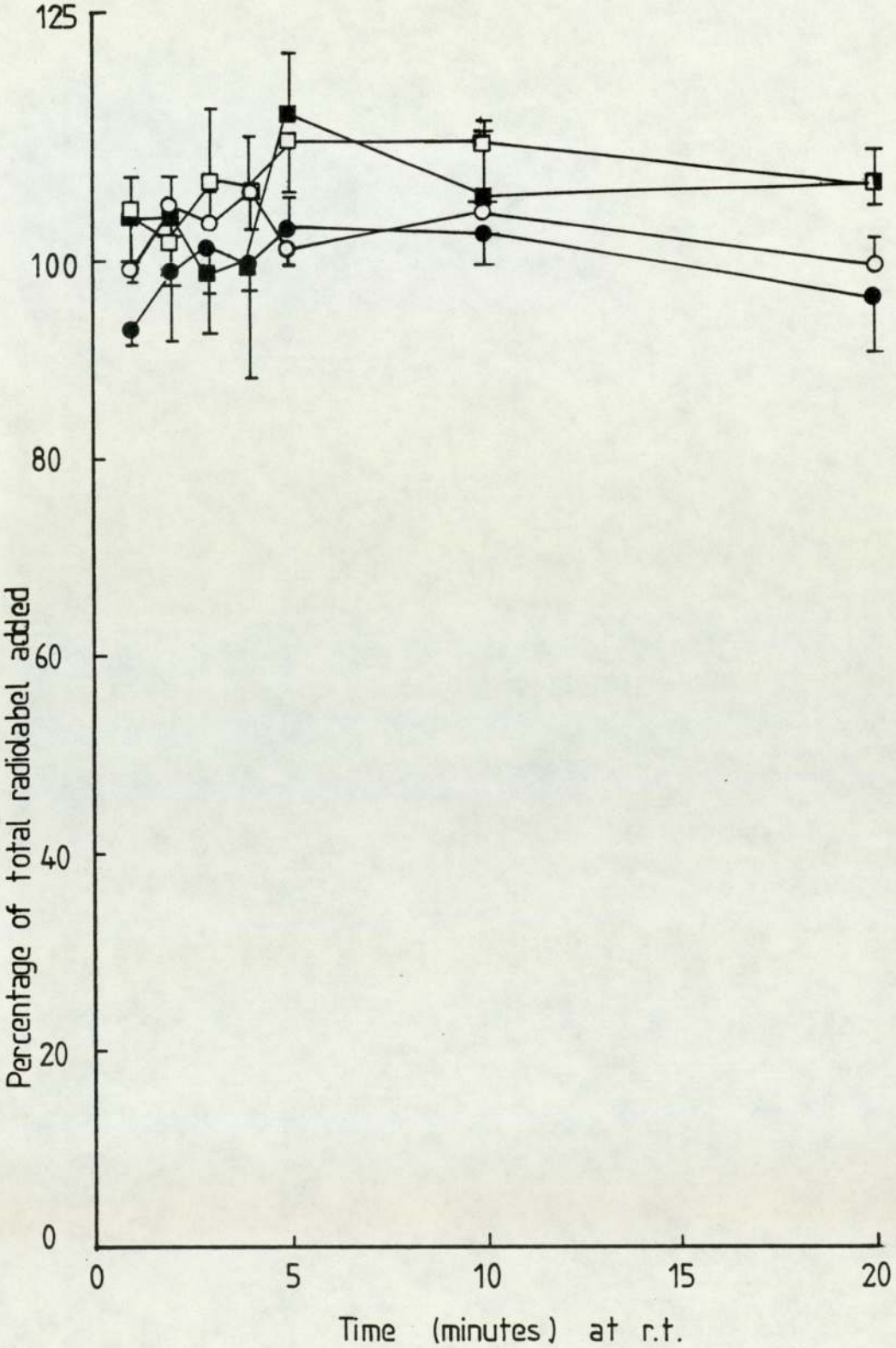
Time (minutes) at r.t.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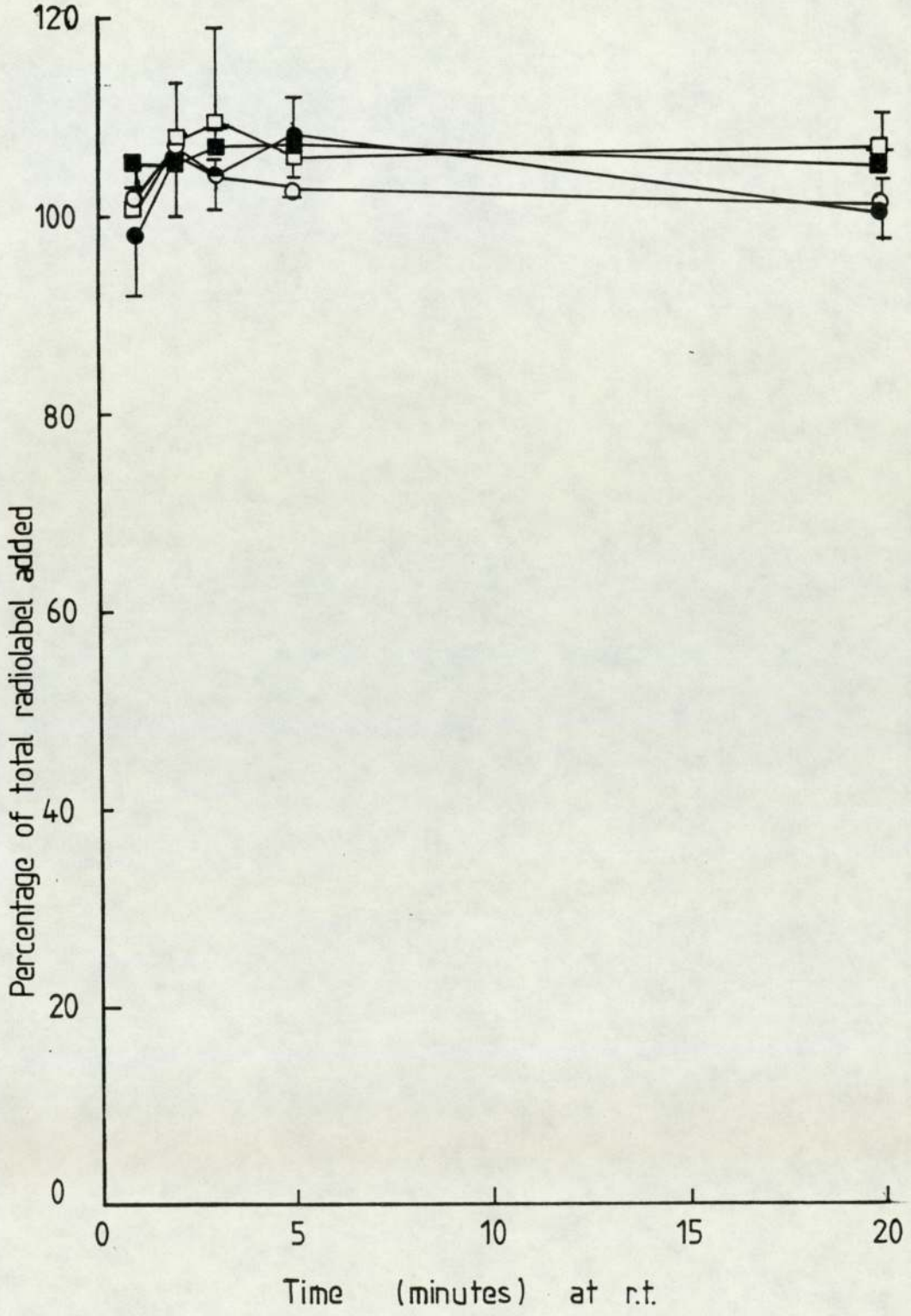
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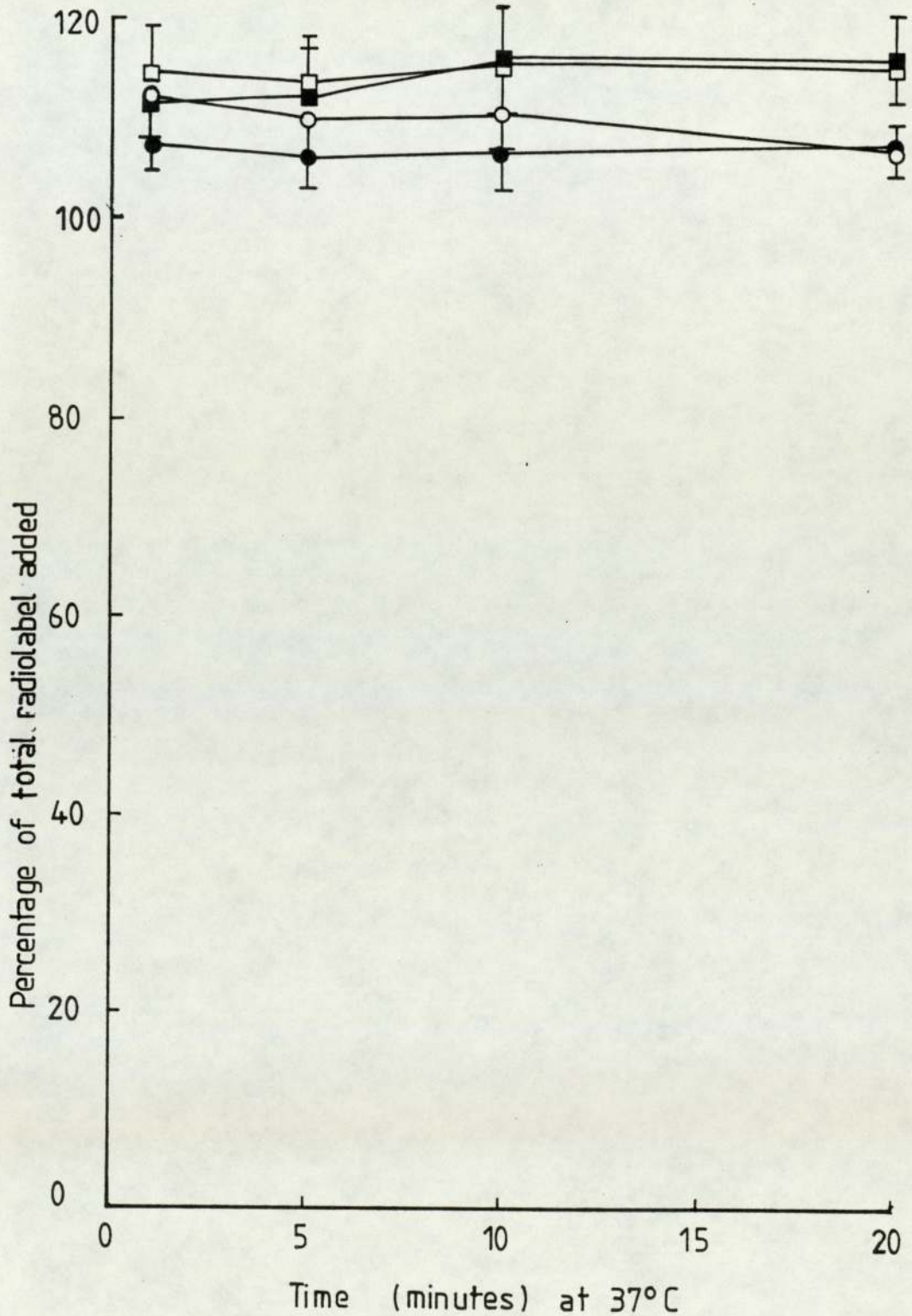
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Percentage of total radiolabel added
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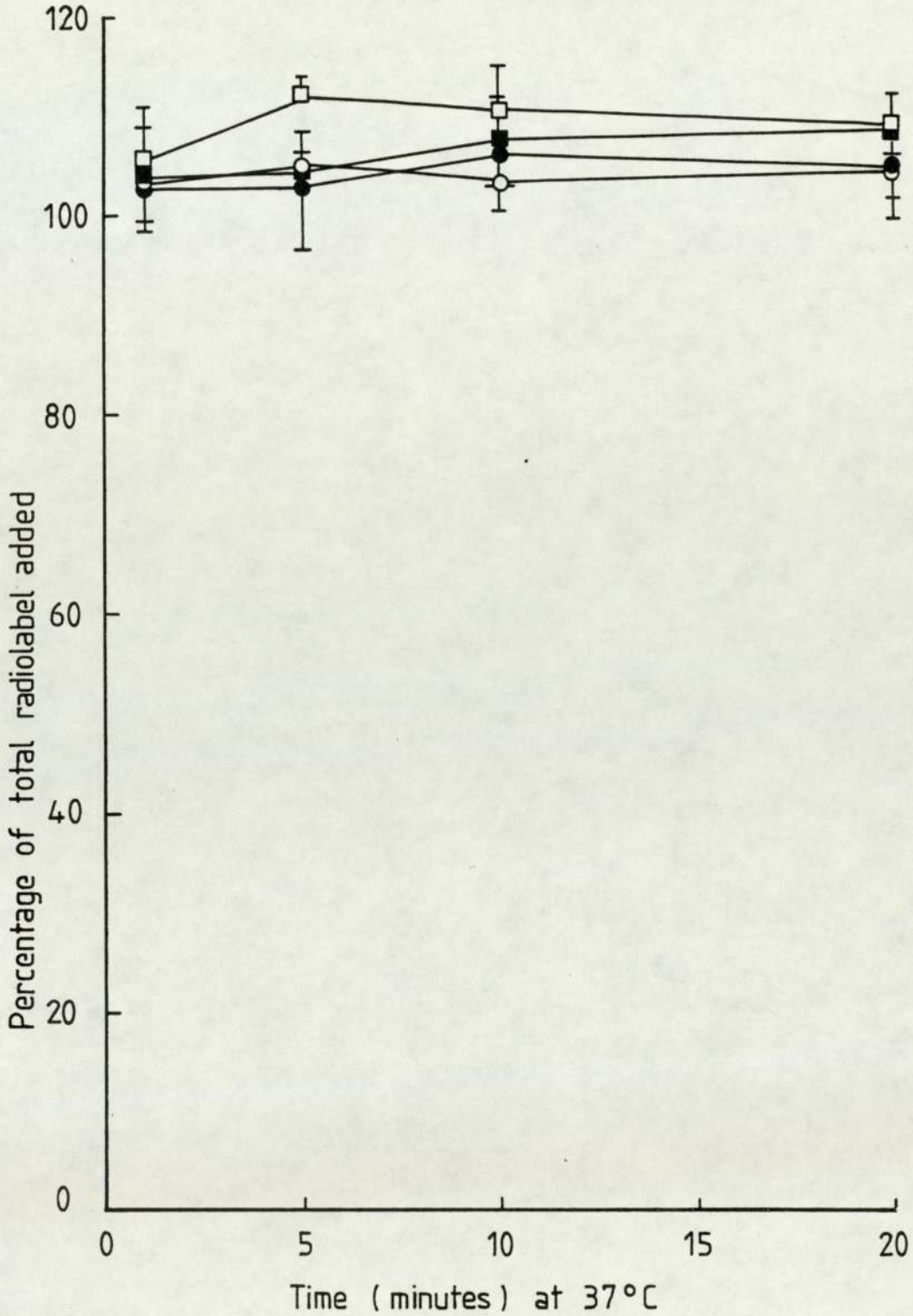
Time (minutes) at 37°C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Percentage of total radiolabel added
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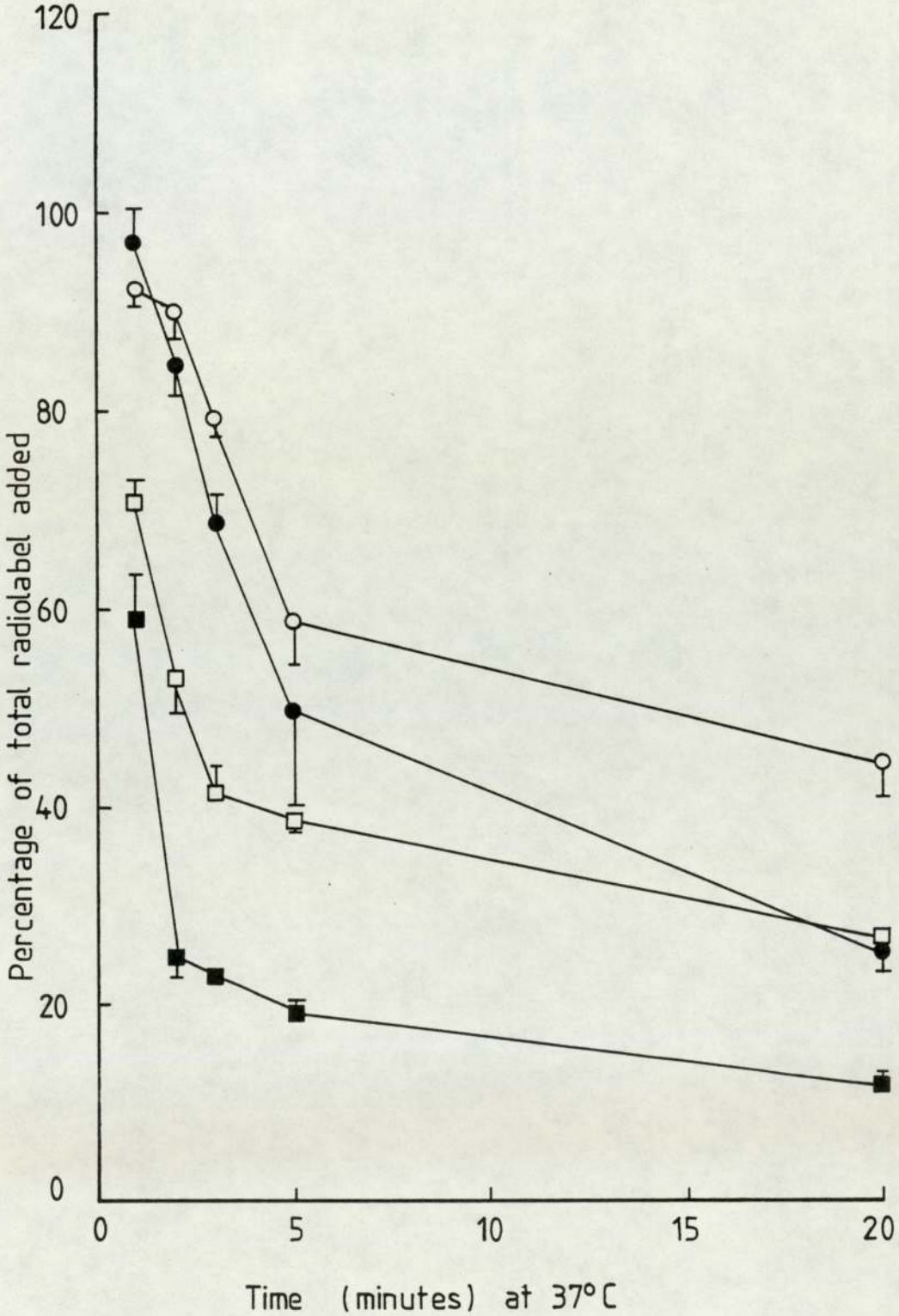
Time (minutes) at 37°C

However, the concentration of OS 346 (Fig. 3.14) remaining in the supernatant decreases significantly with time of incubation with the cells and is influenced by the nature of the added reagents. The smallest reduction in oligosaccharide concentration is seen when plasmolysing agent and maltose were added to the cell suspension. The most significant decrease in the amount of OS 346 present was recorded when no other compounds were added to the cell and labelled sugar mixture. When the effect of adding either maltose or the plasmolysing agent was investigated, the presence of the latter reduced the rate of removal of OS 346 from the supernatant to a greater extent than did the former.

Figures 3.19, 3.20, 3.21 and 3.22 show that the removal of OS 346 from the environment by E. coli is temperature dependent. This is most clearly seen if Figures 3.14, 3.19 and 3.22 are compared, where the experiments were carried out at 37^{o}, 20^{o} and $0^{o}C$ respectively. In experiments above $0^{o}C$ (i.e. 15^{o}, 20^{o}, 30^{o}, $37^{o}C$) the most OS 346 is removed by the cells when neither plasmolysing agent nor additional unlabelled maltose are present. The presence of both these reagents reduces the loss of OS 346 from the supernatant such that, in the same experiments (i.e. all experiments run at temperatures greater than $0^{o}C$), the least OS 346 is removed by E. coli under these conditions. OS 346 is removed more quickly by cells in the presence of maltose, but not plasmolysing agent, but in experiments carried out at 37^{o} and $30^{o}C$ (Figs. 3.19 and 3.20) the final concentration of OS 346 in the two systems is very similar. When the experiment was carried out at $15^{o}C$, the final amount of OS 346 removed by the cells is greater when there is no plasmolysing agent present, only maltose.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Percentage of total radiolabel added
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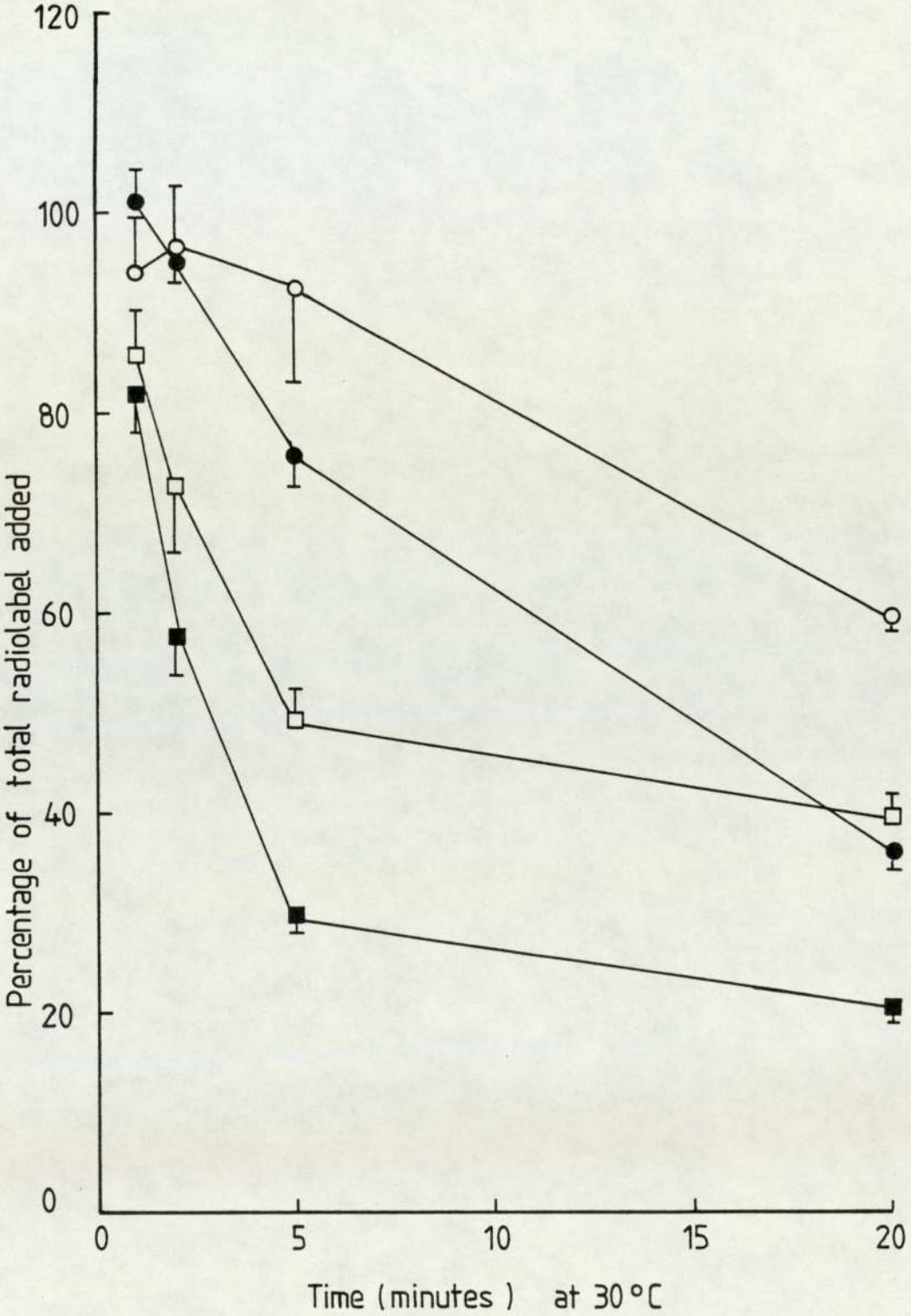
Time (minutes) at 37°C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Percentage of total radiolabel added
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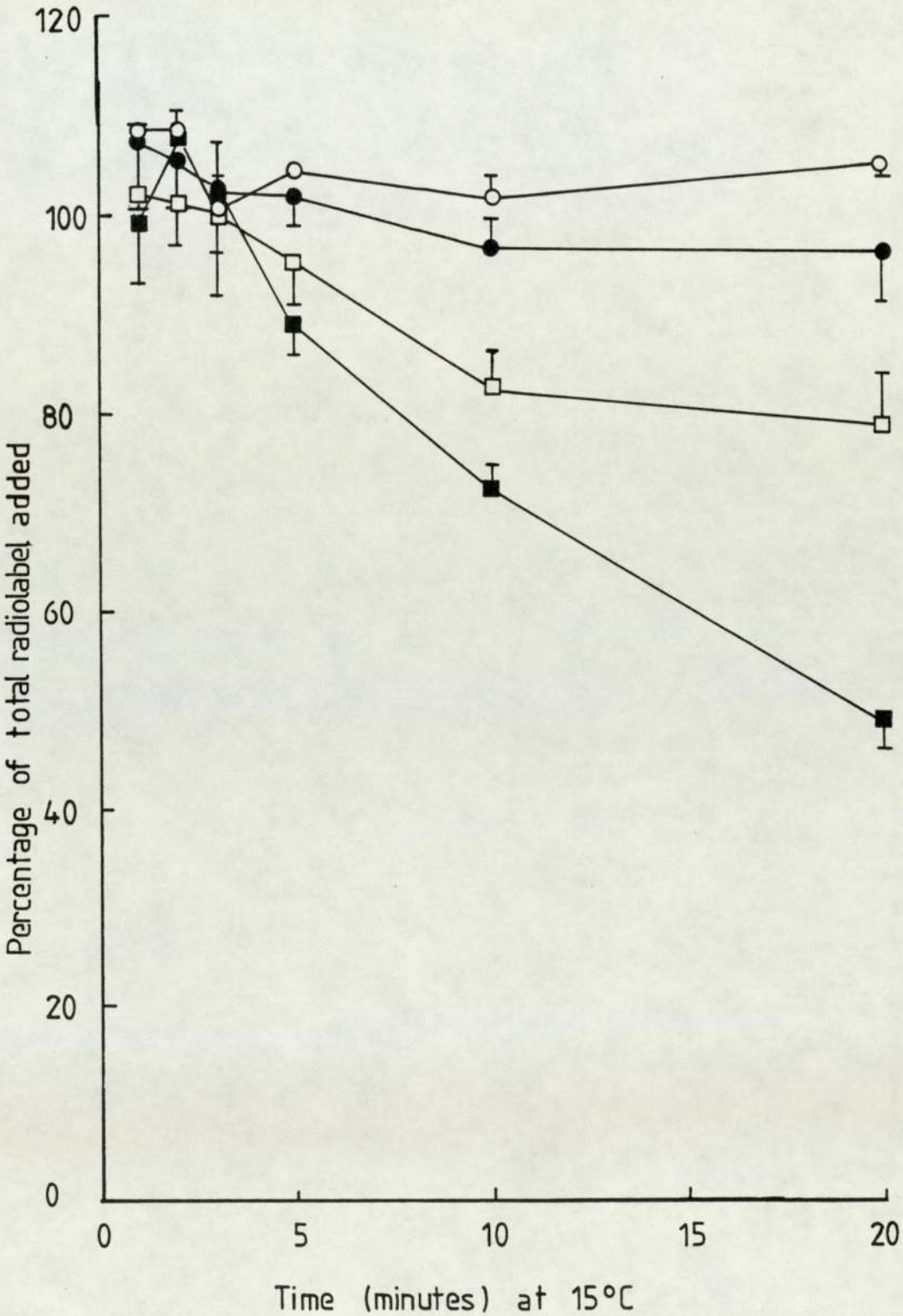
Time (minutes) at 30 °C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Percentage of total radiolabel added
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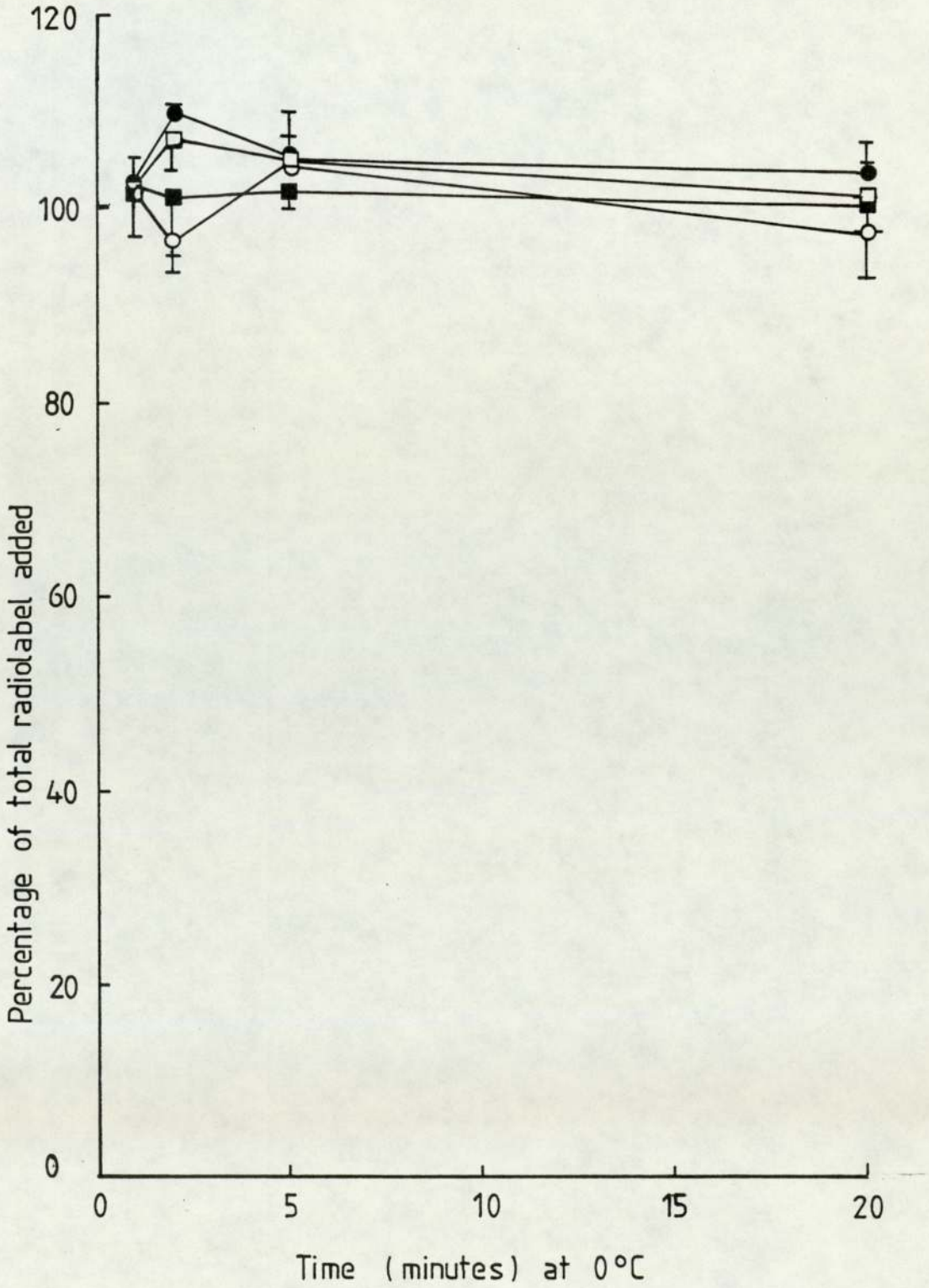
Time (minutes) at 15°C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Percentage of total radiolabel added
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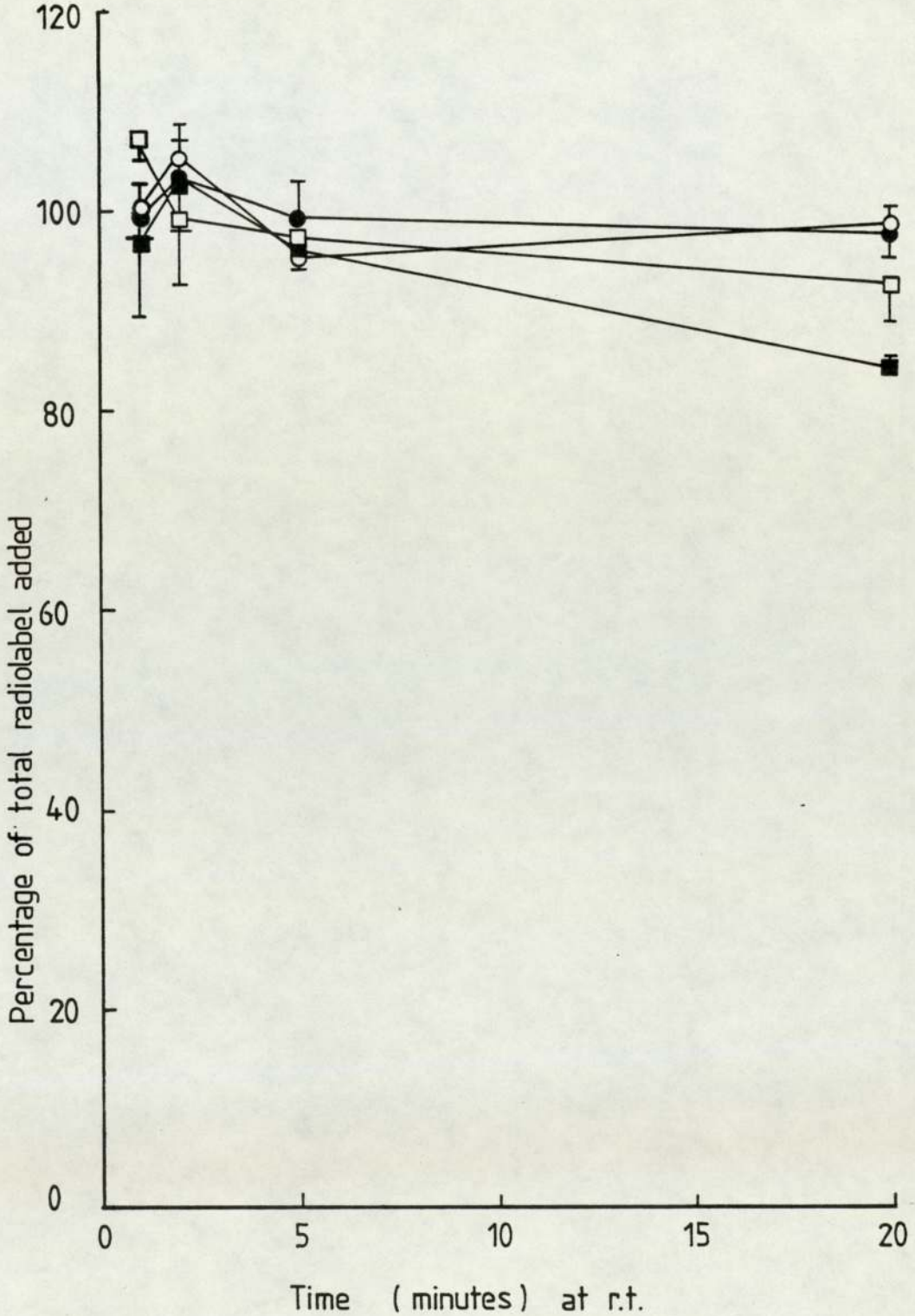
Time (minutes) at 0°C

As a result of these experiments, E. coli was grown up in CDM as opposed to nutrient broth, and the rate of removal of OS 346 was investigated at room temperature as described above. The results (Fig. 3.23) show that although the removal of the oligosaccharide is greatly reduced, as compared with nutrient broth grown E. coli (Fig. 3.14) there is still some reduction in the concentration of the compound in the supernatant with time. This is most noticeable when there are no added reagents, and least noticeable, possibly insignificant, when there is plasmolysing agent present, whether maltose is added or not.

If the uptake of OS 508 and OS 670 by E. coli grown in nutrient broth (Figs. 3.15 and 3.16) and of OS 346 by E. coli grown in CDM (Fig. 3.23) are compared it will be seen that there is a small but significant difference between the change in the concentration of OS 346 as compared with the other two sugars. This is most apparent when the data obtained from the cells incubated in the presence of the oligosaccharides and no other reagent are compared.

The slight decrease in the concentration of OS 346 in the supernatant when the sugar is incubated with CDM grown cells (Fig. 3.23) also contrasts with the relatively constant concentration of OS 346 present in the supernatant when the oligosaccharide is incubated with nutrient broth grown cells at $0^{o}C$ (Fig. 3.22). Again this difference is most clearly seen when the data obtained from cells incubated in the absence of any reagent other than the labelled sugar are compared.

It was ascertained that P. aeruginosa 6750 was unable to grow using maltose as a carbon source by attempting to grow the organism in glucose free CDM, containing maltose as the sole carbon source. At no stage was growth observed.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Percentage of total radiolabel added
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Time (minutes) at r.t.

3.3.5 Discussion

It is apparent from all the data that the removal of isomaltitol (OS 346) by E. coli from the environment is due to an enzymic reaction. The process is markedly temperature dependent, both occurring most rapidly at 37°C and being brought to a complete halt at 0°C. Also, the system is affected by the presence of maltose. When OS 346 and maltose are present together, the amount of OS 346 taken up, and the rate at which this occurs is reduced.

As the maltose operon is induced by the presence of maltose, it is reasonable to assume from the results that nutrient broth must contain, amongst its many ingredients, maltose or maltose analogues capable of inducing the maltose operon. However, the genes are expressed at a very low level under maltose free conditions, as is seen from the results obtained with CDM grown E. coli. In this growth situation, at least the maltose binding protein must be present in the cells at a level adequate to remove some OS 346 from the environment, particularly when no additional maltose is present, and the cells are unplasmolysed. This very low level constitutive expression of the operon is due to the need of the organism to have adequate enzymes for maltose uptake present so that, should maltose be an available compound, it can be taken up and used to stimulate the maltose operon into full expression. Thus it would be expected that E. coli grown on glucose in the absence of maltose would still be capable of utilising low levels of OS 346.

The use of OS 346 by the maltose uptake system is predictable, but it is perhaps a little unexpected that only OS 346 is removed from the environment by the bacteria in any measurable quantity. It is known that maltodextrins larger than maltose can be accumulated

by E. coli (Ferenci, 1980) and that the periplasmic maltose binding protein is accessible to the molecules larger than 600 d mw (Boehler-Kohter, Boos, Dieterle and Benz, 1979), possibly up to 2500 d (Ferenci, 1980). This is due to the larger diameter of the λ pore, an integral part of the maltose uptake system (Ferenci, 1980; Ferenci and Boos, 1980). It has also been observed that maltose, and maltodextrins, bind to the lamB protein, the affinity of the molecules for the outer membrane protein being proportional to the size of the compound (Ferenci and Boos, 1980; Ferenci, Schwentorat, Ullrich and Vilmart, 1980).

It would therefore be expected that molecules larger than OS 346 would be removed by the cells, either due to binding to the λ pore protein, or by binding to the periplasmic maltose binding protein and subsequent transport across the cytoplasmic membrane. However, the results indicate that only OS 346 is removed by the maltose uptake system into the cells. The most straightforward and likely explanation of this observation is that the reduced form of maltose is not as easily recognised by the active sites of the enzymes concerned, and neither are the α1-6 links of isomaltose type molecules which replace the α1-4 bonds of maltose. Also, the three dimensional structure of the isomaltitol series would differ from that of maltodextrins. Together, these factors probably make OS 508 and OS 670 too distinct from maltose, or maltotriose and maltotetrose, to be recognised by the system. But this leaves the question of how OS 346 could be identified by the cell, as the primary differences between it and the larger compounds are those of size, number of the α1-6 links and three-dimensional structure. It is most probable that the solution lies with this last difference. Possibly the spatial conformation of OS 346,

although distinct from that of maltose, is not immensely so, whereas the larger compounds in the OS 346 series adopt three dimensional structures which are markedly different.

The three-dimensional conformation of the molecules is the best explanation of the failure of E. coli to remove OS 508 and OS 670 from the environment enzymatically. The outer membrane is not likely to provide a permeation barrier, in either nutrient broth grown cells, where the λ pore will be present in large numbers (Ferenci, 1980) or in CDM grown E. coli (see section 3.4).

The remaining question is the effect of the presence of plasmolysing agent and maltose on the removal of OS 346 from the environment. Clearly, as the system is enzymatic, the presence of maltose, which will be preferentially taken up by the system, would reduce the rate of removal, and the amount removed, of OS 346. But plasmolysing the cells also affects their ability to take up OS 346, and consequently the least OS 346 is removed from the supernatant by plasmolysed cells incubated with unlabelled maltose as well as OS 346.

It is possible that this phenomenon is due to the leakage of maltose binding protein from the periplasm of plasmolysed cells, but in the light of the results in section 3.2, any holes in the outer membrane are probably not large enough to accommodate such molecules. More likely is that the effect of plasmolysis on the relationship between the outer and inner membranes includes interfering with the relationships between the λ pore, the periplasmic binding protein and the maltose permease. It has been shown that the binding protein has a location in the outer membrane dependent on the λ receptor protein (Ferenci and Boos, 1980) and this relationship may well be damaged by the plasmolysing conditions.

Another possibility is that the osmolarity of the plasmolysing solution is such that some component of the maltose uptake system is conformationally, or in some other way, altered. Equally, due to the slightly hypotonic systems in which the unplasmolysed cells are suspended, it is possible that the outer membrane pores are more open than in plasmolysed cells (see p. 181) enabling the OS 346 to diffuse across the outer membrane more quickly. This would imply, however, that the rate limiting step of the maltose uptake system is the permeation of OS 346 (or maltose) through the outer membrane itself, which would appear unlikely (Nikaido and Rosenberg, 1981).

Despite the reduction in the effective activity of the maltose uptake system when incubated with plasmolysing agent and maltose, or when E. coli was grown in CDM, it was decided that OS 346 could not be used in the double label assay with E. coli. However, OS 508 and larger molecules would be unaffected by the maltose uptake system, and could therefore be used.

3.4 Double label assay to investigate the diffusion of oligosaccharides across the outer membrane of *E. coli* and *P. aeruginosa*

3.4.1 Introduction

Sucrose, a small, hydrophilic disaccharide of molecular weight 342 daltons, is known to diffuse across the outer membrane of Gram-negative bacteria, but is not used as a carbon source by many of these organisms. It is therefore a very suitable plasmolysing agent and is frequently used as such. Sucrose crosses the outer membrane probably by passive diffusion, and in *E. coli* and *P. aeruginosa* no sucrose uptake, or active transport, enzymes have been identified. Sucrose has, therefore, been assumed to traverse the outer membrane through the pores. This is now a common assumption (Decad and Nikaido, 1976; Stock et al., 1977).

Because, in theory, sucrose can diffuse freely into the periplasmic space of both *E. coli* and *P. aeruginosa*, it is possible to investigate the diffusion of other compounds relative to the diffusion of sucrose. This is most conveniently carried out using a double label assay, and in the experiments described below, the diffusion of ^{14}C sucrose is compared with various compounds, all labelled with tritium. Thus it is possible to examine the effect of growth environment of the organisms under investigation on the size of the pores in the outer membrane.

3.4.2 Method

E. coli W3110 R- or *P. aeruginosa* 6750 were grown in 4 x 2 l volumes as described previously (2.2.2.1). Cells were harvested by centrifugation at 8000 g, 10 minutes, room temperature, washed once in 0.1 M sodium phosphate buffer pH 7.2 (phosphate buffer) and

resuspended in the same to a standard cell concentration. The volume of this mixture was calculated by obtaining the wet weight of cells harvested, and dividing this by 0.45. When resuspended to this volume (in ml), a 40% (v/v) cell suspension was obtained. To ascertain that this was correct, the packed cell volume of at least ten samples from the suspension was determined, using a haematocrit.

3.2 ml portions of the cell suspension were placed in test tubes and to each was added 1.6 ml of either plasmolysing agent plus maltose, or maltose. The plasmolysing agent was 0.22 M NaCl + 0.01 M $MgCl_2$ (final concentrations) with 0.02 M maltose (final concentration) in all experiments except those using magnesium limited cells. In such experiments, 0.3 M NaCl with 0.02 M maltose (final concentrations) were used. In those experiments in which the cells were not plasmolysed, 0.02 M maltose (final concentration) was added. 1.6 ml of radiolabel solution was then added (Table 3.9). The experiment was timed from this point. The tubes were mixed thoroughly and allowed to incubate at room temperature (normally 19^{o}-21^{o}C) unless otherwise stated.

A 1 ml sample was removed from each tube after one minute and placed in a 1.5 ml plastic centrifuge tube. The suspensions were centrifuged for 80 seconds, 9980 g, to give a firm pellet, in an Eppendorf bench centrifuge (Model 5412) and the supernatant from each discarded. The cells were then washed once. This was carried out by resuspending those cells which had been plasmolysed in phosphate buffer containing 0.22 M NaCl + 0.01 M $MgCl_2$ or phosphate buffer containing 0.3 M NaCl, whichever was appropriate, using a vortex mixer; unplasmolysed cells were resuspended in phosphate buffer. The cells were centrifuged for 70 seconds to give

a loose pellet, and the supernatants again discarded. 0.7 ml distilled water was then added to each centrifuge tube and the cells resuspended, using a vortex mixer. The suspensions were incubated at room temperature for 30 minutes. After this time, the cells were centrifuged for a further 90 seconds, the supernatants removed and stored, if necessary, at $4^{o}C$. This last supernatant corresponds to the periplasmic contents less the neglected loss during the brief centrifugations.

Three 0.2 ml portions of each supernatant were placed in separate scintillation vials containing 5 ml NE260, and were mixed using a vortex mixer. The vials were counted for 10 minutes, and the separate amounts of ^{14}C and ^{3}H labelled material expressed as dpm. This procedure was repeated after the initial cell suspension had been incubated for 1, 3, 6, 10, 20 and 30 minutes.

In addition, 0.1 ml of each of the solutions of radiolabelled compounds used in the experiment was placed in three vials containing 5 ml NE260 and counted for 10 minutes, to provide controls for each experiment.

In addition to investigating P. aeruginosa 6750, the entire range of sugars used were tested with stationary phase, nutrient broth grown, P. aeruginosa PAO1. This provided a control for results obtained with P. aeruginosa 6750 (see p. 185).

Where whole-cell-free systems were used in this method (i.e. as controls to examine the effect of quenching by cellular material), the steps involving the removal of a supernatant were replaced as follows. After the first incubation period (of between 1 and 30 minutes) and centrifugation, 0.75 ml of the solution was removed. 0.5 ml of the appropriate washing agent was added, and, after mixing, the solution was centrifuged for 70 seconds. 0.5 ml was then

Table 3.9

Stock solutions of radiolabelled compounds used in the double label assay..

^{3}H labelled compound	Molarity[a] (μM)	Specific Activity ($\mu Ci\mu mol^{-1}$)	^{14}C labelled compound	Molarity[a] (μM)	Specific Activity ($\mu Ci\mu mol^{-1}$)
Isomaltitol (OS 346)	120	10 (approx.)	sucrose	2.10	477
Isomaltotriitol (OS 508)	120	10 (approx.)	sucrose	2.10	477
Isomaltotetritol (OS 670)	120	10 (approx.)	sucrose	2.10	477
Isomaltopentitol (OS 833)	120	10 (approx.)	sucrose	2.10	477
Isomaltohexitol (OS 995)	120	10 (approx.)	sucrose	2.10	477
Isomaltoheptitol (OS 1157)	120	10 (approx.)	sucrose	2.10	477
Inulin (5200 d mw)	0.179	5600	sucrose	2.10	477
Sucrose	0.102	9800	sucrose	2.10	477

a. In the double label assay, the radiolabel stock solutions are diluted by four, reducing the concentration, but not the specific activity, by that factor.

discarded and 0.7 ml distilled water added. After 30 minutes incubation at room temperature, the mixture was once again centrifuged as above and the supernatant removed and stored at $4^{o}C$, if necessary, or counted as described above.

3.4.3 Calculation of results

The ratio of the amount of ^{14}C sucrose to the amount of ^{3}H labelled material initially present (I) in each of the 3 vials containing material from a given stock solution of radiolabels was calculated as the $\frac{\text{dpm } ^{14}C}{\text{dpm } ^{3}H}$. The mean and standard deviation for the 3 vials was then calculated, which represented the ratio of the two different compounds in that solution ($I \pm i$).

The ratios of ^{14}C sucrose to ^{3}H labelled material in the final efflux supernatants (E) obtained from the three vials for each time point appropriate to a given initial stock solution were calculated. Each result was then subtracted from the ratio of ^{14}C sucrose to ^{3}H labelled compound in the stock solution (I - E). Because the ratio of compounds in the stock solutions, I, were not always unity (although they were always within the range 0.4-1.2) the data, I-E, were not directly comparable when considering results obtained with different ^{3}H labelled compounds. All values of I-E were therefore divided by the ratio of ^{14}C sucrose to ^{3}H labelled material in the appropriate stock solution $|(I-E)/I|$, and the mean and standard deviations of the three values of this, appropriate to the time point, were calculated ($F \pm f$). The values of $F \pm f$ for each solution of radiolabels were plotted against the duration of the initial incubation of the cells with that radiolabel solution.

3.4.4 Theory

The ratio of dpm ^{14}C to dpm ^{3}H measured in the supernatants obtained after the 30 minute efflux period (E) may be assumed to be representative of the concentrations of the two compounds present in the periplasmic space. This is because after 30 minutes the independent concentrations of the two compounds in the periplasmic space and the environment will have reached equilibrium; the initial period of incubation of the cells with the radiolabel is no longer than 30 minutes. The ratio of the labelled material in the periplasmic space is, in turn, a representation of the different amounts of the compounds which diffused across the outer membrane in the permitted time of initial incubation. It is assumed that the same relative amount of ^{14}C and ^{3}H labelled compounds will leave the cells when they are suspended in water as initially diffused in. This is unless the pores only permit the passage of molecules into the cells and not out again, or there is specific, or non-specific, adhesion of some labelled molecules in preference to others to the cells. This is true with respect to all the compounds used other than those which are taken up actively by enzymes from the periplasmic space, such as the one example of isomaltitol (OS 346) with E. coli used in this study.

Thus, except where compounds taken up by active transport are concerned, a difference between the ratio of dpm ^{14}C to dpm ^{3}H in the initial solution of labelled material and the ratio of the same in the final efflux material from the cells represents the difference in concentrations of the ^{14}C labelled sucrose and the tritiated compound present in the periplasmic space after that time of initial incubation. There can be three overall results: 1) The ratio of the efflux material from the cells may be significantly greater

than the ratio in the initial solution ($I < E$). 2) the two ratios may be approximately the same ($I \simeq E$). 3) the ratio of labelled material from the cells may be less than the ratio in the initial solution ($I > E$). These various results can be interpreted as follows.

If I is less than E, then either relatively more ^{14}C labelled material than ^{3}H labelled material has penetrated into the periplasmic space than was in the original stock solution, or else less ^{3}H labelled material has entered the cells. Either interpretation suggests that the ^{14}C sucrose is more easily able to cross the outer membrane, and that there is a relatively higher concentration of that compound present in the periplasmic space. If I is approximately equal to E, this suggests that there has been little or no change in the relative amounts of ^{14}C labelled and tritiated material. Either both compounds are equally able to cross into the periplasmic space, or neither are able to penetrate the outer membrane. The relative concentrations of the two compounds in the periplasmic space would therefore be the same. Where I is greater than E, it is implied that the ^{3}H labelled compound is more easily able to cross the outer membrane than the ^{14}C labelled material, and that after the period of incubation, relatively more tritiated material diffused into the periplasmic space.

In addition, when $F \pm f$ is expressed against time, the slope of the graph represents the uptake of the ^{3}H labelled molecule relative to that of ^{14}C sucrose and the x axis ($I = E$) represents the uptake of the ^{14}C sucrose. Thus, where the slope of the graph is negative, the tritiated compound is diffusing into the cell more slowly (i.e. E is decreasing) than the ^{14}C labelled compound, whereas a positive slope suggests that the tritiated compound is diffusing

across the outer membrane more quickly than the ^{14}C labelled molecules. A line with no overall slope indicates that the rate of diffusion of the two compounds is the same.

3.4.5 Statistical analysis

In some situations, it was necessary to determine whether the results obtained were statistically significant. In these cases, the null hypothesis was that a given tritiated molecule diffused into the periplasmic space of the organism concerned in a manner similar to or the same as ^{14}C sucrose. Therefore, to determine whether or not this were true, t-tests were carried out, comparing the values of F obtained at each time point for a given tritiated sugar, with F = 0, the value for sucrose. This value is represented by the time axis. A t-test compares the difference between two means and because in the present situation, one of the means is not determined, but is theoretical, the formula used to calculate t is

$$t = \frac{\bar{x}-\mu}{s/\sqrt{n-1}}$$

$\bar{x}$ is the determined mean, or F; μ is the theoretical mean, zero; s is the standard deviation obtained when calculating $\bar{x}$; n is the number of determinations of F which contribute to the calculation of $\bar{x}$ (Caulcott, 1973).

This calculation of t allows for a comparison between a calculated mean and the mean of a population the dimensions of which cannot be known. Where values of t were significant, this indicated that the concentration of the ^{3}H molecule present in the cell after that period of initial incubation was different from the concentration of ^{14}C sucrose.

3.4.6 Results

3.4.6.1 The effect of cellular and other material on the quenching of ^{14}C and ^{3}H

Figure 3.24 shows the results obtained when the double label assay was carried out in the absence of whole cells. ^{3}H and ^{14}C sucrose were mixed with sonicated cell material obtained from either E. coli or P. aeruginosa and the plasmolysing agent plus maltose, or maltose. A control of radiolabelled material with phosphate buffer was also run.

When the data obtained with cell material from E. coli or P. aeruginosa (Figure 3.24) are compared with the phosphate buffer control, it will be seen that they are very similar. In addition, the three lines fall approximately parallel with the zero line, which represents the diffusion of ^{14}C sucrose. In the case of the data obtained with P. aeruginosa cell material present, both lines are slightly displaced above the abcissa. However, the control is similarly altered, and this slight anomaly is probably not significant.

3.4.6.2 E. coli

This, and subsequent sections of the results and discussion, are divided into several parts. Here they are data obtained with plasmolysed cells, those obtained with unplasmolysed cells, the results with isomaltotetritol and the effect of the environment.

Data obtained with plasmolysed cells. There are four main features of the data obtained for the uptake of various compounds into E. coli grown and treated in different ways. The first is the overall pattern of the uptake of the ^{3}H isomaltitol series into those cells which were incubated in the presence of plasmolysing agent plus maltose. In these situations (Figures 3.25-3.28) isomaltotriitol (OS 508) and ^{3}H sucrose diffuse into the periplasmic space in a

Figure 3.24: The effect of cell material obtained from sonicated *E. coli* W3110 or *P. aeruginosa* 6750, the plasmolysing agent and maltose on the quenching of ^{3}H and ^{14}C sucrose in the double label assay.

The double label assay was carried out with ^{3}H sucrose, ^{14}C sucrose and:-

a) *E. coli*

phosphate buffer (control) △

material from sonicated *E. coli* plus

plasmolysing agent plus maltose ○

maltose ●

b) *P. aeruginosa*

phosphate buffer (control ▲

material from sonicated *P. aeruginosa* plus

plasmolysing agent plus maltose □

maltose ■

Plasmolysing agent = 0.22 M NaCl + 0.01 M $MgCl_2$ (final concentrations)

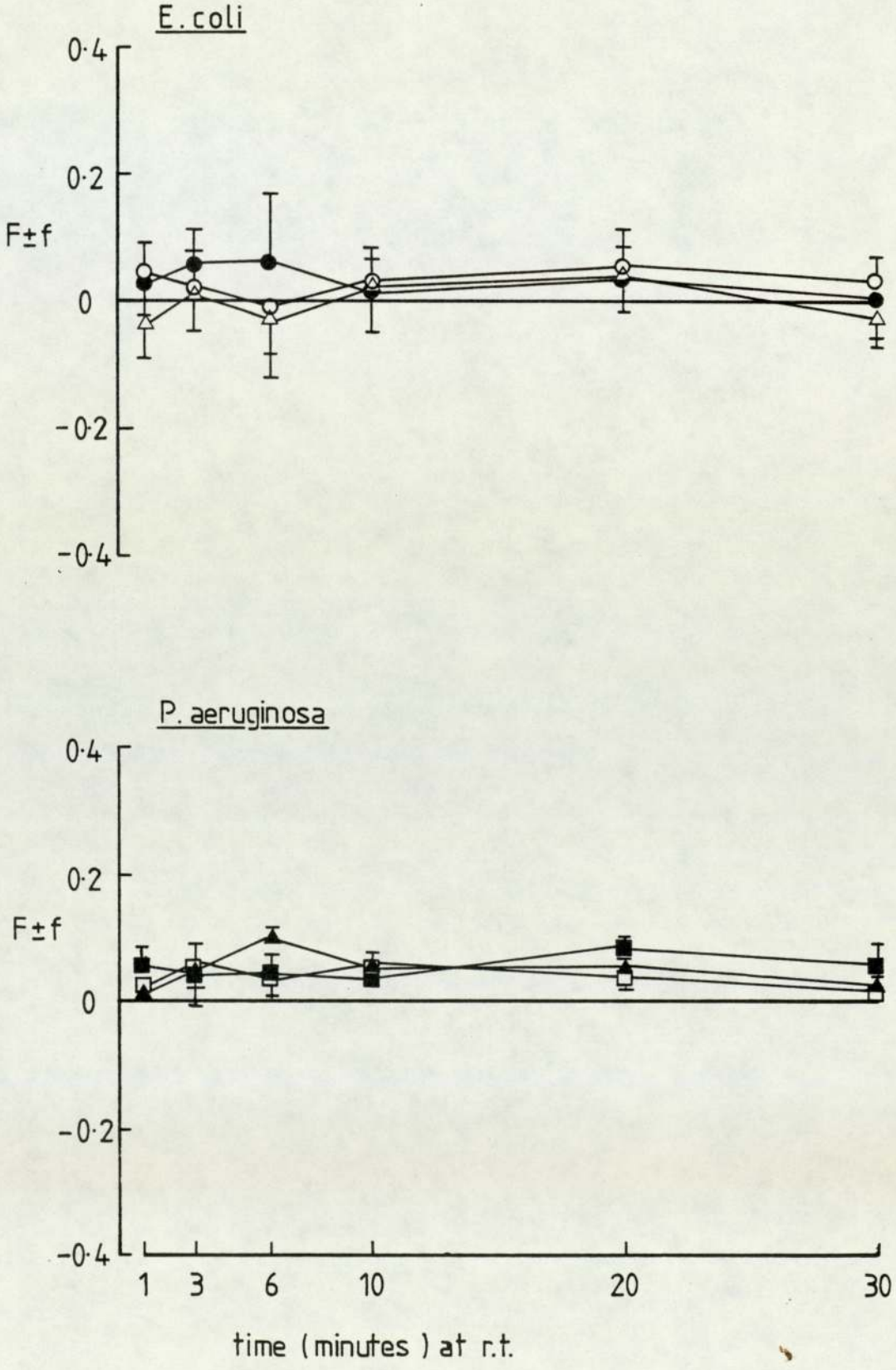
Maltose = 0.02 M maltose (final concentration)

$F = (I - E) / I$ where

$I = \frac{\text{dpm } ^{14}C \text{ sucrose}}{\text{dpm } ^{3}H \text{ sugar}}$ in the initial stock solution

$E = \frac{\text{dpm } ^{14}C \text{ sucrose}}{\text{dpm } ^{3}H \text{ sugar}}$ in the final efflux supernatant

represents the change in the ratio of ^{14}C to ^{3}H material, after incubation with the cells, adjusted for variation in the initial values of I.


E. coli
0·4
0·2
F±f
0
-0·2
-0·4
P. aeruginosa
0·4
0·2
F±f
0
-0·2
-0·4
1
3
6
10
20
30
time (minutes) at r.t.

very similar manner to ^{14}C sucrose, the data for both molecules lying close to the zero line. When t-tests were carried out on the data, the values of t were generally not significant (Tables 3.10-3.13). Isomaltotetritol (OS 670), however, appears to diffuse into the cells initially more quickly than ^{14}C sucrose, a plateau being reached after approximately 10 minutes incubation, and certainly the concentration of the molecule present in the periplasmic space after 10, 20 and 30 minutes initial incubation was significantly higher than those of ^{14}C sucrose.

Isomaltopentitol (OS 833) also diffuses into the cells in a consistent manner whenever incubated with E. coli and plasmolysing agent plus maltose. The initial value of F is generally -0.4 to -0.5 (Figs. 3.25-3.27), the exception being E. coli grown in the presence of tetracycline (Fig. 3.28), when the initial value of F is approximately -0.8. Thus (F) increases with increasing length of initial incubation, as E (dpm $^{14}C \div$ dpm ^{3}H) decreases, i.e. the dpm ^{3}H increase. Correspondingly, the significance of the differences between F and zero tends to decrease with time (Tables 3.10-3.13). The relative rate of penetration of OS 833 is greatest during the first 10 minutes in all situations, thereafter it decreases but the molecule was still apparently able to diffuse into the cells. After 30 minutes incubation of log phase, plasmolysed E. coli (Fig. 3.25) with ^{14}C sucrose and OS 833, the ratio of the two compounds (E) had become approximately that originally present in the stock solution (I). Where E. coli has been grown in other ways and incubated with OS 833 in the presence of plasmolysing agent plus maltose, this pattern is not as dramatic. However, in all three cases (Figs. 3.26-3.28) the value of E is tending towards I, with magnesium limited E. coli most closely resembling the log phase cells.

Figure 3.25: Uptake of tritiated oligosaccharides relative to that of ^{14}C sucrose, in the presence of maltose, across the outer membrane of plasmolysed E. coli W3110 grown to mid log phase in CDM.

Cells were incubated as described in the text with plasmolysing agent, maltose, ^{14}C sucrose and

^{3}H isomaltitol (OS 346)	○
^{3}H isomaltotriitol (OS 508)	●
^{3}H isomaltotetritol (OS 670)	□
^{3}H isomaltopentitol (OS 833)	■
^{3}H isomaltohexitol (OS 995)	△
^{3}H isomaltoheptitol (OS 1157)	▲
^{3}H sucrose	▼

F ± f : see Figure 3.24

Data for standard deviation will be found in Appendix 3.

Figure 3.26: Uptake of tritiated oligosaccharides relative to that of ^{14}C sucrose, in the presence of maltose, across the outer membrane of plasmolysed E. coli W3110 grown to stationary phase (oxygen limitation) in CDM.

Legend as for Fig. 3.25.

Figure 3.25

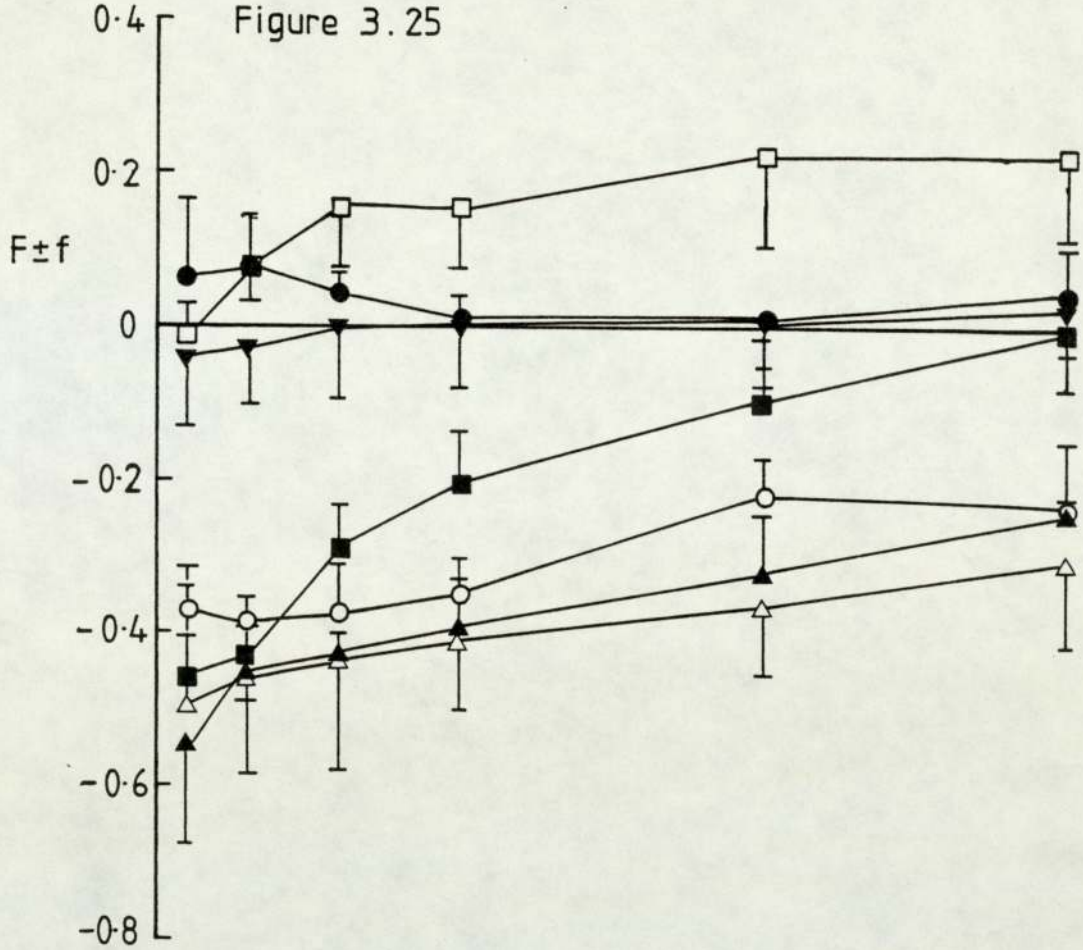


Figure 3.26

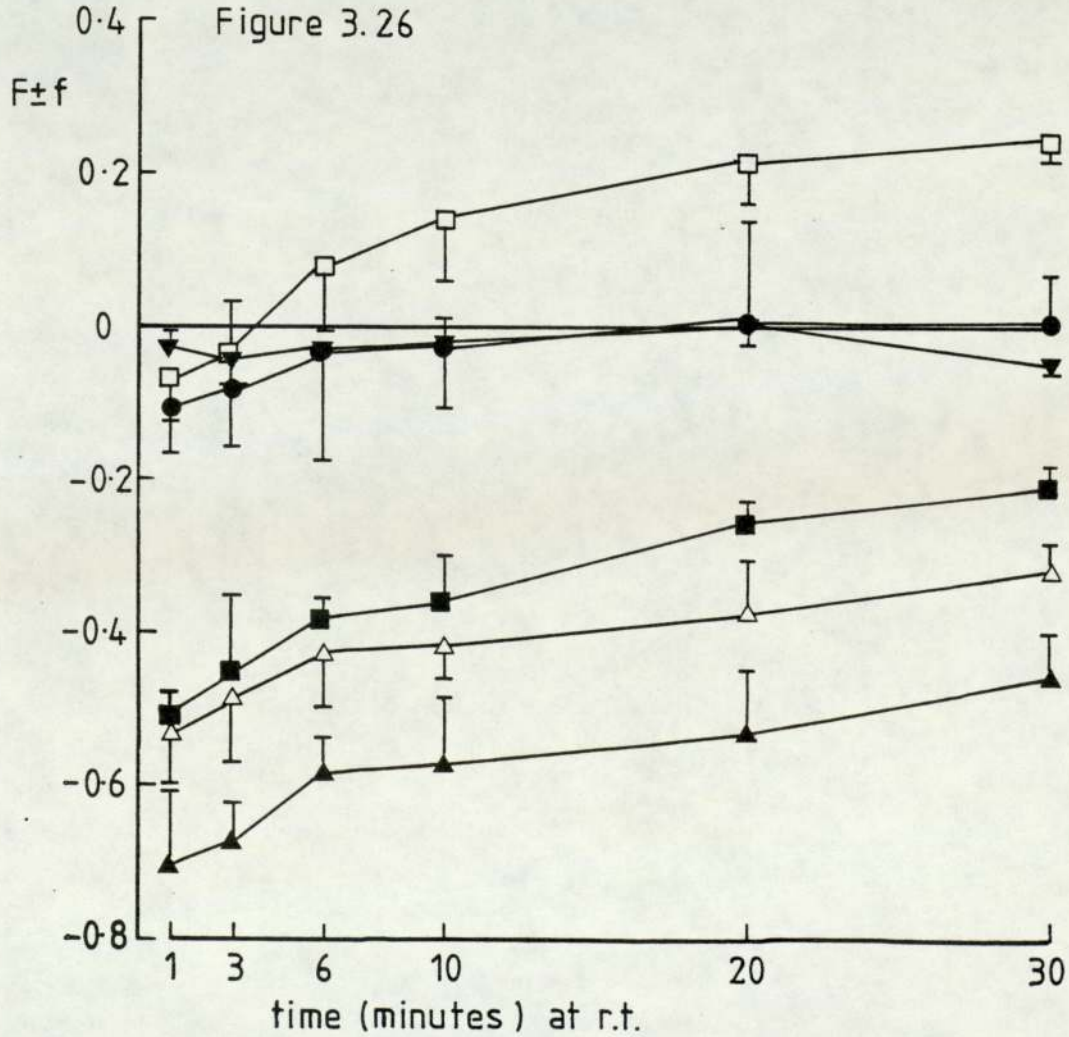


Figure 3.27

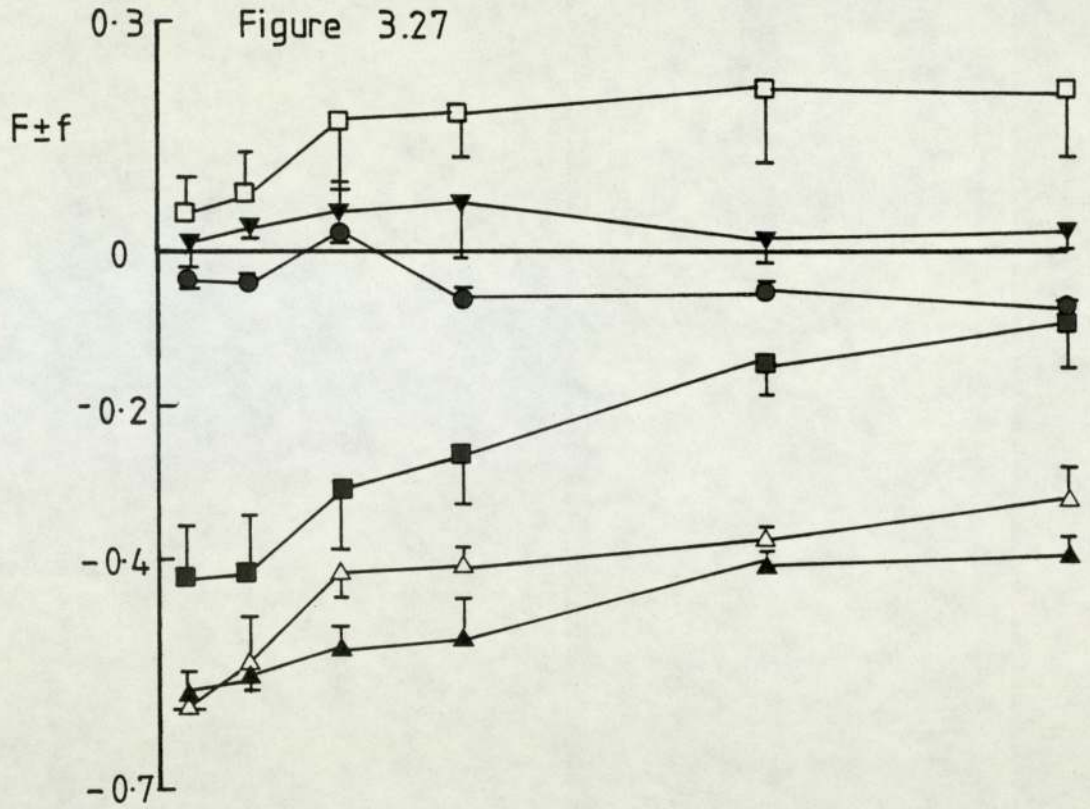


Figure 3.28

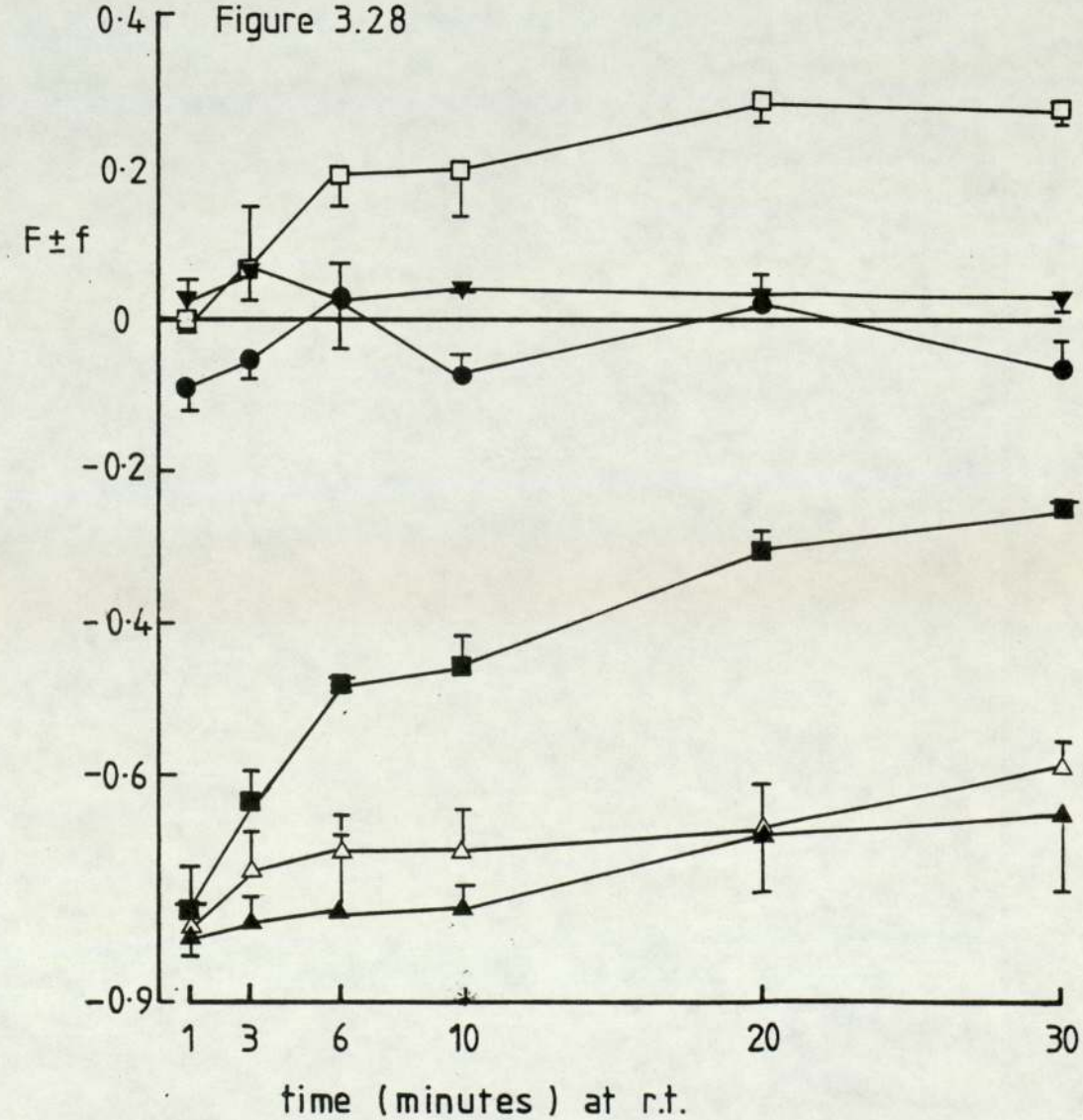


Table 3.10

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars compared with the uptake of ^{14}C sucrose after incubation of the compounds with plasmolysing agent plus maltose and E. coli W3110 grown to log phase in CDM.

Time of initial incubation	^{3}H labelled sugars			
	OS 508	OS 670	OS 833	sucrose
	$t(p=0.05) = 2.306$[a,b]	$t = 2.306$	$t = 2.306$	$t = 2.571$
1	1.763	0.547	24.292	0.998
3	3.607	5.886	25.879	0.750
6	4.185	4.888	15.179	0.056
10	1.282	5.924	8.580	0.027
20	1.280	5.614	5.331	0.238
30	2.470	6.008	0.208	0.557

Table 3.11

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation of the compounds with plasmolysing agent plus maltose and E. coli W3110 grown to stationary phase in CDM.

Time of initial incubation	^{3}H labelled sugars			
	OS 508	OS 670	OS 833	sucrose
	$t(p=0.05) = 2.201$[a,b]	$t = 2.201$	$t = 2.306$	$t = 4.303$
1	6.301	4.828	42.426	2.583
3	3.397	1.659	12.652	2.889
6	0.819	3.120	41.569	1.392
10	1.159	5.912	18.022	0.940
20	0.0418	14.056	26.22	0.428
30	0.103	26.887	21.380	4.143

a. The statistical significance of the data was determined by calculating

$$t = \frac{\bar{x} - \mu}{s / \sqrt{n-1}}$$

where $\bar{x}$ is the mean of the values of I - E/I for a given sugar after a given period of incubation; μ represents the diffusion of ^{14}C sucrose, and always = 0; s is the standard deviation of $\bar{x}$; n is the number of samples used to obtain $\bar{x}$.

b. In all cases, a two-tailed test is used, and p = 0.05.

Table 3.12

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation of the compounds with plasmolysing agent plus maltose and E. coli W3110 grown to magnesium limitation in magnesium deficient CDM.

Time of initial incubation	^{3}H labelled sugars			
	OS 508	OS 670	OS 833	sucrose
	t(p=0.05) = 4.303[a,b]	t = 2.201	t = 2.306	t = 4.303
1	3.887	3.738	16.780	0.485
3	3.932	5.002	12.570	2.785
6	3.018	6.272	12.047	2.047
10	8.009	10.803	9.147	1.342
20	5.410	7.039	10.494	0.602
30	8.842	9.055	4.553	1.824

Table 3.13

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation of the compounds with plasmolysing agent plus maltose and E. coli W3110 grown in CDM and limited with tetracycline.

Time of initial incubation	^{3}H labelled sugars			
	OS 508	OS 670	OS 833	sucrose
	t(p=0.05) = 4.303[a,b]	t = 2.571	t = 4.303	t = 4.303
1	5.836	0.336	20.368	1.489
3	3.663	2.203	24.243	2.546
6	1.310	11.474	52.218	0.622
10	4.680	7.733	20.426	6.094
20	0.139	45.236	17.410	1.380
30	2.909	47.300	35.624	2.198

a. For calculation of t, see Table 3.10.

b. In all cases, a two-tailed test was used, and p = 0.05.

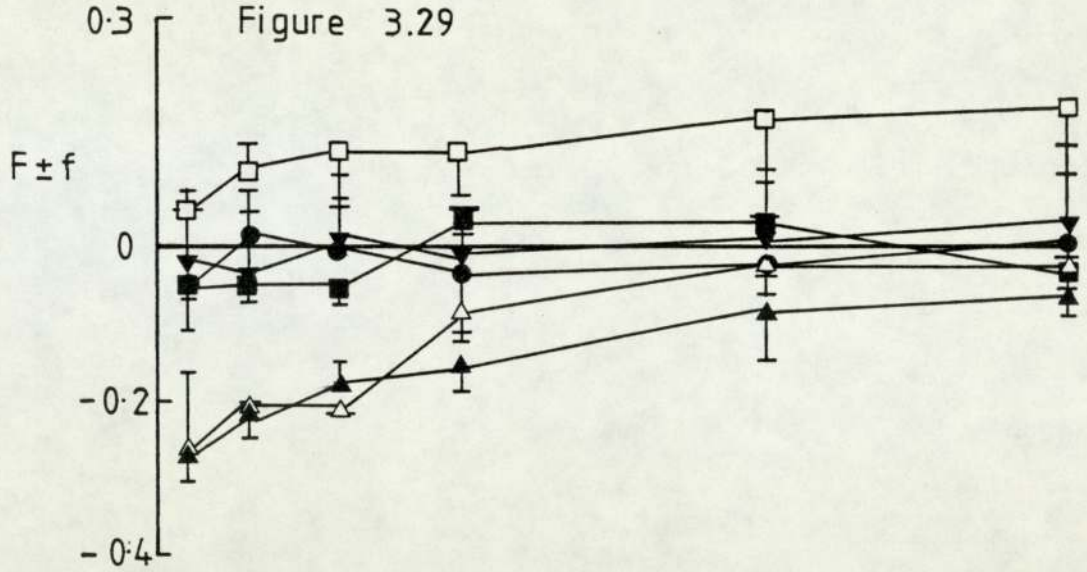


Figure 3.29

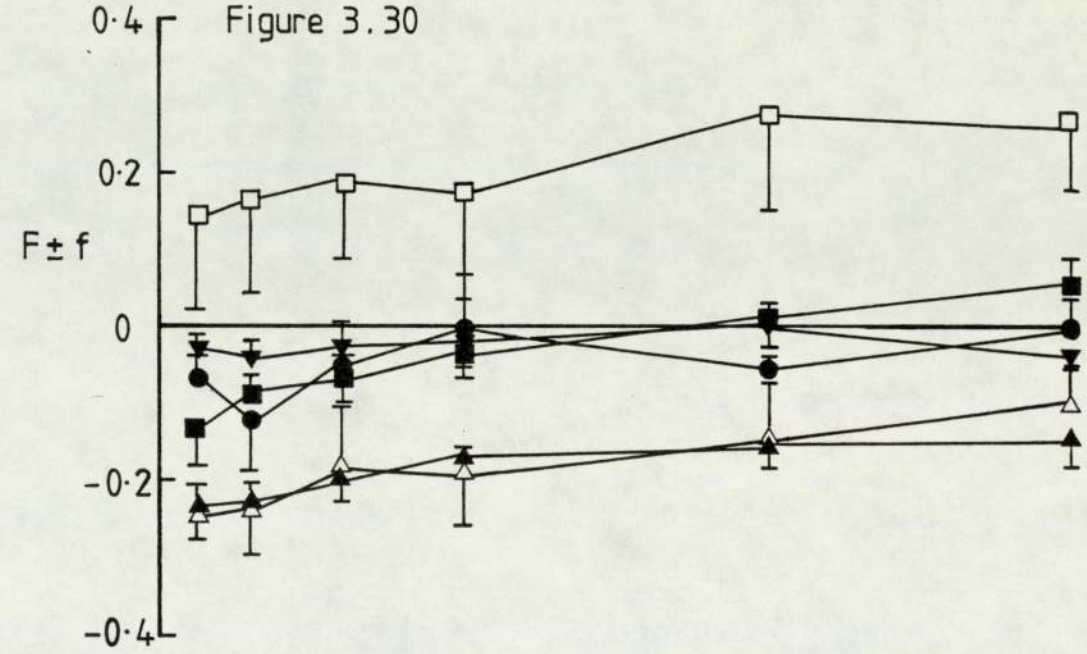


Figure 3.30

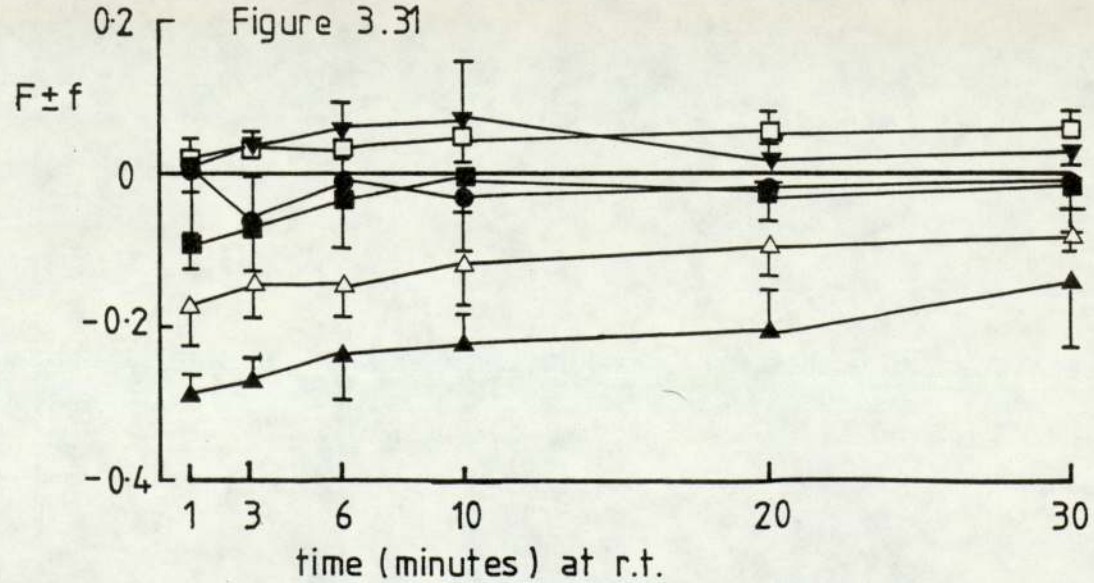


Figure 3.31

Figure 3.29: Uptake of tritiated oligosaccharides relative to that of ^{14}C sucrose in the presence of maltose, across the outer membrane of unplasmolysed E. coli W3110 grown to mid log phase in CDM.

Cells were incubated as described in the text with maltose, ^{14}C sucrose and

OS 508	●
OS 670	□
OS 833	■
OS 995	△
OS 1157	▲
sucrose	▼

F ± f : see Figure 3.24

Data for standard deviation will be found in Appendix 3.

Figure 3.30: Uptake of tritiated oligosaccharides relative to that of ^{14}C sucrose in the presence of maltose, across the outer membrane of unplasmolysed E. coli W3110 grown to stationary phase (oxygen limitation) in CDM.

Legend as for Figure 3.29

Figure 3.31: Uptake of tritiated oligosaccharides relative to that of ^{14}C sucrose in the presence of maltose, across the outer membrane of unplasmolysed E. coli W3110 grown to magnesium limitation in magnesium depleted CDM.

Legend as for Figure 3.29.

Table 3.14

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation of the compounds with maltose and unplasmolysed E. coli W3110 grown to log phase in CDM.

Time of initial incubation	^{3}H labelled sugar					
	OS 508	OS 670	OS 833	OS 995	OS 1157	sucrose
	$t(p=0.05) = 2.306$[a,b]	$t = 2.306$	$t = 2.571$	$t = 4.303$	$t = 2.571$	$t = 2.571$
1	2.009	3.523	4.595	4.052	44.341	0.750
3	0.501	7.331	8.413	20.289	20.909	0.998
6	0.190	5.407	9.626	90.652	15.406	0.027
10	1.135	6.753	2.646	4.124	10.972	0.266
20	1.634	5.771	8.138	1.791	3.93	0.056
30	1.302	6.122	7.084	1.171	7.775	0.557

a. For calculation of t, see Table 3.10.

b. In all cases, a two-tailed test is used, and $p = 0.05$.

Table 3.15

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation with maltose and unplasmolysed E. coli W3110 grown to stationary phase in CDM.

Time of initial incubation	^{3}H labelled sugar					
	OS 508	OS 670	OS 833	OS 995	OS 1157	sucrose
	$t(p=0.05) = 2.571$[a,b]	$t = 2.571$	$t = 2.571$	$t = 2.571$	$t = 4.303$	$t = 4.303$
1	4.174	2.690	6.463	24.266	12.427	2.583
3	4.528	3.040	7.659	12.013	11.687	2.889
6	6.214	4.350	4.827	4.864	17.324	1.399
10	0.106	2.693	3.116	7.108	17.954	1.002
20	25.561	5.018	2.120	4.472	10.542	0.313
30	0.318	7.093	2.979	2.929	14.296	4.143

a. For calculation of t, see Table 3.10

b. In all cases a two-tailed test is used, and $p = 0.05$.

Table 3.16

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation of the compounds with maltose and unplasmolysed E. coli W3110 grown to magnesium limitation in magnesium deficient CDM.

Time of initial incubation	^{3}H labelled sugars				
	OS 508	OS 670	OS 833	OS 995	sucrose
	$t(p=0.05) = 2.571$[a,b]	$t = 2.571$	$t = 2.571$	$t = 2.571$	$t = 4.303$
1	0.245	1.250	6.970	8.174	0.484
3	2.425	6.013	2.839	6.972	2.785
6	1.022	2.426	1.273	7.213	2.047
10	0.779	2.721	0.101	5.583	1.342
20	1.052	4.380	0.863	6.429	0.602
30	1.185	4.372	0.485	4.757	1.824

a. For calculation of t, see Table 3.10

b. in all cases, a two-tailed test is used, and $p = 0.05$.

The uptake patterns of isomaltohexitol (OS 995) and isomaltoheptitol (OS 1157) are relatively similar to each other, and also to some extent to OS 833. When incubated, in the presence of plasmolysing agent plus maltose, with E. coli grown in various ways (Figs. 3.25-3.27) the initial values of F are generally -0.5 to -0.7 for both compounds, the exception again being tetracycline grown E. coli (Fig. 3.28) where the initial value of F for OS 833 is consistently lower. The difference between I and E decreases for both compounds, with increasing length of incubation, but the apparent rate of diffusion of the molecules tends to be consistently linear throughout the 30 minutes initial incubation, without a more rapid phase during the first 10 minutes. After the 30 minutes incubation, some of both molecules had diffused into the cell, but the concentration in the periplasmic space was considerably less than that of ^{14}C sucrose, whatever the growth and harvesting conditions of the organism. Except where E. coli was grown to log phase in CDM (Fig. 3.25) the concentration of OS 995 in the periplasm was slightly greater than that of OS 1157 (Figs. 3.26-3.28). This pattern is reversed when log phase E. coli was used, but the standard deviations of all the points on both lines overlap considerably, implying that this alteration of the size order is not significant in any way.

Data obtained with unplasmolysed cells. The second main feature of the data obtained when incubating E. coli with the different radiolabelled sugars is the overall pattern of uptake of the various tritiated compounds into unplasmolysed cells, incubated in the presence of maltose (Figs. 3.29-3.31). It can be seen that the data tend to fall much closer to the abcissa, values of F being generally larger than those obtained with plasmolysed cells

(i.e. E is larger, as the dpm ^{3}H are reduced). OS 508, ^{3}H sucrose and OS 833 all appear to have penetrated into the periplasmic space at a very similar rate as ^{14}C sucrose, and the relative concentrations of the compounds in the cell (E) were very similar to those in the stock solution (I). The differences between the zero line (uptake of ^{14}C sucrose) and the lines obtained for OS 508 and ^{3}H sucrose tend not to be significant (Tables 3.14, 3.16) the exception being OS 508 penetrating into stationary phase, unplasmolysed E. coli. Here, the errors are small, resulting in several of the values of F being statistically significantly different from the zero line. However, it is possible that biologically this is not relevant.

The uptake pattern of OS 833 is slightly less clear; when incubated with unplasmolysed E. coli the molecule appears to be initially slightly less able to penetrate the periplasmic space, the values of t being significant (Tables 3.14-3.16). But after 10 minutes, the compound was present in the cell at the same relative concentration as ^{14}C sucrose. The pattern of uptake of the molecule obtained with magnesium limited cells (Fig. 3.31; Table 3.16) suggests that after the first few minutes of incubation, OS 833 behaved in a manner identical to ^{14}C sucrose. This is not as apparent with either log or stationary phase E. coli, in which the diffusion patterns show considerable, statistically significant, variation around the abcissa (Figs. 3.29, 3.30; Tables 3.14, 3.15).

OS 670 penetrates into logarithmic and stationary phase, unplasmolysed E. coli (Figs. 3.29, 3.30)with a pattern very similar to that observed with plasmolysed cells; however, this is not apparent with magnesium limited cells (Fig. 3.31).

As with OS 833, the values of F obtained for both OS 995 and OS 1157 are very much reduced, causing the data to fall much closer

to the abcissa (Figs. 2.29-2.31). The rate of increase of the concentration of the two molecules relative to sucrose in the cells would not appear to be linear (unlike in plasmolysed cells), the molecules penetrating into the periplasmic space slightly more quickly during the first 6-10 minutes of the initial incubation (most clearly seen with log phase cells, Fig. 3.29). After 30 minutes incubation with the unplasmolysed cells, the concentration of both molecules in the periplasmic space was much greater than that in plasmolysed cells after the same time, the ratio of the concentrations of ^{14}C sucrose and OS 995 in the periplasm of log phase E. coli being the same as in the stock solution (Fig. 3.29; Table 3.14). There again appears to be a size-order for OS 995 and OS 1157, the former penetrating the outer membrane more easily, although this pattern is undoubtedly not significant in stationary phase E. coli.

The special case of OS 670. OS 670 behaved in a very consistent manner under all conditions other than when incubated with unplasmolysed, magnesium limited cells (Fig. 3.31). In general, the molecule appears to have crossed the outer membrane more quickly than ^{14}C sucrose during the first 10 minutes of the initial incubation. Thereafter the rate of penetration is less great, but still tends to be slightly higher than that of ^{14}C sucrose. This pattern would not in itself be remarkable, (it is the same as that for OS 833 diffusing into plasmolysed E. coli) if it were not for the fact that the relative concentration of OS 670 in the periplasmic space appears to have been greater than that of sucrose, and to have increased with the length of the initial incubation.

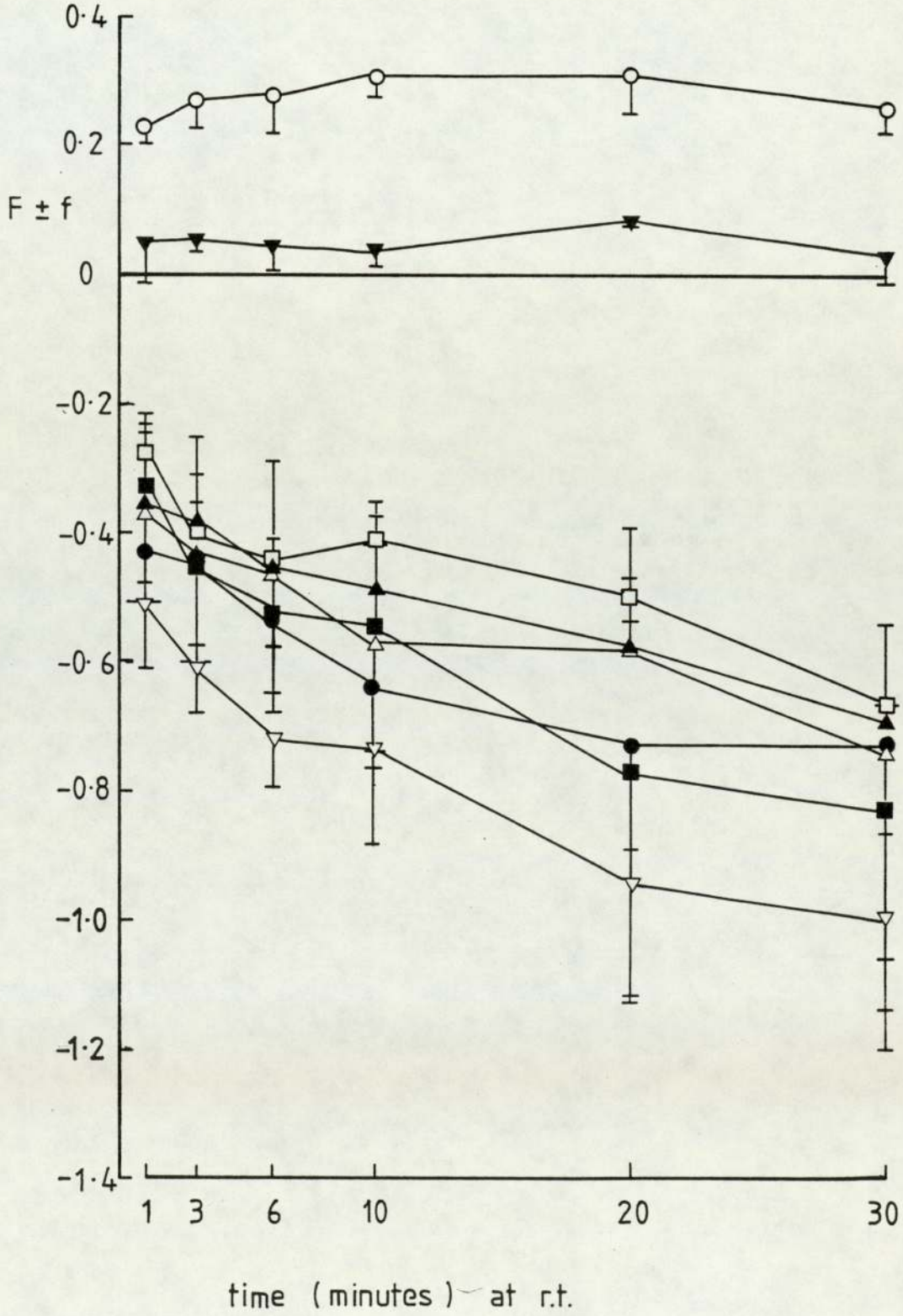
The effect of the environment. When comparing the effect of the growth environment of the organism on the penetration of the various

molecules into the periplasmic space, it would appear that for a given time of incubation the concentrations of OS 833, OS 995 and OS 1157 present in tetracycline growth-limited E. coli (Fig. 3.28) were lower than those of the same molecules diffusing into cells grown in other conditions (Figs. 3.25-3.27). Also, OS 833 (Fig. 3.25) and OS 995 (Fig. 3.26) appear to be able to cross the outer membrane of, respectively, plasmolysed and unplasmolysed, log phase E. coli slightly more easily than the outer membranes of cells grown under different conditions but treated (i.e. plasmolysed or otherwise) in the same manner.

3.4.6.3 P. aeruginosa

The diffusion of sucrose. In general, tritiated sucrose diffused into P. aeruginosa at a similar rate and concentration as ^{14}C sucrose, irrespective of the way in which the cells have been grown, or the conditions of the initial incubation (Figs. 3.32 to 3.39). There are occasions when the values calculated for t are significant (Tables 3.17 to 3.24) such as with nutrient broth grown cells (Tables 3.20, 3.21) or plasmolysed, log phase P. aeruginosa (Table 3.17), but the significant values of t are probably a function of the small standard deviation and number of samples used in determining F.

Results obtained with CDM grown cells. Where the organism was grown in CDM, either to log phase, stationary phase (oxygen limitation) or iron limitation, the overall patterns of the data obtained for the various sugars used are very similar, OS 346 crossing into the cells at a rate which is similar but not identical, to that of sucrose. The slopes for the lines obtained for all the remaining oligosaccharides and inulin are negative. They tend, however, to be steeper (although still negative) during the first 10 minutes of the initial incubation, although there are exceptions, such as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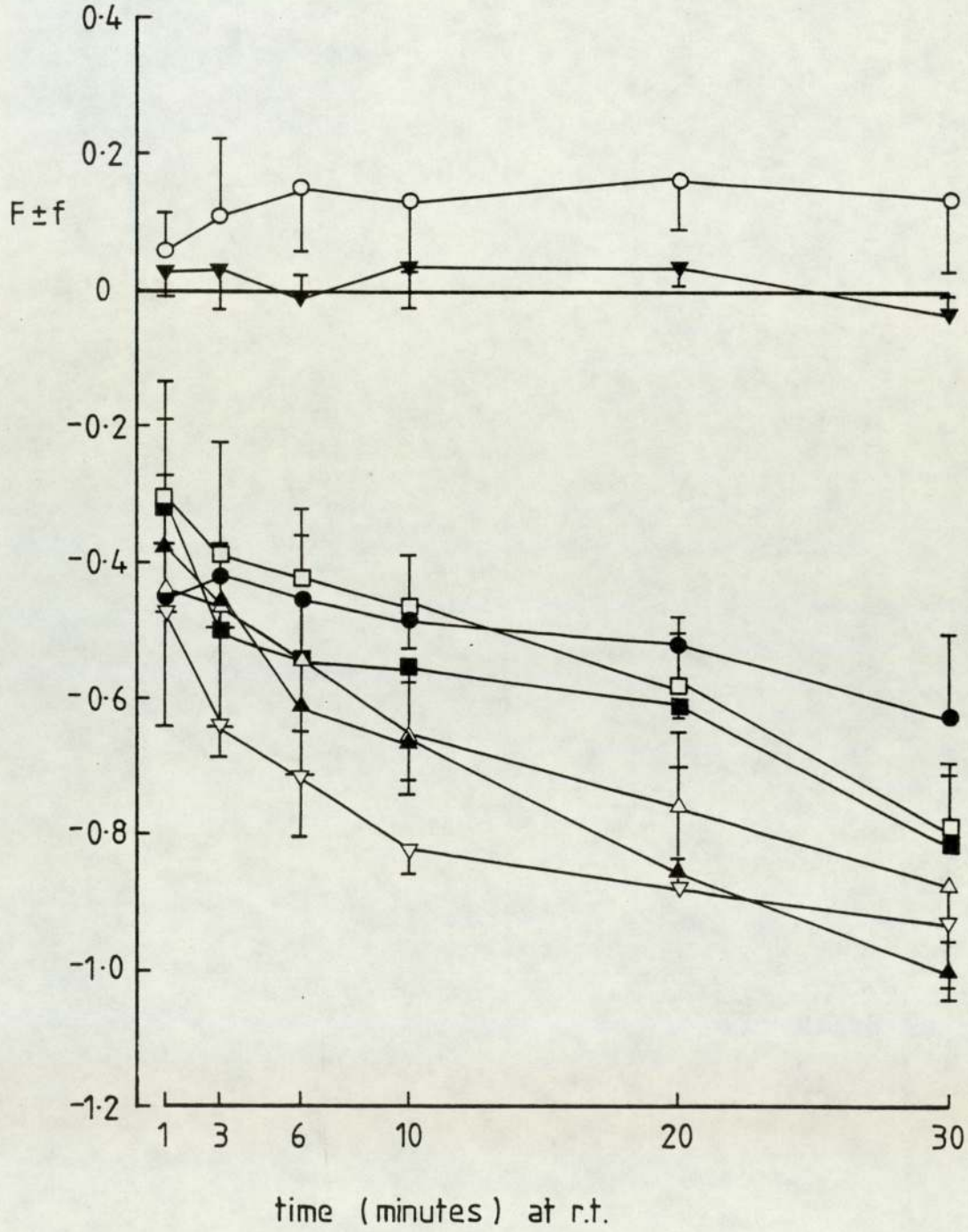
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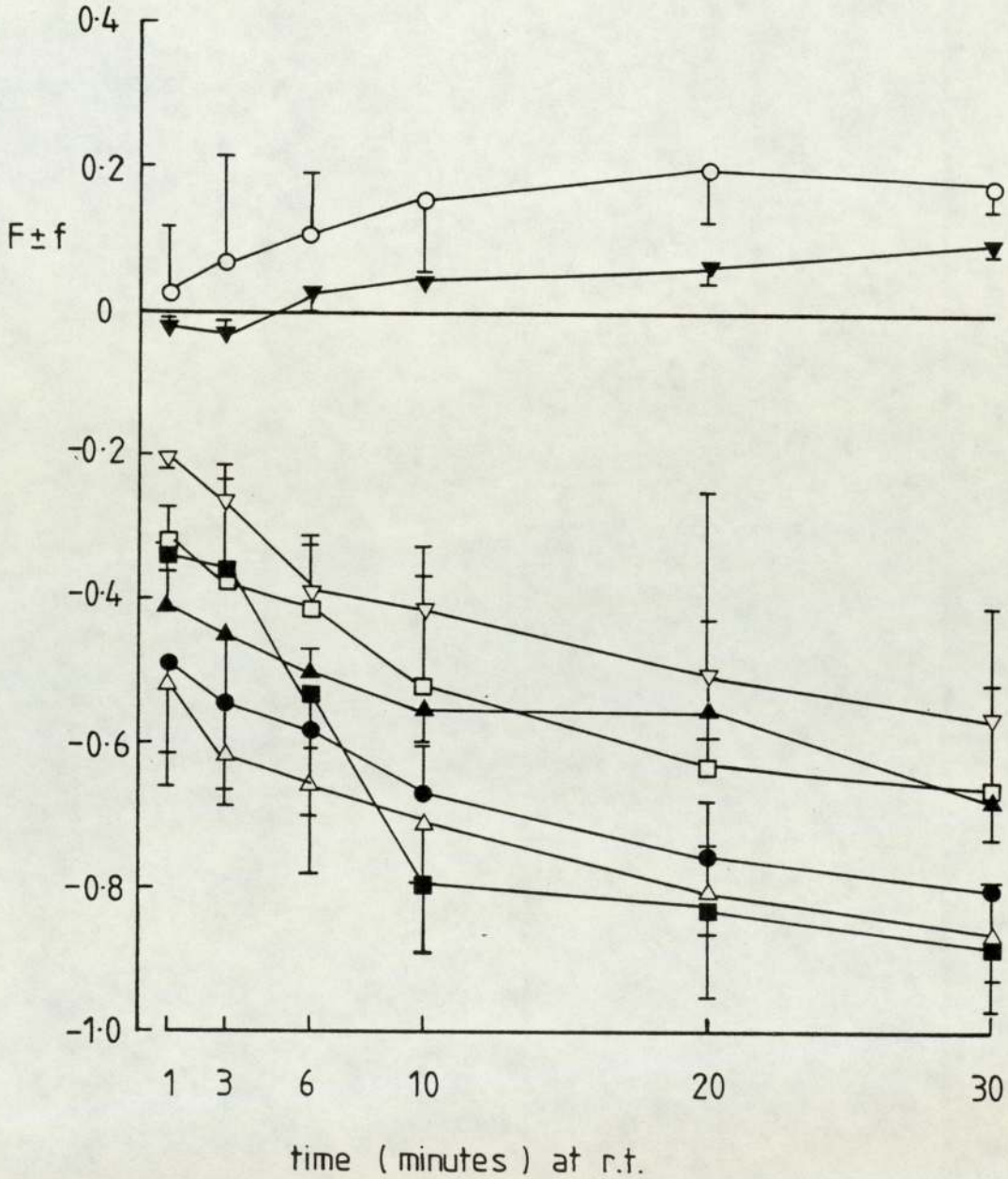
time (minutes) at r.t.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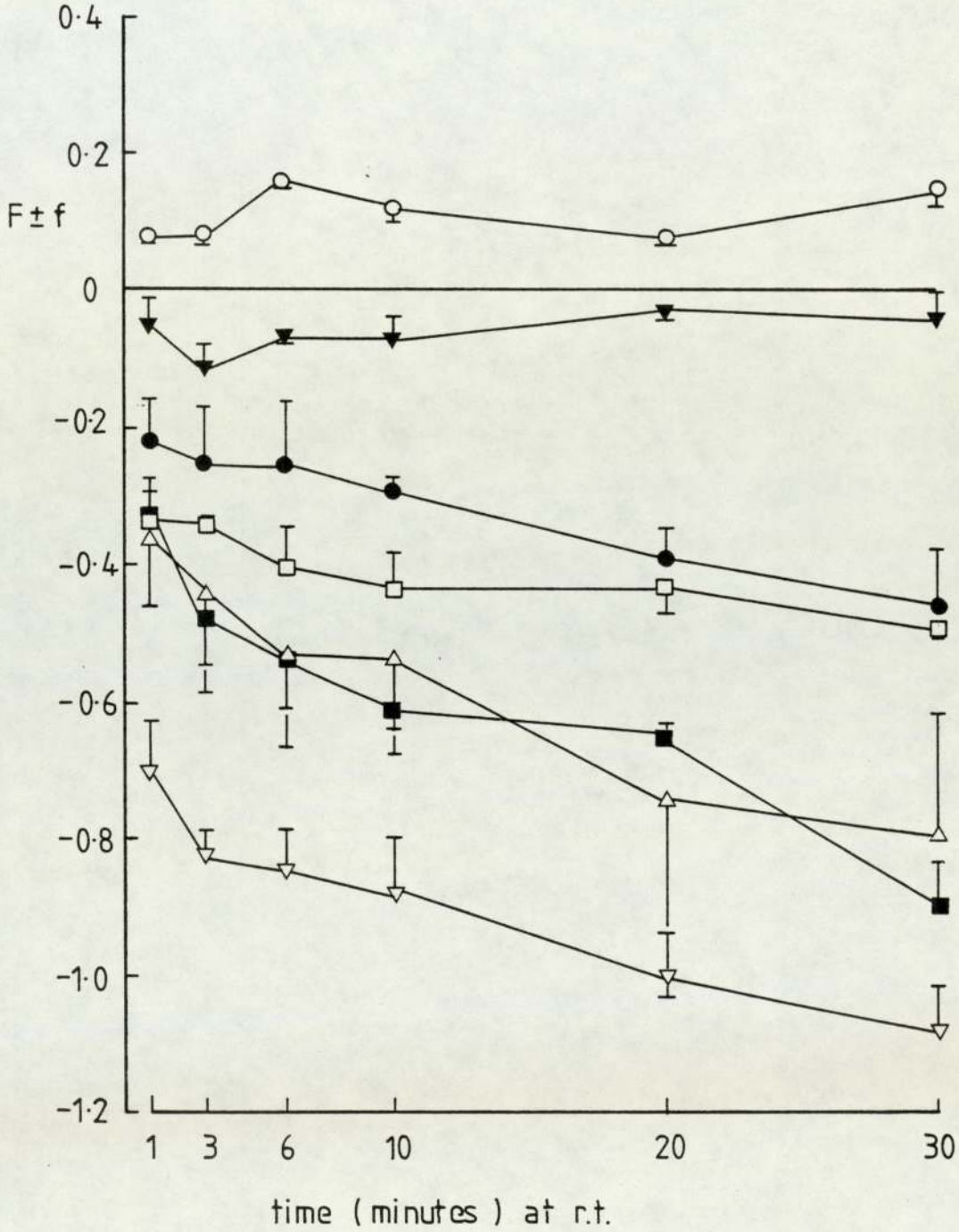
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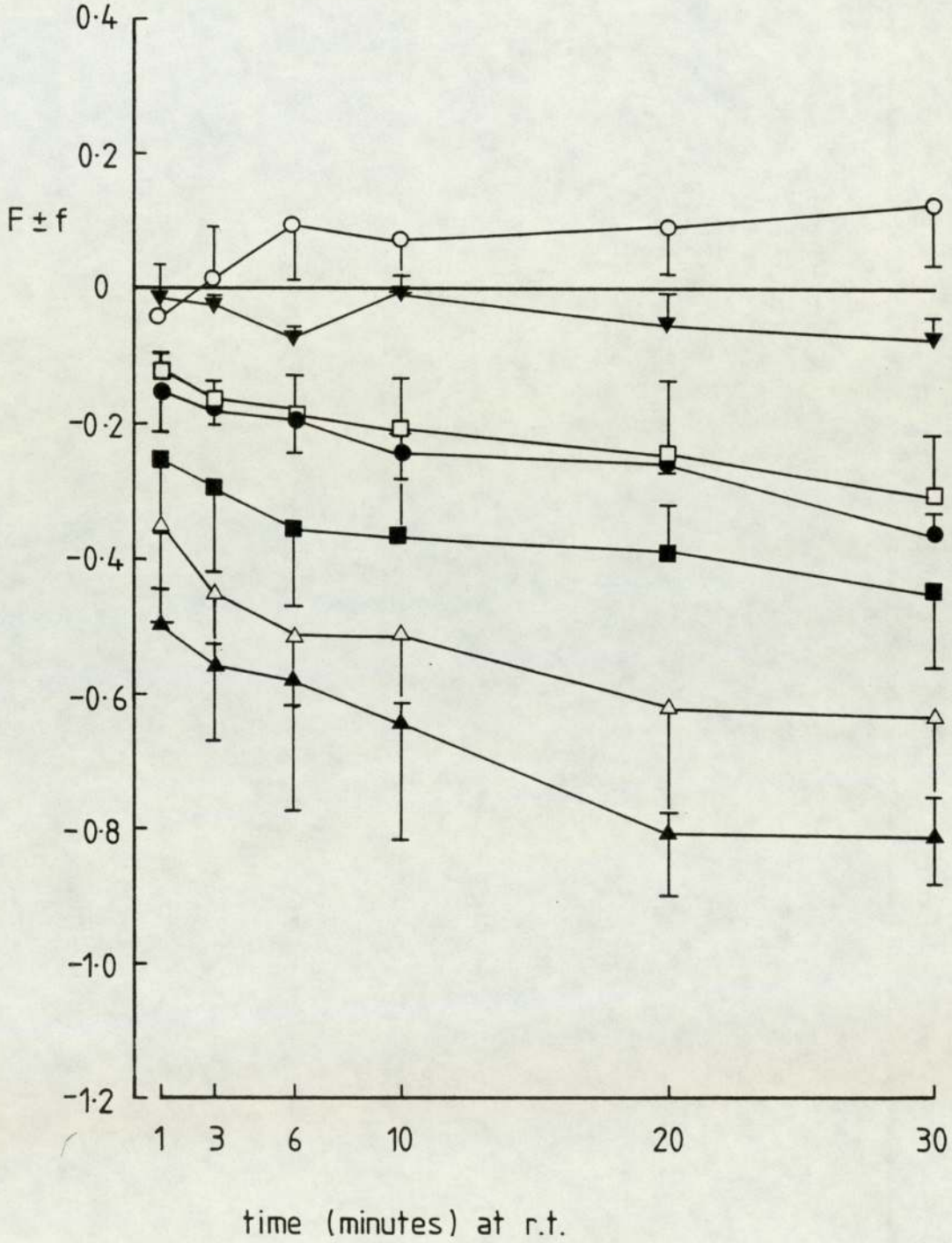
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time (minutes) at r.t.

Table 3.17

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose after incubation of the compounds with plasmolysing agent plus maltose and P. aeruginosa 6750 grown to log phase in CDM.

Time of initial incubation	^{3}H labelled sugars			
	OS 346	OS 670	OS 995	sucrose
	t(p=0.05) = 2.571[a,b]	t = 2.571	t = 2.365	t = 4.303
1	25.659	12.676	7.660	1.261
3	16.546	15.926	6.088	3.848
6	11.700	17.583	7.340	1.478
10	26.601	26.236	8.003	3.043
20	14.683	29.940	8.520	59.821
30	21.014	12.074	4.2593	0.961

Table 3.18

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of sucrose, after incubation of the compounds with plasmolysing agent plus maltose and P. aeruginosa 6750 grown to stationary phase (oxygen limitation) in CDM.

Time of initial incubation	^{3}H labelled sugars				
	OS 346	OS 508	OS 670	OS 833	sucrose
	t(p=0.05) = 2.571[a,b]	t = 2.571	t = 2.306	t = 4.303	t = 2.571
1	2.515	37.139	7.400	2.521	1.755
3	2.158	46.586	6.763	5.361	1.128
6	3.961	9.416	15.741	7.079	0.962
10	2.743	27.603	21.778	4.524	1.357
20	4.866	28.341	23.208	56.060	3.046
30	2.936	11.530	26.731	10.466	2.310

a. For calculation of t, see Table 3.10

b. In all cases, a two-tailed test is used, and p = 0.05.

Table 3.19

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of sucrose, after incubation with plasmolysing agent plus maltose and P. aeruginosa 6750 grown to iron limitation in iron deficient CDM.

Time of initial incubation	^{3}H labelled sugars			
	OS 346	OS 670	inulin	sucrose
	$t(p=0.05) = 2.306$[a,b]	$t = 2.447$	$t = 4.303$	$t = 4.303$
1	0.770	15.529	38.259	2.395
3	1.137	6.284	11.779	2.458
6	2.904	10.943	7.293	1.669
10	4.396	6.936	12.336	4.506
20	7.882	4.802	9.602	0.592
30	18.240	7.390	16.875	12.999

Table 3.20

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of sucrose, after incubation with plasmolysing agent plus maltose and P. aeruginosa 6750 grown to log phase in nutrient broth.

Time of initial incubation	^{3}H labelled sugars		
	OS 346	OS 508	sucrose
	$t(p=0.05) = 4.303$[a,b]	$t = 2.571$	$t = 4.303$
1	13.258	7.856	1.855
3	13.895	6.738	4.960
6	20.712	6.388	9.668
10	13.977	29.088	3.421
20	16.853	21.789	4.112
30	17.265	11.564	1.488

a. For calculation of t, see Table 3.10

b. In all cases, a two-tailed test is used, and p = 0.05.

Table 3.21

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of sucrose, after incubation with plasmolysing agent plus maltose and P. aeruginosa 6750 grown to stationary phase (oxygen limitation) in nutrient broth.

Time of initial incubation	^{3}H labelled sugars			
	OS 346	OS 508	OS 670	sucrose
	$t(p=0.05) = 2.201$ [a,b]	$t = 2.571$	$t = 2.306$	$t = 4.303$
1	1.909	4.840	12.991	0.569
3	0.792	16.717	15.757	2.466
6	3.929	8.709	9.491	9.147
10	2.602	15.633	7.843	1.414
20	4.624	42.601	5.950	1.615
30	4.679	20.516	9.451	5.015

a. For calculation of t, see Table 3.10

b. In all cases, a two-tailed test is used, and p = 0.05.

OS 995 penetrating into unplasmolysed P. aeruginosa grown to stationary phase in CDM (Fig. 3.38). There is some evidence that a "size-order pattern" may exist, with the smaller molecules (OS 508, OS 670) appearing to be present in the cells at a slightly higher concentration than the larger compounds, but this is not consistent, and there are some systems in which inulin (mw 5200 d) features as possibly being present in the cell at a higher concentration than all the other, smaller compounds (Figs. 3.34, 3.37). There are many other inconsistencies in a possible size-related pattern, which are in contrast with the consistency of the overall shape of the uptake patterns observed, and the compactness of these data.

Results obtained with nutrient broth grown cells. Where P. aeruginosa has been grown in nutrient broth, and harvested during either log (Fig. 3.35) or stationary phase (Fig. 3.36) the data are slightly different from that obtained with cells grown in CDM. This is primarily because the values of F for OS 508 and OS 670 are nearer to zero at each time point than the appropriate values obtained with CDM grown cells. Also, there is a slightly more obvious size-order in the uptake patterns of the sugars obtained with nutrient broth grown cells, the larger the molecule concerned, the further the points fall below the zero line. The actual shape of the lines obtained with nutrient broth grown cells is similar to those from CDM grown cells, data obtained with molecules larger than OS 346 giving a negative slope, the gradient of which decreases slightly with time. But when the overall pattern of the data obtained when investigating nutrient broth grown cells is considered, it appears more spread out, the distinctions between the different molecules being greater.

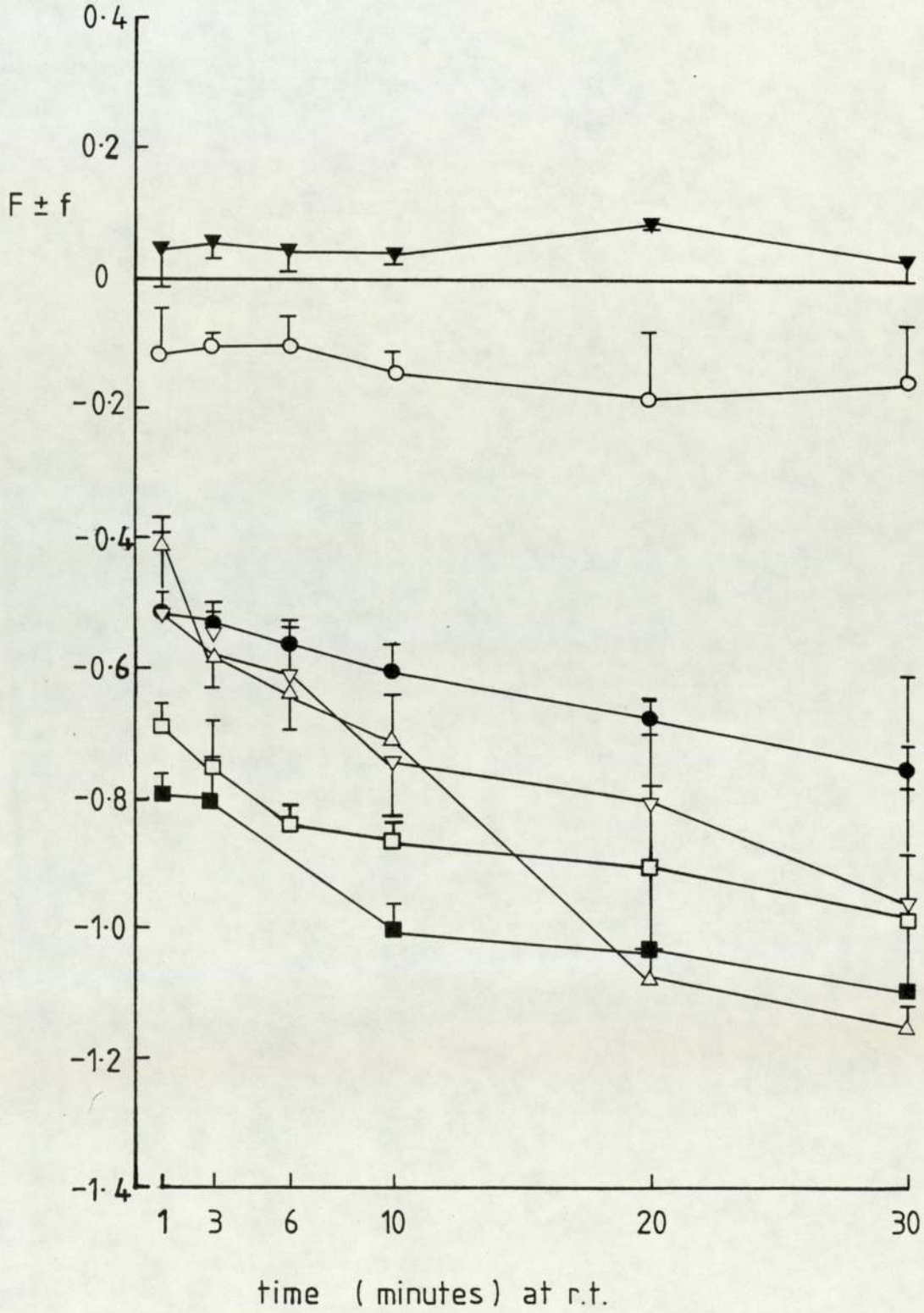
Figure 3.37: Uptake of tritiated oligosaccharides and inulin relative to that of ^{14}C sucrose, in the presence of maltose, across the outer membrane of unplasmolysed P. aeruginosa 6750 grown to mid-log phase in CDM.

Cells were incubated as described in the text with maltose, ^{14}C sucrose and

OS 346	○
OS 508	●
OS 670	□
OS 833	■
OS 995	△
^{3}H inulin	▽
^{3}H sucrose	▼

F $\pm$ f : see Figure 3.24.

Data for standard deviations will be found in Appendix 3.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time (minutes) at r.t.

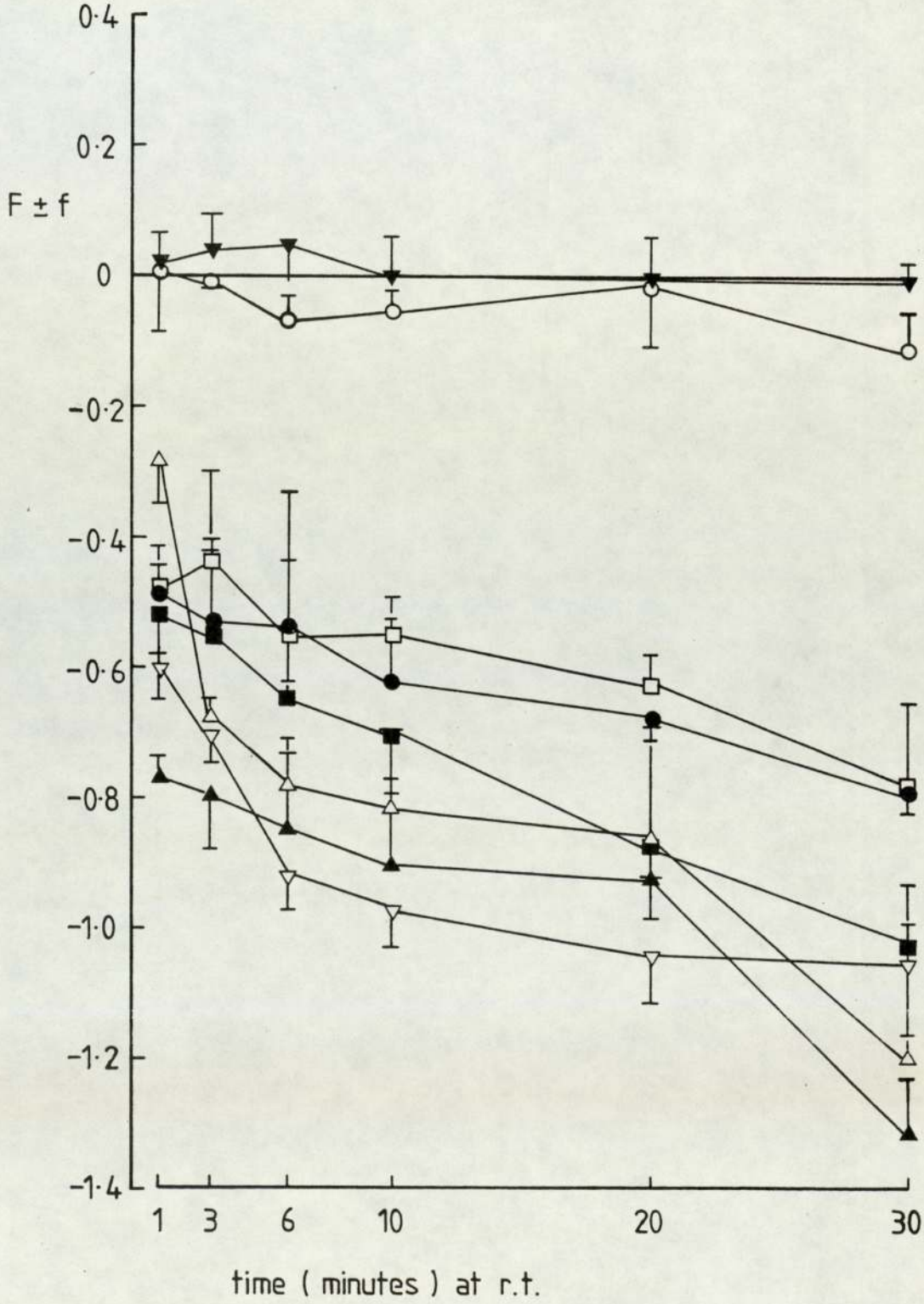
Figure 3.38: Uptake of tritiated oligosaccharides and inulin relative to that of ^{14}C sucrose, in the presence of maltose, across the outer membrane of unplasmolysed P. aeruginosa 6750 grown to stationary phase (oxygen limitation) in CDM.

Cells were incubated as described in the text, with maltose, ^{14}C sucrose and

OS 346	○
OS 508	●
OS 670	□
OS 833	■
OS 995	△
OS 1157	▲
^{3}H inulin	▽
^{3}H sucrose	▼

F ± f : see Figure 3.24.

Data for standard deviations will be found in Appendix 3.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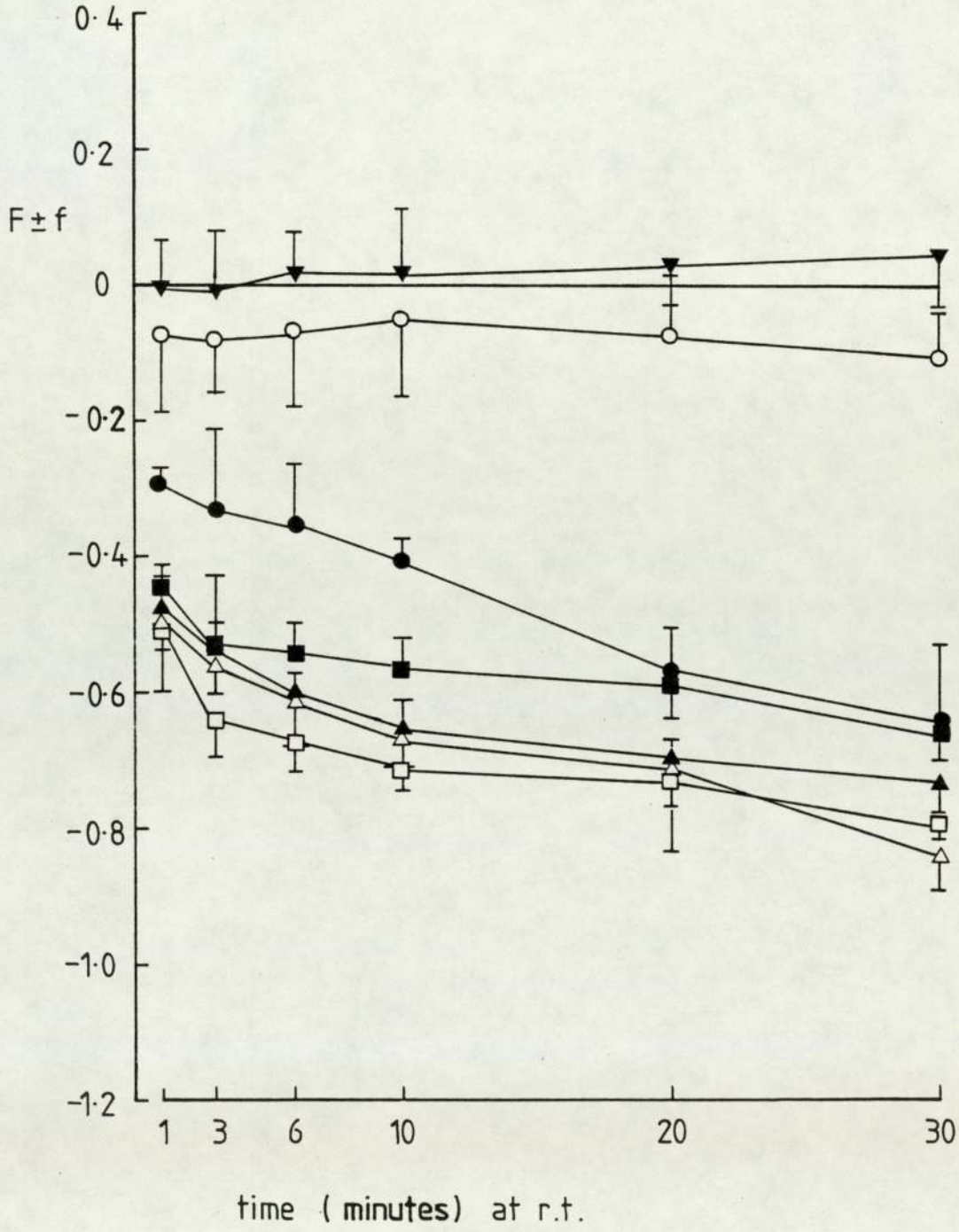
time (minutes) at r.t.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time (minutes) at r.t.

Table 3.22

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of ^{14}C sucrose, after incubation of the compounds with maltose and unplasmolysed P. aeruginosa 6750 grown to log phase in CDM.

Time of initial incubation	^{3}H labelled sugar			
	OS 346	OS 508	OS 670	sucrose
	$t(p=0.05) = 2.571$ [a,b]	$t = 4.303$	$t = 3.182$	$t = 4.303$
1	3.637	25.471	43.635	1.004
3	13.328	28.441	87.090	3.168
6	5.297	20.114	48.534	1.834
10	12.202	23.566	48.083	3.837
20	4.015	33.589	8.932	2.447
30	4.108	28.250	5.299	0.797

Table 3.23

t-tests to determine the significance of the difference between the uptake of ^{3}H labelled sugars as compared with the uptake of sucrose after incubation of the compounds with maltose and unplasmolysed P. aeruginosa 6750 grown to stationary phase (oxygen limitation) in CDM.

Time of initial incubation	^{3}H labelled sugars			
	OS 346	OS 508	OS 670	sucrose
	$t(p=0.05) = 2.306$ [a,b]	$t = 2.571$	$t = 3.182$	$t = 2.571$
1	0.265	17.416	19.380	0.814
3	1.350	10.681	15.982	1.180
6	5.116	5.652	13.422	1.930
10	4.813	10.028	39.866	0.267
20	0.307	29.318	34.496	0.123
30	5.215	75.130	15.058	0.137

a. For calculation of t, see Table 3.10

b. In all cases, a two-tailed test is used, and $p = 0.05$.

Table 3.24

t-tests to determine the significance of the difference between the uptake of various ^{3}H labelled sugars as compared with the uptake of sucrose, after incubation with maltose and unplasmolysed *P. aeruginosa* 6750 grown to iron limitation in iron deficient CDM.

Time of initial incubation	^{3}H labelled sugars		
	OS 346	OS 508	sucrose
	$t(p=0.05) = 2.306$[a,b]	$t = 2.571$	$t = 2.571$
1	2.030	50.295	0.224
3	3.295	6.637	0.179
6	1.952	9.169	0.477
10	1.516	28.512	0.276
20	2.426	20.125	0.981
30	4.775	14.020	1.302

a. For calculation of t, see Table 3.10.

b. In all cases, a two-tailed test is used, and p = 0.05.

As a control for results obtained with P. aeruginosa 6750, P. aeruginosa PAO1 grown in nutrient broth, to stationary phase, and plasmolysed in the presence of maltose was investigated using all the compounds used in the double label assay. The results were similar to those obtained with P. aeruginosa 6750 grown and treated in the same way (see p. 185).

Despite this contrast between the effect of the nutritional richness or otherwise of the growth media of the organism, there seems to be little important difference between the uptake of the molecules into log or stationary phase nutrient broth grown P. aeruginosa, or into log, stationary (i.e. oxygen limited) or iron limited cells grown in CDM. The remaining outstanding difference is the contrast between plasmolysed and unplasmolysed cells.

The effect of plasmolysis. When data obtained for P. aeruginosa grown in a specific way and incubated with the radiolabelled sugars either with (Figs. 3.32-3.36) or without (Figs. 3.37-3.39) plasmolysing agent are compared, it is immediately apparent that the uptake of OS 346 is affected by the state of the cells. The statistical significance of the data varies (Tables 3.17-3.24) but consistently OS 346 was present in the periplasmic space of plasmolysed cells at a higher concentration than ^{14}C sucrose (Figs. 3.32-3.36). The rate at which this molecule crossed into the periplasm of plasmolysed, iron limited, log and stationary phase, CDM grown P. aeruginosa is initially much greater than that of ^{14}C sucrose (Figs. 3.32-3.34) but after approximately 20 minutes incubation appears to have reached a maximum, and then the difference in the concentration of the compounds decreases slightly. This pattern does not repeat itself when the uptake of OS 346 into

plasmolysed, nutrient broth grown cells is investigated (Figs. 3.35 and 3.36).

In contrast, OS 346 appears to have crossed into unplasmolysed P. aeruginosa (Figs. 3.37-3.39) at a rate similar to that of ^{14}C sucrose but at a slightly lower concentration. The values of F in these situations are not always significant (Tables 3.22-3.24) but the concentration of OS 346 always appears to be less than that of sucrose, whilst the overall patterns of uptake are very similar to that of sucrose, irrespective of the conditions in which the cells were grown.

3.4.7 Discussion

The use of a double label assay, comparing the uptake of one compound with that of another eliminates many errors which would otherwise affect the data. This is in particular true of any possible variation in the volume of the total periplasmic space (i.e. that of all the cells present in a given system) available to the radiolabelled molecules under investigation. Were the assay to be carried out using only one labelled molecule (in this situation, those from the ^{3}H isomaltitol series) any variation in the volume within the cells which was available to the compounds would become very important. It is likely that when the organisms are grown in different conditions, the periplasmic space of E. coli or P. aeruginosa would vary considerably, and that plasmolysing the cells would not necessarily bring the periplasmic volume to a consistent percentage of the total volume of the cell. The total number of cells present in the 40% (v/v) cell suspension would also be unlikely to be consistent, providing a further source of variation in the total volume within the cells available to the

radiolabelled molecules. By using a double label assay in which two compounds are compared with each other, such variation in the total space available to a molecule can be ignored, as it will affect both compounds equally, should they be able to penetrate the outer membrane. Therefore, comparisons can be made between the same experiment performed on different days, or between experiments using cells grown in different ways, or different organisms, without needing to consider the effect of variation in the periplasmic space.

Also eliminated by the use of a double label assay are technical errors of measurement, or the removal of supernatants. Variation in these could lead to very considerable differences in the results obtained with a single label assay. However, during a double label assay, any technical error occurs for both the ^{3}H and ^{14}C compounds and on calculation of the ratio would be eliminated.

These practical reasons for using the double label assay formed the background to the way in which it was devised. There are three major steps to be considered; the initial incubation period, the washing and the final efflux period. A calculation of the predicted amount of radiolabel present in each stage of the assay will be found at page 200.

The first stage, in which cells and radiolabelled material were mixed and incubated for 30 minutes was designed partly based on the work of Decad and Nikaido (1976). The cell suspension used there was approximately 20% (v/v). If this were to pertain in the double label assay, the cells had to be resuspended at a higher concentration which could then be diluted to give 20% (v/v) cells. Hence the bacteria are suspended to a concentration of 40% (v/v), and diluted by two with radiolabel and plasmolysing solutions.

The periplasmic volume is generally held to be a small percentage of the total volume of the Gram-negative cell (Decad and Nikaido, 1976) and it was thought to be necessary to plasmolyse the cells in order to increase the size of the periplasm, and hence the sensitivity of the assay. The choice of a plasmolysing agent for this purpose has already been discussed (see section 3.2). However, it was found that plasmolysing the cells was not necessary for increasing the sensitivity of the assay, and the real interest in the data obtained with plasmolysed cells lay in the fact that the bacteria were initially incubated in a hypertonic medium (602 mOsm as opposed to 300 mOsm of the periplasmic space (Stock et al., 1977)). Equally, those cells which were not plasmolysed were incubated in a hypotonic medium (114 m Osm), which is also of interest. The maltose, which slightly affects the osmolarity of the system used, was added in all situations in an attempt to saturate any available non-specific adsorption sites.

The concentration of the manufactured ^{3}H labelled compounds used (120 µM ÷ 4) directly reflects the approximate concentration of the most dilute of the original batch of labelled oligosaccharides to be prepared. Subsequently, it was found that, provided the amount (i.e. µCi) of ^{3}H mixed with a standard amount (µCi) of ^{14}C was of the same order of magnitude, the molarity of the oligosaccharide solutions, and hence the concentration added to the cells, was not of great importance. However, the concentration of the manufactured compounds used was maintained at 120 µM, diluted by four when mixed with the cells.

When the cells were washed, both the pH and the tonicity of the initial incubation system were maintained as far as possible by using either phosphate buffer (for unplasmolysed cells) or

phosphate buffer containing plasmolysing agent. This latter would maintain the plasmolysed cells in that condition, preventing any extra radiolabelled material in the artificially enlarged periplasmic space from being squeezed out owing to the return of the cells to a more normal condition.

However, when the cells were finally resuspended in order to remove the labelled material from the periplasm, it was desirable to extract as much of this as possible. Thus the cells were resuspended in water, in which situation plasmolysed cells cease to be so, and it would be likely that cells (previously plasmolysed or otherwise) would lyse as a result of the very hypotonic conditions. It would not matter that the degree of lysis was probably associated with the original growth conditions of the organism, as the two differently labelled molecules would be equally affected. Unless the pores only function in one direction, permitting material to enter the cells but not to leave, and provided there was no selective binding of one radiolabelled molecule as opposed to the other to structures in or on the cell, the two labelled compounds should have diffused into the environment (water) from the periplasmic space via the pores or as a result of lysis. After a 30 minute final incubation it is presumed that the ratios of the concentrations (dpm) of the ^{14}C sucrose and ^{3}H labelled material in the environment represent those originally in the periplasmic space, and hence reflect the relative amounts of the two molecules which crossed the outer membrane.

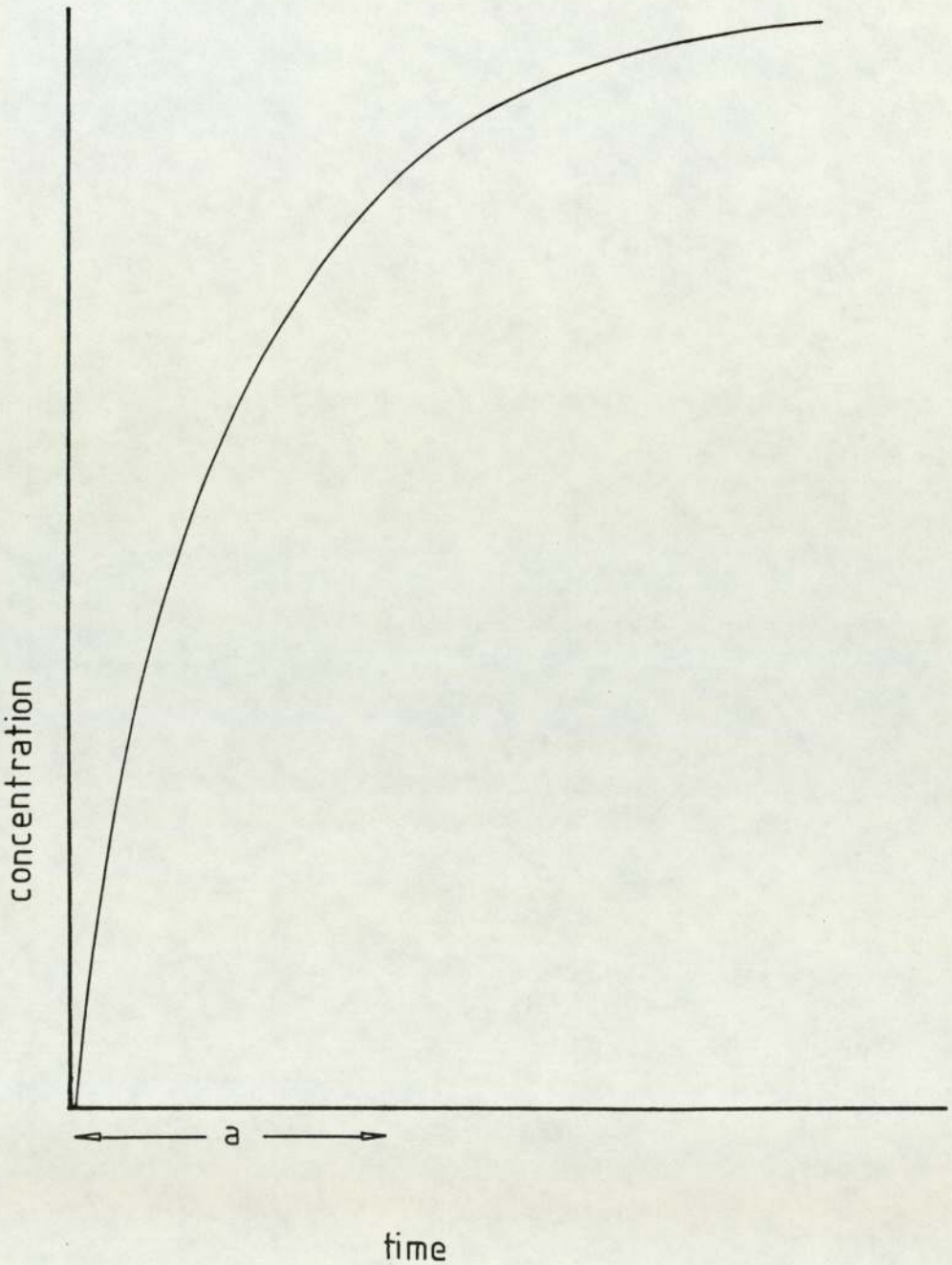
Although the interpretation of the data obtained from the double label assay with E. coli and P. aeruginosa should be considered separately, it is necessary to mention that the results obtained with sonicated cell material rather than whole cells suggest

that there are no problems of differential quenching owing to the presence of cells and cell material. Further, it would appear legitimate to assume that molecules bearing different radiolabels (in this case ^{3}H and ^{14}C) when not affected by the presence of whole cells, do remain at the same relative concentration throughout the assay, neither adhering to any equipment used. Therefore, it is not possible to explain the phenomena observed by such solutions as the quenching of one radiolabel to a greater extent than the other.

3.4.7.1 The diffusion of sucrose

Interpretation of the double label assay is dependent on the way in which sucrose diffuses into the periplasm of E. coli and P. aeruginosa. It is known that the compound does not penetrate beyond the periplasmic space of either organism, and that, owing to their nature, sucrose crosses the outer membranes by passive diffusion through the pores. A method for determining the absolute pattern of the diffusion of sucrose into cells has not been found, and it has been necessary to make various assumptions.

It is accepted (Nikaido, 1979; Nikaido and Rosenberg, 1981) that molecules diffusing passively through the outer membrane of a Gram-negative bacterium will obey first order kinetics, the rate of diffusion being related to the concentration of the diffusing molecule. Thus the rate at which sucrose diffuses across the outer membrane will decrease as the concentration of the compound in the periplasm increases, until equilibrium is reached between the two compartments. The general pattern of such diffusion is represented in Figure 3.40. The period of time required for sucrose to approach equilibrium between the periplasmic space and the environment (a on Fig. 3.40) is not known and various assumptions


concentration
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time

of its length, such as 5 minutes (Decad and Nikaido, 1976) have been made. Recently, it has been suggested that the diffusion rate of lactose (a disaccharide) across the outer membrane (i.e. through the pores) of E. coli B/r is "slow" (Nikaido and Rosenberg, 1981) and this would presumably also be true of sucrose. How "slow" is not made clear, but it may be necessary to alter the more usual assumptions as to the time required for the equilibration of sucrose between the periplasm and the environment. However, it would seem reasonable to assume that the concentration of sucrose in the environment and the periplasmic space has reached, or is near to, equilibrium, after 30 minutes (Stock et al., 1977), and that the most rapid rate of diffusion of the molecules into the cell occurs during the first 1-5 minutes of incubation.

In the data obtained from the double label assay, the diffusion pattern of sucrose in represented topologically by a straight line. The relationship of the determined diffusion patterns to this straight line can therefore be reinterpreted in terms of the true diffusion pattern of sucrose. This will be considered as the data are interpreted, but one point should be made here. If a molecule is unable to cross the outer membrane, whereas sucrose is, the result will be negative values of F which increase with length of incubation. However, as the concentration of sucrose in the periplasmic space gradually reaches equilibrium with that in the external medium, the negative slope of the apparent diffusion data obtained from the molecules unable to penetrate the outer membrane will flatten. Thus, should such a pattern emerge (as it does) those ^{3}H labelled molecules associated with it are probably unable to penetrate the outer membrane.

There is a further consideration. It is possible that sucrose does not diffuse across the outer membrane of an organism in exactly the same way irrespective of how the cells were grown or treated. This could invalidate comparisons between an organism grown in different ways, or between different organisms, and is particularly relevant in this thesis when considering the results obtained with P. aeruginosa, or the effect of growth rate on the pore size in E. coli. However, much previous work with E. coli suggests that the outer membrane would be freely permeable to sucrose (Decad and Nikaido, 1976; Nakae, 1975, 1976; Lugtenberg et al., 1976; Nikaido, 1979; Nikaido et al., 1980) and therefore that any variation in the outer membrane structure should not affect the diffusion of sucrose, unless the pore size is reduced dramatically.

3.4.7.2 The penetration of molecules across the outer membrane of E. coli

Although it is probable that the molecules used in the double label assay cross the outer membrane by passive diffusion, this process has also been described in this thesis as uptake; because, in the correct, general sense of the word, molecules crossing into the periplasmic space of a Gram-negative bacterium are being taken up into the organism. Uptake in this context is not to be confused with active transport of molecules such as that of maltose by the maltose uptake system.

Sucrose. ^{3}H sucrose was found to diffuse into E. coli grown in various conditions, and either plasmolysed or not, in a manner very similar to that of ^{14}C sucrose. The differences which were observed were almost certainly the result of variation in the diffusion of both ^{14}C and ^{3}H sucrose, and are negligible. The important feature is that when presented with E. coli, ^{3}H and

^{14}C sucrose behave as the same molecule in the double label assay, which is as would be predicted.

Isomaltotriitol. From the results, it would appear that OS 508 diffuses across the outer membrane of E. coli as easily as sucrose, the outer membrane pores having a diameter adequate to allow this molecule relatively unimpeded access to the periplasm, unaffected by the manner in which the cells are grown or treated. E. coli and other enteric bacteria are generally reported as having pores permitting the passage of molecules of 600 d mw, and the ability of a molecule of 508 d to cross the outer membrane is therefore not surprising. However, molecules of a weight greater than this could have been predicted as being unable to penetrate the outer membrane. This is belied by the results for all cells with OS 670 and unplasmolysed cells with OS 833.

Isomaltotetritol. OS 670 is clearly able to get into E. coli more easily than sucrose, entering the cells more rapidly and reaching a higher concentration than the ^{14}C labelled compound. The reasons for this phenomenon are unclear, but there are several factors which are not its cause. One is the way in which the cells were grown. Irrespective of the growth conditions OS 670 crosses into the periplasmic space of E. coli in exactly the same manner relative to sucrose every time, except into unplasmolysed magnesium limited cells. Here, the results are possibly a consequence of the limited number of experiments performed. The fact that the data obtained with plasmolysed magnesium limited cells fit into the general trend suggests that the results are not necessarily correct. Further, the effect of the presence or absence of plasmolysing agent does not affect the results obtained with log and stationary phase grown E. coli, suggesting that, again, these components of the system are

not the cause of the effect seen with OS 670. It could possibly have been argued that the presence of Mg^{2+}, a divalent cation which could have an important affect on the outer membrane surface, in the plasmolysing agent, would affect the results in some way. But this would suggest that a difference should be seen between data from plasmolysed and unplasmolysed cells when Mg^{2+} was a component of the plasmolysing agent, and not when it is absent. $MgCl_2$ is not added to the plasmolysing agent used for magnesium limited E. coli.

Another explanation is that the OS 670 is being taken up actively by some enzyme, or is being adsorbed on to some structure within the periplasmic space or on the outer membrane surface. It is difficult to accept the idea that the molecule is taken up by some active transport enzyme, when the data obtained for OS 346 and log phase, plasmolysed cells are considered. Here the pattern is dissimilar from that obtained with OS 670, falling entirely below the abcissa. This is due to the conversion of free OS 346 in the periplasm, available to reach an equilibrium with that in the environment, into bound OS 346 by the periplasmic maltose binding protein. The removal of OS 346 enzymatically, whatever its ultimate fate, would occur initially at least as fast as the molecule could diffuse into the periplasmic space (Nikaido and Rosenberg, 1981) until the available maltose binding proteins were occupied. This would lead to little free OS 346 being available to diffuse out of the periplasmic space during the 30 minute efflux period, and hence results in the double label assay implying that there is present in the cells less OS 346 than sucrose. The gradual increase in the concentration of free OS 346 present in the cells with the time of incubation is probably a reflection on the rate at which free maltose binding proteins are made available, whether by the manufacture

of new proteins, or the disposal of the OS 346 to the maltose permease so that the sugar can cross the cytoplasmic membrane.

However, this is certainly not the effect observed with OS 670. It does remain a possibility that the molecule is in some way selectively bound to a component of the cell, but so loosely that the bonds break when the cells are resuspended in water for the final efflux period. It is to be noted that no compound allowed to cross from the environment into the periplasmic space of a Gram-negative bacterium can reach a higher concentration in the periplasm than is present in the environment unless the cell is able, in some manner, to "concentrate up" that compound. In the double label assay the ratios of the labelled sugars present in the stock solutions and the final efflux supernatants after 30 minutes can be expanded from I and E to ^{14}CI/^{3}HI and ^{14}CE/^{3}HE. Unless the cells can selectively accumulate a specific compound, it is not possible for ^{14}CE to be greater than ^{14}CI, nor ^{3}HE to be greater than ^{3}HI. The reverse is, of course, possible. Hence, either the concentration of ^{14}C sucrose present in the periplasmic space is considerably less than a quarter of that in the initial stock solution, and the concentration of OS 670 has reached equilibrium with the environment, and is thus similar to that in the original stock solution, diluted by four. Or the ^{14}C sucrose has virtually reached equilibrium within the periplasm and environment, and in some way the cell has been able to increase the concentration of free OS 670 within the periplasm to an artificially high level. Either interpretation is somewhat unexpected, but the latter is certainly more acceptable, as it is very unlikely that sucrose has failed to be close to or have reached equilibrium after 30 minutes incubation.

Isomaltopentitol. Although it does not appear that the diffusion of OS 670 into E. coli is affected by the plasmolysed or unplasmolysed condition of the cells, that of OS 833 is affected. The bacteria were originally plasmolysed to increase the size of the periplasmic space, and hence the sensitivity of the assay. However, all the data would suggest that either the assay is more sensitive when the cells are unplasmolysed, or that the tonicity of the assay system affects the results. When incubated with plasmolysed cells, OS 833 appears to penetrate the outer membrane less easily than sucrose. However, the rate at which the molecule is able to cross into the periplasmic space is such that as the cells are exposed to the molecules for increasing periods of time, the concentration of OS 833 in the cell increases very considerably. Although initially the concentration of OS 833 in the cells is much less than that of sucrose, the rate of uptake of the former molecule across the outer membrane is greater than that of ^{14}C sucrose during the time period investigated. This undoubtedly suggests that the sucrose has passed its maximum rate of diffusion during the first minute of the experiment, irrespective of the conditions in which the cells were grown. The gradual decrease in the rate at which the concentration of OS 833 is increasing in the periplasmic space of plasmolysed cells results from the approaching equilibrium of the compound in the environment with that in the periplasmic space.

When unplasmolysed cells are incubated in the presence of OS 833, the outer membrane appears to be virtually freely permeable to this compound irrespective of the way in which the organism was grown. There is a little evidence that sucrose is able to cross the outer membrane slightly more easily, as the concentration of OS 833 in the periplasmic space is marginally lower than that of

sucrose during the first few minutes of the initial incubation. However, the difference is slight, and the data imply that the pores are larger when the cells are present in the hypotonic incubation system.

Isomaltohexitol and isomaltoheptitol. The difference in the ability of larger molecules to penetrate the outer membrane of plasmolysed and unplasmolysed cells is borne out with both OS 955 and OS 1157. With increasing time of incubation the concentration of each molecule in the periplasmic space increases, that of the smaller molecule being generally higher. However, although the rates of uptake of the two are similar to each other, and hardly affected by the tonicity of the system, the concentrations of the compounds is much higher in the periplasm of unplasmolysed cells. As with the data for the diffusion of OS 833, it would appear that in a hypotonic system, the pores are artificially enlarged, or that they are artificially reduced in a hypertonic situation.

The results obtained with E. coli lead to two main considerations. One is the actual size of the pores, and the other the overall effect of the tonicity of the environment of the bacteria on the pore size.

Decad and Nikaido (1976), using whole cells found that the pores in E. coli have an exclusion limit of approximately 600 d (equivalent to a diameter of between 0.6 and 1.0 nm). Even during the 5 minute incubation period allowed, larger molecules were able to penetrate the cells to a limited extent. In the double label assay, after 5 minutes incubation, molecules up to and including OS 670 have diffused into plasmolysed cells (such as those used by Decad and Nikaido, 1976) whereas the difference in concentration of OS 833 and larger molecules, compared with that of ^{14}C sucrose

in the periplasmic space, is great. Thus, had the assay been stopped after 5 minutes, the data would have suggested that the pores in plasmolysed E. coli had an absolute exclusion limit of approximately 670 d. It is however, logical to assume that if a membrane contains pores of a given diameter which will freely permit the passage of molecules up to a certain size, then larger molecules should be able to pass through the pores provided as is likely, some of their dimensions are less than the diameter of the pores, and that the membrane and molecules were incubated together for an adequate period of time. With the longer period of incubation afforded by the double label assay, it can be concluded that when plasmolysed, the E. coli W3110 outer membrane does not have an absolute permeability, but, although permitting the free passage of molecules up to 670 d mw it is also reasonably porous to molecules of greater size, the permeability being inversely related to the size of the compound. However, in contrast, Decad and Nikaido (1976) argue that the outer membrane does have an absolute exclusion limit because the outer membrane must be essentially impermeable to any molecule which, in their assay, was only partially able to penetrate it.

It is possible that the pores are actually larger still, and that they are of a diameter great enough to permit the free passage of OS 833. If this is so, the effect of plasmolysing the cells either reduces the sensitivity of the assay, masking the fact that OS 833 can easily diffuse into the periplasm; or the hypertonic situation forces the pores to close slightly, perhaps as a result of the contraction of the cytoplasm and the resultant stress at the Bayer's points of adhesion. Another possibility is that the magnesium in the plasmolysing agent in some way pulls the pores together,

reducing their size. However, the plasmolysing agent used with magnesium limited E. coli does not contain any magnesium chloride, but despite this, the results closely resemble those of plasmolysed log and stationary phase grown cells.

Alternatively, the results obtained with plasmolysed cells could more closely resemble the in vivo situation. Placing E. coli in a hypotonic medium may, because the cytoplasm expands slightly, force the pores to become slightly more open. It has been observed that low osmolarity of the external medium increases the rate of exit of compounds from bacteria, as a result of stretching the membranes (Davis and Leive, 1973). Also, placing eryrthrocytes in hypotonic media can open up large pores in the red blood cell membrane (20-40 nm diameter) (Seeman, 1967; Bodemann and Passow, 1972) which effect may be a similar phenomenon. This stretching of the pores in hypotonic media is a possible explanation for the results obtained with unplasmolysed E. coli, and additionally means that the data obtained from plasmolysed cells give a more representative picture of the size and state of the pores, which fits in well with previous measurements of the size of pores in whole E. coli cells.

The fact that OS 833, and larger oligosaccharides, appear to be penetrating the outer membrane of E. coli more quickly than ^{14}C sucrose suggests that sucrose has ceased to diffuse rapidly across the outer membrane within a minute of addition to the cell suspension. Hence any slight variation there may be in the diffusion of sucrose into the periplasm of E. coli grown under different conditions is immaterial, and comparisons between the bacteria can be made.

The rate of growth of E. coli at the time at which the cells are harvested, rather than the chemical structure of the cell as affected by the different growth environments, may have a slight

influence on the ability of the molecules close to the size of, and greater than, the apparent exclusion limit of the cells to diffuse through the pores. This can be seen in that molecules of molecular weight greater than 670 d are less able to penetrate the outer membrane of plasmolysed tetracycline limited (i.e. slow growing) E. coli than plasmolysed log phase (i.e. fast growing) cells. Equally, OS 833 is more easily able to penetrate plasmolysed log phase cells than plasmolysed E. coli harvested in any other condition. A similar pattern is seen with unplasmolysed cells, where it appears that OS 995 is slightly more able to cross the outer membrane of log phase cells than those of stationary or magnesium limited cells. It is possible that the results obtained with tetracycline limited cells are a reflection on the effect of the antibiotic on E. coli, which is to inhibit septation causing the organism to grow in filaments. However, the evidence also points to those cells which are growing more quickly having slightly, and only slightly, more open pores, than those pores in the outer membrane of slower growing E. coli.

3.4.7.3 The penetration of molecules across the outer membrane of P. aeruginosa

Sucrose. As with E. coli, the data obtained when ^{3}H and ^{14}C sucrose are incubated together with plasmolysed and unplasmolysed P. aeruginosa, irrespective of the way in which the cells were grown, suggest that the double label assay is valid. The two molecules diffuse at an approximately constant and equal concentration, behaving as one compound. Such differences as are observed are no doubt due to variation in the diffusion of both ^{3}H and ^{14}C labelled sucrose, and are of little importance.

The remaining data observed on any of the graphs showing the

diffusion of ^{3}H labelled molecules into P. aeruginosa as compared with that of ^{14}C sucrose fall into one of two basic patterns. Isomaltose. OS 346 is clearly able to cross the outer membrane, through the pores. The concentration of the compound in the periplasmic space is affected by the presence or absence of plasmolysing conditions during the initial incubation; where the cells were plasmolysed, the concentration of OS 346 in the periplasm is higher than that of ^{14}C sucrose, and, in cells grown in CDM, increases to a maximum after approximately 20 minutes of the initial incubation. In nutrient broth grown cells such a trend is not apparent. The relatively high concentration of OS 346 in the periplasmic space of plasmolysed P. aeruginosa resembles the similarly high concentration of OS 670 in E. coli. However, unplasmolysed P. aeruginosa appears to contain slightly less OS 346 than ^{14}C sucrose, although the actual patterns of uptake are similar to that of sucrose. There are possible explanations for this phenomenon which, unlike OS 670 in E. coli, is undoubtedly the result of the effect of the presence or absence of plasmolysing agent when OS 346 and ^{14}C sucrose are incubated with P. aeruginosa.

OS 346, although of almost identical molecular weight to ^{14}C sucrose (346 d as opposed to 342 d) does not have the same structure, as one of the hexose rings has been reduced and therefore opened out. It is not possible, at present, to know the true three-dimensional conformation adopted by OS 346 or sucrose when passing through the pores, but it may be that OS 346 has slightly smaller dimensions. In a pore of large diameter and high exclusion limit, such as normally proposed for P. aeruginosa (Hancock and Nikaido, 1978 ; Benz and Hancock, 1981) or even pores of the size found in E. coli, this slight difference in size would be immaterial.

But were the P. aeruginosa pores smaller in diameter, with a lower exclusion limit than that of E. coli or S. typhimurium, then the three-dimensional configuration of molecules such as sucrose and OS 346 could become critical. Were this the case, then plasmolysing the cells could actually increase the sensitivity of the assay (as was the original intention), showing that sucrose is at the size limit for the pores in P. aeruginosa, and OS 346's slightly smaller conformation enabled it to cross the outer membrane significantly more easily than sucrose.

Alternatively, rather than increasing the sensitivity of the assay by plasmolysing the cells, incubating the bacteria in hypotonic media may stretch the outer membrane, enabling sucrose to penetrate the cells more easily than when the outer membrane pores are in a more usual condition. Either argument is dependent on sucrose and OS 346 being very close to the exclusion limit of the pores found in P. aeruginosa, which is in complete contrast to previous data (Hancock and Nikaido, 1978 ; Benz and Hancock, 1981). There are other possibilities; e.g. the presence of Mg^{2+} in the plasmolysing agent in some way increases the non-specific adsorption of OS 346 to components on the surface of, or within, the periplasmic space. If so then, as with OS 670 in E. coli, the binding is very weak and on resuspension of the cells in water this bound OS 346 becomes free, and able to diffuse from the cells. Alternatively, the presence of plasmolysing agent could be causing ^{14}C sucrose to bind irreversibly to the cell, after removal from the initial incubation system, thus reducing the apparent amount of the molecule which has been able to cross the outer membrane.

The most likely solution, however, remains that sucrose and OS 346 are at the limit of the pore diameter, and that the results

are the consequence of the three-dimensional size of the molecules combined with the stretching of the outer membrane when the cells are in a hypotonic environment. There are flaws in this, some of which will be considered in section 3.5. But an important feature of the data obtained with P. aeruginosa 6750 is that irrespective of the way in which the organism was grown, and of how it was treated during the initial incubation with the radiolabelled sugars, molecules larger than OS 346 do not appear to be able to cross the outer membrane via the pores. As a control, P. aeruginosa PAO1 (strains of which have been used by Nikaido and other workers) grown to stationary phase in nutrient broth was also investigated, to determine whether or not results obtained with P. aeruginosa 6750 were unique to that strain. The results obtained with P. aeruginosa PAO1 were substantially the same as those obtained with P. aeruginosa 6750 for all eight ^{3}H labelled compounds used in the double label assay.

All other ^{3}H labelled compounds. It will be recalled that if the diffusion of sucrose, which really follows the pattern outlined in Fig. 3.40, is represented by a straight line, than a molecule unable to cross the outer membrane, which in reality would give data falling on the abcissa (y = concentration = 0) of Figure 3.40, would in the double label assay give a pattern which could be seen as the mirror image of the true diffusion of sucrose, below the abcissa. The overall shape of the curve should reflect the rate of diffusion of sucrose, except that the sign of the slopes would be reversed. This is what is seen in the data obtained for all tritiated compounds inclduing inulin, other than OS 346 and sucrose when incubated in the presence of P. aeruginosa 6750.

The data are not artefacts resulting from the inability of sucrose to diffuse across the outer membrane. If this were the case, all the curves obtained would be parallel with and close to the zero line as, none of the compounds being able to penetrate the outer membrane, the molecules would all behave exactly as sucrose. If the results were to be the consequence of all the molecules freely penetrating the outer membrane, as was predicted, then again the data would lie close to and parallel with the abcissa. It is a more acceptable suggestion that no molecules can cross the outer membrane but that the results are the direct effect of adsorption of ^{14}C sucrose and OS 346 to accessible components of the cells, with release of both these compounds when the cells are resuspended in water. However, there are various problems associated with this theory, including the very clearly proven existence of glucose induced pores in P. aeruginosa. These, composed of protein D1, a glucose induced protein, were shown to permit the passage of glucose and sucrose across membrane vesicles comprised of LPS, phospholipid and D1 (Hancock and Carey, 1980).

The lack of a clear size-order pattern amongst molecules other than OS 346 would be explained by their inability to pass through the outer membrane. Further, both the large standard deviations observed in this data and the overall compactness of the results obtained with CDM grown cells would be explained by the pores being too small to allow the molecules to cross the outer membrane. Thus it would be expected that there would be little difference between the diffusion pattern of, for example, OS 670, OS 995 and inulin.

As has been observed, there is a slight contrast between the data obtained with CDM and nutrient broth grown cells. With the

latter cells, the data are more spread out towards the abcissa, and there does appear to be a size-order pattern. Also, the difference between the uptake of OS 346 and ^{14}C sucrose into plasmolysed, nutrient broth grown cells is less significant than into plasmolysed CDM grown cells. Unfortunately, these results could be due either to the pores being slightly smaller or slightly larger as a result of growth in nutritionally rich media, although the difference is very small, whichever direction it is in. This problem will be further considered in section 4.

3.5 Investigation of the final location of radiolabelled material in the double label assay

3.5.1 Introduction

It was possible that the results obtained for the diffusion of molecules across the outer membrane of *E. coli* and *P. aeruginosa* were a consequence of, or affected by, the adsorption of the radiolabelled compounds to the outer surface of the cells. It was therefore necessary to determine how much radiolabelled material was located in each stage of the assay, and how much had adhered to the cellular material remaining at the end of the experiment.

3.5.2 Method

E. coli W3110 and *P. aeruginosa* 6750 were grown in 4 x 2 litre volumes as described previously (2.2.2.1). Cells were harvested by centrifugation, washed and resuspended as for the double label assay (3.4.2).

0.5 ml portions of cell suspension were placed in 1.5 ml centrifuge tubes and to each was added 0.25 ml of plasmolysing agent plus maltose (0.22 M NaCl plus 0.01 M $MgCl_2$ with 0.02 M maltose (final concentrations) in all experiments other than those carried out with magnesium limited *E. coli*, where 0.3 M NaCl with 0.02 M maltose (final concentrations) were used). 0.25 ml of each of the radiolabel solutions to be investigated (see Table 3.9) were then added to the separate tubes, in triplicate. The contents of the tubes were mixed thoroughly and incubated at room temperature for either 10 or 30 minutes, with futher mixing every 5 minutes.

The tubes were then centrifuged (80 seconds, 9980 g) and each initial supernatant removed and placed in a scintillation vial

with 10 ml NE260. The cells were resuspended in 0.5 ml phosphate buffer with plasmolysing agent, and centrifuged (70 seconds, 9980 g). The washing supernatants were removed and placed in separate vials with 5 ml NE260. The cells were resuspended in 0.7 ml water and incubated at room temperature for 30 minutes. At the end of this period, the tubes were centrifuged to obtain a very firm pellet (90 seconds, 9980 g) and the final (efflux) supernatants removed and each mixed with 10 ml NE260. The cell pellets were again resuspended in 0.7 ml water. In some experiments the cells were then sonicated (2.2.7). The resuspended cells, or broken cell material, were then mixed with 15 ml NE260 in scintillation vials. Finally the tops of the 1.5 ml plastic centrifuge tubes were cut off, and both the tube and top were placed, together, in a scintillation vial and 10 ml NE260 were added.

The total amount of each labelled compound originally mixed with the cells was determined by mixing 0.25 ml of each solution of radioactive material used with 5 ml NE260, in triplicate.

All vials were counted for 5 minutes, and the results obtained were in dpm.

3.5.3 Calculation of results

The average dpm of each label found in the three different supernatants were calculated and expressed as a mean and standard deviation. These values were in turn expressed as a percentage of the total amount of ^{14}C or ^{3}H material, whichever was appropriate, added to the cells at the beginning of the experiment.

To calculate the amount of labelled material remaining in the cell pellet, the dpm of ^{3}H or ^{14}C material found in the cellular material and the related centrifuge tube were summed. The average

from the three cell pellets was calculated, and in turn expressed as a percentage of the total dpm of the appropriate radiolabel originally added.

3.5.4 Results

In general, when the results obtained with the various radiolabelled sugars after incubation with *E. coli* or *P. aeruginosa* grown under different conditions were compared, there was no difference in the data due to the possible effect of the manner in which the organism had been grown, except on one occasion when nutrient broth grown *P. aeruginosa*, after incubation with any combination of ^{3}H labelled sugars other than ^{3}H sucrose, and ^{14}C sucrose, retained very high levels of ^{14}C sucrose in the cell pellet (20-50% of the total label added). However, on repetition of the entire experiment on three separate occasions, data identical with the more usual pattern were obtained. Therefore, rather than presenting all the data, a typical set of results for all the sugars used with each organism is given (Tables 3.25 and 3.26) and the remaining material will be found in Appendix 4.

When considering *E. coli* (Table 3.25) and *P. aeruginosa* (Table 3.26) grown to stationary phase in CDM (oxygen limitation) and incubated with the various sugars, the overall patterns are very similar. In the initial supernatants, between 75% and 85% of the total radiolabel originally added are removed, and a further 8-12% are removed by the washing process. In general either the percentage of ^{3}H and ^{14}C material removed after this stage are approximately the same, or less of the tritiated sugar remains. This latter situation particularly applies to the larger sugars in *E. coli* (greater than four glucose rings) and molecules of molecular

Table 3.25

Data giving the final location of radiolabelled material in the double label assay after an initial incubation of 10 or 30 minutes with E. coli W3110 grown to stationary phase in CDM

Stage of assay analysed[a]	^{3}H material (% of total added[b] ± standard deviation)		^{14}C material (% of total added[b] ± standard deviation)	
	Initial incubation of 10 min[c]	30 min[d]	10 min[c]	30 min[d]
Cells incubated with ^{3}H isomaltotriitol (OS 508) and ^{14}C sucrose				
Initial supernatant (first discard)	79.2 ± 2.3	78.7 ± 3.1	77.0 ± 2.3	76.0 ± 3.2
Washing (second discard)	11.2 ± 0.9	12.5 ± 1.2	11.6 ± 1.0	12.9 ± 1.3
Final supernatant (efflux after 30 min)	3.2 ± 0.8	3.5 ± 0.9	4.3 ± 6.9	4.1 ± 1.5
Cell pellet	1.6 ± 0.8	1.0 ± 1.1	6.9 ± 1.2	6.3 ± 1.0
Total label recovered	93.2 ± 4.8	95.7 ± 6.3	99.8 ± 5.5	99.3 ± 1.0
Cells incubated with ^{3}H isomaltotetritol (OS 670 and ^{14}C sucrose				
Initial supernatant (first discard)	84.9 ± 1.5	78.4 ± 1.6	80.6 ± 3.0	78.0 ± 2.7
Washing (second discard)	9.4 ± 1.1	11.6 ± 1.1	12.0 ± 2.0	14.0 ± 1.8
Final supernatant (efflux after 30 min)	3.7 ± 0.9	4.9 ± 0.9	3.9 ± 1.7	4.3 ± 1.7
Cell pellet	1.8 ± 0.9	1.5 ± 0.9	4.4 ± 1.6	4.1 ± 1.7
Total label recovered	99.8 ± 4.2	96.4 ± 4.5	100.9 ± 1.6	100.4 ± 7.9
Cells incubated with ^{3}H isomaltopentitol (OS 833) and ^{14}C sucrose				
Initial supernatant (first discard)	79.7 ± 1.7	80.8 ± 1.8	74.6 ± 1.3	75.7 ± 1.8
Washing (second discard)	11.5 ± 1.3	9.7 ± 1.3	14.0 ± 0.6	12.0 ± 0.5
Final supernatant (efflux after 30 min)	2.7 ± 1.3	2.6 ± 1.2	4.4 ± 0.6	3.7 ± 0.4

Table 3.25 (continued)

Stage of assay analysed[a]	^{3}H material (% of total added[b] ± standard deviation)		^{14}C material (% of total added[b] ± standard deviation)	
	Initial incubation of 10 min[c]	30 min[d]	10 min[c]	30 min[d]
Cell pellet	1.5 ± 1.3	1.6 ± 1.3	4.0 ± 0.7	4.0 ± 0.7
Total label recovered	95.4 ± 5.6	93.9 ± 5.6	97.0 ± 3.2	95.4 ± 3.4
Cells incubated with ^{3}H isomaltohexitol (OS 995) and ^{14}C sucrose				
Initial supernatant (first discard)	84.7 ± 1.8	85.3 ± 3.2	80.0 ± 2.1	81.4 ± 2.4
Washing (second discard)	9.5 ± 1.1	9.4 ± 1.3	12.2 ± 1.7	11.9 ± 2.0
Final supernatant (efflux after 30 min)	2.5 ± 1.1	2.2 ± 1.0	3.7 ± 1.2	3.1 ± 1.7
Cell pellet	0.7 ± 1.1	0.9 ± 1.1	0.9 ± 1.0	1.1 ± 1.7
Total label recovered	97.4 ± 5.1	97.8 ± 7.6	96.8 ± 6.0	97.5 ± 7.8
Cells incubated with ^{3}H isomaltoheptitol (OS 1157) and ^{14}C sucrose				
Initial supernatant (first discard)	84.4 ± 3.4	84.6 ± 2.7	75.3 ± 2.3	76.2 ± 4.8
Washing (second discard)	9.9 ± 1.9	10.1 ± 2.0	11.7 ± 1.2	12.3 ± 1.5
Final supernatant (efflux after 30 min)	1.9 ± 1.8	2.0 ± 1.9	3.8 ± 1.3	4.2 ± 1.3
Cell pellet	0.6 ± 1.8	0.7 ± 1.8	5.6 ± 1.4	5.8 ± 1.2
Total label recovered	96.8 ± 8.9	97.4 ± 8.4	96.4 ± 6.2	98.8 ± 8.8
Cells incubated with ^{3}H sucrose and ^{14}C sucrose				
Initial supernatant (first discard)	81.0 ± 2.6	82.7 ± 2.3	78.4 ± 2.5	81.1 ± 4.1
Washing (second discard)	11.8 ± 1.2	11.7 ± 1.5	11.4 ± 1.6	11.6 ± 2.3

Table 3.25 (continued)

Stage of assay analysed[a]	^{3}H material (% of total added[b] ± standard deviation)		^{14}C material (% of total added[b] ± standard deviation)	
	Initial incubation of 10 min[c]	30 min[d]	10 min[c]	30 min[d]
Final supernatant (efflux after 30 min)	3.7 ± 1.0	3.2 ± 1.3	3.6 ± 1.6	3.1 ± 1.9
Cell pellet	1.2 ± 1.2	1.2 ± 1.1	1.0 ± 1.7	0.9 ± 1.5
Total label recovered	97.7 ± 6.0	98.8 ± 6.2	94.4 ± 7.4	96.7 ± 9.8

a. These refer to the four stages of the double label assay: the supernatant obtained when the cells are centrifuged for the first time, immediately after incubation with the radiolabelled material; the supernatant obtained after the cells have been resuspended in fresh buffer and again centrifuged; to wash the cells; the supernatant obtained after the cells have been resuspended in water and incubated for 30 minutes, to allow the radiolabelled material in the periplasmic space to efflux out (the ratio is normally determined at this stage); and the cell pellet remaining after removal of the final supernatant.

b. The total radioactivity originally added from the stock solution to the cell suspension was determined in triplicate, and the two values calculated (dpm ^{3}H, dpm ^{14}C) were used as 100%. The dpm of each label in each stage of the assay were measured and expressed as a percentage of the 100% value dpm.

c. Before mixing with NE260, the cell pellets were sonicated.

d. The cell pellets were mixed directly in NE260, without sonication.

weight higher than OS 346 with P. aeruginosa. However, in many cases the error is such that this observation could be irrelevant.

The remaining 3-16% of the two radiolabels are not distributed in any very clear pattern between the final (efflux) supernatant and the cell pellet material. In experiments carried out with P. aeruginosa there is frequently more ^{14}C sucrose remaining in the cell pellet than ^{3}H labelled material (Tables 3.26 and 3.28) and it is notable that the amount of ^{3}H material present in the efflux supernatant is almost invariably greater than that retained in the cell pellet. There is no such clear pattern when comparing the amounts of ^{14}C sucrose located in the cellular material and the efflux supernatant.

With P. aeruginosa (Tables 3.26 and 3.28), after incubation with ^{14}C sucrose and any labelled sugars other than OS 346 or ^{3}H sucrose, the amount of ^{14}C material present in the final supernatant is always significantly higher than that of the ^{3}H labelled compound. In the case of ^{3}H sucrose the amounts are very similar whereas after incubation with OS 346 and ^{14}C sucrose there tends to be more of the OS 346 present in the supernatnat. A similar trend, although not as marked, was apparent with E. coli (Table 3.27) where the amount of OS 833 and other compounds of greater molecular weight, present in the efflux supernatant is significantly less than the amount of ^{14}C sucrose.

In general, 95% of the total amount of both ^{3}H and ^{14}C labelled material added to the system is recovered. In this context it is important to say that no difference was found when measuring the amount of radiolabelled material found in the sonicated or unsonicated cell pellet, as is shown in Table 3.25.

Table 3.26

Data giving the final location of radiolabelled material in the double label assay after an initial incubation of 10 or 30 minutes with P. aeruginosa 6750 grown to stationary phase in CDM.

Stage of assay analysed[a]	^{3}H material (% of total added[b] ± standard deviation)		^{14}C material (% of total added[b] ± standard deviation)	
	Initial incubation of 10 min	30 min	10 min	30 min
Cells incubated with ^{3}H isomaltitol (OS 346) and ^{14}C sucrose				
Initial supernatant (first discard)	81.4 ± 3.1	82.6 ± 2.5	78.7 ± 3.2	80.2 ± 1.8
Washing (second discard)	9.8 ± 2.3	6.8 ± 2.3	9.0 ± 0.9	6.2 ± 0.9
Final supernatant (efflux after 30 min)	3.5 ± 1.8	4.0 ± 1.8	3.1 ± 0.4	3.4 ± 0.5
Cell pellet[c]	1.0 ± 1.7	1.4 ± 1.8	3.1 ± 0.5	3.2 ± 0.7
Total label recovered	95.7 ± 8.9	94.8 ± 8.4	93.9 ± 0.5	93.0 ± 3.9
Cells incubated with ^{3}H isomaltotriitol (OS 508) and ^{14}C sucrose				
Initial supernatant (first discard)	83.8 ± 1.5	83.7 ± 3.0	77.3 ± 6.3	76.6 ± 6.1
Washing (second discard)	8.8 ± 1.1	6.4 ± 1.0	8.4 ± 5.4	6.1 ± 5.3
Final supernatant (efflux after 30 min)	1.9 ± 0.9	1.8 ± 0.8	4.0 ± 7.8	3.4 ± 5.3
Cell pellet	0.8 ± 0.8	1.0 ± 0.8	4.3 ± 5.7	3.9 ± 5.3
Total label recovered	95.2 ± 4.3	92.9 ± 5.6	94.0 ± 25.2	90.0 ± 22.0
Cells incubated with ^{3}H isomaltotetritol (OS 670) and ^{14}C sucrose				
Initial supernatant (first discard)	84.0 ± 1.4	82.2 ± 1.8	82.3 ± 2.8	80.2 ± 3.1
Washing (second discard)	8.6 ± 0.7	10.1 ± 1.4	8.4 ± 2.3	10.0 ± 2.9
Final supernatant (efflux after 30 min)	1.6 ± 0.3	1.9 ± 0.4	3.0 ± 1.9	3.6 ± 2.2

Table 3.26 (continued)

Stage of assay analysed[a]	^{3}H material (% of total added[b] ± standard deviation)		^{14}C material (% of total added ± standard deviation)	
	Initial incubation of 10 min	30 min	10 min	30 min
Cell pellet	1.0 ± 0.4	1.0 ± 0.4	3.6 ± 1.9	3.5 ± 2.3
Total label recovered	95.2 ± 2.8	95.2 ± 4.0	97.3 ± 1.9	97.3 ± 10.5
Cells incubated with ^{3}H isomaltopentitol (OS 833) and ^{14}C sucrose				
Initial supernatant (first discard)	89.0 ± 2.6	86.1 ± 2.3	87.4 ± 2.6	86.2 ± 2.6
Washing (second discard)	7.4 ± 1.2	8.2 ± 1.4	7.2 ± 1.3	8.1 ± 1.7
Final supernatant (efflux after 30 min)	1.5 ± 0.9	1.6 ± 1.0	2.4 ± 1.1	2.7 ± 1.3
Cell pellet	1.0 ± 1.1	0.7 ± 0.8	1.2 ± 1.1	1.4 ± 1.1
Total label recovered	98.9 ± 5.8	96.6 ± 5.5	98.2 ± 6.1	98.4 ± 6.7
Cells incubated with ^{3}H isomaltohexitol (OS 995) and ^{14}C sucrose				
Initial supernatant (first discard)	85.1 ± 3.1	83.0 ± 4.6	85.5 ± 2.3	85.3 ± 5.7
Washing (second discard)	9.9 ± 1.3	11.6 ± 1.4	8.9 ± 2.4	10.4 ± 2.6
Final supernatant (efflux after 30 min)	2.2 ± 0.9	2.4 ± 0.9	2.9 ± 2.0	3.5 ± 2.1
Cell pellet	1.4 ± 0.8	1.4 ± 0.8	2.0 ± 1.9	2.0 ± 1.9
Total label recovered	98.6 ± 6.1	98.4 ± 7.4	99.3 ± 8.6	101.2 ± 12.3
Cells incubated with ^{3}H isomaltoheptitol (OS 1157) and ^{14}C sucrose				
Initial supernatant (first discard)	84.7 ± 2.2	83.5 ± 2.2	81.5 ± 3.0	79.4 ± 3.1
Washing (second discard)	8.1 ± 1.3	9.4 ± 1.7	7.8 ± 2.8	8.8 ± 3.0

Table 3.26 (continued)

Stage of assay analysed[a]	^{3}H material (% of total added[b] ± standard deviation)		^{14}C material (% of total added[b] ± standard deviation)	
	Initial incubation of 10 min	30 min	10 min	30 min
Final supernatant (efflux after 30 min)	1.4 ± 1.1	1.4 ± 1.3	2.6 ± 2.6	3.5 ± 2.3
Cell pellet	1.2 ± 3.5	1.1 ± 1.0	3.5 ± 2.5	3.6 ± 2.8
Total label recovered	95.4 ± 8.1	95.4 ± 6.2	95.4 ± 10.9	95.5 ± 11.2
Cells incubated with ^{3}H inulin and ^{14}C sucrose				
Initial supernatant (first discard)	82.6 ± 3.9	78.3 ± 8.2	81.5 ± 3.1	81.5 ± 1.9
Washing (second discard)	9.5 ± 3.3	9.4 ± 3.0	10.1 ± 1.7	9.6 ± 1.5
Final supernatant (efflux after 30 min)	2.4 ± 3.0	2.2 ± 2.8	4.0 ± 1.2	3.6 ± 1.5
Cell pellet	1.3 ± 2.9	1.4 ± 2.9	1.7 ± 1.2	2.0 ± 1.4
Total label recovered	95.8 ± 13.1	91.3 ± 16.9	97.3 ± 7.2	96.7 ± 6.3
Cells incubated with ^{3}H sucrose and ^{14}C sucrose				
Initial supernatant (first discard)	86.7 ± 3.4	85.0 ± 1.9	87.4 ± 6.4	86.2 ± 4.6
Washing (second discard)	7.3 ± 1.3	7.3 ± 1.3	7.4 ± 3.5	7.2 ± 3.6
Final supernatant (efflux after 30 min)	2.3 ± 1.0	2.0 ± 1.0	2.5 ± 3.3	2.2 ± 3.3
Cell pellet	1.3 ± 1.2	1.3 ± 1.2	1.0 ± 3.3	1.1 ± 3.5
Total label recovered	97.6 ± 6.9	95.6 ± 5.4	98.3 ± 16.5	96.7 ± 15.0

Table 3.26 (continued)

a. These refer to the four stages of the double label assay: the supernatant obtained when the cells are centrifuged for the first time, immediately after incubation for 10 or 30 minutes with the radiolabelled material; the supernatant obtained after the cells have been resuspended in fresh buffer and again centrifuged, to wash the cells; the supernatant obtained after the cells have been resuspended in water and incubated for 30 minutes to allow the radiolabelled material in the periplasmic space to efflux out (the ratio is normally determined at this stage); and the cell pellet remaining after removal of the final supernatant.

b. The total radioactivity originally added from the stock solution to the cell suspension was determined, in triplicate, and the two values calculated (dpm ^{3}H, dpm ^{14}C) were used as 100%. The dpm of each label in each stage of the assay were measured and expressed as a percentage of the 100% value dpm.

c. All cell pellets were mixed directly in NE260, without sonication.

Table 3.27

t-tests to compare the amount of ^{3}H radiolabel found in the final supernatant or the cell pellet with the amount of ^{14}C sucrose found in the same in the double label assay carried out with E. coli W3110.

^{14}C sucrose compared with	Value of t[a] For the final supernatant (efflux after 30 minutes)	For the cell pellet
OS 508	1.887	1.550
OS 670	1.006	0.942
OS 833	2.439	0.112
OS 995	4.349	1.579
OS 1157	3.262	1.760
^{3}H sucrose	0.545	0.699
$t(p = 0.05)$[b]	2.032	2.032

Table 3.28

t-tests to compare the amount of ^{3}H radiolabel found in the final supernatant or the cell pellet with the amount of ^{14}C sucrose found in the same in the double label assay carried out with P. aeruginosa 6750.

^{14}C sucrose compared with	Value of t[a] For the final supernatant (efflux after 30 minutes)	For the cell pellet
OS 346	1.102	2.283
OS 508	4.026	2.789
OS 670	4.153	2.541
OS 833	4.704	1.461
OS 995	3.320	2.057
OS 1157	4.693	0.985
^{3}H inulin	3.379	0.095
^{3}H sucrose	0.167	0.821
$t(p = 0.05)$[b]	2.010	2.010

a. The formula for t will be found at Table 3.7. Calculations of F will be found in Appendix 4.

b. t(at p = 0.05) was determined for a two-tailed test.

3.5.5 Discussion

It is possible to calculate the theoretical approximate amounts of radiolabel which would be found in the various supernatants and the cell pellet as a percentage of the total label originally added. An amount of radiolabel, 100%, is added to the cell suspension plus plasmolysing agent to give a total volume of 100%. However, because the cytoplasm of the cells is not penetrable by the molecules used in the double label assay, not all of the 100% volume is available to the radiolabelled material. 20% of the total volume is occupied by the cells, and it is reasonable to assume that to some molecules, none of this cell volume is available to the labelled compounds, whereas with others, a generous estimate for the total volume of the periplasmic space being half of that of the cell, 50% of the cell volume is available to radiolabelled compounds. Thus between 80 and 90% of the total volume of the system is available to the radiolabelled material. In the double label assay, after the first centrifugation, approximately 70% of the total volume is removed, thus removing 77.7-87.5% of the total radiolabel. After washing the cells a further 7.8-13.9% of the total radiolabel originally added is removed, leaving between 4.7 and 8.4%. The centrifugation to obtain the final (efflux) supernatant (at which stage the ratio of ^{14}C dpm : ^{3}H dpm would be measured in the double label assay) gives a supernatant of approximately 70% of the volume, containing 3.3-5.9% of the total radiolabel originally added. In the remaining 30% of the volume (i.e. the cell pellet) would be 1.4-2.5% of the total radiolabel originally added.

This calculation fits in very well with the observed data suggesting that there is little adhesion of any of the labelled compounds used to the cells. However, it is possible that the

^{14}C sucrose is adhering very slightly to the cellular material, particularly of P. aeruginosa. If this were so, it would in fact, not affect the measurement of the ratio. The effect of a small quantity of ^{14}C sucrose adhering to the cells would be to reduce the true value of I, as the amount of ^{14}C originally added which was available for diffusion would be less than in the control measurement of the dpm. ^{14}C added (from which I is calculated). Therefore $F|=(I-E)/I|$ would in fact be slightly greater. However, this would not alter the overall pattern of the diffusion of the molecules into the two organisms, nor of which molecules were able to penetrate the outer membrane and how easily. This is particularly true as ^{14}C sucrose does not adhere to cells grown under any specific conditions, nor in the presence of any specific tritiated compound.

Although it would appear that slightly more ^{14}C sucrose than ^{3}H sugars, other than ^{3}H sucrose, remains adhered to the cell pellet, this pattern changes when ^{14}C and ^{3}H sucrose are incubated together. It would be expected that, if sucrose does adhere in some manner to some part of the cell, then the amount of both types of sucrose retained in the cell pellet would be greater than the predicted 2.5% of the total originally added. However, it is only so on two occasions (out of eleven) and the errors in both cases are adequate to explain this. Equally the amounts of both types of sucrose in the cell pellet are very similar, suggesting that it is not some aspect of the ^{14}C label influencing the results obtained with ^{3}H sugars other than sucrose. It is in fact not at all clear why ^{14}C sucrose should apparently adhere to the cell material in the presence of tritiated compounds other than ^{3}H sucrose, but it may be that there are a number of adhesion sites available for sucrose in and on the cells. That less ^{14}C material is retained within the cell

pellet when ^{3}H sucrose is mixed with ^{14}C sucrose would be due to the presence of more sucrose, labelled with tritium, which could also occupy any adhesion sites. It is significant that this trend is more apparent with P. aeruginosa, and that in general the adhesion of sucrose to cellular material from E. coli is no greater than the adhesion of any other compound, including OS 833.

The data could be interpreted as supporting the finding that P. aeruginosa 6750 has outer membrane pores smaller than E. coli. This is suggested in the observation that the relative amount of any tritiated compound larger than sucrose present in the efflux supernatant is less than the relative amount of ^{14}C sucrose. This is further supported by the data for the tritiated material in general closely resembling the data calculated on the assumption that only 80% of the initial total volume of the system is available to the radiolabelled material, i.e., where the compound was unable to penetrate the cells. This pattern is not particularly supported by the data for the labelled material remaining in the cell pellet.

However, the results obtained with P. aeruginosa could equally be used to support the argument that none of the molecules, including sucrose, can penetrate the organism, but that sucrose and OS 346 are able to adhere to the outside surface of the outer membrane. The first flaw in this is the improbability that only sucrose and OS 346 could adhere to the exposed structures of the cells, which would primarily be LPS. However, it might be that the adhesion sites are only accessible to small molecules. Secondly, it is unlikely that sucrose would be totally unable to penetrate CDM grown P. aeruginosa unless protein D were absent, as this provides the glucose pore which has been shown to be large enought to allow the passage of sucrose.

With the data obtained for E. coli, it appears that there is less OS 995 or OS 1157 than ^{14}C sucrose in the efflux supernatant, and in many cases this is also true of OS 833. This also tends to support the findings of the double label assay, that molecules smaller than OS 833 can penetrate the outer membrane easily. With OS 833 the situation is less clear, as after 30 minutes incubation the double label assay suggests that this molecule has penetrated the outer membrane considerably more than the larger ones, although less than ^{14}C sucrose and other, smaller tritiated compounds. The difference between the relative amounts of ^{14}C sucrose and OS 833 in the final supernatant is only just significant which tends to add to the argument that OS 833 does penetrate the outer membrane, but not easily.

This investigation was set up to determine whether there was any high level of adhesion to the cells of one or more of the compounds used in the double label assay. If there were, this would have been reflected in higher than predicted amounts of the compounds concerned being located in the final supernatant and the cell pellet, or just in the cell pellet. This is not so, suggesting that the double label assay is measuring the diffusion of molecules into the cells through the outer membrane pores, and not the adhesion of molecules to structures on the surface of the outer membrane. However, the results are partially inconclusive, as it is still possible that the data for P. aeruginosa in the double label assay is the consequence of the adhesion of only sucrose and OS 346, no larger molecules, to the outer surface of the cells. But if it is possible for molecules to adhere to P. aeruginosa, it is also possible for such adhesion to occur with E. coli, and if the assumption is made that the results obtained for P. aeruginosa are the consequence

of adhesion, the same assumption should also be made with E. coli. The similarity of the size of the pores in E. coli determined using the double label assay and other methods (Decad and Nikaido, 1976) suggest that adsorption is not interfering with the data. In turn, this suggests that adhesion will not be playing an important role in experiments with P. aeruginosa.

There is a further piece of evidence against the data in the double label assay resulting from adhesion of radiolabelled compounds to the cells. Hancock and Nikaido (1978) working with outer membrane vesicles of P. aeruginosa, and Decad and Nikaido (1976) working with whole cells of E. coli and P. aeruginosa found that non-specific adsorption of the compounds they used (which included sucrose and inulin) was negligible. This suggests that the data from the double label assay with both organisms can be interpreted as the result of molecular diffusion and not adhesion.

3.6 Outer membrane protein composition of *E. coli* W3110 and *P. aeruginosa* 6750

3.6.1 Introduction

The pores in the outer membrane of *E. coli* and *P. aeruginosa* are primarily constructed of proteins, porins, which in turn comprise a large proportion of the protein located in the outer membrane. It has been observed that the presence of various porins, and other outer membrane proteins, is affected by the growth environment of the organism (Lugtenberg et al., 1976; Ferenci, 1980; Hancock and Carey, 1980). It was therefore necessary to determine the protein composition of the outer membranes of *E. coli* W3110 and *P. aeruginosa* 6750 grown under the same conditions as those for the double label assay, so that any relationship between the nature of the porin protein and the determined pore size could be examined.

3.6.2 Method

E. coli W3110 and *P. aeruginosa* 6750 were grown up in 2 litre volumes as described in 2.2.2.1. The outer membrane proteins were extracted and separated as described in 2.2.8 and 2.2.9 respectively.

3.6.3 Results

The various major proteins present on the gels were identified by calculating their R_f values and determining the molecular weights from a calibration curve of log mw against R_f value of known standards run on the same gel. Also, the *P. aeruginosa* 6750 outer membrane proteins were compared with a sample of *P. aeruginosa* PAO1 outer membrane proteins, again run on the same gel.

Figure 3.41 SDS polyacrylamide slab gel of outer membrane proteins found in *P. aeruginosa* PAO1, *E. coli* W3110 and *P. aeruginosa* 6750 grown in various environments.

a *P. aeruginosa* PAO1 grown to stationary phase in nutrient broth

E. coli W3110

b grown to log phase in CDM

c grown to stationary phase in CDM

d grown in magnesium deficient CDM

e grown in CDM, limited with tetracycline

P. aeruginosa 6750

f grown to log phase in CDM

g grown to stationary phase in CDM

h grown in iron deficient CDM

i grown to log phase in nutrient broth

j grown to stationary phase in nutrient broth

Molecular weights are given in K daltons.

Proteins were identified as described in the text.


a
b
c
d
e
f
g
h
i
j
49
44·6
41·4
25·7
21·5
19·9
lam B
Omp F
Omp A
tsx
protein III
66·0
D
E
45·0
F
24·0
G
H1
H2
18·4
14·3

Figure 3.42. SDS polyacrylamide slab gel of outer membrane proteins found in *E. coli* W3110.

E. coli W3110

a grown in CDM, limited with tetracycline

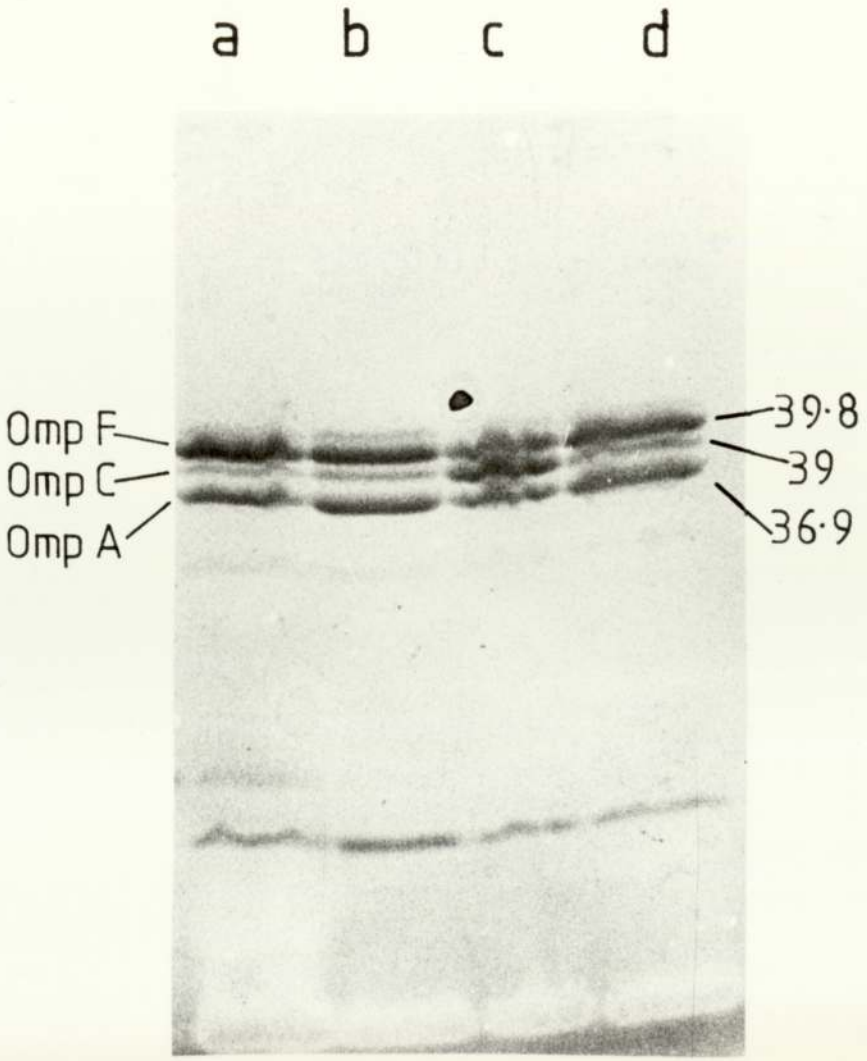
b grown in magnesium deficient CDM

c grown to stationary phase in CDM

d grown to log phase in CDM

Molecular weights are given in K daltons.

Proteins were identified as described in the text.


a
b
c
d
Omp F
Omp C
Omp A
39·8
39
36·9

3.6.3.1 *E. coli* W3110

Figures 3.41 and 3.42 show the outer membrane proteins found in *E. coli* W3110 after growth in various ways. It is clear that the dominant proteins in all the different conditions investigated are OmpF, OmpC and OmpA, so much so that, in order to detect most of the other outer membrane proteins, the concentrations of these three proteins are too high for OmpC and OmpF to be distinct in Figure 3.41. However, in Figure 3.42 it can be clearly seen that although all three major outer membrane proteins are present, the concentration of OmpC is much lower relative to that of OmpF and OmpA in all the various outer membrane preparations.

In addition to the three Omp proteins, the other identified outer membrane proteins which are clearly major constituents of *E. coli* W3110 include the *tsx* gene product and protein III. These proteins, like OmpA, C and F, are present under all growth conditions. The only difference would seem to be the relatively slightly higher concentration of protein III present in magnesium limited cells. Also slightly different in the magnesium limited cells is the almost total absence of the high molecular weight iron binding proteins.

The *lamB* gene product is present under all the growth conditions, but only as a very minor constituent of the outer membrane proteins. It appears on these gels as a doublet, and it is not clear whether one, or both, are the λ protein.

Other than the slight difference with magnesium limited *E. coli*, there seems to be little effect of the growth environment of the cells on their major outer membrane protein composition. Some of the minor proteins are slightly altered, for example there is a relatively higher concentration of the iron binding proteins in

log phase cells than in other cells. There also appear to be more minor proteins of molecular weight 40-50 Kd in tetracycline limited cells than in the others.

3.6.3.2 P. aeruginosa 6750

The major outer membrane proteins in P. aeruginosa 6750 are very similar to those found in P. aeruginosa PAO1. Using this system for separating the different molecules, D, E, F, G, H1 and H2 can be easily identified. D1 and D2 cannot be separated.

Although in all cases protein F is a major outer membrane protein, it is not the dominant one in log and stationary phase nutrient broth grown cells. In these organisms protein H2 is present in at least an equal concentration to F. However, it (H2) appears to be absent from cells grown in CDM. The relative amount of H1 also varies; there is more in nutrient broth grown cells than in CDM grown cells, and certainly more H1 in log phase than stationary phase nutrient broth grown cells. This is also possibly true for log and stationary CDM grown cells.

The amount of protein G relative to the other outer membrane proteins also varies slightly. In all the nutrient broth grown cells (including P. aeruginosa PAO1) there is relatively more than in the CDM grown cells, in which it has become a minor protein. Further, the protein is virtually absent in iron limited P. aeruginosa 6750.

In P. aeruginosa PAO1, protein F is the major outer membrane protein. But, although this is true for the cells grown in CDM, the dominance of protein F is not as marked, and there are several other proteins of almost equal concentration. In nutrient broth grown cells this includes H1 and H2, and in CDM grown cells D and E.

Protein E is present in the organism under all growth conditions, although there would appear to be relatively slightly more in log phase, CDM grown cells than in the other bacteria. Growth of the organism in the presence of glucose as the sole carbon source does not noticeably alter the amount of protein E in the cells.

This is also apparently true with protein D. Unfortunately, as this separation system cannot distinguish between D1 and D2, there is no means of determining whether the growth environment has affected the relative amount of D1 in the outer membrane. It does appear that the concentration of protein D is relatively constant, irrespective of growth conditions, possibly a little higher (again) in log phase CDM cells.

The only other major difference is the high concentration of iron binding proteins present in iron limited cells, and their virtual absence in nutrient broth grown cells, particularly P. aeruginosa 6750 (as compared with P. aeruginosa PAO1).

3.6.4 Discussion

3.6.4.1 E. coli W3110

E. coli W3110 contains those major outer membrane proteins usually found in other strains of E. coli K12 (Nikaido and Nakae, 1979; Osborn and Wu, 1980; Lugtenberg, 1981) in similar relative concentrations. Thus, the two porins OmpF and OmpC are both present, the former being the dominant outer membrane protein. It has generally been observed that the concentration of OmpF in the outer membrane is greater than that of OmpC, particularly when the organism is grown in minimal media such as the CDM used in this thesis (Lugtenberg et al., 1976), although this may be an effect caused by osmolarity (van Alphen and Lugtenberg, 1977; Kawaiji et al., 1979).

In view of the apparent greater efficiency of OmpF as a pore (Nikaido et al., 1980) it is perhaps not surprising to find that this protein is present in the outer membrane in greater concentrations than OmpC when the organism is growing in the nutritionally poor CDM environment.

Both OmpA and the tsx gene product have been implicated as proteins forming specific pores (Manning et al., 1977 ; Hantke, 1976; McKeown et al., 1976; van Alphen et al., 1978; Heuzenroeder and Reeves, 1981). It is therefore significant that both are present under all growth conditions (as are OmpF and OmpC) and that the concentration of OmpA is almost as great as that of OmpF. Schnaitman (1974) had observed that protein 1 (equivalent to OmpF and OmpC together) was present in a higher concentration than protein 3 (OmpA) when E. coli K12, grown in batch culture, was in exponential phase, but that this was reversed in stationary phase. Such a pattern is not seen in these results, but this may be the result of differences in the strains of E. coli used. However no other reports on the difference between log and stationary phase cells have mentioned such a change in the major outer membrane proteins, and it is possible that such a difference does not actually exist.

OmpF (forming a non-specific pore) and OmpA (possibly forming a pore specific for amino acids) are the dominant outer membrane proteins irrespective of growth conditions (Hindennach and Henning, 1975; Lugtenberg et al., 197 ; Lugtenberg et al., 1975). OmpC (forming a non-specific pore) and protein III (of unknown function) are the proteins present in the next highest concentration. The tsx gene product (possibly forming a specific pore for nucleotides) is present as a major outer membrane protein, but in a slightly

lower concentration still. These five proteins could probably be classified as the major outer membrane proteins in E. coli W3110 which can be identified and compared with proteins found in other strains of E. coli. It is interesting to find that the proteins involved in pore formation are present in such high concentrations, but it is not particularly surprising, as their functions, both structurally and in terms of the permeability of the outer membrane, are very important.

There are several minor outer membrane proteins, one of which is, in the growth conditions used here, the lamB gene product. This polypeptide is present as a minor component of the E. coli W3110 outer membrane protein pattern, but is distinct, due to the lack of other proteins of similar apparent molecular weight. It is important to appreciate that the λ receptor is only present in very small quantities, as this has a bearing on the interpretation of the double label assay.

There is surprisingly little effect of growth environment on the outer membrane protein pattern of the organism, the main changes being amongst the minor proteins. Amongst these, the most marked is the effect of insufficient environmental Mg^{2+}, most clearly seen in the reduction in the amount of iron binding proteins. Presumably, these require magnesium for a correct structure and are not manufactured when this is in short supply. Equally, the presence of relatively higher concentrations of iron binding proteins in log phase (complete CDM) cells may reflect the need of the organism to obtain increased quantities of iron when growing quickly.

E. coli W3110 limited in its growth by the addition of tetracycline appears to accumulate small amounts of 40-50 Kd mw proteins. This is probably a result of the inability of the

organism to divide, due to the action of tetracycline. Possibly these are proteins normally associated with the triggering and carrying through of cell division. If division could not occur, such proteins might accumulate in the outer membrane.

The outer membrane protein patterns of *E. coli* W3110 closely resemble those observed in other strains of *E. coli*, particularly *E. coli* K12. Thus comparisons between the size of pores found in *E. coli* W3110 and strains of *E. coli* used by other workers should not be affected by differences in the outer membrane composition of the various bacteria.

3.6.4.2 *P. aeruginosa* 6750

The outer membrane of *P. aeruginosa* contains only one protein so far identified as having non-specific pore forming properties, protein F. Under all growth conditions, this protein is present in high concentrations, but it does not dominate the outer membrane protein pattern in the manner in which OmpF and OmpA dominate that of *E. coli*. This is true of *P. aeruginosa* PAO1 (here grown to stationary phase in nutrient broth) as well as *P. aeruginosa* 6750. It has been observed (P. Lambert, personal communication) that growing *P. aeruginosa* in double strength nutrient broth causes the relative concentration of protein F to increase dramatically.

In addition to protein F, protein D1 forms a glucose-specific pore (Hancock and Carey, 1980). This protein, and to a lesser extent proteins D2 and E, are induced by the presence of glucose in the growth environment (Hancock and Carey, 1979; 1980). As D1 and D2 are not separated, it is not possible to determine whether the presence of glucose as the sole carbon source in the CDM used does include increased concentrations of protein D1 in the outer membrane. However, the relative concentration of protein D does

not appear to vary very much. Just as there is probably maltose present in nutrient broth (p. 125) there is also likely to be glucose, or some analogue capable of inducing the production of protein D1 and other components of the high affinity (low Km) glucose uptake system in P. aeruginosa.

In addition to the constant appearance of both proteins F and D, irrespective of the growth environment, there are various other, previously identified, proteins present in the outer membrane of P. aeruginosa 6750. These include proteins G, H1 and H2, which, unlike proteins F and D, do vary in relation to the growth environment. The distinctions appear to be primarily that of nutritional richness (i.e. CDM or nutrient broth); but the growth rate (here represented by log or stationary phase cells) also affects the relative concentration of protein H1.

CDM grown cells appear to contain no protein H2, and very little protein G, which in these conditions becomes a minor protein. Further, the concentration of H1 is lower than that of protein F, whereas protein H2 is present in approximately equal concentration to protein F in nutrient broth grown P. aeruginosa 6750 and PAO1. Further, the relative concentration of both proteins H1 and G is higher in such cells than in CDM grown cells. This contrast between the effect of the different growth environments on proteins G, H1 and H2 was not observed by Hancock and Carey (1979). They found protein H2 in outer membrane preparations from cells grown in both minimal media (with glucose as carbon source) and proteose peptone media. They also found that there was little variation in any of the other proteins other than D1, which could be separated from D2 in their system. Thus, in contrast to the results obtained with P. aeruginosa 6750, P. aeruginosa H103 (a strain of PAO1) was found

to contain proteins G, H1 and H2 in relatively constant concentrations under nutritionally rich and poor conditions, as were proteins F, E and D2. Interestingly, under the two different environmental conditions investigated, protein F was of approximately equal concentration with protein H2, and these were the dominant proteins (Hancock and Carey, 1979).

When the cells are growing quickly, the relative concentration of protein H1 in P. aeruginosa 6750 is higher than that found in stationary phase cells grown in the same media. Protein H1 has been identified as being involved in resistance to polymyxin, EDTA and gentamicin (Nicas and Hancock, 1980), high levels of the protein being associated with a raised resistance of the organism to these agents. Why the organism should require an increased concentration of this polypeptide under conditions of rapid growth, is not clear. Under conditions of magnesium limitation, protein H1 becomes the dominant outer membrane protein and substitutes for Mg^{2+}; the amount of cell envelope bound magnesium has been found to be inversely proportional to the concentration of protein H1 (Nicas and Hancock, 1980). Possibly, under conditions of rapid growth H1 is used in place of Mg^{2+} at sites on the LPS (Nicas and Hancock, 1980), thus releasing magnesium for use elsewhere, and providing the rapidly growing cells with extra protection against various cationic antibiotics and EDTA.

It is also unknown why proteins G and H2 should vary in quite the way they do. The former is apparently absent from iron limited cells (or present in exceedingly low concentrations). This probably reflects a requirement for iron in the molecule's structure. But despite this, were the protein essential, the organism would continue to manufacture it, or some similar replacement. Its absence from

the outer membrane of iron limited cells, and the fact that as yet no function has been identified for the protein suggests that in fact it is a non-essential outer membrane protein, in P. aeruginosa 6750 at least. If this is so, its reduced concentration in CDM grown cells would also be explicable. Here, the available nutrients would be used for the production of, first and foremost, essential cell components. Production of useful but non-essential macromolecules would only occur after this. In CDM grown cells, the energy and materials available for synthesis of non-essential cell components would be considerably less than in nutrient broth grown cells. Therefore, were it such a molecule, the amount of protein G in the outer membranes of nutrient broth grown cells would be considerably greater than that in CDM grown cells.

The explanation of the presence or otherwise of protein H2 may be similar. But it is surprising to find it apparently absent from CDM grown P. aeruginosa 6750. Protein H2 has been identified as being a lipoprotein (Mizuno, 1979), associated non-covalently with the peptidoglycan (Hancock et al., 1981). However, no other function has been identified with this protein, nor is there any correlation between proteins H2 and I (the Braun's lipoprotein) (Mizuno, 1979). Because of the striking contrast in the occurrence of protein H2 in strains of P. aeruginosa grown in minimal media by Hancock and Carey (1979) as compared with P. aeruginosa 6750 grown in CDM, it is difficult to draw any conclusions as to why H2 is apparently absent in the latter strain of bacteria grown in CDM but clearly present in high concentration in the outer membrane of the same organism grown in nutrient broth.

Despite the variation caused by the nutritional environment of the organism, protein F is always present as a major protein,

in concentrations relative to the other outer membrane proteins similar both to those observed by other workers (Hancock and Carey, 1979; Mizuno and Kageyama, 1979b; Hancock et al., 1981) and to those found in P. aeruginosa PAO1 as examined with this system. Comparisons of studies on the size of pores found in P. aeruginosa should therefore be unaffected by any consideration of the outer membrane proteins. However, in the light of the results obtained with the double label assay it is necessary to bear in mind the possibility that protein D1 is present in cells grown in CDM but not in nutrient broth (Hancock and Carey, 1979; 1980).

4. GENERAL DISCUSSION

4.1 *E. coli* and other enteric bacteria

The bulk of the work carried out on the pores found in the outer membrane and on the nature of the outer membrane itself, has used *E. coli* or *S. typhimurium* as the experimental organism. This has led to a considerable accumulation of knowledge about the outer membranes of such enteric bacteria.

As has been mentioned (section 1.3), several approaches have been taken in determining the size and mode of function of the outer membrane pores. The important division between the methods is that of *in vivo* and *in vitro* experimentation. Much of the work done to determine the exclusion limit of pores in *E. coli* has used membrane vesicle techniques, which do not permit the pores to be investigated in their natural environment. Data obtained from such methods, although very useful, can only be legitimately considered in the light of data obtained from experiments with whole cells. However, the methods utilising whole cells should and can be compared with each other in order to determine the probable size of the pores, and results obtained with membrane vesicles may support such conclusions.

4.1.1 Comparison of the double label assay with other methods used for determining the outer membrane pore exclusion limit

Decad and Nikaido (1976), after incubating radiolabelled sugars with plasmolysed *E. coli* or other Gram-negative bacteria for 5 minutes found that raffinose (a trisaccharide, mw 504 d) could penetrate the outer membrane whereas stachyose (a tetrasaccharide, mw 667 d) could not. These results were similar to those obtained by Gilvarg and Katchalski (1965) and Payne and Gilvarg (1968b). Here, mutants of *E. coli*, unable to manufacture various amino acids,

were grown in the presence of oligopeptides of different lengths. It was found that the mutants could utilise tetralysine (mw 600 d) and tetraalanine (mw 306 d) but not the pentapeptides. Although the reasons for pentaalanine not crossing the outer membrane are not entirely apparent, it would appear from the studies with oligopeptides that, where the charged nature of the molecules did not interfere, the outer membrane of intact, whole cells, was freely permeable to oligopeptides of approximately 600 d mw, as was found with oligosaccharides (Decad and Nikaido, 1976). This is a slightly simplistic view of the results of Decad and Nikaido (1976), which indicate that molecules of 800 d mw were able to penetrate the outer membrane to some extent, certainly more than molecules of weights greater than 1000 d.

When Kawaji et al. (1979) were investigating the effect of medium osmolarity on the synthesis of porins OmpC and OmpF in E. coli K12, they grew the bacterium in the presence of various concentrations of different sized sugars. It was found that the larger sugars, unable to penetrate the outer membrane, were more efficient at switching the porin synthesis than raffinose and smaller molecules, which were known to be able to pass through the pores. However, stachyose, and a dextran fraction of 700 d mw, were of intermediate efficiency in effecting the alteration in porin production, suggesting that these molecules were able to penetrate the outer membrane, although not as readily as the smaller compounds.

When the data obtained from the double label assay used with E. coli are compared with other data from whole cells, it would appear that the pores are slightly larger than suggested by Decad and Nikaido (1976). The main difficulty is caused by the problems of comparing different types of molecules, the absolute sizes of

which are unknown. It is therefore valuable at this stage to consider the results obtained with membrane vesicles. These would suggest that molecules larger than 700 d mw are unable to penetrate membrane vesicles composed of phospholipid, LPS and porins, obtained either from E. coli (Nakae, 1976b) or S. typhimurium (Nakae, 1976a; Nakae and Ishii, 1978). However, molecules of 600-700 d mw, although able to cross the vesicle membranes, did not do so as easily as sucrose.

The work carried out with membrane vesicles cannot be seen as ideal, and it is possible that the results are partly a consequence of the in vitro situation in which the pores are present. The membranes formed from outer membrane components are not guaranteed to adopt the asymmetric conformation of their native outer membrane and will lack much of the structural support of the underlying complete peptidoglycan layer and the cytoplasmic membrane. Further, without the molecules present in the periplasmic space or the cytoplasm, it is unlikely that the normal pressure exerted on the outer membrane from inside the cell would exist (Stock et al., 1977). A Donnan equilibrium is said to exist across the outer membrane, maintained by the high level of anionic material within the periplasmic space (Stock et al., 1977) and this too would be absent from membrane vesicles.

Despite these objections, membrane vesicles are a form of the outer membrane, and provided they include protein, LPS and phospholipid, they probably resemble the native outer membrane (Nakamura and Mizushima, 1975). They can, and do, provide useful information about the pores. Even in whole cell assays there is no guarantee that the organism is being retained in the normal state, the cells are simply closer to normality than membrane vesicles.

Results obtained by Nakae (1976a, b) and Nakae and Ishii (1978) with membrane vesicles are similar to those obtained by Decad and Nikaido (1976) and Kawaji et al. (1979) in that the pores appear to be partially permeable to molecules of 600-700 d mw. This is slightly in contrast to the results obtained in this thesis with E. coli W3110 in the double label assay, where OS 670 appears to be freely permeable through the outer membrane, more so than sucrose. However, without knowing the absolute three dimensional size of the different molecules used in the various assays, it is not easy to define an absolute exclusion limit for the pores of E. coli, and presumably in other enteric bacteria. By combining all the different results, it would appear that molecules of 600-700 d mw diffuse through the outer membrane, and that slightly larger molecules can, given time, permeate through the pores.

4.1.2 Interference by the maltose uptake system

It should be considered whether the results obtained with the double label assay for the larger molecules are a consequence of their involvement with the maltose uptake system. The λ pore is only a major outer membrane protein when induced by growth of E. coli on maltose and is not found in any important quantity in the organism when grown under the conditions used in this thesis (section 3.6). However, should any lamB protein be present in the outer membrane it has been shown that only isomaltitol (OS 346) is taken up by the maltose transport system (section 3.3) and this molecule was not used in experiments to determine the pore size of E. coli. It is known (Ferenci, 1980; Ferenci and Boos, 1980) that the maltose uptake system has a higher affinity for maltodextrins than maltose, and were isomaltotriitol (OS 508) and larger molecules to be involved

with such enzymatic uptake, this would have been observed when investigating the active uptake of OS 346. Here the results suggest that whatever specific mechanism distinguishes between maltose and non-maltose compounds also differentiates between maltose and OS 580 and OS 670. It is therefore unlikely that the apparently slightly larger than usual pore size of E. coli W3110 when investigated with the double label assay is a consequence of the maltose-like structure of the compound used, which might have been able to cross the outer membrane through the λ receptor pore.

Perhaps the best evidence comes from studies carried out by Luckey and Nikaido (1980), investigating the ability of various sugars to penetrate liposomes comprised of E. coli phospholipids and the λ protein. This was done by suspending the liposomes in isotonic media containing the sugar under investigation, and measuring their rate of swelling by following the change in turbidity of the suspension.

It was found that both maltotriose and isomaltose penetrated the liposomes at about 65% of the rate of maltose, but the rate of permeation of isomaltotriose was only 13% that of maltose. This suggests that isomaltotriitol (OS 508) would be even less able to utilise the λ pore as a means of crossing the outer membrane than isomaltotriose; which does imply that such a method of membrane permeation is not interfering with the double label assay.

4.1.3 Determinations of the rate at which molecules cross the outer membrane

Most of the work on the Gram-negative outer membrane pores has been about the size of the molecules able to penetrate the outer membrane, but the double label assay also investigates the

rate of penetration of the molecules relative to that of sucrose. It has been reported (Nikaido and Rosenberg, 1981) that sucrose, although able to cross the outer membrane, diffuses through the pores slowly. Lactose (a disaccharide) was found to cross the outer membrane of whole cells at a rate 60 times more slowly than arabinose, which itself crossed the outer membrane five times more slowly than glycerol. These results were determined using a method initially developed by Bavoil, Nikaido and von Meyenberg (1977). E. coli B/r ompB mutants were grown in liquid media containing various carbon sources (such as glycerol, glucose, arabinose, lactose) which were present in the media as the growth limiting nutrient. The rate of diffusion of the carbon compounds was, as a result, equal to the rate of transport across the cytoplasmic membrane and hence the rates could be determined by measuring the yield of cells and the half maximal growth rate appropriate to each carbon source. These could be used to calculate the rate of diffusion of the different molecules through the outer membrane which could in turn be used in Fick's Law:

$$V = P.A(Co-Cp)$$

which does theoretically describe the diffusion of molecules across the outer membrane. P is the permeability coefficient, A the surface area of the cell and Co and Cp the concentration of solute in the external and the periplasmic space. By using ompB mutants it was assumed that Co would be very much greater than Cp, which could consequentially be ignored, as Cp would be very difficult to measure. The data obtained in this manner were compared with data obtained by measuring the rate at which porin and phospholipid liposomes increased in volume when suspended in isotonic media containing various different sugars (including sucrose). Although neither

method gave absolute rates of diffusion, the results were clearly related; making it possible for statements to be made about compounds, such as sucrose, which could not be used in the whole cell growth assay. Hence Nikaido and Rosenberg (1981) concluded that sucrose would diffuse across the outer membrane slowly. They also pointed out that maltose would only cross the outer membrane with a rapid rate of diffusion when the λ receptor pore was present, otherwise maltose would also diffuse through the outer membrane slowly. This observation, if correct, supports the conclusions drawn from the results of the double label assay, namely that molecules other than OS 670 are not diffusing through the λ pore. If they were, then molecules would appear to diffuse more quickly than sucrose. It does not provide any explanation for the results obtained with OS 670; there is no reason why only this molecule should be able to find and then diffuse through, the λ receptor pore. That would, in fact, be a highly improbable explanation of those observations.

Bavoil et al. (1977) had also found that hexoses diffused very slowly across the outer membrane of ompB mutants, and von Meyenberg (1971) had observed that lactose had an even higher K_m value (i.e. diffused even more slowly than found by Bavoil et al. (1977)) in such ompB mutants. This is not surprising, in that small molecules would diffuse more quickly than larger ones, and it supports the findings of Nikaido and Rosenberg (1981) which are more detailed than previous work.

If Nikaido and Rosenberg (1981) are correct in suggesting that, although able to cross the outer membrane pore, sucrose does so slowly, then it may have been a poor choice of standard diffusing compound for the double label assay, and the results of the assay could be misleading. For example, if sucrose is diffusing slowly

then so too is OS 508, and OS 670 is not actually diffusing quickly across the outer membrane. Further, OS 833, although able to cross the outer membrane, would be doing so exceedingly slowly, the number of molecules penetrating the cell being almost negligible. This would not, however, alter the basic interpretation of the double label assay, that the exclusion limit of the pores in E. coli W3110 was approximately 700 d.

The work of Nikaido and Rosenberg (1981) is open to question, partly because of the assumptions made about the growth of E. coli on the various carbon sources, partly because using ompB mutants (which theoretically have very low levels of porin) does not necessarily solve the problem of calculating Cp. Furthermore, it would be logically expected that glycerol would diffuse more quickly across the outer membrane than lactose or sucrose, and it is not apparent from the work how fast any of the molecules do cross the membrane. Thus the absolute rate of diffusion of sucrose, or any other compound, across the outer membrane still has to be determined. It is worth commenting that the double label assay with P. aeruginosa suggests that sucrose is able to diffuse reasonably quickly through the pores, rapid diffusion having ceased after 10-15 minutes. If the double label assay results are correct, sucrose should diffuse more quickly into E. coli than into P. aeruginosa. Also, Stock et al. (1977) assume that sucrose diffusing from the environment to the periplasmic space will have reached, or at least be near to, equilibrium after 30 minutes.

4.1.4 A reconsideration of the pore size

The problem of the exclusion limit of the pores in E. coli could be considered in terms of an attempt to determine the absolute

diameter of the pores. However reports have suggested that E. coli pores are between 0.8 nm (Benz et al., 1979) to 1.4 nm (Benz and Hancock, 1981) diameter. These methods all measured the pore size by determining the increase in conductance of black lipid films when the porin was added. Heasley (1980) when investigating the pore size of N. gonorrheae, calculated that molecules of 565 d mw (easily small enough to cross through the E. coli outer membrane pores) had a diameter of 1.8 nm. It is therefore clear that measuring the size of the pore using this method is unsatisfactory and very confusing in terms of the results obtained.

Perhaps part of the problem when attempting to define the exclusion limit of the pores in E. coli, or other Gram-negative bacteria, is the tendency to assume that molecules of the same approximate molecular weight are spherical, with identical diameters. Or that only molecules of less than 700 d mw can pass through the pores, larger molecules being totally unable to penetrate the outer membrane. Non-specific pores would not determine the molecular weights of the potential permeant molecules, rather their primary form of discrimination would be the dimensions of the molecule, coupled with its hydrophobicity (Harder et al., 1981).

Specific pores (such as the lamB protein) would obviously discriminate between potentially permeant compounds using other criteria, but this would not be detected with the molecules used in the double label assay. In particular, it is not possible, using uncharged sugars, to investigate whether the porin pores do have a slight selectivity in favour of cationic compounds (Benz et al., 1979).

4.1.5 The relationship between the environment and the exclusion limit of the pores

One of the features of pores which might have been observed was that the size of the pore responded to a change in growth conditions. This was thought possible in part because of the known effect of environmental conditions on the composition of the bacteria (section 1.5). But it would appear that growth at different rates (i.e. log phase cells as compared with any other cultures) and in different growth environments has little or no important effect on the pore size, nor on the major outer membrane protein composition of E. coli W3110. Although this could be seen as being surprising, it is possible that the fixed pore size is the optimum compromise, giving both reasonable protection against deleterious compounds and reasonable access to nutrients, any adjustment to improve one of these two features bringing a far greater impairment of the other.

Nikaido and Rosenberg (1981) have proposed that molecules would diffuse very slowly through the pores, even when small enough to pass freely through membrane vesicle pores. This would suggest that antibiotics and other compounds, although using such pathways, would not penetrate the outer membrane rapidly. This would also be true of essential nutrients, transport of which across the outer membrane could easily become the rate limiting step of uptake (Nikaido and Rosenberg, 1981). But when molecules which can be used in metabolism by E. coli have crossed the outer membrane into the periplasm, they are taken up by enzymes, either to be transported directly across the cytoplasmic membrane or to be altered prior to such transport. This enzymatic removal of the molecules would, should passage across the outer membrane be rate limiting, prevent

any build-up of the compound concerned in the periplasmic space. Thus the rate of uptake of the molecule into the periplasmic space would not gradually decrease, whereas any deleterious compound would gradually reach an equilibrium between periplasm and environment. It could well be that such a situation is tolerable for an organism, whereas restricting access to essential nutrients, for whatever benefits, is not.

Although the various CDM environments investigated do appear to have had little effect on the pore size, there are two major considerations. One is the effect of nutritionally rich environments, and the other of the specificity of the pores.

4.1.5.1 Effect of the nutritional richness and osmolarity of the environment

It has been shown (section 1.5) that both the osmolarity and nutritional richness of the media influence the OmpF to OmpC ratio. Primarily, it would appear that OmpF is dominant in the outer membranes of E. coli grown in isotonic, poor nutritional conditions, whereas this is altered in higher osmolarity, nutritionally rich conditions, OmpC and OmpF at least being equal in concentration, or OmpC becoming dominant (Lugtenberg et al., 1976; Bassford et al., 1977; van Alphen and Lugtenberg, 1977; Kawaji et al., 1979; Hall and Silhavy, 1981a). Associated with this are the relative efficiencies of OmpF and OmpC as pores (Nikaido et al., 1981; Harder et al., 1981). If OmpC is ten times less efficient than OmpF, then it could possibly be that in media with an osmolarity higher than isotonic (300 mOsm in E. coli and S. typhimurium (Stock et al., 1977)) a less efficient pore is needed. The increased osmolarity could indicate to the organism a higher concentration of necessary nutrients, or, possibly the appearance of compounds detrimental to

the cells. There certainly is a mechanism for the recognition of medium osmolarity changes, which has been partially identified by Hall and Silhavy (1981a, b). OmpC has been shown to reduce the penetration of antibiotics, particularly hydrophobic species, across the outer membrane as compared with OmpF (Harder et al., 1981). There is undoubtedly potential for further work on the relationship of medium osmolarity, the porins expressed in the outer membrane of E. coli and the resistance of the organism to antibiotics.

4.1.5.2 Role of the specificity of the pores

Specificity has been claimed for both the porin pores and those composed of other outer membrane proteins. In particular, it has been proposed that the tsx gene product (Hantke, 1976; McKeown et al., 1976; van Alphen et al., 1978; Heuzenroeder and Reeves, 1981) and OmpA (Manning et al., 1977) act as pores facilitating diffusion of amino acids (both proteins) nucleotides and nucleosides (tsx gene product). The double label assay would, of course, not investigate such possibilities nor would it be capable of determining whether features such as the dicarboxylate binding protein existed (Lo and Bewick, 1981; sections 1.3.3, 1.3.4). Because of this it is possible that some form of control of the pore size is not being observed, although it is occurring. For example, the pores might be able, as a result of a given stimulus, to prevent slightly hydrophobic molecules which would normally have crossed the outer membrane via the pores from so doing. This might apply to positively charged molecules, or molecules with some other feature which could represent a non-useful, even detrimental, group of compounds. The mechanism could be the consequence of some opening and closing system (e.g. Stock et al., 1977; Nikaido et al., 1981) although

this would appear unlikely (Nikaido and Rosenberg, 1981). But more possibly, there could be the production of binding proteins facilitating entry of useful compounds (Bewick and Lo, 1979; Lo and Bewick, 1981), or perhaps the mechanism links back to a change from OmpF to OmpC. The possibilities are many, and probably none are being measured by any assay, *in vitro* or *in vivo*, so far devised.

As yet, inadequate information has been obtained as to the relationship between the pores and the resistance of *E. coli* or other enteric bacteria, to antibiotics. Thus, any hypotheses are essentially informed prediction, rather than conclusions drawn from a large body of data. As ideas are put forward, it is essential to remember that the organisms are not simply showing off for the benefit of those carrying out research. Rather, the first consideration of any bacterium, irrespective of the growth environment, is survival; the second is growth to an adequate cell size for division to occur. To achieve such ends, the bacterium may not necessarily behave as human beings would anticipate.

4.2 *P. aeruginosa*

4.2.1 Comparison of the exclusion limit determined for pores in *P. aeruginosa* obtained with various assays

Investigation of the size of the pores found in *P. aeruginosa* has primarily depended on *in vitro* examination of the porin protein, F. Only one study to determine the size of the pores carried out with whole cells of *P. aeruginosa* has been reported (Decad and Nikaido, 1976), and the results obtained were ambiguous (Hancock and Nikaido, 1978; Hancock et al., 1979). This technique involved a similar method to the double label assay, incubating plasmolysed cells with ^{3}H sugars + ^{14}C sucrose for 5 minutes, removing the cells and then resuspending them for 10 minutes in fresh buffer, the relationship between the radioactivity present in the two supernatants being used to determine the extent to which the ^{3}H sugar was able to penetrate to the periplasmic space. Trisaccharides were found to diffuse as easily as sucrose across the outer membranes of *E. coli* and *S. typhimurium*, but the results obtained with *P. aeruginosa* were unclear. However, at the time, Decad and Nikaido interpreted the data as implying that the pore size was very similar to that of enteric bacteria such as *E. coli*. Subsequently, using membrane vesicle techniques, it was found that pores in *P. aeruginosa* probably had an exclusion limit such that molecules of 6000 ± 3000 d mw could penetrate to the periplasm (Hancock and Nikaido, 1978; Hancock et al., 1979). The disadvantages of membrane vesicles have already been mentioned (p. 220) but in some respects are even greater with *P. aeruginosa*. This organism, although very resistant to a large number of chemicals, has a more fragile outer membrane than many Gram-negative bacteria which is

very susceptible to physical stress, such as cold shock or osmotic stress. It is also more dependent than such organisms as E. coli on divalent cations for maintaining the integrity of the outer membrane (Rogers et al., 1969; Roberts et al., 1970; also see p. 27). These additional facts tend to imply that there would be very great problems encountered in manufacturing outer membrane vesicles of P. aeruginosa, despite the claims that this has been successfully achieved (Hancock and Nikaido, 1978; Hancock et al., 1979).

Thus, measurements of the P. aeruginosa outer membrane pore exclusion limit made using membrane vesicles are not necessarily accurate or correct. But, prior to any investigation using the double label assay, it was a reasonable assumption that P. aeruginosa did have larger pores than E. coli, possibly ten times as large. This was supported by the findings of Benz and Hancock (1981) who measured the diameter of P. aeruginosa pores formed by protein F in artificial lipid bilayers, and compared these results with measurements of E. coli and S. typhimurium porin pore sizes in similar systems. They found that P. aeruginosa had pores of 2.2 nm diameter, as opposed to the smaller diameter of enteric bacterial pores (1.4 nm). There are problems associated with these calculations (p. 226) as Heasley (1980) showed that dextrans of an approximate mw of 870 d had a diameter of 2.5 nm, greater than the diameter calculated for pores of P. aeruginosa. Further, these experiments were carried out using artificial lipid bilayers, which undoubtedly do not resemble the outer membrane of any organism. However, these alone are not adequate reasons for ignoring the results.

One reason for the measured difference in the diameters of the pores of E. coli and P. aeruginosa could be the problem of divalent cations. As has been observed, these are essential for

both organisms, but the outer membranes of P. aeruginosa are generally held to be more anionic than those of E. coli (Gray and Wilkinson, 1965). In the absence of divalent cations, LPS and bacterial phospholipids, it could be that the negative charges of the porin molecules prevent them from adopting their normal, more compact, configuration.

In the light of this background, the double label assay, using molecules of 200-1200 d mw, was expected simply to show that all these molecules crossed the outer membrane at the same rate, or at least not very different from that of sucrose. Even a 1200 d molecule should be able freely to diffuse through pores of the size proposed by Hancock and Nikaido (1978). Therefore, the results indicating that only OS 346 diffuses across the outer membrane with sucrose are a complete reversal of the predicted data.

Because so little work has been carried out with P. aeruginosa, it is difficult to assess the validity of results which are totally contradictory. There are, however, two pieces of information about P. aeruginosa which are relevant when considering the possible size of the outer membrane pores.

P. aeruginosa is known to be more resistant than many Gram-negative bacteria to many antibiotics and other compounds. These include compounds which probably cross the outer membrane of S. typhimurium via the pores (Nikaido, 1976). Were the pores to be large enough to accommodate molecules of 6000 d mw, it would be surprising to find that molecules of less than 1000 d mw (as many antibiotics are) were less able to penetrate these large pores than the ten-fold smaller pores of E. coli.

Hancock and Carey (1980) observed that the glucose induced protein D1 forms outer membrane pores specific for glucose but large enough to allow the passage of sucrose. It is not explained

why P. aeruginosa, with huge pores, also requires a relatively small (smaller than E. coli pores), specific pore for glucose. However, one of the important natural habitats of P. aeruginosa is water, and in such an environment it might be very important for the organism to have some specific mechanism for procuring glucose, which is a very valuable energy (and carbon) source. Were the non-specific outer membrane pores in fact smaller than those of E. coli, as the double label assay implies, such a system, with a high affinity for glucose, would be useful, possibly essential, as the protein F pores would not allow rapid passage of glucose across the outer membrane (Nikaido and Rosenberg, 1981).

4.2.2 Factors which potentially affect the results of the double label assay

4.2.2.1 The D1 protein pore

Despite these problems, the published data suggest that P. aeruginosa has large pores. It is therefore necessary to consider whether the results of the double label assay, which have been taken as implying that P. aeruginosa has very small pores (3.4.7) could be due to the presence of protein D1 pores, or to adhesion of the molecules used to cell structures. It is possible that the data obtained from the double label assay with P. aeruginosa are the result of all the molecules used diffusing through the large protein F pores, and sucrose and OS 346 also being able to diffuse through the D1 protein pores. If this were so, sucrose and OS 346 would be able to cross into the periplasmic space more quickly than other compounds, probably giving results similar to those observed (3.4.6). The D1 protein pores are undoubtedly present in cells

grown in CDM using glucose as a carbon source (Hancock and Carey, 1979, 1980) but it is not possible to determine here whether P. aeruginosa 6750 or P. aeruginosa PAO1 grown in nutrient broth have large amounts of protein D1 present in their outer membranes. However, if the glucose pore is specific, the presence or absence of the protein would have little influence on the double label assay. As already observed, isomaltotriitol (OS 508) is not recognised by the maltose uptake system in E. coli (3.3) and this molecule is more similar to maltose than either sucrose or OS 346 are to glucose. In such a situation it is unlikely that the D1 pores would be capable of enabling sucrose and OS 346 to penetrate the outer membrane and reach the periplasmic space. This is made more probable by the fact that neither sucrose or maltose (and hence OS 346) are used as carbon sources by P. aeruginosa.

However, Hancock and Carey (1980) showed that when in LPS-dioleoyl phosphatidyl choline membrane vesicles the D1 protein pores were equally permeable to sucrose as to glucose, which if true leads to several possible interpretations of the data in the double label assay. Both sucrose and OS 346 might be able to penetrate the outer membrane through protein F and D1 pores, whereas, as suggested above, the larger molecules could only cross the outer membrane through the 6000 d mw exclusion limit of the protein F pores. But, given this high exclusion limit, there would probably be little difference in the apparent rates of penetration. Equally, such a situation could pertain for OS 346 and sucrose, in that these molecules could cross the outer membrane via one or both of the pore types, but larger molecules would be unable to pass through either species of pore. A further possibility is that one of either sucrose or OS 346 is able to utilise the D1 protein pore

more than the other as a means of crossing the outer membrane. If this is so, it would probably be reflected in the results, unaffected by the presence or absence of plasmolysing agent.

Although there are these possibilities, the evidence is that even when glucose is present, P. aeruginosa grown in nutritionally rich media (e.g. casamino acids, proteose peptone) does not have protein D1 amongst its outer membrane proteins (Hancock and Carey, 1980). This fact implies that where the double label assay was carried out with nutrient broth grown P. aeruginosa there was negligible protein D1 present in the outer membrane. Assuming that this is true, there would therefore be a difference between data obtained with CDM and nutrient broth grown cells. This could be very great, with all the different molecules used appearing to cross the outer membrane of nutrient broth grown cells. If so, the implication would be that P. aeruginosa had large pores, but that there were relatively many more protein D1 pores than protein F pores (Hancock and Carey, 1980; Angus, Carey, Caron, Kropinski and Hancock, 1982), giving results with CDM grown cells which suggested that the pores were permeable only to sucrose and OS 346. On the otherhand, the difference in the results from CDM and nutrient broth grown P. aeruginosa could be very slight, as observed. This would imply that protein D1 pores either played no role at all in the double label assay, or that both pore species were of very similar size, molecules larger than sucrose being unable to pass through them.

4.2.2.2 The influence of adsorption on the double label assay

It is unlikely that the results obtained with the double label assay are affected by adsorption of sucrose and OS 346 onto cell surface structures, only for these compounds to be released

subsequently; or by the adsorption of all molecules larger than OS 346 onto cell components, without their release later on in the experiment. This is supported by the results of the double label assay itself, and the determination of the fate of the radiolabelled material during the assay. Furthermore, Decad and Nikaido (1976) and Hancock and Nikaido (1978) found that there was no adsorption to cell structures of any one compound they used to a greater extent than any other. Hence it seems highly probable that the results are unaffected by non-specific adsorption of compounds used in the double label assay to cell structures.

4.2.3 The relationship between the pores and the resistance of *P. aeruginosa* to antibiotics

Given the conclusions of the last few paragraphs, the double label assay suggests that *P. aeruginosa* has pores composed of protein F which are permeable to sucrose and OS 346, but not to larger members of the isomaltitol series, or inulin. Had the size of the protein F pores not been determined, but rather their possible exclusion limit predicted primarily on the basis of *P. aeruginosa*'s high resistance to many compounds, it is likely that it would have been assumed that the organism had pores of a diameter similar to or smaller than those of other Gram-negative bacteria, such as *S. typhimurium* and *E. coli*. The results of the double label assay are far more aligned with the knowledge of *P. aeruginosa*'s physiology and biochemistry than are those data previously obtained.

Because of the marked contrast between the high exclusion limit of the outer membrane pores of *P. aeruginosa* 6750 and the organism's resistance to antibiotics, Benz and Hancock (1981) and Angus et al. (1982) have proposed hypotheses which would explain the difference without compromising either type of result. It had

been found that the pore activity per unit weight of purified porin protein of protein F was approximately 100 times lower than that of the porins in E. coli and S. typhimurium (Benz and Hancock, 1981). Also, it had been suggested that the pores in E. coli have some form of "gating" by which they can be opened and closed in response to some specific stimulus, possibly membrane voltage, although this has recently been rejected (Nikaido and Rosenberg, 1981). The results of Benz and Hancock (1981) implied that the P. aeruginosa pores were not gated by a membrane voltage stimulus, nor probably did they possess any specific mechanism for regulating their opening closing. A more likely explanation was that at any one time the majority of the protein F pores were non-functional. This appeared a useful theory which could help explain the difference in the resistance of E. coli and P. aeruginosa to antibiotics, as there would be approximately one hundred times as many active pores in the E. coli outer membrane. The theory is partially supported by the findings of Harder et al. (1981) that E. coli mutants with increased resistance to certain beta lactam antibiotics lacked OmpF, although OmpC, probably ten times less efficient than OmpF as a pore, was present in the outer membrane; i.e. a less efficient porin did increase the resistance of the organism to some compounds.

Recently, an antibiotic-supersusceptible strain of P. aeruginosa has been isolated and used to investigate why there was this loss of resistance (Angus et al., 1982). It was found that there was neither an alteration in protein F, nor a loss or appearance of some novel outer membrane protein (Irvin et al., 1981). It was suggested that the increased susceptibility of the mutant relative to the wild type stemmed from the lack of functional pores in the wild type organism (there being possibly only 100 to 300 functional

pores in a wild type P. aeruginosa cell); whereas, in the mutant, they calculated a permeability parameter (based on that calculated by Zimmermann and Rosselet (1977)) which suggested that five to ten times more pores were functional in such cells giving a greater area available for molecules to diffuse through. It was proposed that the number of available functional protein F pores in the outer membrane was influenced by the state of the LPS, partly because the only major difference observed between the outer membrane conformations and compositions of the mutant and wild type organisms was the chemical structure of the LPS (Kropinski, Kuzio, Angus and Hancock, 1982), and also because of the observed special relationship between various outer membrane protein and the LPS (Angus et al., 1982).

Thus it was proposed that P. aeruginosa had large pores (with an exclusion limit of 6000 d mw) only a fraction of which (one in one hundred) were functional in wild type strains of the organism. In the antibiotic-supersusceptible mutant P. aeruginosa Z61 more of the available pores were functional. This difference was due to a chemical alteration in the LPS, which could not be switched on or off by the organism.

However, these data could be easily explained by assuming that the protein F pores are in fact smaller than those of E. coli, hence the high antibiotic resistance of P. aeruginosa. The mutant, P. aeruginosa Z61, did not have an altered pore but rather, altered LPS. This could make the pores functionally larger, by pulling the protein monomers apart from each other, out of their correct conformation. Alternatively, Kropinski et al. (1982) describe P. aeruginosa Z61 as being a leaky rough mutant, and there might literally be breaks in the outer membrane, although this is not

very likely, despite the fragility of the outer membrane.

Another possible explanation of the pore size of P. aeruginosa is that there is some instantaneous mechanism by which P. aeruginosa pores are closed when the environment changes, which has been lost by the antibiotic-supersusceptible mutant. If this were so, then, with the wild type organism growing in reasonable conditions, the pores could be large, perhaps with an exclusion limit of 6000 d mw. But an alteration of the environment detrimental to the cells such as the addition of an antibiotic or removal of the bacteria from the growth vessel into centrifuge tubes (lower temperature, little oxygen) could in some way trigger the pores so that the exclusion limit was drastically reduced. This property could be dependent on the whole cell envelope (as opposed to the three major components of the outer membrane used in membrane vesicles) and therefore membrane vesicle assays would detect only the large pore size, whereas an assay such as the double label assay would measure only the very small pore size.

The high resistance of P. aeruginosa to many hydrophilic compounds which is conferred on the organism by the outer membrane is best explained by the findings that the pores in the outer membrane only permit the passage of very small molecules, the exclusion limit being lower than that of E. coli and other enteric bacteria. It is, however, difficult to be certain, given the contrasts in the evidence. Many differences will have to be resolved, in particular the reasons for the larger diameter of the protein F pore than E. coli porin pores in black lipid films and how the organism obtains nutrients through virtually impermeable pores (Nikaido and Rosenberg, 1981). But it would seem possible, given the additional information of the double label assay, that the

pores found in P. aeruginosa are smaller than those found in enteric bacteria, having an exclusion limit approximately half, or less, than that found in E. coli, certainly as measured with oligosaccharides.

If this is the case, it provides a reasonable solution to the problem of the role of the outer membrane pores in bacterial resistance to antibiotics (p. 55). The very small pores of P. aeruginosa would confer the great protection against antibiotics and dyes which the organism appears to have, whereas the relatively large E. coli pores would permit the passage of many antibiotics across the outer membrane. The presence of the different sized pores in the two organisms would explain both the contrast in the resistance of the bacteria to antibiotics and, in particular, help to explain the problem of the almost freak resistance which P. aeruginosa exhibits against so many different bactericidal compounds.

4.3 Suggestions for further work

There are several ways in which the double label assay could be extended as a future project. These can be divided into three areas; improving the actual assay, continuing work on *E. coli* and *P. aeruginosa* and investigating other organisms using the double label assay.

4.3.1 Improvement of the double label assay

Sucrose may possibly have been a poor choice of standard in the double label assay (p. 224), and it might be possible to improve the assay by the choice of an alternative control molecule. However, any such molecule would have to be considerably smaller than sucrose (Nikaido and Rosenberg, 1981), hydrophilic, uncharged and unable to cross the cytoplasmic membrane by any mechanism. In addition, any compound chosen would have to be capable of carrying a low molecular weight, stable radiolabel (not necessarily ^{14}C).

Alternatively, the absolute diffusion of sucrose could be determined. In fact, whatever molecule were chosen as the standard, its absolute diffusion ought to be measured in order to determine the diffusion of the test molecules.

Finally, alterations in the choice of the homologous series of test molecules could be made. Such changes would partly be dictated by the organism being investigated and what aspect of the pores (e.g. size, specificity) was of interest.

4.3.2 Continuation of work with *E. coli* and *P. aeruginosa*

Although the results obtained from the double label assay with *E. coli* and *P. aeruginosa* are different, and only those with *E. coli* are similar to published data, the approaches which could

best be taken with the two organisms are similar in many aspects. With both organisms it would be interesting to compare the uptake across the outer membrane of suitable labelled antibiotics and sucrose. This could give important information as to the ability of the antibiotics to cross the permeability barrier, particularly how this is affected by the chemical nature, such as hydrophobicity or charge, of the molecules. An extension of such work would be the use of mutants such as E. coli B/r ompF and E. coli K12 ompF mutants (the former having virtually no porin, the latter only OmpC in the outer membrane). From this information would be obtained as to the effect changes in the outer membrane protein composition could have on the permeability of the membrane.

Using such mutants would be valuable, both with the double label assay as it already is, and with any antibiotics chosen for investigation. In P. aeruginosa investigation of antibiotic-sensitive mutants using the double label assay might help to elucidate the problems associated with the pore size of the organism. It is essential that this area is further investigated. A clear path forward would be to obtain a mutant such as the antibiotic-supersusceptible mutant of Angus et al. (1982) and investigate the outer membrane pore size with the double label assay. It would also be valuable to isolate mutants of P. aeruginosa which lacked protein F, although such organisms would undoubtedly be partially crippled in their ability to obtain nutrients and to grow (Bavoil et al., 1977), and investigate the permeability of the outer membrane in these strains.

With E. coli, it might be possible to investigate the specificity of various pores, both those composed of porins and those of the tsx gene product or OmpA. For example, the diffusion of charged

and uncharged molecules relative to sucrose could be determined in E. coli (Benz et al., 1979). Perhaps dicarboxylate sugars could also be investigated in an attempt to determine whether there is some mechanism facilitating the diffusion of these compounds (Bewick and Lo, 1979; Lo and Bewick, 1981).

In addition to varying the compounds used in the double label assay and the genetic makeup (and hence phenotype) of E. coli and P. aeruginosa, further investigation of the effect of the environment could be carried out. In particular, it would be worth comparing the pore size of E. coli grown in nutritionally rich and poor media, and media of varying osmotic strengths. With P. aeruginosa, growing the organism such that protein F is more dominant than observed in this thesis would be useful. Further, investigating the effect of growth rate on the pore size by growing the cells in chemostats could be extremely valuable.

4.3.3 Investigations with other bacteria

The possession of outer membrane pores is not restricted to E. coli and P. aeruginosa and there are many other organisms worth examining in an attempt to determine whether they have outer membrane pores, and if so, what their characteristics are. The double label assay would be very useful in such an exploratory role, as the molecules are unlikely to be used as a carbon source by many organisms, particularly when grown in conditions which would provide catabolite repression of equivalents of the mal operon. Other organisms could also be investigated in the various different ways proposed for P. aeruginosa and E. coli using radiolabelled antibiotics and, if they were available, antibiotic resistant and sensitive mutants.

4.4 Conclusions

There are several conclusions which can be drawn from the work described in this thesis. These form two categories, those resulting from the double label assay and those resulting from other studies such as the uptake of isomaltitol.

4.4.1 Studies other than the double label assay

1. It was found that the nature of the plasmolysing agent affected the viability of *E. coli* and *P. aeruginosa* as compared with unplasmolysed cells. The resistance of both organisms was reduced after pre-incubation with plasmolysing agent followed by growth on nutrient agar plates containing rifampicin. After pre-incubation with plasmolysing agent the resistance of *P. aeruginosa* grown on nutrient agar containing bacitracin was also reduced. The addition of 0.01 M $MgCl_2$ (final concentration) to the plasmolysing agent reduced its deleterious effect, particularly that of NaCl.

2. When grown in nutrient broth it was found that the maltose uptake system of *E. coli* was expressed. Isomaltitol (OS 346) was recognised by this system and removed from the environment via the λ pore and the maltose binding protein. Larger molecules in the isomaltitol series (OS 508, OS 670) were not affected by the maltose uptake system.

3. The various CDM environments used for growing *E. coli* in the double label assay did not significantly affect the major outer membrane proteins. However, there were changes in the relative proportions of the major outer membrane proteins found in *P. aeruginosa*, primarily associated with the nutritional richness of the media. In nutrient broth grown cells, protein H2 is of

equal dominance with protein F, whereas the former protein is absent from CDM grown cells. Protein G is also expressed relatively poorly in CDM grown cells. Protein H1 is expressed more strongly in log phase cells than in those grown to stationary phase in the same medium.

4.4.2 The double label assay

4. It was found to be possible to design an *in vivo* assay to determine the exclusion limit of pores in the outer membrane of Gram-negative bacteria based on a comparison of ^{3}H labelled molecules from an isomaltitol series with ^{14}C sucrose. The assay can also be used to measure the rate of uptake of molecules relative to the standard molecule. The results of the double label assay were found to be unaffected by any adhesion of the radiolabelled molecules to components of the cells.

5. Using the double label assay it was found that *E. coli* had pores in its outer membrane with an exclusion limit of approximately 700 d mw. However, given time, larger molecules did penetrate the outer membrane to some extent.

6. The tonicity of the test environment appeared to affect the size of the *E. coli* pores. When present in a hypotonic medium, the pores were found to have an exclusion limit of approximately 900 d mw.

7. Variation in the CDM growth environment of the cells had little effect on the size of the pores. Possibly growth of *E. coli* in the presence of tetracycline caused the pores to become marginally smaller than those found in the outer membrane of *E. coli* grown without the antibiotic.

8. Using the double label assay the outer membrane pores in *P. aeruginosa* were found to be very small, with an exclusion limit

of approximately 360 d mw or possibly less. No molecules larger than sucrose or OS 346 appeared able to cross the outer membrane.

9. The tonicity of the test environment appeared to affect the ability of OS 346 relative to sucrose to cross the outer membrane of P. aeruginosa. In hypertonic environments OS 346 penetrated the outer membrane more easily than sucrose, whereas in hypotonic environments the two compounds were far more similar in their behaviour.

10. The growth environment appeared to have little effect on the pore size of P. aeruginosa, although the pores might be marginally smaller in cells grown in nutrient broth as compared with those grown in CDM.

Appendix 1

Chemical nomenclature of ^{3}H labelled oligosaccharides

Trivial name	Systematic name
^{3}H isomaltitol	O-α-D-glucopyranosyl-(1→6)-1-tritio-D-glucitol.
^{3}H isomaltotriitol	O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-1-tritio-D-glucitol.
^{3}H isomaltotetritol	O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-1-tritio-D-glucitol.
^{3}H isomaltopentitol	O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyransoyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-1-tritio-D-glucitol.
^{3}H isomaltohexitol	O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-1-tritio-D-glucitol.
^{3}H isomaltoheptitol	O-α-D-glucopyransoyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(⟶6)-O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl-(1→6)-1-tritio-D-glucitol.

Appendix 2

Data for the response of P. aeruginosa 6750 to rifampicin and bacitracin after plasmolysis

Antibiotic concentration	Percentage survivors ± standard deviation[a]					F(p = 0.05)[b]	
Rifampicin µg ml^{-1}	Control (unplasmolysed)	Sucrose plasmolysed	NaCl plasmolysed	NaCl & $MgCl_2$ plasmolysed	Sucrose & $MgCl_2$ plasmolysed		
20	0	0	0	0	0	0	
10	8.0 ± 6.1	0	0	8.3 ± 3.7	0	12.233	Degrees of freedom:
5	22.4 ± 8.0	0	0	26.1 ± 1.8	3.7 ± 0.7	136.873	numerator = 4
2.5	54.3 ± 6.2	1.1 ± 1.1	0	55.3 ± 16.3	30.0 ± 1.6	80.481	denominator = 25
1.25	68.5 ± 7.0	12.0 ± 7.0	4.8 ± 3.2	77.3 ± 3.8	60.9 ± 7.9	196.101	F(p = 0.05) = 3.3500
0.625	77.0 ± 4.8	17.9 ± 7.9	7.0 ± 3.8	81.4 ± 4.9	67.8 ± 7.8	211.855	
0.313	79.1 ± 4.3	30.9 ± 10.3	31.4 ± 3.3	83.3 ± 4.0	68.2 ± 3.6	149.916	
Bacitracin IU ml^{-1}							
400	7.7 ± 5.7	0	1.3 ± 0.6	18.4 ± 8.7	0.7 ± 0.4	11.415	
200	37.4 ± 8.8	7.6 ± 3.7	5.8 ± 2.7	52.8 ± 7.2	19.1 ± 4.9	53.837	Degrees of freedom:
100	68.1 ± 4.6	19.3 ± 8.4	28.0 ± 6.8	76.9 ± 6.1	39.6 ± 11.8	64.804	numerator = 4
50	84.7 ± 3.4	49.9 ± 7.9	51.9 ± 1.3	91.8 ± 2.2	56.3 ± 5.9	90.778	denominator = 17
25	81.2 ± 5.2	56.2 ± 10.5	77.6 ± 3.7	104.6± 3.1	70.6 ± 7.3	37.923	F(p = 0.05) = 3.6600
12.5	93.1 ± 3.5	64.1 ± 9.9	82.3 ± 1.0	93.0 ± 3.3	79.0 ± 6.6	24.897	
6.25	96.9 ± 1.9	72.0 ± 7.2	82.4 ± 2.6	102.2± 0.2	98.0 ± 0.5	70.767	

Appendix 2 (continued)

Antibiotic concentration	Percentage survivors ± standard deviation[a]					F(p = 0.05)[b]	
Rifampicin µg ml^{-1}	Control (unplasmolysed)	Sucrose plasmolysed	NaCl plasmolysed	NaCl & $MgCl_2$ plasmolysed	Sucrose & $MgCl_2$ plasmolysed		
2.5	0	0	0	1.0 ± 0.5	0.9 ± 0.4	9.951	
1.25	9.6 ± 4.4	2.8 ± 0.7	2.8 ± 1.3	15.8 ± 5.8	16.5 ± 3.3	10.130	Degrees of freedom:
0.625	48.8 ± 6.5	10.2 ± 3.1	9.9 ± 3.0	53.2 ± 8.2	69.3 ± 5.9	66.511	numerator = 4
0.313	80.1 ± 3.2	40.6 ± 3.2	34.5 ± 5.0	63.8 ± 5.1	92.6 ± 3.4	112.02	denominator = 10
0.157	93.2 ± 0.8	66.4 ± 5.7	64.2 ± 3.8	95.5 ± 0.9	104.5± 4.6	68.389	F(p = 0.05) = 4.4700
0.079	100.9± 2.7	89.8 ± 1.6	101.4± 5.2	111.2± 2.3	104.6± 3.4	16.812	
Bacitracin IU ml^{-1}							
50	1.1 ± 0.4	0	4.6 ± 2.0	2.1 ± 0.7	13.6 ± 4.5	18.095	
25	47.4 ± 6.9	39.6 ± 5.6	61.4 ± 8.7	50.9 ± 10.3	50.1 ± 8.0	2.836	Degrees of freedom:
12.5	91.3 ± 4.5	81.5 ± 1.5	96.5 ± 3.2	92.7 ± 6.3	84.4 ± 4.1	6.398	numerator = 4
6.25	100.5± 1.9	96.1 ± 2.8	107.3± 3.4	97.4 ± 0.9	97.3 ± 3.4	2.674	denominator = 10
3.13	94.1 ± 1.1	105.8± 0.4	102.2± 4.3	103.0± 3.5	89.3 ± 3.1	17.124	F(p = 0.05) = 4.4700
1.57	105.6± 2.9	94.2 ± 0.2	109.0± 0.8	101.1± 2.0	109.1± 1.6	37.820	
0.79	102.0± 0.6	93.6 ± 3.4	104.8± 3.5	101.7± 6.7	107.3± 1.1	5.684	

Appendix 2 (continued)

a. Percentage survivors: The average number of cells from a given suspension on agar containing each concentration of the relevant drug is expressed as a percentage of the average number of cells from that suspension surviving on drug free nutrient agar.

b. F is calculated using the following formula:-

$$F = \frac{\text{variance between the sample means}}{\text{variance within the samples}}$$

$$= \frac{n\Sigma(\bar{x}_i - \bar{x})^2 / (K - 1)}{\Sigma s_i^2 / K}$$

degrees of freedom = $(K - 1)$

degrees of freedom = $K(n - 1)$

or, if the sample sizes are unequal:

$$F = \frac{\Sigma n_i(\bar{x}_2 - \bar{x})^2 / (K - 1)}{\Sigma s_i^2 / K}$$

degrees of freedom = $(K - 1)$

degrees of freedom = $(\Sigma n_i - K)$

For determination of the significance of calculated values of F, a two tailed test was used.

Appendix 3

Data obtained with E. coli W3110 and P. aeruginosa 6750 grown under different conditions and incubated with various radiolabelled sugars in the double label assay.[a]

(A) E. coli

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and[b]					
	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H sucrose
	CDM grown, log phase, plasmolysed cells					
1	0.0655 ±0.1051	-0.0082 ±0.0424	-0.4612 ±0.0537	-0.4961 ±0.1856	-0.5480 ±0.1219	-0.0400 ±0.0896
3	0.0797 ±0.0625	0.0770 ±0.0370	-0.4346 ±0.0475	-0.4609 ±0.1252	-0.4415 ±0.0447	-0.0227 ±0.0677
6	0.0401 ±0.0271	0.1538 ±0.0890	-0.2839 ±0.2078	-0.4424 ±0.1373	-0.4315 ±0.0350	0.0024 ±0.0958
10	0.0126 ±0.0278	0.1575 ±0.0752	-0.2078 ±0.0685	-0.4119 ±0.0833	-0.3949 ±0.0605	0.0010 ±0.0820
20	0.0119 ±0.0263	0.2209 ±0.1113	-0.0901 ±0.0478	-0.3657 ±0.0796	-0.3249 ±0.0780	-0.0112 ±0.0941
30	0.0490 ±0.0561	0.2209 ±0.1040	0.0021 ±0.0285	-0.3069 ±0.1044	-0.2456 ±0.1016	0.0255 ±0.1023
	CDM grown, log phase, unplasmolysed cells					
1	-0.0488 ±0.0687	0.0406 ±0.0326	-0.0487 ±0.0237	-0.2868 ±0.1001	-0.2796 ±0.0141	-0.0227 ±0.0677
3	0.0102 ±0.0576	0.0946 ±0.0365	-0.0459 ±0.0122	-0.2152 ±0.0150	-0.2160 ±0.0231	-0.0400 ±0.0896
6	-0.0048 ±0.0716	0.1256 ±0.0657	-0.0508 ±0.0188	-0.2085 ±0.0023	-0.1874 ±0.0272	0.0010 ±0.0820
10	-0.0347 ±0.0865	0.1220 ±0.0511	0.0355 ±0.0300	-0.0837 ±0.0287	-0.1580 ±0.0322	-0.0112 ±0.0941
20	-0.0227 ±0.0393	0.1616 ±0.0792	0.0313 ±0.0086	-0.0214 ±0.0169	-0.0887 ±0.0503	0.0024 ±0.0958
30	0.0093 ±0.0202	0.1816 ±0.0839	-0.0396 ±0.0125	-0.0260 ±0.0314	-0.0685 ±0.0197	0.0255 ±0.1023

Appendix 3 (continued)

(A) E. coli

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and					
	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H sucrose
	CDM grown, stationary phase, plasmolysed cells					
1	-0.1062 ±0.0559	-0.0722 ±0.0496	-0.5115 ±0.0341	-0.5308 ±0.0636	-0.7098 ±0.0928	-0.0316 ±0.0173
3	-0.0824 ±0.0686	-0.0373 ±0.0636	-0.4538 ±0.0949	-0.4886 ±0.0817	-0.6764 ±0.0520	-0.0476 ±0.0233
6	-0.0329 ±0.1333	0.0808 ±0.0859	-0.3880 ±0.0264	-0.4268 ±0.0664	-0.5841 ±0.0456	-0.0279 ±0.0282
10	-0.0251 ±0.0718	0.1378 ±0.0773	-0.3683 ±0.0578	-0.4184 ±0.0433	-0.5777 ±0.0877	-0.0222 ±0.0334
20	0.0016 ±0.1269	0.2132 ±0.0429	-0.2596 ±0.0280	-0.3727 ±0.0667	-0.5328 ±0.0777	0.0085 ±0.0281
30	0.0018 ±0.0629	0.2424 ±0.0255	-0.2109 ±0.0279	-0.3224 ±0.0412	-0.4665 ±0.0672	-0.0416 ±0.0142
	CDM grown, stationary phase, unplasmolysed cells					
1	-0.0700 ±0.0375	0.1421 ±0.1181	-0.1318 ±0.0456	-0.2496 ±0.0230	-0.2399 ±0.0273	-0.0316 ±0.0173
3	-0.1219 ±0.0602	0.1638 ±0.1205	-0.0959 ±0.0208	-0.2407 ±0.0448	-0.2347 ±0.0284	-0.0476 ±0.0233
6	-0.0603 ±0.0217	0.1922 ±0.0988	-0.0626 ±0.0290	-0.1886 ±0.0867	-0.2156 ±0.0176	-0.0279 ±0.0282
10	-0.0037 ±0.0783	0.1714 ±0.1423	-0.0393 ±0.0282	-0.1955 ±0.0615	-0.1752 ±0.0138	-0.0222 ±0.0384
20	-0.0663 ±0.0058	0.2778 ±0.1238	0.0146 ±0.0154	-0.1458 ±0.0729	-0.1558 ±0.0209	-0.0085 ±0.0281
30	-0.0064 ±0.0450	0.2636 ±0.0831	0.0509 ±0.0382	-0.1047 ±0.0715	-0.1486 ±0.0147	-0.0416 ±0.0142
	Magnesium limited, plasmolysed cells					
1	-0.0437 ±0.0159	0.0506 ±0.0449	-0.4263 ±0.0719	-0.5898 ±0.0068	-0.5747 ±0.0229	0.0123 ±0.0359
3	-0.0431 ±0.0155	0.0757 ±0.0502	-0.4160 ±0.0740	-0.5336 ±0.0500	-0.5516 ±0.0119	0.0321 ±0.0163
6	0.0286 ±0.0134	0.1702 ±0.0900	-0.3139 ±0.0737	-0.4171 ±0.0309	-0.5195 ±0.0273	0.0537 ±0.0371
10	-0.0606 ±0.0107	0.1780 ±0.0521	-0.2668 ±0.0656	-0.4131 ±0.0226	-0.5081 ±0.0519	0.0683 ±0.0720
20	-0.0547 ±0.0143	0.2118 ±0.0998	-0.1447 ±0.0390	-0.3777 ±0.0142	-0.4084 ±0.0161	0.0123 ±0.0289
30	-0.0769 ±0.0123	0.2045 ±0.0749	-0.0940 ±0.0584	-0.3219 ±0.0435	-0.3987 ±0.0243	0.0285 ±0.0221

Appendix 3 (continued)

(A) E. coli

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and					
	OS 508	OS 670	OS 833	OS 955	OS 1157	^{3}H sucrose
	Magnesium limited, unplasmolysed cells					
1	0.0095 ±0.0868	0.0147 ±0.0263	-0.0952 ±0.0306	-0.1718 ±0.0470	-0.2970 ±0.0334	0.0123 ±0.0359
3	-0.0643 ±0.0593	0.0320 ±0.0119	-0.0664 ±0.0533	-0.1425 ±0.0457	-0.2728 ±0.0395	0.0321 ±0.0163
6	-0.0144 ±0.0315	0.0396 ±0.0365	-0.0308 ±0.0541	-0.1403 ±0.0389	-0.2376 ±0.0598	0.0537 ±0.0371
10	-0.0268 ±0.0769	0.0498 ±0.0366	-0.0019 ±0.0419	-0.1191 ±0.0477	-0.2253 ±0.0402	0.0683 ±0.0720
20	-0.0144 ±0.0306	0.0521 ±0.0266	-0.0156 ±0.0404	-0.0966 ±0.0336	-0.2079 ±0.0575	0.0123 ±0.0289
30	-0.0160 ±0.0302	0.0553 ±0.0253	-0.0163 ±0.0751	-0.0819 ±0.0385	-0.1490 ±0.0734	0.0285 ±0.0221
	CDM, tetracycline limited, plasmolysed cells					
1	-0.0978 ±0.0237	0.0031 ±0.0206	-0.7835 ±0.0544	-0.8031 ±0.0372	-0.8120 ±0.0159	0.0238 ±0.0226
3	-0.0632 ±0.0244	0.0741 ±0.0752	-0.6394 ±0.0373	-0.7320 ±0.0523	-0.7990 ±0.0327	0.0740 ±0.0411
6	0.0362 ±0.0352	0.1955 ±0.0281	-0.4837 ±0.0131	-0.7097 ±0.0208	-0.7867 ±0.1313	0.0231 ±0.0525
10	-0.0728 ±0.0220	0.1940 ±0.0561	-0.4564 ±0.0316	-0.7026 ±0.0536	-0.7829 ±0.0308	0.0418 ±0.0097
20	0.0217 ±0.0255	0.2812 ±0.0139	-0.3053 ±0.0248	-0.6722 ±0.0592	-0.6783 ±0.0737	0.0283 ±0.0290
30	-0.0720 ±0.0350	0.2771 ±0.0131	-0.2519 ±0.0100	-0.5941 ±0.0300	-0.6660 ±0.0921	0.0244 ±0.0157

Appendix 3 (continued)

(B) P. aeruginosa

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and							
	OS 346	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H inulin	^{3}H sucrose
	CDM grown, log phase, plasmolysed cells							
1	0.2295 ±0.0200	-0.4365 ±0.0405	-0.2806 ±0.0495	-0.3285 ±0.0836	-0.3740 ±0.1381	-0.3554 ±0.1306	-0.5167 ±0.0988	0.0492 ±0.0552
3	0.2694 ±0.0358	-0.4420 ±0.1296	-0.4081 ±0.0573	-0.4565 ±0.1428	-0.4349 ±0.1890	-0.3840 ±0.0795	-0.6127 ±0.0776	0.0566 ±0.0208
6	0.2768 ±0.0529	-0.5488 ±0.0347	-0.4451 ±0.0358	-0.5213 ±0.1323	-0.4686 ±0.1689	-0.4569 ±0.2154	-0.7190 ±0.0660	0.0416 ±0.0398
10	0.3093 ±0.0260	-0.6379 ±0.0993	-0.4106 ±0.0313	-0.5478 ±0.1939	-0.5747 ±0.1900	-0.4866 ±0.0652	-0.7374 ±0.1495	0.0383 ±0.0178
20	0.3060 ±0.0466	-0.7297 ±0.1540	-0.5000 ±0.0334	-0.7715 ±0.3356	-0.5829 ±0.1935	-0.5714 ±0.0316	-0.9453 ±0.1750	0.0846 ±0.0020
30	0.2575 ±0.2740	-0.7203 ±0.1354	-0.6631 ±0.1228	-0.8264 ±0.3676	-0.7465 ±0.3919	-0.6944 ±0.0335	-0.9990 ±0.0614	0.0229 ±0.0337
	CDM grown, log phase, unplasmolysed cells							
1	-0.1137 ±0.0699	-0.5187 ±0.0288	-0.6986 ±0.0358	-0.7945 ±0.0229	-0.4014 ±0.0185		-0.5233 ±0.1520	0.0392 ±0.0552
3	-0.1055 ±0.0177	-0.5249 ±0.0261	-0.7513 ±0.0122	-0.8006 ±0.1203	-0.5814		-0.5475 ±0.0362	0.0466 ±0.0208
6	-0.1066 ±0.0450	-0.5675 ±0.0399	-0.8408 ±0.0245		-0.6370 ±0.0573		-0.6187 ±0.0784	0.0516 ±0.0398

Appendix 3 (continued)

(B) P. aeruginosa

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and							
	OS 346	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H inulin	^{3}H sucrose
10	-0.1648 ±0.0302	-0.6049 ±0.0363	-0.8670 ±0.0255	-1.0284 ±0.0418	-0.7177 ±0.0729		-0.7402 ±0.0891	0.0483 ±0.0178
20	-0.1826 ±0.1017	-0.6769 ±0.0285	-0.9012 ±0.2256	-1.0359 ±0.2490	-1.0766 ±0.0456		-0.8019 ±0.1032	0.0348 ±0.0200
30	-0.1580 ±0.0860	-0.7591 ±0.0380	-0.9833 ±0.3711	-1.0996 ±0.3107	-1.1598 ±0.0321		-0.9671 ±0.0788	0.0230 ±0.0408
	CDM grown, stationary phase, plasmolysed cells							
1	0.0586 ±0.0521	-0.4501 ±0.0271	-0.3210 ±0.1227	-0.3195 ±0.1792	-0.4455 ±0.0643	-0.3844 ±0.1120	-0.4729 ±0.1647	0.0277 ±0.0353
3	0.1077 ±0.1116	-0.4216 ±0.0181	-0.3900 ±0.1631	-0.5023 ±0.1325	-0.4679 ±0.0390	-0.4367 ±0.0854	-0.6407 ±0.0414	0.0279 ±0.0553
6	0.1465 ±0.0827	-0.4595 ±0.0976	-0.4281 ±0.0902	-0.5461 ±0.1091	-0.5488 ±0.1010	-0.6134 ±0.1102	-0.7195 ±0.0824	-0.0166 ±0.0386
10	0.1276 ±0.1040	-0.4839 ±0.0392	-0.4695 ±0.0715	-0.5598 ±0.1750	-0.6516 ±0.0757	-0.6532 ±0.0536	-0.8228 ±0.0344	0.0361 ±0.0595
20	0.1630 ±0.0149	-0.5260 ±0.0415	-0.5857 ±0.0837	-0.6065 ±0.0153	-0.7638 ±0.0580	-0.8579 ±0.0066	-0.8778 ±0.2366	0.0316 ±0.0232
30	0.1334 ±0.1010	-0.6260 ±0.1214	-0.7947 ±0.0986	-0.8178 ±0.1105	-0.8740 ±0.0728	-1.0006 ±0.0164	-0.9300 ±0.1006	-0.0374 ±0.0362

Appendix 3 (continued)

(B) P. aeruginosa

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and							
	OS 346	OS 508	OS 670	OS 833	OS 955	OS 1157	^{3}H inulin	^{3}H sucrose
	CDM grown, stationary phase, unplasmolysed cells							
1	0.0088 ±0.0938	-0.4899 ±0.0629	-0.4851 ±0.0354	-0.5237 ±0.1203	-0.2834 ±0.0689	-0.7716 ±0.0320	-0.6073 ±0.0263	0.0146 ±0.0401
3	-0.0146 ±0.0306	-0.5374 ±0.1125	-0.4396 ±0.0389	-0.5555 ±0.2531	-0.6793 ±0.0113	-0.7962 ±0.0820	-0.7084 ±0.0454	0.0303 ±0.0574
6	-0.0700 ±0.0387	-0.5404 ±0.2138	-0.5514 ±0.0581	-0.6513 ±0.2159	-0.7852 ±0.0478	-0.8471 ±0.1282	-0.9231 ±0.0455	0.0499 ±0.0578
10	-0.0582 ±0.0342	-0.6265 ±0.1397	-0.5501 ±0.0239	-0.7071 ±0.0849	-0.8113 ±0.1154	-0.9021 ±0.1857	-0.9704 ±0.0543	-0.0076 ±0.0636
20	-0.0103 ±0.0949	-0.6779 ±0.0327	-0.6248 ±0.0405	-0.8778 ±0.0480	-0.8556 ±0.1725	-0.9302 ±0.0416	-1.0428 ±0.0772	-0.0034 ±0.0619
30	-0.1187 ±0.0509	-0.7963 ±0.0237	-0.7751 ±0.1151	-1.0290 ±0.0312	-1.2054 ±0.2640	-1.3132 ±0.0802	-1.0512 ±0.1052	-0.0019 ±0.0309
	Iron limited, plasmolysed cells							
1	0.0276 ±0.0948	-0.4894 ±0.1250	-0.3271 ±0.0471	-0.3414 ±0.0154	-0.5154 ±0.1322	-0.4081 ±0.0805	-0.2029 ±0.0075	-0.0210 ±0.0124
3	0.0627 ±0.1564	-0.5493 ±0.1395	-0.3830 ±0.1724	-0.3724 ±0.0194	-0.6115 ±0.0514	-0.4479 ±0.0980	-0.2707 ±0.0325	-0.0358 ±0.0206
6	0.1014 ±0.0924	-0.5856 ±0.1123	-0.4137 ±0.0926	-0.5314 ±0.2415	-0.6595 ±0.1058	-0.5029 ±0.0307	-0.3950 ±0.0766	0.0203 ±0.0172

Appendix 3 (continued)

(B) P. aeruginosa

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and							
	OS 346	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H inulin	^{3}H sucrose
10	0.1506 ±0.0969	-0.6705 ±0.1173	-0.5238 ±0.1998	-0.7933 ±0.0937	-0.7127 ±0.1036	-0.5531 ±0.0365	-0.4126 ±0.0473	0.0376 ±0.0118
20	0.1931 ±0.0693	-0.7579 ±0.1969	-0.6322 ±0.3483	-0.8308 ±0.0304	-0.8095 ±0.1287	-0.5519 ±0.0398	-0.5024 ±0.0740	0.0607 ±0.0145
30	0.1767 ±0.0274	-0.8058 ±0.1664	-0.6600 ±0.2526	-0.8803 ±0.0495	-0.8691 ±0.0693	-0.6793 ±0.0513	-0.5632 ±0.0472	0.0910 ±0.0099
	Iron limited, unplasmolysed cells							
1	-0.0788 ±0.1098	-0.2969 ±0.0132	-0.5163 ±0.0886	-0.4489 ±0.0300	-0.5000 ±0.0397	-0.4782 ±0.0438		-0.0068 ±0.0679
3	-0.0868 ±0.0745	-0.3366 ±0.1134	-0.6424 ±0.0529	-0.5263 ±0.0949	-0.5619 ±0.0400	-0.5375 ±0.0363		-0.0060 ±0.0751
6	-0.0717 ±0.1039	-0.3502 ±0.0854	-0.6733 ±0.0415	-0.5423 ±0.0493	-0.6124 ±0.0608	-0.6010 ±0.0302		0.0127 ±0.0595
10	-0.0556 ±0.1037	-0.4144 ±0.0325	-0.7113 ±0.0306	-0.5633 ±0.0324	-0.6709 ±0.0419	-0.6664 ±0.0503		0.0116 ±0.0939
20	-0.0790 ±0.0921	-0.5742 ±0.0638	-0.7333 ±0.0306	-0.5883 ±0.0487	-0.7162 ±0.1204	-0.7067 ±0.0296		0.0261 ±0.0595
30	-0.1108 ±0.0699	-0.6439 ±0.1027	-0.7966 ±0.0185	-0.6526 ±0.0359	-0.8468 ±0.0438	-0.7358 ±0.0403		0.0450 ±0.0773

Appendix 3 (continued)

(B) P. aeruginosa

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and OS 346	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H inulin	^{3}H sucrose
	Nutrient broth grown, log phase, plasmolysed cells							
1	0.0750 ±0.0080	-0.2210 ±0.0629	-0.3327 ±0.0417	-0.3275 ±0.0423	-0.3611 ±0.0946		-0.7036 ±0.0732	-0.0522 ±0.0398
3	0.0786 ±0.0080	-0.2501 ±0.0830	-0.3451 ±0.0149	-0.4843 ±0.0613	-0.4415 ±0.1408		-0.8327 ±0.0408	-0.1196 ±0.0341
6	0.1611 ±0.0110	-0.2574 ±0.0901	-0.4058 ±0.0588	-0.5416 ±0.0107	-0.5324 ±0.1328		-0.8439 ±0.0583	-0.0752 ±0.0110
10	0.1186 ±0.0120	-0.2979 ±0.0229	-0.4356 ±0.0508	-0.6097 ±0.0300	-0.5391 ±0.1565		-0.8833 ±0.0737	-0.0762 ±0.0315
20	0.0715 ±0.0060	-0.3966 ±0.0407	-0.4354 ±0.0325	-0.6566 ±0.0216	-0.7474 ±0.2847		-1.0133 ±0.0598	-0.0314 ±0.0108
30	0.1465 ±0.0120	-0.4639 ±0.0897	-0.4913 ±0.0074	-0.9022 ±0.0699	-0.7987 ±0.1764		-1.0826 ±0.0672	-0.0426 ±0.0405
	Nutrient broth grown, stationary phase, plasmolysed cells							
1	-0.0434 ±0.0754	-0.1537 ±0.0550	-0.1286 ±0.0280	-0.2514 ±0.1063	-0.3509 ±0.1472	-0.5003 ±0.0519		-0.0198 ±0.0492
3	0.0181 ±0.0758	-0.1727 ±0.0231	-0.1688 ±0.0303	-0.2928 ±0.1214	-0.4534 ±0.2108	-0.5512 ±0.0280		-0.0279 ±0.0160
6	0.0917 ±0.0774	-0.1924 ±0.0494	-0.1869 ±0.0557	-0.3491 ±0.1174	-0.5176 ±0.2542	-0.5787 ±0.0405		-0.0705 ±0.0109

Appendix 3 (continued)

(B) P. aeruginosa

Length of incubation (minutes)	F ± f for cells incubated with ^{14}C sucrose and							
	OS 346	OS 508	OS 670	OS 833	OS 995	OS 1157	^{3}H inulin	^{3}H sucrose
10	0.0712 ±0.0774	-0.2447 ±0.0350	-0.2099 ±0.0757	-0.3872 ±0.1590	-0.5126 ±0.3082	-0.6409 ±0.0248		-0.0100 ±0.0262
20	0.0955 ±0.0685	-0.2572 ±0.0135	-0.2459 ±0.1169	-0.3849 ±0.0612	-0.6208 ±0.2719	-0.8048 ±0.0283		-0.0530 ±0.0464
30	0.1226 ±0.0869	-0.3615 ±0.0394	-0.3054 ±0.0914	-0.4486 ±0.1081	-0.6302 ±0.2489	-0.8106 ±0.0569		-0.0773 ±0.0218

a. The methods used for obtaining the data will be found in section 3.4.

b. All results represent the mean of data obtained on at least two and generally three or four separate occasions.

Appendix 4

Data giving the final location of radiolabelled material in the double label assay after an initial incubation of 10 or 30 minutes with E. coli W3110 or P. aeruginosa 6750 grown under various conditions.[a]

(A) E. coli W3110

Stage of assay analysed[b]	^{3}H material (% of total added[c] ± standard deviation)			^{14}C material (% of total added[c] ± standard deviation)		
	Log phase 30 min[d]	Magnesium limited 10 min	30 min	Log phase 30 min	Magnesium limited 10 min	30 min
Cells incubated with ^{3}H isomaltotriitol and ^{14}C sucrose						
Initial supernatant (first discard)	77.1 ± 4.2	80.5 ± 5.2	81.0 ± 2.9	78.6 ± 1.5	81.8 ± 4.7	82.7 ± 1.1
Washing (second discard)	14.1 ± 3.2	12.5 ± 2.3	10.3 ± 1.8	13.3 ± 1.6	13.1 ± 0.8	10.4 ± 1.0
Final supernatant (efflux after 30 mins)	4.0 ± 2.7	3.4 ± 1.9	2.9 ± 1.9	4.7 ± 0.9	3.4 ± 0.6	2.9 ± 0.7
Cell pellet	1.3 ± 2.6	1.9 ± 1.9	1.5 ± 1.9	2.1 ± 0.5	1.0 ± 0.5	0.7 ± 0.5
Total label recovered	96.5 ± 12.7	98.3 ± 10.3	95.7 ± 8.5	98.7 ± 4.5	99.3 ± 6.6	96.7 ± 3.3
Cells incubated with ^{3}H isomaltotetritol and ^{14}C sucrose						
Initial supernatant (first discard)	78.8 ± 5.5	79.7 ± 1.9	81.4 ± 3.8	72.8 ± 3.3	81.8 ± 2.5	84.8 ± 4.2
Washing (second discard)	11.8 ± 3.8	10.6 ± 2.1	9.6 ± 2.3	12.7 ± 2.5	13.1 ± 2.1	11.5 ± 1.7
Final supernatant (efflux after 30 mins)	4.9 ± 3.3	4.2 ± 1.7	4.1 ± 2.0	4.5 ± 1.4	3.6 ± 1.5	3.2 ± 1.6
Cell pellet	2.4 ± 3.3	3.0 ± 2.0	2.0 ± 2.5	3.5 ± 2.5	0.9 ± 1.4	0.8 ± 1.4
Total label recovered	96.9 ± 15.9	97.5 ± 8.7	97.1 ± 10.6	93.5 ± 9.7	99.4 ± 8.5	100.3± 8.9

Appendix 4 (continued)

(a) E. coli W3110

Stage of assay analysed[b]	^{3}H material (% of total added[c] ± standard deviation)			^{14}C material (% of total added[c] ± standard deviation)		
	Log phase	Magnesium limited		Log phase	Magnesium limited	
	30 min[d]	10 min	30 min	30 min	10 min	30 min
Cells incubated with ^{3}H isomaltopentitol and ^{14}C sucrose						
Initial supernatant (first discard)	76.7 ± 3.1	82.0 ± 2.1	83.4 ± 1.7	88.5 ± 3.3	78.7 ± 4.2	80.7 ± 4.7
Washing (second discard)	11.8 ± 1.3	10.6 ± 2.2	10.7 ± 2.4	7.5 ± 2.8	13.5 ± 3.8	13.0 ± 4.1
Final supernatant (efflux after 30 mins)	4.0 ± 1.4	3.2 ± 1.8	3.5 ± 1.9	4.5 ± 2.1	3.5 ± 3.6	3.2 ± 3.7
Cell pellet	6.0 ± 2.1	1.4 ± 1.9	1.9 ± 1.9	6.0 ± 5.9	0.9 ± 3.5	1.1 ± 3.6
Total label recovered	98.5 ± 7.9	97.2 ± 8.0	99.5 ± 7.9	103.5 ± 14.1	96.6 ± 15.1	98.0 ± 16.1
Cells incubated with ^{3}H isomaltohexitol and ^{14}C sucrose						
Initial supernatant (first discard)	81.0 ± 4.4	83.2 ± 2.5	83.0 ± 2.7	79.5 ± 3.7	81.8 ± 4.2	82.3 ± 4.6
Washing (second discard)	12.3 ± 3.0	10.4 ± 1.5	10.6 ± 2.1	11.2 ± 2.6	13.6 ± 3.1	13.1 ± 3.1
Final supernatant (efflux after 30 mins)	3.0 ± 2.4	2.5 ± 1.5	2.8 ± 1.6	5.6 ± 1.4	3.7 ± 2.7	3.6 ± 2.7
Cell pellet	2.6 ± 2.4	1.3 ± 1.5	1.6 ± 1.6	5.0 ± 3.3	1.0 ± 2.6	1.4 ± 2.7
Total label recovered	98.9 ± 12.2	97.4 ± 7.0	98.0 ± 8.0	101.3 ± 11.0	100.1 ± 12.6	100.4 ± 13.1

Appendix 4 (continued)

(A) *E. coli* W3110

Stage of assay analysed[b]	^{3}H material (% of total added[c] ± standard deviation)			^{14}C material (% of total added[c] ± standard deviation)		
	Log phase 30 min[d]	Magnesium limited 10 min	30 min	Log phase 30 min	Magnesium limited 10 min	30 min
Cells incubated with ^{3}H isomaltoheptitol and ^{14}C sucrose						
Initial supernatant (first discard)	83.2 ± 6.5	74.2 ± 2.1	78.8 ± 1.0	77.0 ± 0.6	68.9 ± 0.6	71.7 ± 1.6
Washing (second discard)	9.8 ± 4.0	17.2 ± 0.5	13.1 ± 1.9	12.0 ± 1.5	17.3 ± 1.4	13.4 ± 0.9
Final supernatant (efflux after 30 mins)	2.3 ± 3.4	5.0 ± 0.9	4.5 ± 0.7	3.7 ± 0.8	5.5 ± 1.0	4.9 ± 0.5
Cell pellet	1.3 ± 3.4	1.7 ± 0.5	1.8 ± 0.7	2.6 ± 0.8	2.0 ± 0.6	2.2 ± 0.7
Total label recovered	96.6 ± 17.3	98.1 ± 4.0	98.2 ± 4.2	95.3 ± 3.7	93.7 ± 2.6	92.2 ± 3.7
Cells incubated with ^{3}H sucrose and ^{14}C sucrose						
Initial supernatant (first discard)	78.8 ± 5.1	77.9 ± 2.3	74.2 ± 1.5	78.3 ± 2.7	77.1 ± 2.8	73.7 ± 1.8
Washing (second discard)	13.6 ± 4.5	14.2 ± 1.3	16.4 ± 2.1	13.1 ± 1.2	14.2 ± 1.1	16.2 ± 0.7
Final supernatant (efflux after 30 mins)	4.1 ± 4.2	4.7 ± 1.1	5.4 ± 0.9	4.0 ± 0.6	4.7 ± 0.8	5.3 ± 0.5
Cell pellet	4.6 ± 4.2	2.5 ± 1.1	2.5 ± 0.9	5.5 ± 0.7	2.1 ± 0.6	2.2 ± 0.5
Total label recovered	101.0± 18.0	99.3 ± 5.8	98.5 ± 5.4	100.9± 4.2	98.1 ± 5.3	97.5 ± 2.5

Appendix 4 (continued)

(B) *P. aeruginosa* 6750

Stage of assay analysed	^{3}H material (% of total added ± standard deviation)				^{14}C material (% of total added ± standard deviation)			
	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins
Cells incubated with ^{3}H isomaltitol and ^{14}C sucrose								
Initial supernatant (first discard)	81.5 ± 2.5	85.4 ± 2.6	83.6 ± 4.3	79.4 ± 2.7	79.8 ± 0.9	84.2 ± 1.4	86.2 ± 1.3	83.9 ± 2.7
Washing (second discard)	9.1 ± 1.6	8.9 ± 1.8	8.6 ± 1.9	9.8 ± 1.4	8.0 ± 1.4	8.4 ± 0.5	8.8 ± 1.7	10.0 ± 1.6
Final supernatant (efflux after 30 mins)	4.2 ± 0.8	3.7 ± 2.0	2.5 ± 1.6	3.4 ± 1.5	3.6 ± 0.8	3.7 ± 0.9	2.1 ± 1.4	2.7 ± 1.3
Cell pellet	1.4 ± 0.9	1.2 ± 1.5	1.2 ± 2.3	1.4 ± 1.5	5.2 ± 1.7	0.9 ± 0.5	1.1 ± 1.4	1.2 ± 1.3
Total label recovered	96.2 ± 5.8	99.2 ± 7.9	95.9 ± 10.1	94.0 ± 7.1	96.6 ± 4.8	97.2 ± 0.5	98.2 ± 5.8	97.8 ± 6.9
Cells incubated with ^{3}H isomaltotriitol and ^{14}C sucrose								
Initial supernatant (first discard)	83.3 ± 1.6	85.3 ± 3.1	85.6 ± 4.2	82.8 ± 4.9	81.0 ± 3.3	79.4 ± 2.7	86.4 ± 2.3	83.2 ± 3.7
Washing (second discard)	8.8 ± 0.6	8.8 ± 2.0	10.0 ± 1.6	10.1 ± 1.4	7.7 ± 2.6	8.3 ± 1.9	9.6 ± 1.4	10.0 ± 1.4
Final supernatant (efflux after 30 mins)	2.1 ± 0.1	3.1 ± 2.1	2.0 ± 1.5	2.1 ± 1.4	3.6 ± 3.1	3.9 ± 2.1	2.3 ± 1.5	2.6 ± 1.3
Cell pellet	1.3 ± 0.3	1.2 ± 1.9	0.8 ± 1.4	0.8 ± 1.5	5.0 ± 3.6	4.5 ± 2.2	1.1 ± 1.4	1.2 ± 1.5
Total label recovered	95.5 ± 2.6	98.4 ± 9.1	98.4 ± 8.7	95.8 ± 9.2	97.3 ± 11.6	96.1 ± 8.9	99.4 ± 6.6	97.0 ± 7.9

Appendix 4 (continued)

(B) *P. aeruginosa* 6750

Stage of assay analysed	^{3}H material (% of total added ± standard deviation)				^{14}C material (% of total added ± standard deviation)			
	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins
Cells incubated with ^{3}H isomaltotetritol and ^{14}C sucrose								
Initial supernatant (first discard)	83.7 ± 3.8	86.8 ± 6.3	86.9 ± 5.7	86.4 ± 6.1	76.3 ± 2.1	77.6 ± 1.3	87.8 ± 3.4	84.4 ± 2.4
Washing (second discard)	8.6 ± 1.8	9.3 ± 2.2	9.9 ± 5.1	10.8 ± 4.9	7.6 ± 1.6	8.2 ± 1.1	9.8 ± 1.4	10.6 ± 1.5
Final supernatant (efflux after 30 mins)	2.0 ± 1.6	3.0 ± 1.7	2.1 ± 4.6	2.2 ± 4.6	3.6 ± 1.3	4.8 ± 1.1	2.4 ± 1.1	2.8 ± 1.1
Cell pellet	1.8 ± 1.7	1.4 ± 1.6	0.7 ± 4.6	0.8 ± 4.7	2.9 ± 3.3	3.9 ± 1.5	0.9 ± 1.1	1.3 ± 1.1
Total label recovered	96.1 ± 8.9	100.5 ± 11.8	99.6 ± 20.0	100.2 ± 20.3	90.4 ± 8.3	94.5 ± 5.0	100.9 ± 7.0	99.1 ± 6.1
Cells incubated with ^{3}H isomaltopentitol and ^{14}C sucrose								
Initial supernatant (first discard)	80.6 ± 3.3	83.3 ± 1.9	84.7 ± 3.7	82.7 ± 6.5	76.0 ± 6.4	83.4 ± 3.3	83.2 ± 3.3	82.2 ± 4.1
Washing (second discard)	9.1 ± 2.6	8.3 ± 1.5	9.7 ± 1.9	10.2 ± 1.1	9.7 ± 3.5	8.6 ± 2.6	9.6 ± 2.4	10.2 ± 2.2
Final supernatant (efflux after 30 mins)	2.2 ± 2.2	2.8 ± 1.6	1.8 ± 1.1	2.0 ± 1.1	2.8 ± 3.0	3.5 ± 2.3	2.2 ± 1.7	2.7 ± 1.7
Cell pellet	5.4 ± 2.8	1.4 ± 1.8	0.6 ± 1.2	0.8 ± 1.2	3.1 ± 3.6	1.9 ± 2.5	0.9 ± 1.7	1.2 ± 1.7
Total label recovered	97.3 ± 10.9	95.8 ± 6.8	96.8 ± 7.9	95.7 ± 10.9	91.6 ± 16.6	97.4 ± 10.7	95.9 ± 9.1	96.3 ± 9.7

Appendix 4 (continued)

(B) *P. aeruginosa* 6750

Stage of assay analysed	^{3}H material (% of total added ± standard deviation)				^{14}C material (% of total added ± standard deviation)			
	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins
Cells incubated with ^{3}H isomaltohexitol and ^{14}C sucrose								
Initial supernatant (first discard)	86.8 ± 3.5	87.5 ± 2.1	84.6 ± 4.8	81.2 ± 3.8	79.2 ± 2.9	81.3 ± 1.8	83.5 ± 1.0	83.0 ± 1.7
Washing (second discard)	8.9 ± 1.0	9.4 ± 1.3	9.6 ± 3.9	11.2 ± 3.8	6.6 ± 3.3	8.8 ± 1.2	9.3 ± 1.4	11.2 ± 1.1
Final supernatant (efflux after 30 mins)	2.4 ± 0.8	2.9 ± 0.9	1.8 ± 3.5	2.5 ± 3.3	3.3 ± 2.8	4.3 ± 1.0	2.4 ± 0.9	3.4 ± 0.8
Cell pellet	3.1 ± 0.9	0.9 ± 0.9	0.8 ± 3.6	0.6 ± 3.4	2.2 ± 3.4	2.5 ± 1.0	1.1 ± 1.1	1.0 ± 0.8
Total label recovered	101.2 ± 6.2	100.7 ± 5.2	96.8 ± 15.7	95.5 ± 14.3	91.3 ± 12.4	96.9 ± 5.0	96.3 ± 4.4	98.6 ± 4.4
Cells incubated with ^{3}H isomaltoheptitol and ^{14}C sucrose								
Initial supernatant (first discard)	83.9 ± 2.0	82.9 ± 1.0	83.3 ± 1.5	79.6 ± 1.1	75.6 ± 5.9	84.0 ± 3.9	85.9 ± 3.9	81.6 ± 2.2
Washing (second discard)	9.5 ± 1.1	8.8 ± 1.0	9.2 ± 1.0	10.9 ± 1.4	6.9 ± 2.3	8.3 ± 1.5	9.4 ± 2.5	11.1 ± 2.7
Final supernatant (efflux after 30 mins)	1.8 ± 0.9	2.8 ± 0.3	1.8 ± 0.8	2.6 ± 0.7	3.8 ± 2.3	3.8 ± 1.2	2.4 ± 2.3	3.5 ± 2.3
Cell pellet	4.8 ± 1.4	3.2 ± 0.2	0.9 ± 0.6	0.5 ± 0.6	3.0 ± 2.7	3.4 ± 1.3	1.2 ± 2.2	1.0 ± 2.2
Total label recovered	100.0 ± 5.4	97.7 ± 2.5	95.2 ± 3.9	93.6 ± 3.8	89.3 ± 13.2	99.5 ± 7.9	98.9 ± 10.9	97.2 ± 9.4

Appendix 4 (continued)

(B) *P. aeruginosa* 6750

Stage of assay analysed	^{3}H material (% of total added ± standard deviation)				^{14}C material (% of total added ± standard deviation)			
	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins	Log phase 30 mins	Fe limited 30 mins	Nutrient broth stationary phase 10 mins	Nutrient broth stationary phase 30 mins
Cells incubated with ^{3}H inulin and ^{14}C sucrose								
Initial supernatant (first discard)	82.7 ± 2.9	92.0 ± 7.1	83.2 ± 4.5	86.1 ± 2.5	77.2 ± 3.8	83.2 ± 3.0	76.6 ± 3.7	78.4 ± 2.9
Washing (second discard)	9.0 ± 1.2	9.6 ± 5.7	10.8 ± 0.3	10.0 ± 0.8	6.4 ± 2.4	8.9 ± 3.1	10.4 ± 2.3	9.4 ± 2.5
Final supernatant (efflux after 30 mins)	1.7 ± 1.2	2.4 ± 5.0	2.8 ± 0.3	2.6 ± 0.4	3.8 ± 2.4	3.8 ± 2.6	3.6 ± 1.9	3.4 ± 1.9
Cell pellet	2.9 ± 1.6	1.7 ± 5.1	4.2 ± 1.0	4.1 ± 0.5	3.2 ± 4.0	2.0 ± 2.6	5.4 ± 3.0	4.2 ± 2.0
Total label recovered	96.3 ± 6.9	105.7 ± 22.9	101.0 ± 6.1	102.8 ± 4.2	90.6 ± 12.6	97.9 ± 11.3	96.0 ± 10.9	95.4 ± 9.3
Cells incubated with ^{3}H sucrose and ^{14}C sucrose								
Initial supernatant (first discard)	83.3 ± 2.1	81.0 ± 4.2	77.1 ± 2.0	82.7 ± 3.6	82.1 ± 2.5	81.4 ± 3.7	78.5 ± 3.9	82.4 ± 6.4
Washing (second discard)	10.0 ± 1.5	8.7 ± 2.6	11.8 ± 1.5	10.5 ± 1.9	8.5 ± 2.8	8.4 ± 2.9	11.4 ± 2.5	9.6 ± 2.4
Final supernatant (efflux after 30 mins)	3.3 ± 1.5	4.3 ± 2.3	3.9 ± 1.1	3.4 ± 1.0	3.2 ± 2.2	4.3 ± 2.6	3.6 ± 2.4	3.1 ± 2.6
Cell pellet	4.4 ± 1.9	2.1 ± 2.2	4.5 ± 0.9	4.6 ± 1.1	4.3 ± 2.3	2.2 ± 2.5	4.2 ± 2.6	3.8 ± 3.2
Total label recovered	101.0 ± 7.0	96.1 ± 11.3	97.3 ± 5.5	101.2 ± 7.6	98.1 ± 9.8	96.3 ± 11.7	97.7 ± 11.4	98.9 ± 14.6

Appendix 4 (continued)

Values of F determined when comparing the average amount of each radiolabelled sugar located in the final supernatant or the cell pellet obtained with E. coli or P. aeruginosa.

Stage of assay	Calculated F[e]	F(P = 0.05)
E. coli		
Final supernatant	6.88	2.74
Cell pellet	4.27	2.74
P. aeruginosa		
Final supernatant	14.46	2.41
Cell pellet	3.93	2.41

a. The method used in obtaining this data is described in 3.5.2.

b. These refer to the four stages of the double label assay; the supernatant obtained when the cells were centrifuged for the first time, immediately after incubation with the radiolabelled material; the supernatant obtained after the cells have been resuspended in fresh phosphate buffer and again centrifuged, to wash the cells; the supernatant obtained after the cells have been resuspended in water and incubated for 30 minutes to allow radiolabelled material in the periplasmic space to efflux out (the ratio is normally determined at this stage); and the cell pellet remaining after removal of the final supernatant.

c. The total radioactivity originally added to the cell suspension was measured separately, in triplicate, and the two values calculated (dpm ^{3}H, dpm ^{14}C) and used as 100% for the measurement of the appropriate label. The dpm of each label in every stage of the assay was measured and expressed as a percentage of the 100% value for dpm.

d. The time values represent the length of the initial incubation period.

e. The formula for F will be found in Appendix 2. The means for calculating F were determined by calculating the average percentage of a given molecule found in a specific part of the assay, hence 7 overall means were obtained for E. coli, and 9 for P. aeruginosa. A two tailed test was used for values of F($p = 0.05$).

References

Aldea, M., E. Herrero, M. I. Esteve and R. Guerrero. 1980. Surface density of major outer membrane proteins in Salmonella typhimurium in different growth conditions. J. Gen. Microbiol. 120: 355-368.

Ames, G. F., E. N. Spudick and H. Nikaido. 1974. Protein composition of the outer membrane of Salmonella typhimurium: effect of lipopolysaccharide mutations. J. Bacteriol. 117: 406-416.

Angus, B. A., A. M. Carey, D. A. Caron, A. M. B. Kropinski and R. E. W. Hancock. 1982. Outer membrane permeability of Pseudomonas aeruginosa: comparison of a wild type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. 21: 299-309.

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

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

Bayer, M. E. 1968a. Areas of adhesion between wall and membrane of Escherichia coli. J. Gen. Microbiol. 53: 395-404.

Bayer, M. E. 1968b. Adsorption of bacteriophages to adhesions between wall and membrane of Escherichia coli. J. Virol. 2: 346-356.

Bayer, M. E., J. Koplow and H. Goldfine. 1975. Alterations in envelope structure of heptose-deficient mutants of Escherichia coli as revealed by freeze-etching. Proc. Natl. Acad. Sci. U.S.A. 72: 5145-5149.

Begg, K. J. 1978. Cell surface growth in Escherichia coli: distribution of matrix protein. J. Bacteriol. 135: 307-310.

Begg, K. J. and W. D. Donachie. 1977. Growth of the Escherichia coli cell surface. J. Bacteriol. 129: 1524-1536.

Benz, R. and R. E. W. Hancock. 1981. Properties of the large ion-permeable pores formed from protein F of Pseudomonas aeruginosa in lipid bilayer membranes. Biochim. Biophys. Acta 646: 298-308.

Benz, R., J. Ishii and T. Nakae. 1980. Determination of ion permeability through the channels made of porins from the outer membrane of Salmonella typhimurium in lipid bilayer membranes. J. Membr. Biol. 56: 19-29.

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

Bewick, M. A. and T. C. Y. Lo. 1979. Dicarboxylic acid transport 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.

Bewick, M. A. and T. C. Y. Lo. 1980. Localisation of the dicarboxylate binding protein in the cell envelope of Escherichia coli K12. Can. J. Biochem. 58: 885-897.

Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

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

Booth, B. R. and N. A. C. Curtis. 1977. Separation of the cytoplasmic and outer membranes of Pseudomonas aeruginosa PAO1. Biochem. Biophys. Res. Commun. 74: 1168-1176.

Boyd, A. and I. B. Holland. 1979. Regulation of the synthesis of surface protein in the cell cycle of E. coli B/r. Cell 18: 287-296.

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

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

Braun, V., R. E. W. Hancock, K. Hantke and A. Hartmann. 1976. Functional organisation of the outer membrane of Escherichia coli: phage and colicin receptors as components of iron uptake systems. J. Supramotec. Struct. 5: 37-58.

Braun, V. and H.-J. Krieger-Brauer. 1977. Interrelationship of the phage λ receptor protein and maltose transport in mutants of Escherichia coli K12. Biochim. Biophys. Acta 469: 89-98.

Braun, V., K. Rehn and H. Wolff. 1970. Supramolecular structure of the rigid layer of the cell wall of Salmonella, Serratia, Proteus and Pseudomonas fluorescens. Number of lipoprotein molecules in a membrane layer. Biochemistry 9: 5041-5049.

Brown, M. R. W. 1975. A question of resistance. Pharmaceut. J. 215: 239-242.

Brown, M. R. W., J. A. Farwell and S. A. Rosenbluth. 1969. Use of membrane filters for measurement of 260 mμ-absorbing substances from bacterial cultures. Annal. Biochem. 27: 484-491.

Brown, M. R. W. and E. R. Garrett. 1964. Kinetics and mechanisms of action of antibiotics on microorganisms. 1. Reproducibility of Escherichia coli growth curves and dependence upon tetracycline concentration. J. Pharm. Sci. 53: 179-183.

Brown, M. R. W. and J. Melling. 1969. Role of divalent cations in the action of polymyxin B and EDTA on Pseudomonas aeruginosa. J. Gen. Microbiol. 59: 263-274.

Brown, M. R. W. and W. M. Watkins. 1970. Low magnesium and phospholipid content of cell walls of Pseudomonas aeruginosa resistant to polymyxin. Nature 227: 1360-1361.

Caulcott, E. 1973. Significance Tests. Routledge and Kegan Paul, London.

Chai, T. J. and J. Foulds. 1979. Isolation and partial characterisation of protein E, a major protein found in certain Escherichia coli K12 mutant strains: relationship to other membrane proteins. J. Bacteriol. 139: 418-423.

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

Cronan, J. E. 1979. Phospholipid synthesis and assembly, p. 35-65. In M. Inouye (ed.) Bacterial Outer Membranes: Biogenesis and Functions. John Wiley-Interscience, New York.

Cronan, J. E. and E. P. Gelmann. 1975. Physical properties of membrane lipids: biological relevance and regulation. Bacteriol. Revs. 39: 232-256.

Datta, D. B., B. Arden and U. Henning. 1977. Major proteins of the Escherichia coli outer cell envelope membrane as bacteriophage receptors. J. Bacteriol. 131: 821-829.

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

Davis, B. D. and L. Leive. 1973. Bacterial physiology: the cell envelope and spores, p. 107-146. In B. D. Davis, R. Dulbecco, H. N. Gisen, H. S. Ginsberg and W. B. Wood Jr. (eds.) Microbiology. Harper and Row, USA.

Dawes, E. A., M. Midgley and P. H. Whiting. 1976. Control of transport systems for glucose, gluconate and 2-oxo-gluconate, and of glucose metabolism in Pseudomonas aeruginosa, p. 195-207. In A. C. R. Dean, D. C. Ellwood, C. G. T. Evans and J. Melling (eds.), Continuous Culture 6: Applications and New Fields. Ellis Horwood, London.

Decad, G., T. Nakae and H. Nikaido. 1974. Permeability of Escherichia coli and Salmonella typhimurium cell wall to oligosaccharides. Fed. Proc. 33: 1240.

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

DeMartini, M. and M. Inouye. 1978. Interaction between two major outer membrane proteins of Escherichia coli: the matrix protein and the lipoprotein. J. Bacteriol. 133: 329-335.

DePetris, S. 1967. Ultrastructure of the cell wall of Escherichia coli and chemical nature of its constituent layers. J. Ultrastruc. Res. 19: 45-83.

DiRienzo, J. M., K. Nakamura and M. Inouye. 1978. The outer membrane proteins of Gram-negative bacteria: biosynthesis, assembly and functions. Annu. Rev. Biochem. 47: 481-532.

Ferenci, T. 1980. The recognition of maltodextrins by Escherichia coli. Eur. J. Biochem. 108: 631-636.

Ferenci, T. and W. Boos. 1980. The role of the Escherichia coli λ receptor in the transport of maltose and maltodextrins. J. Supramolec. Struct. 13: 101-116.

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

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

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

Funahara, Y. and H. Nikaido. 1980. Asymmetric localisation of lipopolysaccharides on the outer membrane of Salmonella typhimurium. J. Bacteriol. 141: 1463-1465.

Fung, J., J. T. MacAlister and L. I. Rothfield. 1978. Role of murein lipoprotein in morphogenesis of the bacterial division septum: phenotypic similarity of IkyD and Ipo mutants. J. Bacteriol. 133: 1467-1471.

Garten, W. and U. Henning. 1974. Cell envelope and shape of Escherichia coli K12. Isolation and preliminary characterisation of the major ghost-membrane proteins. Eur. J. Biochem. 47: 343-352.

Gilleland, H. E. Jr., J. D. Stinnett, I. L. Roth and R. G. Eagon. 1973. Freeze-etching study of Pseudomonas aeruginosa: localisation within the cell wall of an ethylenediaminetetraacetate-extractable component. J. Bacteriol. 113: 417-432.

Gilvarg, C. and E. Katchalski. 1965. Peptide utilisation in Escherichia coli. J. Biol. Chem. 240: 3093-3098.

Gmeiner, J., H.-P. Kroll and H. H. Martin. 1978. The covalent rigid-layer lipoprotein in cell walls of Proteus mirabilis. Eur. J. Biochem. 83: 227-233.

Gray, G. W. and S. G. Wilkinson. 1965. The effect of ethylene-diaminetetraacetic acid on the cell walls of some Gram-negative bacteria. J. Gen. Microbiol. 39: 385-399.

Gustafsson, P., K. Nordstrom and S. Normark. 1973. Outer penetration barrier of Escherichia coli K-12: kinetics of the uptake of gentian violet by wild type and envelope mutants. J. Bacteriol. 116: 893-900.

Halegoua, S., A. Hirashima and M. Inouye. 1974. Existence of a free form of a specific membrane lipoprotein in Gram-negative bacteria. J. Bacteriol. 120: 1204-1208.

Hall, M. N. and T. J. Silhavy. 1979. Transcriptional regulation of Escherichia coli K-12 major outer membrane protein 1b. J. Bacteriol. 140: 342-350.

Hall, M. N. and T. J. Silhavy. 1981a. The ompB locus and the regulation of the major outer membrane porin proteins of Escherichia coli K12. J. Mol. Biol. 145: 1-22.

Hall, M. N. and T. J. Silhavy. 1981b. Genetic analysis of the ompB locus in Escherichia coli K12. J. Mol. Biol. 151: 1-16.

Hamilton Miller, J. M. T. 1965. Effect of EDTA upon bacterial permeability to benzyl penicillin. Biochem. Biophys. Res. Commun. 20: 688-691.

Hancock, R. E. W. and A. M. Carey. 1979. Outer membrane of Pseudomonas aeruginosa - heat and 2 mercaptoethanol modifiable proteins. J. Bacteriol. 140: 902-910.

Hancock, R. E. W. and A. M. Carey. 1980. Protein D1 - a glucose inducible pore forming protein from the outer membrane of Pseudomonas aeruginosa. FEMS Microb. Lett. 8: 105-109.

Hancock, R. E. W., G. M. Decad and H. Nikaido. 1979. Identification of the protein producing transmembrane diffusion pores in the outer membrane of Pseudomonas aeruginosa PAO1. Biochim. Biophys. Acta 554: 323-331.

Hancock, R. E. W., K. Hantke and V. Braun. 1976. Iron transport in Escherichia coli K12: involvement of the colicin B receptor and of a citrate-inducible protein. J. Bacteriol. 127: 1370-1375.

Hancock, R. E. W., R. T. Irvin, J. W. Costerton and A. M. Carey. 1981. Pseudomonas aeruginosa outer membrane peptidoglycan associated proteins. J. Bacteriol. 145: 628-631.

Hancock, R. E. W. and H. Nikaido. 1978. Outer membranes of Gram-negative bacteria. XIX. Isolation from Pseudomonas aeruginosa PAO1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. 136: 381-390.

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

Hantke, K. and V. Braun. 1975. Membrane receptor dependent iron transport in Escherichia coli. FEBS Lett. 49: 301-305.

Harder, K. J., H. Nikaido and M. Matsuhashi. 1981. Mutants of Escherichia coli that are resistant to certain beta lactam compounds lack the ompF porin. Antimicrob. Agents Chemother. 20: 549-552.

Hatt, B. W. 1970. The continuous separation of carbohydrates. Ph.D. Thesis, University of Birmingham.

Heasley, F. A. 1980. Reconstitution and characteristics of the N. gonorrhoea outer membrane permeability barrier. EMBO Workshop, Genetics and Immunobiology of Pathogenic Neisseria, Proceedings S29-32, Hemaran, Sweden.

Henning, U. and I. Haller. 1975. Mutants of Escherichia coli K-12 lacking all "major" proteins of the outer cell envelope membrane. FEBS Lett. 55: 161-164.

Henning, U., W. Schmidmayr and I. Hindennach. 1977. Major proteins of the outer cell envelope membrane of Escherichia coli K12: multiple species of protein 1. Mol. Gen. Genet. 154: 293-298.

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

Heuzenroeder, M. W. and P. Reeves. 1981. The Tsx protein of Escherichia coli can act as a pore for amino acids. J. Bacteriol. 147: 1113-1116.

Hindennach, I. and U. Henning. 1975. The major proteins of the Escherichia coli outer cell envelope membrane. Preparative isolation of all major membrane proteins. Eur. J. Biochem. 59: 207-213.

Ichihara, S. and S. Mizushima. 1978. Characterisation of major outer membrane proteins O-8 and O-9 of Escherichia coli K12. Evidence that structural genes for the two proteins are different. J. Biochem. (Tokyo) 83: 1095-1100.

Inouye, M. 1979. What is the outer membrane? p. 1-12. In M. Inouye (ed.), Bacterial Outer Membranes: Biogenesis and Functions. John Wiley - Interscience, New York.

Inouye, M., J. Shaw and C. Shen. 1972. The assembly of a structural lipoprotein in the envelope of Escherichia coli. J. Biol. Chem. 247: 8154-8159.

Irvin, R. T., J. W. R. Govan, J. A. M. Fyfe and J. W. Costerton. 1981. Heterogeneity of antibiotic resistance in mucoid isolates of Pseudomonas aeruginosa obtained from cystic fibrosis patients: role of outer membrane proteins. Antimicrob. Agents Chemother. 19: 1056-1063.

John, M., G. Trénel and H. Dellweg. 1969. Quantitative chromatography of homologous glucose oligomers and other saccharides using polyacrylamide gel. J. Chromatog. 42: 476-484.

Jones, N. C. and M. J. Osborn. 1977a. Interaction of Salmonella typhimurium with phospholipid vesicles. Incorporation of exogenous lipid into intact cells. J. Biol. Chem. 252: 7398-7404.

Jones, N. C. and M. J. Osborn. 1977b. Translocation of phospholipids between the outer and inner membranes of Salmonella typhimurium. J. Biol. Chem. 252: 7405-7412.

Kamio, Y. and H. Nikaido. 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: 2561-2570.

Kamio, Y. and H. Nikaido. 1977. Outer membrane of Salmonella typhimurium. Identification of proteins exposed on the cell surface. Biochim. Biophys. Acta 464: 589-601.

Kawaji, H., T. Mizuno and S. Mizushima. 1979. Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of Escherichia coli K12. J. Bacteriol. 140: 843-847.

Kenward, M. A. 1975. Ph.D. Thesis. University of Aston in Birmingham.

Kenward, M. A., M. R. W. Brown and J. J. Fryer. 1979. The influence of calcium or manganese on the resistance to EDTA, polymyxin B or cold shock, and the composition of Pseudomonas aeruginosa grown in glucose- or magnesium-depleted batch cultures. J. Appl. Bacteriol. 47: 489-503.

Klemperer, R. M. M., N. T. A. J. Ismail and M. R. W. Brown. 1979. Effect of R plasmid RP1 on the nutritional requirements of Escherichia coli in batch culture. J. Gen. Microbiol. 115: 325-331.

Konisky, J. 1979. Specific transport systems and receptors for colicins and phages, p. 319-359. In M. Inouye (ed.), Bacterial Outer Membranes. Biogenesis and Functions. John Wiley - Interscience, New York.

Koplow, J. and H. Goldfine. 1974. Alterations in the outer membrane cell envelopes of heptose-deficient mutants of Escherichia coli. J. Bacteriol. 117: 527-543.

Kropinski, A. M., J. Kuzio, B. L. Angus and R. E. W. Hancock. 1982. Chemical and chromatographic analysis of lipopolysaccharide from an antibiotic-supersusceptible mutant of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 21: 310-319.

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

Leive, L. 1965. Release of lipopolysaccharide by EDTA treatment of Escherichia coli. Biochem. Biophys. Res. Commun. 21: 290-296.

Leive, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. 243: 2373-2380.

Leive, L. 1974. The barrier function of the Gram-negative envelope. Ann. N.Y. Acad. Sci. 235: 109-129.

Leytus, S. P., L. K. Bowles, J. Konisky and W. F. Mangel. 1981. Activation of plasminogen to plasmin by a protease associated with the outer membrane of Escherichia coli. Proc. Natl. Acad. Sci. USA 78: 1485-1489.

Lo, T. C. Y. and M. A. Bewick. 1981. Use of a non-penetrating substrate analogue to study the molecular mechanism of the outer membrane dicarboxylate transport system in Escherichia coli K12. J. Biol. Chem. 256: 5511-5517.

Luckey, M. and H. Nikaido. 1980a. Diffusion of solutes through channels produced by phage lambda receptor protein of Escherichia coli: inhibition by higher oligosaccharides of maltose series. Biochem. Biophys. Res. Commun. 93: 166-171.

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

Lugtenberg, B. 1981. Composition and function of the outer membrane of Escherichia coli. TIBS - October 1981: 262-266.

Lugtenberg, B., J. Meijers, R. Peters, P. van der Hock and L. van Alphen. 1975. Electrophoretic resolution of the "major outer membrane proteins" of Escherichia coli K-12 into four bands. FEBS Lett. 58: 254-258.

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

Lugtenberg, B., R. van Boxtel, C. Verhoef and W. van Alphen. 1978. Pore protein e of the outer membrane of Escherichia coli K-12. FEBS Lett. 96: 99-105.

Lundrigan, M. and C. F. Earhart. 1981. Reduction in the iron-regulated outer membrane proteins and protein a by the Escherichia coli K-12 perA mutation. J. Bacteriol. 146: 804-807.

Lutkenhaus, J. F. 1977. Role of a major outer membrane protein in Escherichia coli. J. Bacteriol. 131: 631-637.

Manning, P. A. and M. Achtman. 1979. Cell-to-cell interactions in conjugating Escherichia coli: the involvement of the cell envelope, p. 409-447. In M. Inouye (ed.) Bacterial Outer Membranes: Biogenesis and Functions. John Wiley - Interscience, New York.

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

Manning, P. A., A. Puspurs and A. Reeves. 1976. Outer membrane of Escherichia coli K12: isolation of mutants with altered protein 3A by using host range mutants of bacteriophage B3. J. Bacteriol. 127: 1080-1084.

Mariello, J. M., H. Heymann and F. W. Adair. 1979. Isolation of atypical lipopolysaccharide from purified cell walls of Pseudomonas cepacia. J. Gen. Microbiol. 112: 397-400.

Matsushita, K., O. Adachi, E. Shnagawa and M. Ameyama. 1978. Isolation and characterisation of outer and inner membranes from Pseudomonas aeruginosa and effect of EDTA on the membranes. J. Biochem. 83: 171-181.

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

Meyer, J. M., M. Mock and M. A. Abdallah. 1979. Effect of iron on the protein composition of the outer membrane of fluorescent pseudomonads. FEMS Microbiol. Lett. 5: 395-398.

Midgley, M. and E. A. Dawes. 1973. The regulation of transport of glucose and methyl α-glucoside in Pseudomonas aeruginosa.and J. Biochem. 132: 141-154.

Mizuno, T. 1979. A novel peptidoglycan-associated lipoprotein found in the cell envelope of Pseudomonas aeruginosa and Escherichia coli. J. Biochem. 86: 991-1000.

Mizuno, T. and M. Kageyama. 1978. Separation and characterisation of the outer membrane of Pseudomonas aeruginosa. J. Biochem. 84: 179-191.

Mizuno, T. and M. Kageyama. 1979a. Isolation and characterisation of a major outer membrane protein of Pseudomonas aeruginosa. Evidence for the occurrence of a lipoprotein. J. Biochem. 85: 115-122.

Mizuno, T. and M. Kageyama. 1979b. Isolation and characterisation of major outer membrane proteins of Pseudomonas aeruginosa PAO1 with special reference to peptidoglycan-associated protein. J. Biochem. 86: 979-989.

Muhlradt, P. E. and J. R. Golecki. 1975. Asymmetrical distribution and artifical reorientation of lipopolysaccharide in the outer membrane bilayer of Salmonella typhimurium. Eur. J. Biochem. 51: 343-352.

Muschel, L. H. and L. Gustafson. 1968. Antibiotic, detergent and complement sensitivity of Salmonella typhimurium after ethylene-diaminetetraacetic acid treatment. J. Bacteriol. 95: 2010-2013.

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

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

Nakae, T., J. Ishii and M. Tokunuga. 1979. Subunit structure of functional porin oligomers that form permeability channels in the outer membrane of Escherichia coli. J. Biol. Chem. 254: 1457-1461.

Nakae, T. and H. Nikaido. 1975. Outer membrane as a diffusion barrier in Salmonella typhimurium. Penetration of oligo- and polysaccharides into isolated outer membrane vesicles and cells with degraded peptidoglycan layer. J. Biol. Chem. 250: 7359-7365.

Nakamura, K. and S. Mizushima. 1976. Effects of heating in dodecyl sulphate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from Escherichia coli K12. J. Biochem. (Tokyo) 80: 1411-1422.

Nesmeyanova, M. A., I. M. Tsfasman and I. S. Kulaev. 1981. Changes in the membrane protein composition in the mutants on regulatory genes of Escherichia coli alkaline phosphatase. FEBS Lett. 124: 210-214.

Nicas, T. I. and R. E. W. Hancock. 1980. Outer membrane protein H1 of Pseudomonas aeruginosa: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B and gentamicin. J. Bacteriol. 143: 872-878.

Nikaido, H. 1976. Outer membrane of Salmonella typhimurium: transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta 433: 118-132.

Nikaido, H. 1979. Nonspecific transport through the outer membrane, p. 361-407. In M. Inouye (ed.) Bacterial Outer Membranes: Biogenesis and Functions. John Wiley - Interscience.

Nikaido, H., M. Luckey and E. Y. Rosenberg. 1980. Nonspecific and specific diffusion channels in the outer membrane of Escherichia coli. J. Supramolec. Struct. 13: 305-314.

Nikaido, H. and T. Nakae. 1973. Permeability of model membranes containing phospholipids and lipopolysaccharides: some preliminary results. J. Infect. Dis. 128S: 30-34.

Nikaido, H. and T. Nakae. 1979. The outer membrane of Gram-negative bacteria. Adv. Microbiol. Physiol. 20: 163-250.

Nikaido, H. and E. Y. Rosenberg. 1981. Effect of solute size on diffusion roles through the transmembrane pores of the outer membrane of Escherichia coli. J. Gen. Physiol. 77: 121-135.

Nikaido, H., A. Song, L. Shaltiel and M. Nurminen. 1977. Outer membrane of Salmonella XIV: reduced transmembrane diffusion rates in porin-deficient mutants. Biochem. Biophys. Res. Commun. 76: 324-330.

Nishijima, M., S. Nakaike, Y. Tamori and S. Nojima. 1977. Detergent-resistant phospholipase A of Escherichia coli K12. Purification and properties. Eur. J. Biochem. 73: 115-124.

Ohkawa, I., S. Shiga and M. Kageyama. 1980. Effect of iron concentration in the growth medium on the sensitivity of Pseudomonas aeruginosa to pyocin S2. J. Biochem. 87: 323-331.

Ombaka, E. A. 1980. The effect of environment on virulence factors and resistanceof mucoid strains of Pseudomonas aeruginosa. Ph.D. Thesis, University of Aston in Birmingham.

Osborn, M. J., J. E. Gander and J. Parisi. 1972. Mechanism of assembly of the outer membrane of Salmonella typhimurium. Site of synthesis of lipopolysaccharide. J. Biol. Chem. 247: 3973-3986.

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

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

Overbeeke, N., G. van Scharrenberg and B. Lugtenberg. 1980. Antigenic relationships between pore proteins of Escherichia coli K12. Eur. J. Biochem. 110: 247-254.

Palva, E. T. 1978. Major outer membrane protein in Salmonella typhimurium induced by maltose. J. Bacteriol. 136: 286-294.

Palva, E. T. 1979. Arrangement of protein 2, a phage-directed major outer membrane protein in Escherichia coli. FEMS Microbiol. Lett. 5: 73-76.

Palva, E. T. and L. L. Randall. 1976. Nearest-neighbour analysis of Escherichia coli outer membrane proteins, using cleavable cross-links. J. Bacteriol. 127: 1558-1560.

Palva, E. T. and L. L. Randall. 1978. Arrangement of protein 1 in Escherichia coli outer membrane: cross-linking study. J. Bacteriol. 133: 279-286.

Payne, J. W. and C. Gilvarg. 1968a. The role of the terminal carboxyl group in peptide transport in Escherichia coli. J. Biol. Chem. 243: 335-340.

Payne, J. W. and C. Gilvarg. 1968b. Size restriction on peptide utilisation in Escherichia coli. J. Biol. Chem. 243: 6291-6299.

Pugsley, A. P. and C. A. Schnaitman. 1978a. Outer membrane proteins of Escherichia coli. VII: Evidence that bacteriophage-directed protein 2 functions as a pore. J. Bacteriol. 133: 1181-1189.

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

Raetz, C. R. H. 1978. Enzymology, genetics and regulation of membrane phospholipid synthesis in Escherichia coli. Microbiol. Revs. 42: 614-659.

Reithmeier, R. A. F. and P. D. Bragg. 1977. Cross-linking of the proteins in the outer membrane of Escherichia coli. Biochim. Biophys. Acta 466: 245-256.

Robbie, J. P. and T. H. Wilson. 1969. Transmembrane effects of β galactosides on thiomethyl-β-galoctoside transport in Escherichia coli. Biochim. Biophys. Acta 173: 234-244.

Roberts, N. A., G. W. Gray and S. G. Wilkinson. 1970. The bactericidal action of ethylenediaminetetraacetic acid on Pseudomonas aeruginosa. Microbios 2: 189-208.

Rogers, S. W., H. E. Gilleland Jr. and R. G. Eagon. 1969. Characterisation of a protein-lipopolysaccharide complex released from cell walls of Pseudomonas aeruginosa by ethylenediaminetetra-acetic acid. Can. J. Microbiol. 15: 743-748.

Rosenbusch, J. P. 1974. Characterisation of the major envelope protein from Escherichia coli: regular arrangement of the peptidoglycan and unusual dodecyl sulphate binding. J. Biol. Chem. 249: 8019-8029.

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

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

Schindler, H. and J. P. Rosenbusch. 1978. Matrix protein from Escherichia coli outer membrane forms voltage controlled channels in lipid bilayers. Proc. Natl. Acad. Sci. USA 75: 3751-3755.

Schmitges, C. J. and U. Henning. 1976. The major proteins of the Escherichia coli outer cell-envelope membrane. Heterogeneity of protein 1. Eur. J. Biochem. 63: 47-52.

Schnaitman, C. A. 1973. Outer membrane proteins of Escherichia coli. 1. Effect of preparative conditions on the migration of proteins in polyacrylamide gels. Arch. Biochem. Biophys. 157: 541-552.

Schnaitman, C. A. 1974. Outer membrane proteins of Escherichia coli. IV. Differences in outer membrane proteins due to strain and cultural differences. J. Bacteriol. 118: 454-464.

Schweizer, M., H. Schwarz, I. Sonntag and U. Henning. 1976. Mutational change of membrane architecture: mutants of Escherichia coli K12 missing major proteins of the outer cell envelope membrane. Biochim. Biophys. Acta 448: 474-491.

Shands, J. W. 1966. Localisation of somatic antigen on Gram-negative bacteria using ferritin antibody conjugates. Ann. N.Y. Acad. Sci. 133: 292-298.

Smit, J., Y. Kamio and H. Nikaido. 1975. Outer membrane of Salmonella typhimurium: chemical analysis and freeze fracture studies with lipopolysaccharide mutants. J. Bacteriol. 124: 942-958.

Smit, J. and H. Nikaido. 1978. Outer membrane of Gram-negative bacteria XVIII. Electron microscope studies on porin insertion sites and growth of cell surface of Salmonella typhimurium. J. Bacteriol. 135: 687-702.

Sonntag, I., H. Schwarz, Y. Hirota and U. Henning. 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.

Stein, W. D. 1967. The movement of molecules across cell membranes. Academic Press, New York.

Steven, A. C., B. ten Heggeler, R. Muller, J. Kistler and J. P. Rosenbusch. 1977. Ultrastructure of a periodic protein layer in the outer membrane of Escherichia coli. J. Cell Biol. 72: 292-301.

Stinnett, J. D. and R. G. Eagon. 1973. Outer (cell wall) membrane proteins of Pseudomonas aeruginosa. Can. J. Microbiol. 19: 1469-1471.

Stinnett, J. D., H. E. Gilleland Jr. and R. G. Eagon. 1973. Proteins released from cell envelopes of Pseudomonas aeruginosa on exposure to ethylenediaminetetraacetate: comparison with dimethylformamide-extractable proteins. J. Bacteriol. 114: 399-407.

Stinson, M. W., M. A. Cohen and J. M. Merrick. 1977. Purification and properties of the periplasmic glucose-binding protein of Pseudomonas aeruginosa. J. Bacteriol. 131: 672-681.

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

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

Tamaki, S., T. Sato and M. Matsuhashi. 1971. Role of lipopolysaccharide in antibiotic resistance and bacteriophage adsorption of Escherichia coli K-12. J. Bacteriol. 105: 968-975.

Taylor, R. K., M. N. Hall, L. Enquist and T. J. Silhavy. 1981. Identification of OmpR: a positive regulatory protein controlling expression of the major outer membrane matrix proteins of Escherichia coli K12. J. Bacteriol. 147: 255-258.

Tokunuga, H., M. Tokunuga and T. Nakae. 1979. Characterisation of porins from the outer membrane of Salmonella typhimurium. Eur. J. Biochem. 95: 433-439.

Tokunuga, M., H. Tokunuga, Y. Okajima and T. Nakae. 1979. Characterisation of porins from outer membrane of Salmonella typhimurium. 2. Physical properties of functional oligomers aggregates. Eur. J. Biochem. 95: 441-448.

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

Tommassen, I. and B. Lugtenberg. 1981. Localisation of phoE, the structural gene for outer membrane protein e in Escherichia coli K12. J. Bacteriol. 147: 118-123.

Van Alphen, L., B. Lugtenberg, R. van Boxtel, A. M. Hack, C. Verhoef and L. Havekes. 1979. meoA is the structural gene for outer membrane protein c of Escherichia coli K12. Mol. Gen. Genet. 169: 147-155.

Van Alphen, W. and B. Lugtenberg. 1977. Influence of osmolarity of the growth medium on the outer membrane protein pattern of Escherichia coli. J. Bacteriol. 131: 623-630.

Van Alphen, W., R. van Boxtel. N. van Selm and B. Lugtenberg. 1978. Pores in the outer membrane of Escherichia coli K-12. Involvement of proteins b and c in the permeation of cephaloridine and ampicillin. FEMS Microbiol. Lett. 3: 103-106.

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

Van Alphen, L., A. Verkleij, J. Leunissen-Bijvelt and B. Lugtenberg. 1978. Architecture of the outer membrane of Escherichia coli III. Protein-lipopolysaccharide complexes in intramembraneous particles. J. Bacteriol. 134: 1089-1098.

Verkleij, A., L. van Alphen, J. Bijvelt and B. Lugtenberg. 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.

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

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

Yu, F. and S. Mizushima. 1977. Stimulation by lipopolysaccharide of the binding of outer membrane proteins O-8 and O-9 to the peptidoglycan layer of Escherichia coli K-12. Biochem. Biophys. Res. Commun. 74: 1397-1402.

Zimmermann, W. and A. Rosselet. 1977. Functions of the outer membrane of Escherichia coli as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. 12: 368-372.

Bodemann, H. and H. Passow. 1972. Factors controlling resealing of membrane of human erythrocyte ghosts after hypotonic haemolysis. J. Membr. Biol. 8: 1-26.

Hofnung, M. and M. Schwarz. 1972. Mutations allowing growth on maltose of Escherichia coli K12 strains with a deleted malT gene. Mol. Gen. Genet. 112: 117-132.

Seeman, P. 1967. Transient holes in erythrocyte membrane during hypotonic hemolysis and stable holes in membrane after lysis by saponin and lysolecithin. J. Cell Biol. 32: 55-70.

Nakae, T. and J. Ishii. 1978. Transmembrane permeability channels in vesicles reconstituted from single species of porins from Salmonella typhimurium. J. Bacteriol. 133: 1412-1418.

Filip, C., G. Fletcher, J. L. Wulff and C. F. Earhart. 1973. Solubilisation of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115: 717-722.