

Cell Surface of *Enterobacter aerogenes*

oo0oo

Gordon Daniel Cumming



30150 007186692

Presented for the Degree of Ph.D.
University of Edinburgh
1981



CONTENTS

	PAGE
Summary	i
Acknowledgment	ii
Abbreviations	iii
INTRODUCTION.	1
I The diversity of Microbial Cell Envelopes.	1
II Biosynthesis, Genetics and Control of Cell Envelope Polymers.	8
Peptidoglycan	8
Lipopolysaccharide	14
Exopolysaccharides	23
Lipids	31
Membrane Derived Oligosaccharide	35
The Outer Membrane Protein of Gram Negative Bacteria	36
Major Non-Porin Proteins	39
Protein <u>a</u>	40
Matrix Proteins	41
New Membrane Proteins	44
Murein Lipoprotein	46
A new murein-associated lipoprotein	55
Minor Proteins	56
III Assembly of Polymers into the Outer Membrane	62
IV Molecular Organisation of the Outer Membrane	71
MATERIALS & METHODS.	74
RESULTS	95
I Mutagenesis	95
II Growth of Strains	100
III Growth Curves	105
a Exopolysaccharide	105
b Lipopolysaccharide	116

IV	Cell Envelope Proteins of <i>Enterobacter aerogenes</i> .	129
	Sarkosyl isolation of outer membrane	129
	Drug Resistance	141
	Nutrient limitation	144
	Effect of Supplements to YE broth	149
	Fe ³⁺ - Uptake Proteins	153
	Effect of Age of a Culture	156
	Effect of gel solubilising temperature	158
	Pronase treatment of sphaeroplasts	160
	Sucrose-gradient density centrifugation	163
	French Press Procedure	163
	Lithium acetate isolation of outer membrane	163
	Yield of outer membrane	167
	Glycoproteins of the outer membrane	170
	'Protein K' Extraction	170
	Acid Butan-1-ol Extraction	170
	Two-dimension polyacrylamide gel electrophoresis	176
	Ammonium sulphate precipitable protein	176
	Cytoplasmic Membrane Proteins	177
	DISCUSSION	179
	I Outer Membrane Proteins of <i>Enterobacter aerogenes</i> .	179
	Ion limitations	183
	Membrane Active Agents	186
	II Polysaccharide Production	188
	III Outer Membrane Protein and Exopolysaccharide	191
	References	193

SUMMARY

Enterobacter aerogenes mutants altered in capsular polysaccharide synthesis have been selected by mutagenesis and resistance to a variety of agents.

Cells grown under nutrient limitation or supplemented with membrane active agents showed altered ratios of proteins in the outer membrane and changes in exopolysaccharide yield.

Outer membrane proteins have been prepared by a number of procedures; sucrose gradient density centrifugation, sarkosyl treatment, French press, Lithium acetate/shearing and by Cetavlon or Acid-Butanol extraction. The proteins were examined by SDS - polyacrylamide gel electrophoresis on gradient and non-gradient gels, by chromatographic column and at different solubilisation temperatures.

Two major outer membrane proteins (34K and 38K: major non-porin and major porin proteins respectively) were tentatively identified on the basis of properties they exhibited which are common to members of the Enterobacteriaceae.

Cytoplasmic membrane proteins have been visualised.

Gel electrophoresis of lipopolysaccharide and paper chromatography of the hydrolysate of low molecular weight supernatant polysaccharide were performed and a possible source for the low molecular weight polysaccharide has been suggested.

A possible correlation between outer membrane proteins and exopolysaccharide production has been postulated.

ACKNOWLEDGMENTS

I would like to thank Dr. I.W. Sutherland for his encouragement and helpful criticism throughout this work; Prof. J.F. Wilkinson for allowing me to work in the Department of Microbiology and Gordon Finnie and Jackie Calder for photography.

I acknowledge the receipt of a Research Studentship from the Science Research Council.

DECLARATION

I declare that this thesis was composed by myself and that the work herein presented is my own.

August 1981.

ABBREVIATIONS

Abe	:	Abequose
ACP		Acyl Carrier Protein
ADP		Adenosine-diphosphate
Ala		Alanine
AMP		Adenosine-monophosphate
Arab		Arabinose
ATP		Adenosine-triphosphate
CDP		Cytidine-diphosphate
DAP		Diaminopimelic acid
EDTA		Ethylenediaminetetra acetic acid
EtN		Ethanolamine
FA		Fatty Acid
Fuc		Fucose
Gal		Galactose
GalUA		Galacturonic acid
GDP		Guanosine-diphosphate
Glc		Glucose
GlcUA		Glucuronic acid
GlcNAc		N-acetylglucosamine
Glu		Glutamic acid
Gly		Glycine
Gul		Guluronic acid
Hep		Heptose
IP		Isoprenoid alcohol phosphate
KDO		Ketodeoxyoctonate
LPS		Lipopolysaccharide
Lys		Lysine

ABBREVIATIONS (contd.)

Mal	:	Maltose
Man		Mannose
MurNAc		N-Acetyl Muramic acid
NADP		Nicotinamide adeninedinucleotide phosphate (oxidised)
NADPH +		Nicotinamide adenine dinucleotide phosphate (reduced)
NDP		Nucleoside-diphosphate
O - Ac		O - Acetyl
P		Phosphate
PPi		Pyrophosphate
PE		Phosphatidylethanolamine
PEP		Phosphoenolpyruvate
PG		Phosphatidylglycerol
Rha		Rhamnose
SDS		Sodium dodecyl sulphate
Ser		Serine
UDP		Uridine-diphosphate
UTP		Uridine-triphosphate

INTRODUCTION

INTRODUCTION

The cell envelopes of microbial cells are capable of great variation in structure to accomplish similar functional aims. Common to most microbes is the possession of at least one single unit membrane and a rigid polysaccharide lattice to maintain the cell integrity. They may also possess extensive surface layers.

The yeasts and fungi and bacteria with the exception of the Halobacteriaceae can be considered to fall into such a category. The yeasts and fungi, both eukaryotes, can be distinguished on the gross morphological criterion that the former is unicellular and the latter multicellular. The prokaryotic bacteria, excluding *Halobacteria* spp., *Mycobacteria* spp. and the methanogenic bacteria, can be sub-divided into two basic classes using the staining technique of Christiaan Gram, a Danish physician of the 19th century. One class, Gram positive cells, retain a crystal violet-iodine complex despite extraction with polar solvents such as alcohol and acetone. The second class, Gram negative, can be decolourised and counterstained.

These classes of organisms, while possessing similar functional and structural requirements are invested with a variety of cell envelope structures. These are examined below.

THE DIVERSITY OF MICROBIAL CELL ENVELOPES

The cell envelope organelles of most protists contain structural components which confer an element of rigidity on the envelope, thereby protecting the cytoplasmic (plasma) membrane from rupture by alterations in osmotic pressure. Examples are available from the fungi and bacteria.

The mature wall of the fungus *Neurospora crassa* is approximately 125 nm thick and overlies the fluid plasma membrane. An inner chitin layer extends for 20 nm, surrounded by two successive layers composed of protein and glycoprotein and on the outside a layer of α and β -linked glucans arranged coaxially (Gooday and Trinci, 1980). Burnett (1979) has shown that these layers are not discrete but can be considered to be merged together.

The cell walls of yeasts as visualised in electron-micrographs show these structures to be distinct from but apposed to the plasma membrane (Matile *et al*, 1969). The wall is composed largely of polysaccharide with glucosamine, protein and lipids. Mannan and glucan are the main polysaccharide components. Chitin forms bud scars, and can be regarded as localised to these sites alone. Mannan is a polymer of 1—6 and 1—2 linked mannose molecules covalently bonded to protein and is most probably differentiated in location, being interspersed with glucan. Of the glucan obtained from *Saccharomyces cerevisiae*, three classes (based on solubility properties in alkali or acetic acid) were determined by Manners and co-workers (cited by Rose, 1978). However, enzymes such as invertase, which is associated with mannan, are located in the periplasmic zone,

while glucanases are located within the wall structure glucan, suggesting a greater accumulation of the mannan protein complex at the plasma membrane side of the envelope of *Saccharomyces cerevisiae* (Ballou, 1976). The plasma membrane of this yeast is a lipid bilayer containing protein, the two being in approximately equal proportions. Such a structure, common in eukaryotes, offers little protection against rupture in a hypotonic environment.

The *Mycobacteria* spp. like most other prokaryotes contain peptidoglycan as a wall component. These organisms also contain a glycolipid composed of mycolic acid esterified to arabinogalactan, 'cord' factor chemically defined as trehalose 6, 6' dimycolate, mycosides, and sulphatides. Glucan and polyglutamine acid polymers are also present (Reviewed by Rogers *et al*, 1980).

Exceptions to the 'general' trend exhibited by protists in possessing a boundary wall are found among the Mycoplasmas, L-forms and Halobacteria. L-forms, extreme halophiles (the Halococci) and salt dependent halophiles (the Halobacteria) lack peptidoglycan or a formal rigid structural polymer. The former organisms can be obtained from vegetative cells of many organisms. The resulting stabilised cells possess only a cytoplasmic membrane and are extremely susceptible to osmotic pressure. The salt-dependent halophiles similarly lacking in peptidoglycan, are extremely unstable in low-salt concentrations. The extreme halophiles such as *Halococcus morrhuae* lack peptidoglycan but possess a sulphated polysaccharide which substitutes as the structural polymer.

The *Mycoplasmas* can be sub divided into two genera: those requiring sterols for growth, *Mycoplasma*, and those not requiring (but able to incorporate) sterols, *Acholeplasma*. Both genera lack peptidoglycan. They are quite resistant to alterations in the osmotic pressure of their environment. This is attributed to stabilising of the membrane by cholesterol. (Rogers *et al*, 1980).

The above envelope structures from the complex fungal to the simpler but nevertheless effective organelle of the *Mycoplasma*, may be compared to those of the Gram-positive and Gram-negative bacteria.

THE GRAM-POSITIVE CELL ENVELOPE

Analysis of electron micrographs of the Gram-positive cell envelope revealed a trilamellar-like appearance: the cytoplasmic membrane surrounded by a single electron dense track, the retaining wall structure, peptidoglycan, with outer layer material exterior to the peptidoglycan. The thickness of the envelope is variable according to growth conditions and growth stage. Exponentially grown *Bacillus subtilis* cells have a cell envelope thickness of approximately 27 nm. The thickness of the envelope can be radically altered by the addition of

chloramphenicol to prevent protein synthesis: *Staphylococcus aureus* cell envelope thickness can be increased from 30 to 100nm. An exception to the normal division by the Gram stain is *Bacillus fibrisolvens*, which has a Gram-positive envelope structure but stains negatively. It also has a very thin envelope, 15 nm (Rogers *et al*, 1980).

The trilamellar image obtained in Gram-positives may be accounted for by an arrangement of cell envelope polymers external to the peptidoglycan. Weibull (1973) provided evidence for the concentration of a phosphorous containing molecule, teichoic acid, in the outer layer. However, such a hypothesis was rejected by Millward and Reavely (1974) who suggested that teichoic acid was present throughout the envelope and that Weibull's results could be explained as variable packing of wall material.

Peptidoglycan, the cell wall, is a major component of the envelope comprising up to 90% of the dryweight. The biosynthesis of both peptidoglycan and teichoic acids was shown to be co-ordinated (Rogers and Garrett, 1965; Boylen and Ensign, 1968).

The amino-acid composition of peptidoglycan is subject to control by availability of glycine and L-alanine (Schleifer *et al*, 1969) while teichoic acid may be substituted by teichuronic acid when the cell changes from magnesium, potassium, nitrogen or sulphur limitation to phosphate limitation (Ellwood, 1970).

Attachment of the cytoplasmic membrane to the wall is apparent from electron micrographs showing close apposition of the two structures, but the physical bonds between them are as yet tentative. Ring-shaped discs can be visualised on the outer limit of the cytoplasmic membrane in staphylococci, while 'peg'-like structures are noted connecting the cytoplasmic membrane deep into the cell wall (Burdett and Rogers, 1970; Giesbrecht *et al*, 1977).

SURFACE LAYERS OF GRAM-POSITIVE CELLS

Negatively stained *Bacillus* and *Clostridium* cells frequently present a regularly patterned outer layer. These layers can be removed from the cell surface by treatment with guanidine hydrochloride or urea and are released in sheet form. Therefore they are unlikely to be covalently bonded to the remaining envelope components.

The sheets usually consist of protein but may have a carbohydrate substituent. A protein made of sub-units with molecular weight of 150,000 was reported for *Bacillus sphaericus* outer layer material, while clostridial species have been found that possessed sub-units of molecular weight 140,000.

THE GRAM-NEGATIVE CELL ENVELOPE

In contrast to the Gram-positive cell envelope the Gram-negative bacterium possesses two trilamellar unit membranes; the cytoplasmic and outer membranes separated by an electron dense track, peptidoglycan. Various staining procedures have been used to demonstrate the presence of outer layers, exterior to the outer membrane (See figure 1).

Unlike the Gram-negative cells in which peptidoglycan comprises much of the envelope, the peptidoglycan rigid layer, of Gram-negative *E coli* is a thin layer, 2—3 nm thick. It is separated from the cytoplasmic membrane in electron micrographs by an electron transparent zone. Values for most Gram-negative organisms vary from 2—10 nm. These organisms show variability in their reaction to incubation with chloramphenicol. The *E coli* rigid layer does not vary in thickness whereas *Acinetobacter* varies substantially, like Gram-positive cells (Rogers *et al*, 1980).

The electron-transparent region between cytoplasmic and outer membranes, called the periplasmic zone is of indeterminate thickness since variation in fixing techniques can alter values significantly. Burdett and Murray (1974) obtained sections lacking a formal periplasmic zone. However, the existence of a 'space' seems probable since it could occupy the same zone as a peptidoglycan lattice structure. Indirect evidence for such a zone comes from the finding that enzymes, binding proteins and pigments are released from the cell on osmotic shock and none of these components is known to be localised in either membrane (Costerton *et al*, 1974).

The cytoplasmic membrane is closely akin to that found in Gram-positive cells and has a chemical composition similar to other biological membranes (Martin and MacLeod, 1971).

A "unit membrane" 6—10 nm thick, the outer membrane, is distinguishable from the cytoplasmic membrane in many respects. It possesses a lipid-containing polymer lipopolysaccharide, whose presence alters the buoyant density of the membrane substantially and allows a mechanical method, based on buoyant density, of separating the membrane fractions from crude envelope preparations (Sucrose-gradient density centrifugation—see section on "Materials and Methods"). The outer membrane also lacks the biosynthetic capabilities associated with the cytoplasmic membrane and a comparison of the polypeptides of inner and outer membrane proteins on SDS-polyacrylamide gels indicates a considerable difference in patterns and in the number of polypeptides present. The outer membrane may possess up to approximately 20 polypeptides while the cytoplasmic membrane possesses several times this figure.

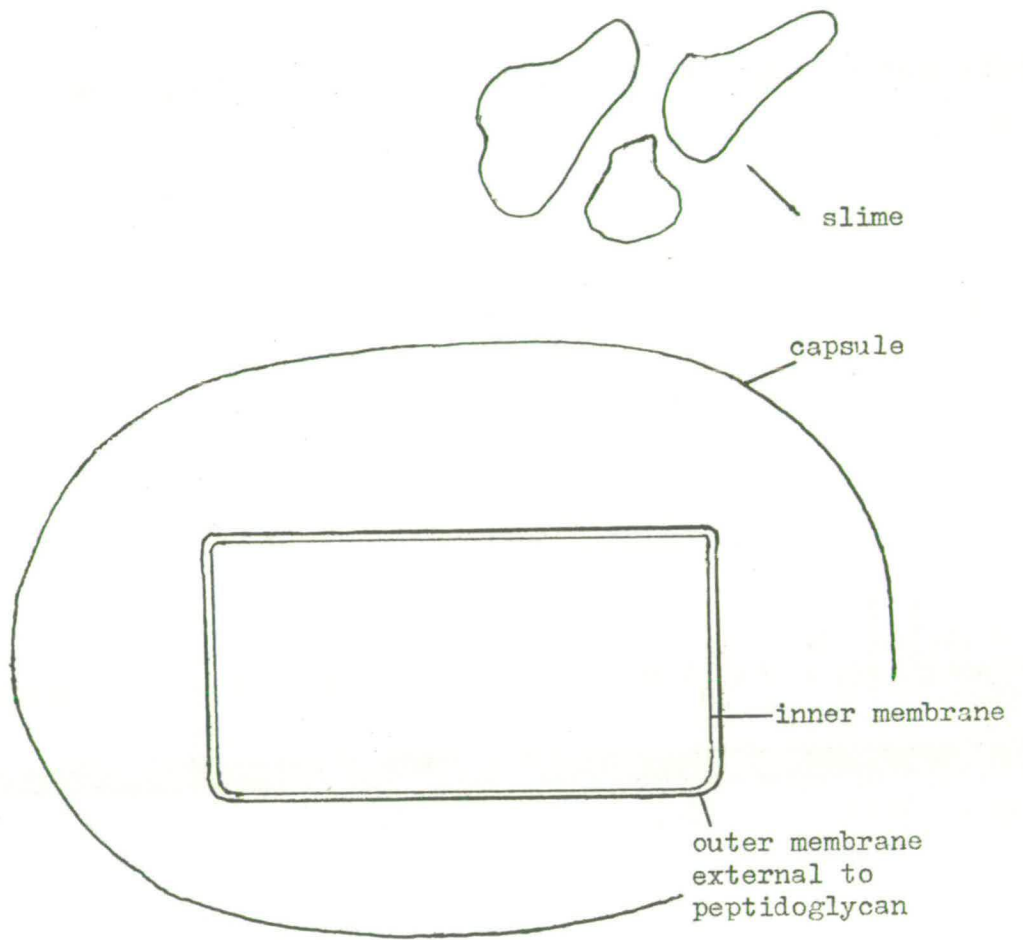


Figure 1 The cell surface of the Gram-negative bacterium.

The association of the rigid layer with the outer layer is mediated by a lipoprotein (Braun and Rehn, 1969). Loss of this protein is reflected in a loosening of the outer membrane link with the cell and finally in loss of viability (Sonntag *et al*, 1978).

OUTER LAYERS OF GRAM-NEGATIVES

Glauert and Thornley (1969) found that the surface of certain Gram-negative cells possess a regular pattern external to the LPS zone. The cell surface of aquatic and rumen bacteria, in particular, is covered by a paracrystalline layer of molecules.

Spirillum serpens can lose such a layer by gentle chemical extraction or by mutation without loss of viability under laboratory conditions. Costerton *et al* (1974) suggested that these layers, prevalent in species living in highly competitive environments such as the rumen, give protection from antibiotics and enzymes produced by competitors. Possession of the extra barrier function provided by such a protective layer may also act to protect a species such as *Spirillum serpens* from attack by *Bdellovibrio* in nature.

THE CAPSULE LAYER

The capsule, according to a definition set out by Duguid (1951), is a demonstrable layer external to the cell wall which has a definite boundary and remains attached to the cell in aqueous suspension. Slime is loosely bound to the cell or found as an amorphous mass in the culture fluid.

Capsules, which are found in both Gram-negative and positive cells, are extremely hydrophilic organelles, up to 99% water (Sutherland, 1972). They may be visualised under the light microscope using the Indian Ink method or under the electron microscope using Ruthenium red staining. The former highlights the capsule as a discrete organelle surrounding the cell and the latter can determine structure to a fibrillar level. Slime may be visualised by both techniques (Duguid, 1951; Pate and Ordal, 1967).

The "Quellung" or "swelling" reaction whereby specific anti-capsular antibodies are used to increase the capsule size and refractility under the light microscope may also be used to demonstrate the presence of capsule.

The chemical composition of capsules varies considerably. Most are polysaccharides but those of some bacilli such as *Bacillus megaterium* may incorporate proteinaceous material.

Slime is chemically identical to the capsular polysaccharide produced by a single strain. Capsular or slime polysaccharides are referred to as 'exopolysaccharide'.

The structure and function of capsules and the mode of attachment of the capsule to the cell envelope are the subject of investigation. A great many capsular polysaccharides have been chemically defined, especially those of the *Enterobacteriaceae* (Grant *et al*, 1969). However, these determinations have not yet provided significant insights into the possible ultrastructure of the capsule or mode of attachment.

It is suggested that the latter may be achieved by covalent linkages between polysaccharide and outer membrane, or, more remotely, cytoplasmic membrane components.

Possible functions for the capsule in protection against phagocytosis, bactericidal serum factors, desiccation and bacteriophage attack as well as a role in the adhesion of pathogenic and aquatic species are documented.

A comprehensive series of reviews explored the aforementioned aspects of the capsule (Sutherland, 1977^{a, b}).

BIOSYNTHESIS, GENETICS AND CONTROL OF CELL ENVELOPE POLYMERS

PEPTIDOGLYCAN

Peptidoglycan is composed of a rigid glycan backbone comprising β 1—4 linked N-acetylglucosamine and N-acetylmuramic acid substituted with stem and cross-linking peptides (Sharon *et al*, 1966; Ghuysen, 1968) (See figures 2,3) In *E. coli* and most Gram-negative organisms the peptidoglycan may account for 5-20% of the cell envelope dry weight (Ghuysen, 1968; Costerton *et al* 1974).

The glycan chain length has been estimated by measuring the ratio of total amino sugar alcohol ^{after borohydride reduction} to total hexosamine and a value of 80 disaccharide units determined for the walls of a lytic deficient *Bacillus licheniformis*. It is important to note that peptidoglycan lytic enzymes (autolysins) are liable to reduce chainlength and extent of peptide crosslinking during extraction of the sacculus and that these estimates must be regarded as minimum chainlength (Ward, 1973).

Ghuysen (1968) studied the peptides substituted on the glycan backbone. The majority of peptidoglycans possess a short tetrapeptide of L-alanine-D-isoglutamic acid-meso-diaminopimelic acid-D-alanine, or a tri-peptide lacking the terminal D-alanine residue, substituted on to the N-acetyl muramic acid (Weidel and Pelzer, 1964). The peptides may be linked to a similar unit on another glycan backbone by means of a crosslinking peptide. These structures can be grouped in four main types indicated by Ghuysen (1968). Schleifer and Kandler (1972) proposed a tri-digital system based on the class of cross-linking, the type of bridge or lack of it, and the amino acid at position 3 of the stem peptide. By this system *E coli* peptidoglycan is classified as A1 γ .

The extent of transpeptidation has been examined (Oldmixon *et al*, 1976; Fordham and Gilvarg, 1974) and the observations of these groups support possible growth of the peptidoglycan from the peptide chain; continued peptide crosslinking after incorporation of precursor subunits in peptidoglycan.

An increasing volume of literature cites a role for peptidoglycan, lipoprotein or outer membrane proteins in determining and maintaining cell shape (Chatterjee and Young, 1972; Lazdunski and Shapiro, 1972). Mutants of *E coli* lacking one or several proteins but maintaining normal morphology tend to support a combined role for these polymers (Henning and Haller, 1975).

The relationship between the degree of cross-linking in peptidoglycan and cell morphology and division is uncertain. Rogers *et al* (1971) using a *Bacillus subtilis* Rod⁻ mutant grown in low and high NaCl concentrations and comparing the morphology and degree of cross-linking found distorted morphology and low cross-linking at low salt concentrations. The normal phenotype parent strain,

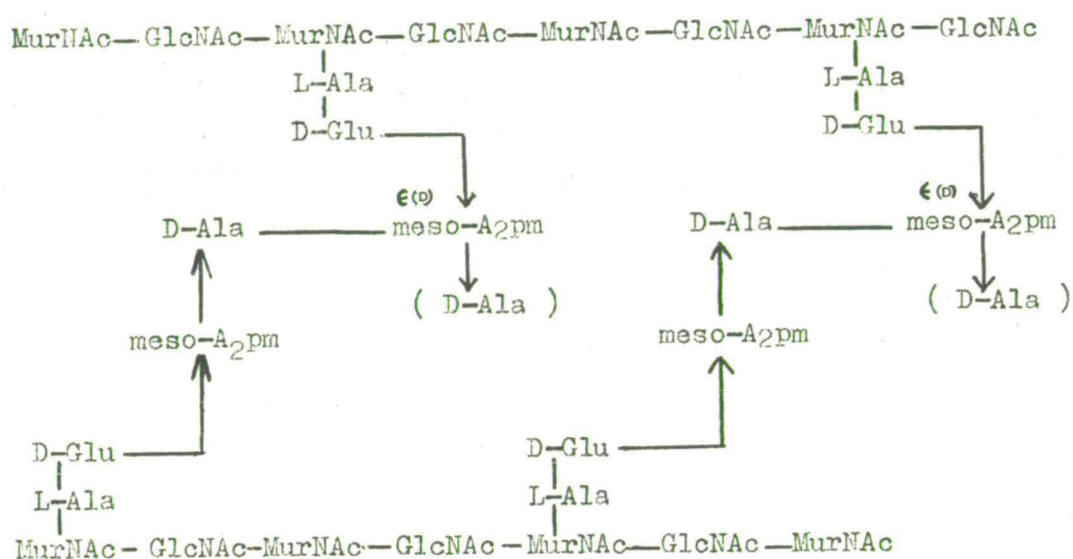


Figure 2 The peptidoglycan backbone linked by stem and cross-linking peptides, in *E. coli*. Schleifer and Kandler (1972) classify such a link as A18

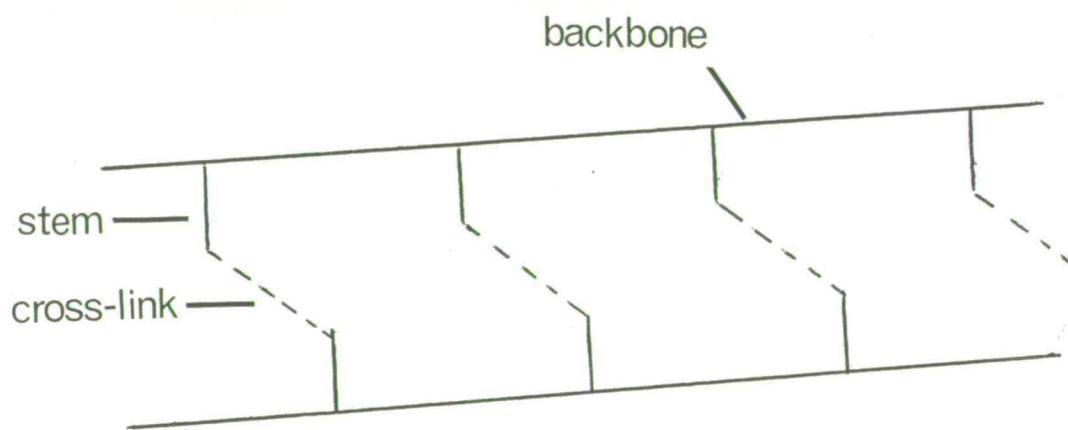


Figure 3 Matrix structure of the peptidoglycan polymer.

however, gave normal morphology and a similarly low degree of cross-linking. Similarly, Schwartz and co-workers (Schwartz *et al*, 1969) did not show any correlation between filamentation and bulging of cells and the degree of cross-linking.

Recently Fontana and co-workers (Satta *et al*, 1979, 1980; Fontana *et al*, 1979) provided evidence for two sites for peptidoglycan assembly in rods, using pH conditional mutants of *Klebsiella pneumoniae*. As proposed, one site would be responsible for lateral wall extension and one for septum formation. Botta and Park (1981) using temperature sensitive division mutants of *E. coli*, one possessing a thermolabile penicillin binding protein 3 (PBP3) found that antibiotics known to affect peptidoglycan synthesis by binding to PBP3 had no effect on peptidoglycan synthesis during formation of non-septate filaments. They concluded that PBP3 is required for peptidoglycan synthesis during septation but not elongation.

BIOSYNTHESIS

Biosynthesis of peptidoglycan has been thoroughly reviewed recently by Rogers *et al* (1980). The biosynthetic process can be sub-divided into three stages:

- a) synthesis of sugar nucleotides: UDP-N-acetyl glucosamine and UDP-N-acetyl muramyl-L-Ala-D-isoGlu-mesoDAP-D-Ala-D-Ala, catalysed by soluble cytoplasmic enzymes.
- b) formation and polymerisation of undecaprenol derivatives.
- c) transpeptidation of stem peptides of translocated soluble sub-units rendering them into an insoluble matrix.

(See figures 4, 5 and 6).

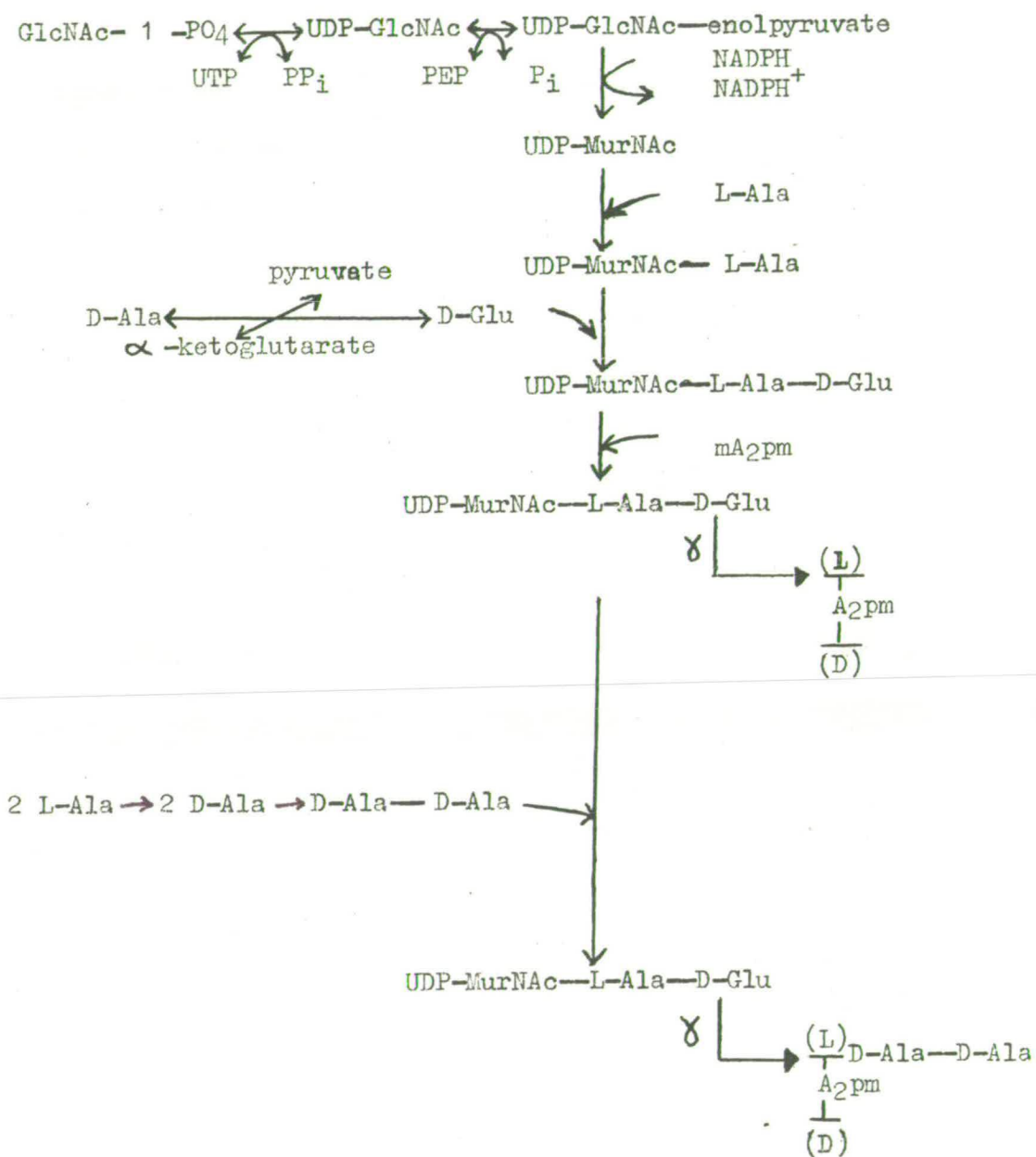


Figure 4 The biosynthesis of UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide. The reaction sequence is common to most Gram-negative bacteria.

From Rogers *et al*, 1980

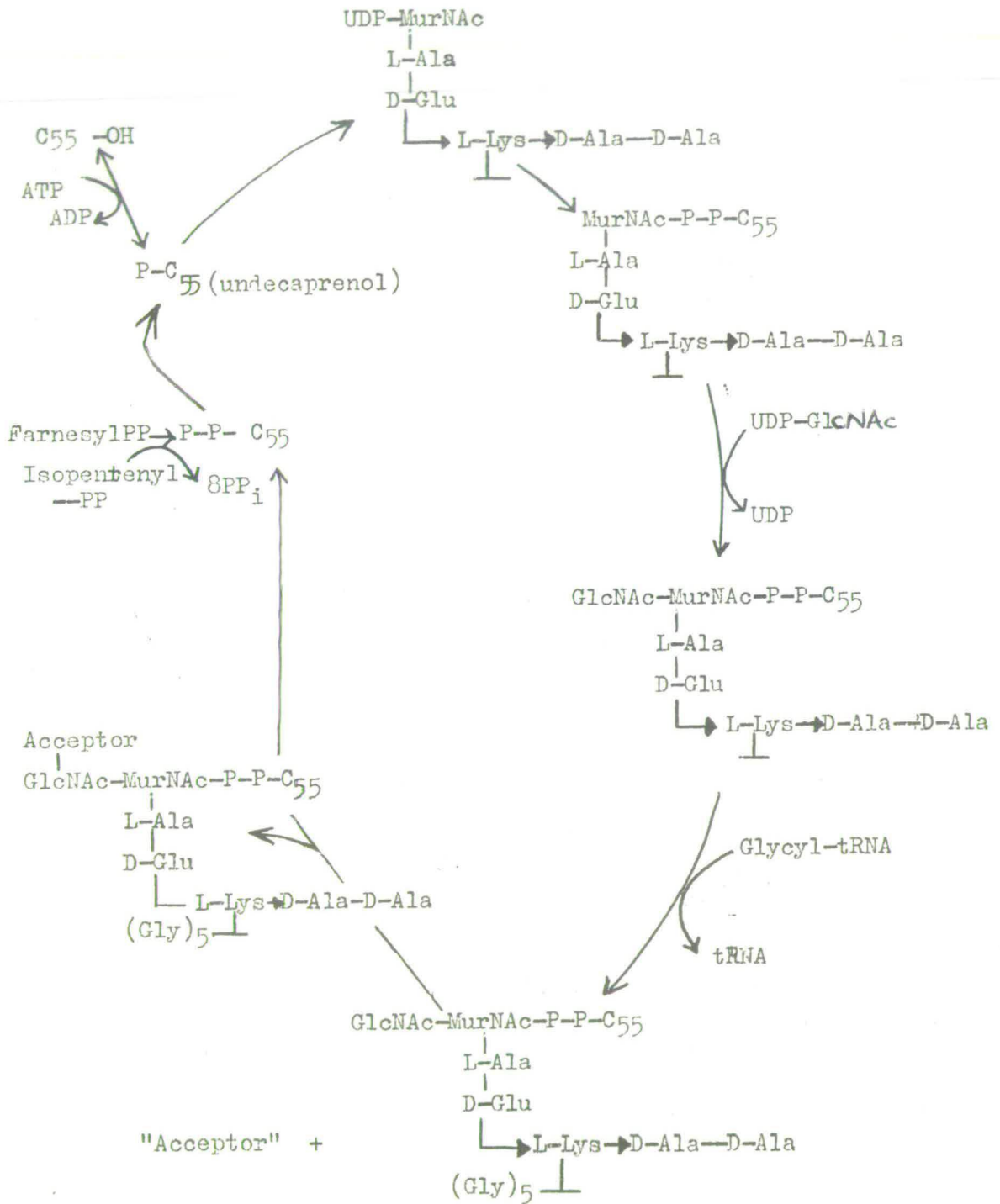


Figure 5 The biosynthesis of linear, uncrosslinked peptidoglycan on the carrier lipid undecaprenol.

From Rogers *et al*, 1980

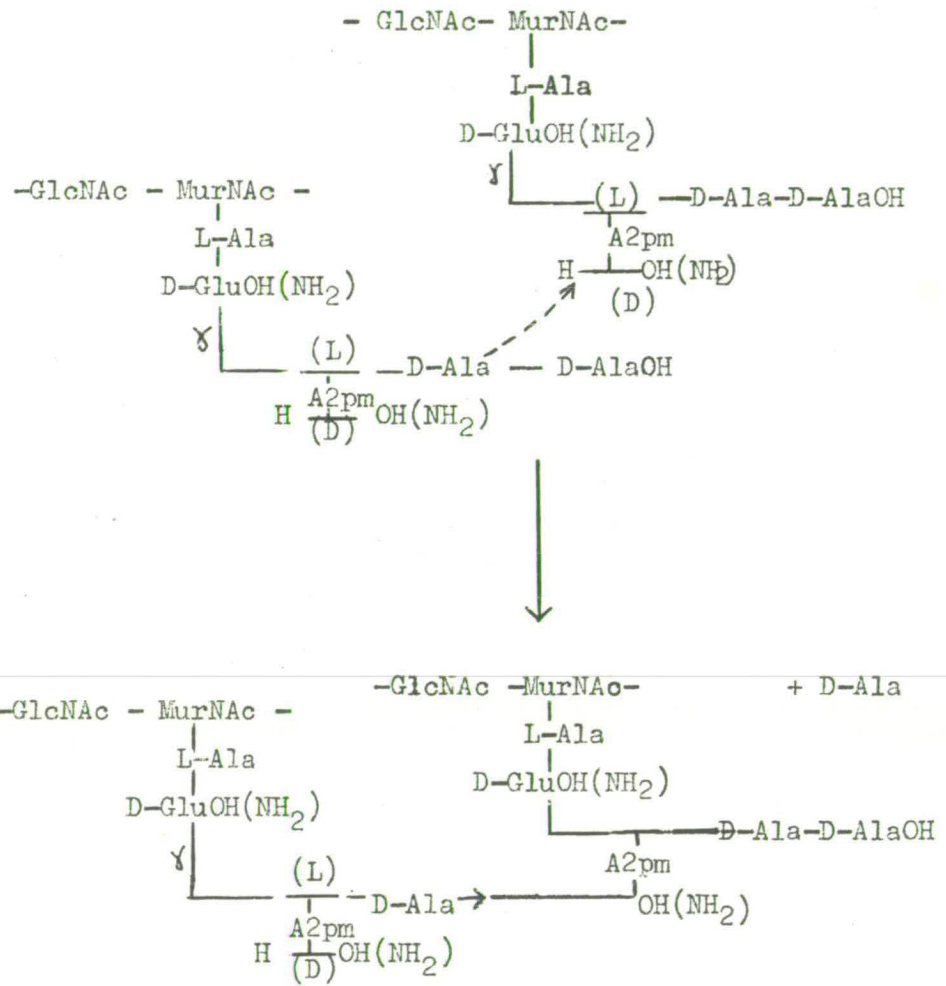


Figure 6 Postulated transpeptidation reactions for synthesis of peptidoglycan.

LIPOPOLYSACCHARIDE

As a molecule common to the envelopes of Gram-negative bacteria and implicated in the endotoxin activity of these cells, lipopolysaccharide structure, biosynthesis and function have been investigated and reviewed widely (Wilkinson, 1977; Luderitz *et al*, 1971; Nikaido, 1973).

Lipopolysaccharide, a complex polar lipid, comprises three distinct covalently linked regions (lipidA, core, 'O' Antigen) has been shown to reside in the outer membrane. A recent report ascribed an asymmetric location of molecules in the outer leaflet of the membrane (Leive, 1968; Schnaitman, 1971; Funahara and Nikaido, 1980). By using E.D.T.A. to chelate Mg^{2+} and Ca^{2+} , approximately 90% of LPS molecules can be released from the membrane. The remaining 10% appears to be in equilibrium such that it can be converted with time and released on treatment with E.D.T.A. (Leive, 1968; Leive *et al* 1968). Leive suggested that the environments surrounding these two fractions differed such that the releasable form required divalent cations for stabilisation, for example, if it was in close association with protein molecules.

Electronmicrographs of the cell surface of EDTA-treated cells showed "elevated plateau" areas which Bayer (1975) suggested may represent the non-releasable LPS fraction.

The LPS molecule consists of a lipid fraction (Lipid A) covalently linked to a polysaccharide. The polysaccharide molecule can be sub-divided into core and 'O' antigen regions. The core is a non-repeating series of repeated sugars while the 'O' antigen is a series of oligosaccharides. (See figure 7).

Enterobacter aerogenes Lipid A fraction is comprised of 3-hydroxytetradecanoic acid, dodecanoic, tetradecanoic and hexadecanoic acids (Gallin and O'Leary, 1968). Commonly in the enterobacteria the fatty acids are linked to a glucosamine moiety by ester and amide bonds.

Nimmich and Korten (1970) presented evidence suggesting the possession of a common core structure in *Klebsiella* and *Enterobacter* species. Compared to other enterobacteria the ratio of core hexoses to KDO is low (Wilkinson, 1977).

The 'O' specific side chains for *Klebsiella* and *Enterobacter* were determined by several groups of workers, predominantly Lindberg's group (cited in Wilkinson, 1977) (See Table 1). Furanoside residues are present in many *Klebsiella* 'O' antigens. Galactans are found in serogroups 01 (= 06), 02, and in the O-acetylated form in 08 and 09. Remarkable on the identity of 'O' antigens 03 and 05 of *Klebsiella* with 08 and 09 of *E coli*, Lindberg speculated that the cores may also be common to both species. Jann and Jann (1977) postulated that these antigens may be related through bacteriophage conversion.

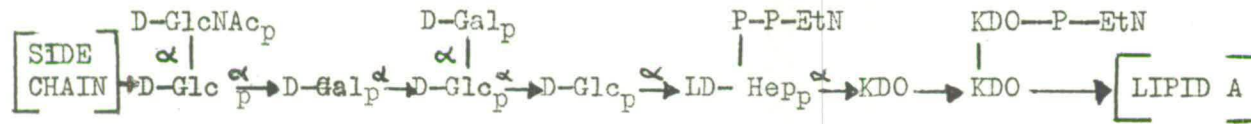


Figure 7 The composition of the lipopolysaccharide molecule, representative of *Salmonella* spp.

From Wilkinson, 1977

01 = 06	$\text{---}^3 \text{Gal} \frac{1}{\alpha}$	Bjorndal <i>et al</i> (1971)
04 = 011	$\text{---}^2 \text{Rib}_f \frac{1}{\beta} \text{---}^4 \text{Gal} \frac{1}{\alpha}$	Bjorndal <i>et al</i> (1972)
05	$\text{---}^2 \text{Man} \frac{1}{\alpha} \text{---}^2 \text{Man} \frac{1}{\alpha} \text{---}^3 \text{Man} \frac{1}{\alpha} \text{---}^2 \text{Man} \frac{1}{\alpha} \text{---}^3 \text{Man} \frac{1}{\alpha}$	Lindberg <i>et al</i> (1972b)
07	$\text{---}^2 \text{Rha} \frac{1}{\alpha} \text{---}^2 \text{Rib}_f \frac{1}{\beta} \text{---}^3 \text{L-Rha} \frac{1}{\alpha} \text{---}^3 \text{L-Rha} \frac{1}{\alpha}$	Lindberg <i>et al</i> (1973)
08	$\text{---}^3 \text{Gal} \frac{1}{\alpha} \text{---}^3 \text{Gal}_f \frac{1}{\alpha} \text{---}^3 \text{Gal}_p \frac{1}{\alpha}$ $\begin{array}{ccc} \text{2,6} & & \text{2,6} \\ & & \\ \text{OAc} & & \text{OAc} \end{array}$	Curvall <i>et al</i> 1973
09	$\text{---}^3 \text{Gal}_f \frac{1}{\alpha} \text{---}^3 \text{Gal} \frac{1}{\alpha} \text{---}^3 \text{Gal}_f \frac{1}{\alpha} \text{---}^2 \text{Gal} \frac{1}{\alpha}$ $\begin{array}{cccc} & & & \text{OAc} \\ & & & \\ & & & 6 \\ \text{2,6} & & 6 & & \text{2,6} & & \text{3} \\ & & & & & & \\ \text{OAc} & & \text{OAc} & & \text{Gal} & & \alpha \\ & & & & & & \\ & & & & \text{OAc} & & 6 \end{array}$	Lindberg <i>et al</i> (1972a)
010	$\text{---}^3 \text{Rha} \frac{1}{\alpha} \text{---}^3 \text{Rib}_f \frac{1}{\beta} \text{---}^4 \text{Rha} \frac{1}{\alpha} \text{---}^3 \text{Rib}_f \frac{1}{\beta} \text{---}^4 \text{Rha} \frac{1}{\alpha}$	Bjorndal <i>et al</i> (1970)
012	$\text{---}^3 \text{GlcNAc} \frac{1}{\beta} \text{---}^3 \text{Rha} \frac{1}{\alpha}$	Erbing <i>et al</i> (1977)

Table : 1 Klebsiella (Enterobacter) ' O ' - Antigen Structures.

Rough mutants, those lacking segments of the polysaccharide fraction, were thoroughly examined in the genus *Salmonella*. The structures and genetics of the mutation from 'smooth' to 'roughness' were discussed in reviews by Lüderitz *et al.* (1971), and Stocker and Makela (1978) (See figure 8).

The molecule was believed to consist of three units covalently bonded. Malchow *et al.* (1969) appeared to demonstrate the presence of three polysaccharide chains per molecule in two *Salmonella* species. Romeo *et al.* (1970) used sedimentation velocities of acylated molecules from *S. typhimurium* G-30 to determine a molecular weight of $10\,300 \pm 650$ for non-acylated molecules. This was interpreted as indicating the presence of three molecules, each of an expected molecular weight of 3350.

Recent work by Rosner *et al.* (1979), Rosner and Khorana (1979) with *E. coli* K-12 and Muhlradt *et al.* (1977) with *Salmonella* spp. supported a monomeric structure for LPS in the membrane. Previous results were explained as non-specific aggregation of the molecules during extraction.

Several papers in recent years presented data which indicated the presence of more than a single chemical type of lipopolysaccharide per strain. It remains to be established whether these are distinct species of lipopolysaccharide or merely represent degradative products of cell enzymes or extraction procedures (Di Rienzo and MacLeod, 1978; Di Rienzo *et al.*, 1978; Le Dur *et al.*, 1980).

BIOSYNTHESIS

The biosynthesis of lipopolysaccharide may be described most conveniently by discussing the process in each of the four components; lipid A, inner core, outer core and 'O' antigen, separately.

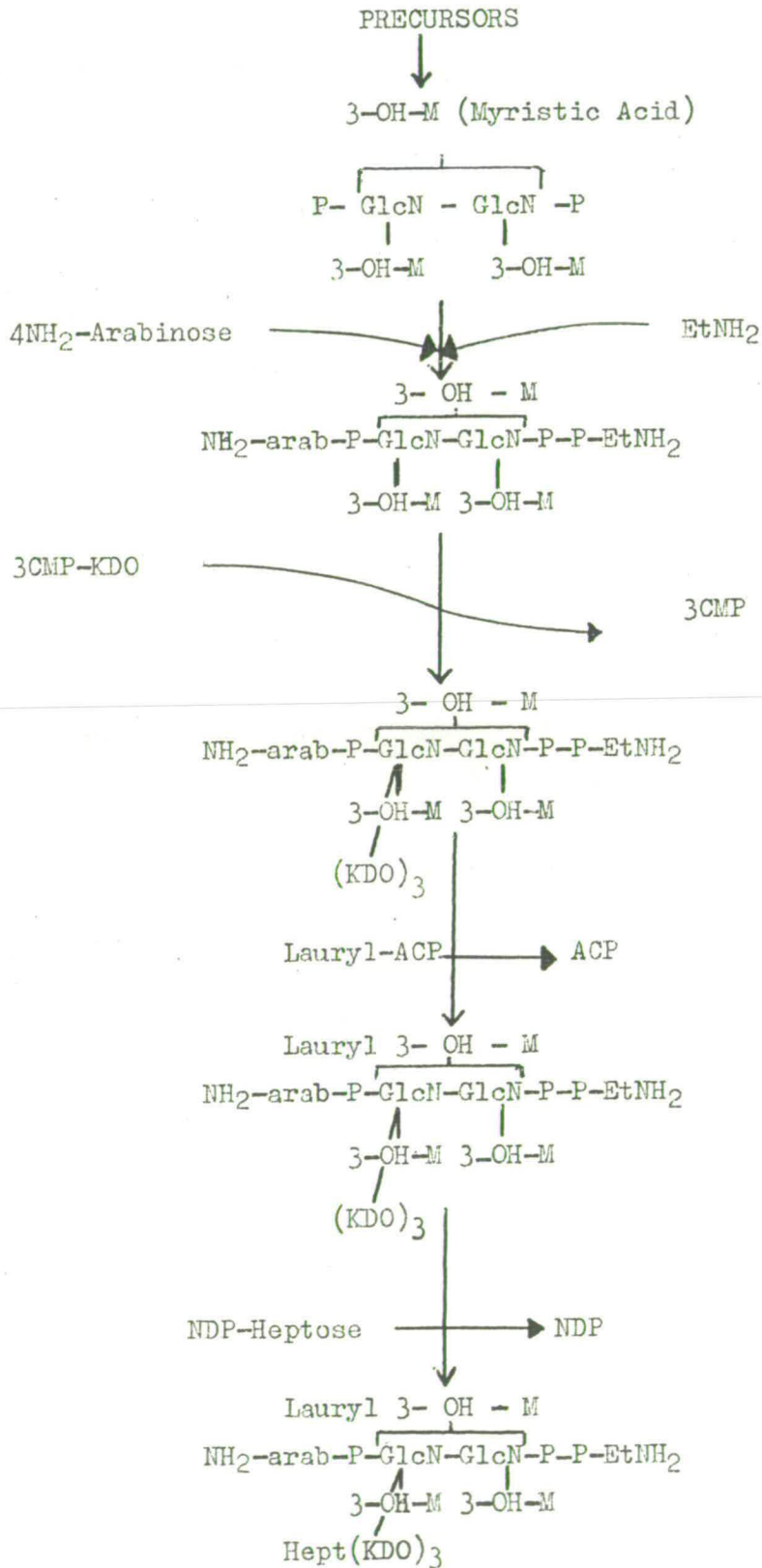
Lipid A and inner core biosynthetic reactions remain largely unknown.

More recently *S. typhimurium* temperature sensitive mutants of KDO biosynthesis were isolated. By growing the cells at the restrictive temperature, lipid A precursors were accumulated. These precursors were composed of glucosamine disaccharides substituted with two phosphate groups (as phosphomonoesters), two amide groups and one or two ester-linked β -hydroxy-myristic acid (Lehmann, 1977).

Studies by Lehmann and co-workers, and Muhlradt on the biosynthesis of Lipid A and the inner core has led to the former group proposing a possible sequential mechanism (Lehmann *et al.*, 1978; Muhlradt, 1969, 1971) (see figure 9). In this sequence the precursor molecule is substituted with 3 KDO molecules, from CMP-KDO, prior to substitution of the molecules with further fatty acid residues.

The outer core of the molecule has been subject to much examination.

Figure 9 Postulated reaction sequence for the biosynthesis of Lipid A and a section of the inner core of *S. typhimurium*.



From Rogers et al, 1980

Salmonella spp. mutants lacking UDP-galactose-4-epimerase showed that the synthesis of the outer core was achieved by step-wise addition of glycosyl moieties. Rogers *et al* (1980) outline a model for outer core biosynthesis based on the findings of several groups (see figure 10).

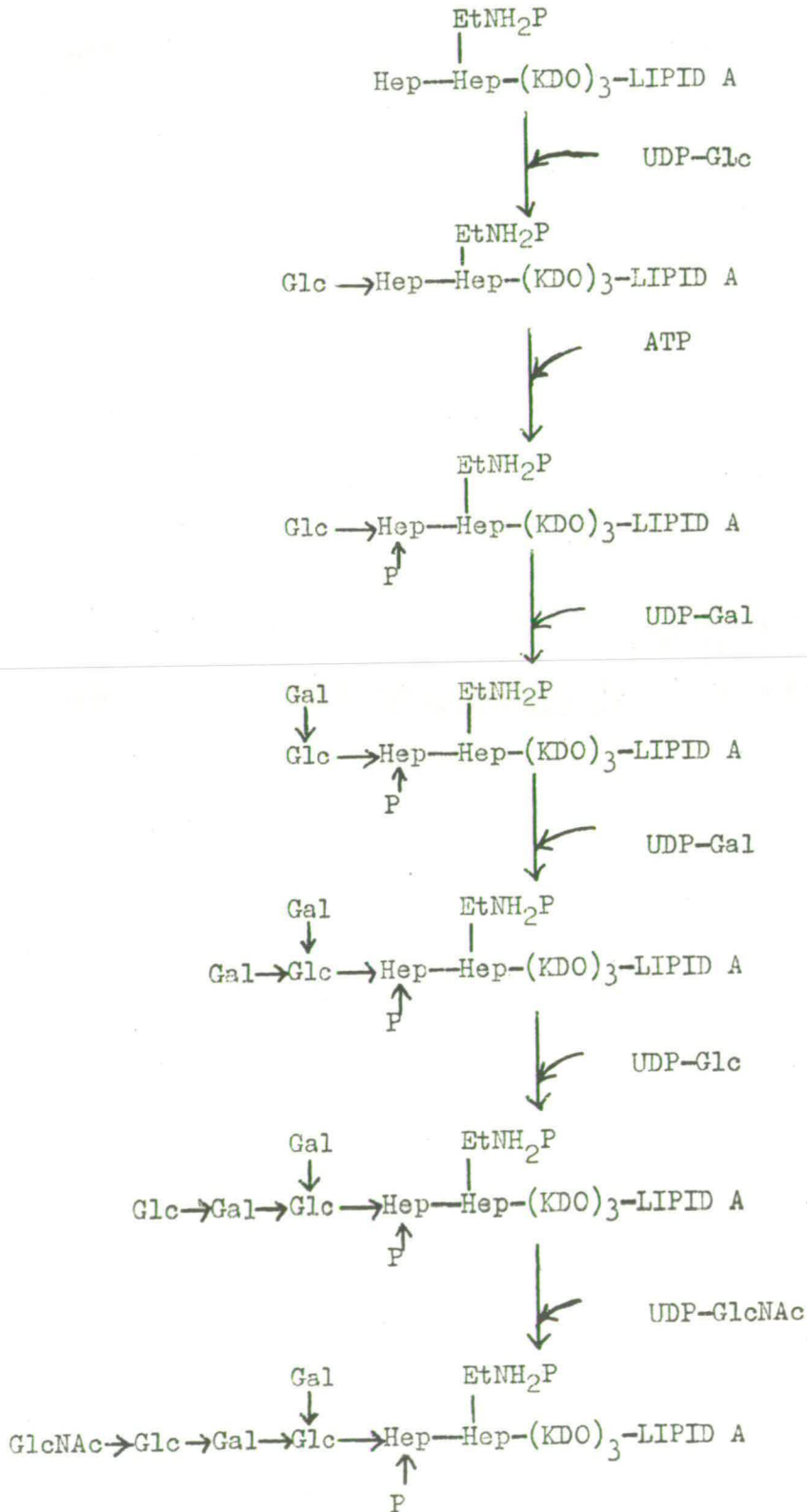
The 'O' antigen is, perhaps, the most studied and best understood pathway. A repeating unit is formed, polymerised, modified (if required) and linked to the outer core by a ligase.

Rogers *et al* (1980) described this process in detail in a review (see figure 11).

The completed molecule must then be transferred to the outer membrane.

Translocation of the molecule to the outer membrane location is complex. It is known from studies using a phage conversion labelling system and direct electron microscopy that LPS is inserted into the membrane at a number (approximately 50) on *S. anatum* of randomly distributed sites (Bayer, 1975). Bayer interprets such results as evidence for adhesion sites, sites of contact between the inner and outer membrane. Further data for a translocation process will be presented in the section on "Assembly."

Figure 10 Postulated mechanism for the biosynthesis of the core of the lipopolysaccharide in *S. typhimurium*.



From Rogers *et al*, 1980

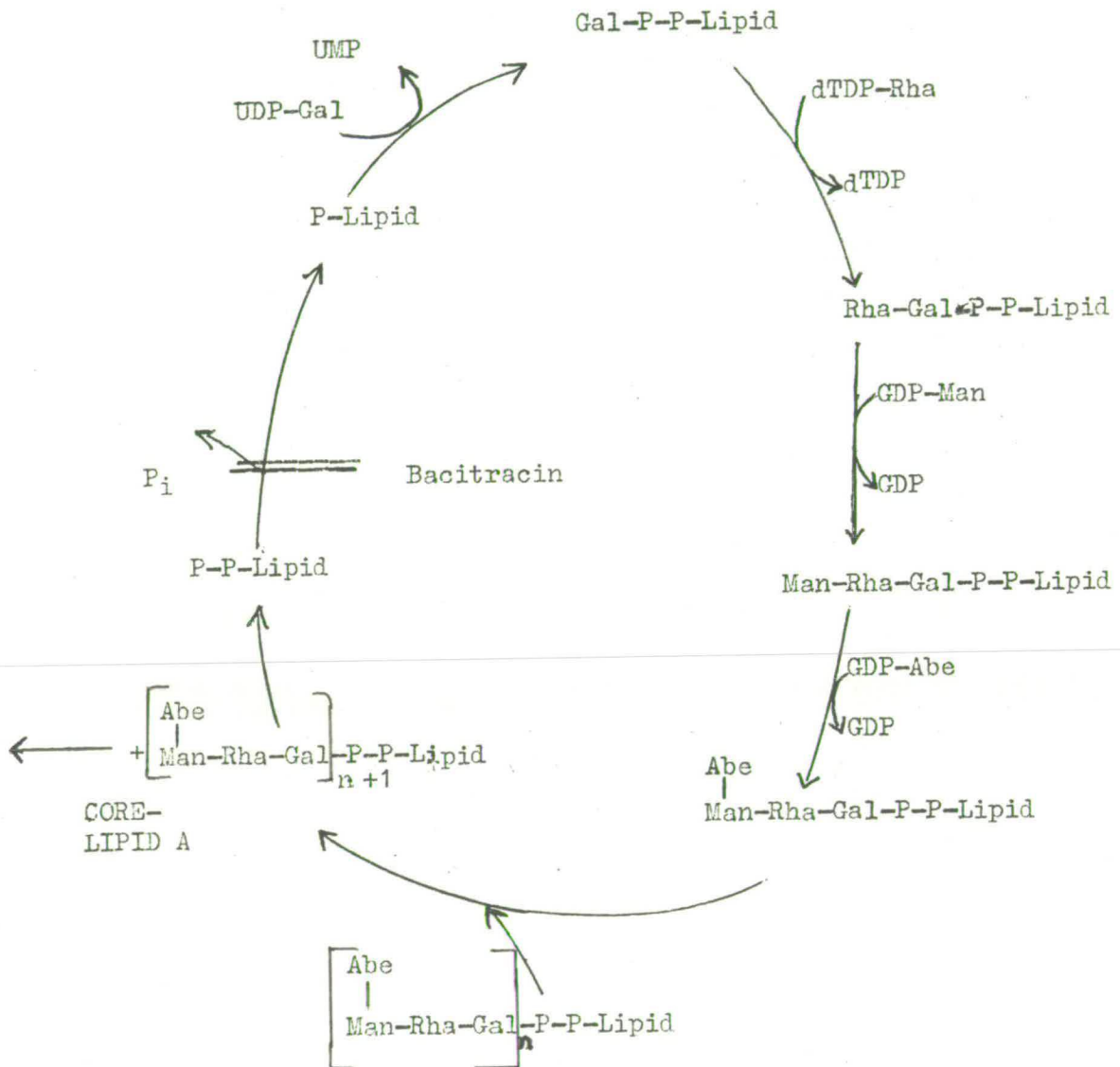


Figure 11 Proposed pathway for the biosynthesis of 'O'-antigen side chains in *S. typhimurium*.

From Rogers *et al*, 1980

EXOPOLYSACCHARIDES

The extracellular polysaccharides, exopolysaccharides, can be classified into four distinct groups: linear or branched homopolysaccharides, heteropolysaccharides and alginates (See figure 12). Examples of homopolysaccharides include cellulose (a linear polymer of β 1-4 linked glucose) which lacks any structural function in the bacterial context and dextrin (a polymer of glucose, branching at carbon atoms two, three or four) which is formed extracellularly and similarly lacks a structural function. Alginates, although chemically classed as heteropolymers (being polymers of D-mannuronic and L-guluronic acids) differ from most heteropolysaccharides in that they tend to consist of sequences of single sugars and sequences of alternating sugars, and lack regularity.

The heteropolysaccharides normally consist of a repeated oligosaccharide unit. Side-chain substitutions are found (Sutherland, 1977).

In the *Enterobacteriaceae*, two genera, Klebsiella (*Enterobacter*) and *Escherichia* provided many capsulate strains on which pioneering studies on structure and biosynthesis of exopolysaccharide were conducted.

Thus, *Klebsiella aerogenes* type 54 (now, *Enterobacter aerogenes*) exopolysaccharide was re-examined by Conrad and co-workers (1966) and the existence of single monosaccharide sidechains discovered. The structures of most of the *Klebsiella* exopolysaccharides were determined and the possession of monosaccharide, acetylated or pyruvylated groups found to be a common feature within this genus (See figure 13).

The type of exopolysaccharide synthesised is normally independent of the carbon substrate (Wilkinson *et al*, 1955).

The *Enterobacter* species are capsulate, exhibiting mucoidness on Yeast Extract Agar, especially at lower incubation temperatures (Sutherland 1977).

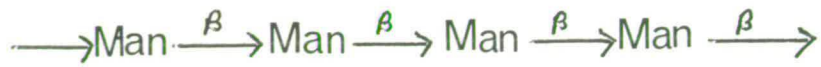
Mutation from encapsulation to slime formation are uncommon and back-mutations extremely rare. Non-mucoid mutants can be obtained spontaneously or by use of mutagens. These are referred to as 'O' mutants possessing only LPS.

CR mutants, the *crenated* mutants of *Enterobacter* described by Norval and Sutherland (1969) exhibited temperature-dependent synthesis of exopolysaccharide and possessed reduced levels of LPS (See figure 14).

The degree of attachment of the capsule to the cell varies from species to species. Accordingly mild agitation or more extreme treatment such as boiling or alkali treatment may be required to release the capsule. Released capsule and slime may then be precipitated from suspension by the addition of polar solvents such as acetone or ethanol.

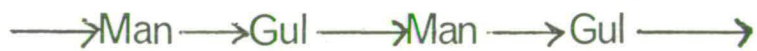
Figure 12

Homopolysaccharide

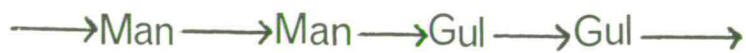


Polymannuronic acid

Heteropolysaccharides



mixed polymer of
D-mannuronic and L-guluronic acids



From Sutherland, 1977a

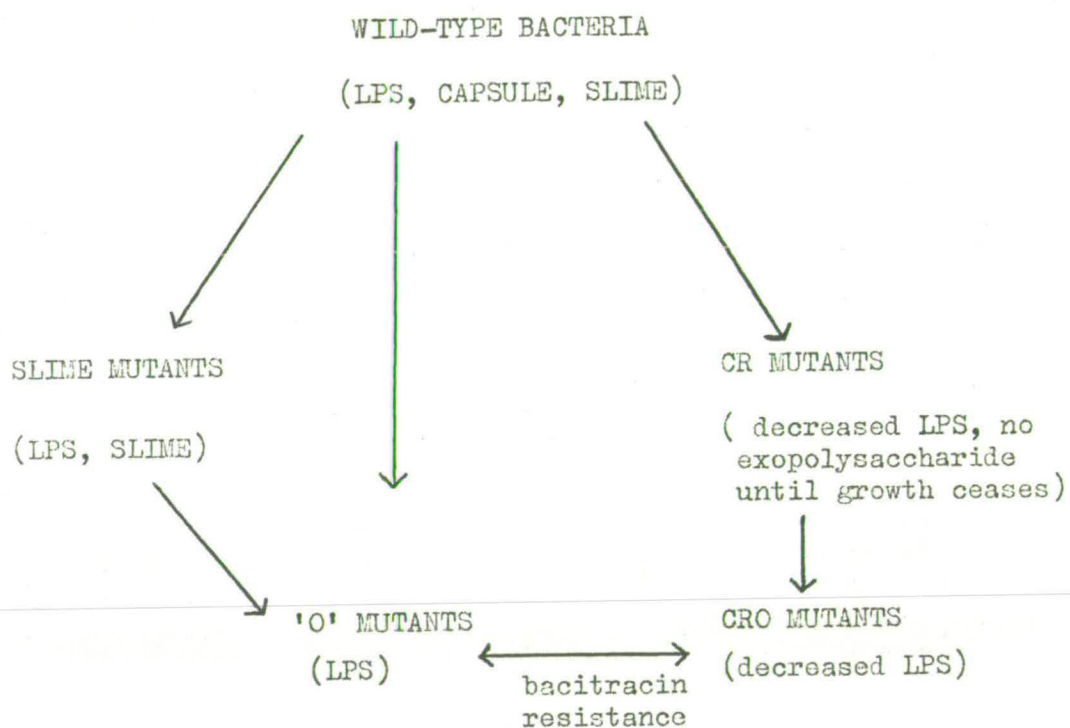


Figure 14 Mutations affecting exopolysaccharide production.

After Sutherland (1977b)

In an elegant microscopic study Bayer and Thurow (1977) described the locations of synthesis and export, and the filamentous substructure of the exopolysaccharide of a temperature-sensitive *E coli* Bi 161/42. 'Knob'-like structures were visualised at the ends of isolated filaments and the authors speculated that this might perform a function in attachment to the cell. They also noted that the exopolysaccharide was exported at a number of random sites on the cell surface, the proposed Bayer adhesion sites (See Sections on Assembly and Molecular Organisation).

The time-course for exopolysaccharide production varies from species to species. Thus, *Enterobacter* spp, produce exopolysaccharide continuously during the cell cycle whereas a *Pseudomonas* spp examined by Williams and Wimpenny (1977) did not synthesise the polymer until late log phase.

BIOSYNTHESIS

It is important to distinguish between the synthesis of exopolysaccharides such as the dextrans and levans, and capsular-type exopolysaccharides. The mechanisms are different. The former polymers are synthesised extracellularly. Substrate molecules and the catalysing enzymes (lipoproteins) are present in the culture fluid. Thus the dextrans and levans are synthesised from the glucose and fructose moieties of sucrose, respectively (Barker *et al*, 1957) (See figure 15).

Acetobacter xylinum synthesised cellulose from UDP-glucose in the presence of a cellodextrin primer (Glaser, 1958). Zaar (1977), examining the biogenesis of cellulose, found that cellulose 'ribbon' assembly was restricted to only a single part of the cell surface.

In contrast the biosynthesis of capsular-type exopolysaccharides is intracellular, and requires membrane bound enzymes, sugar nucleotides and a lipid intermediate.

Much of the pioneering work on biosynthesis was conducted on *Streptococcus pneumoniae* and *Enterobacter aerogenes*. This survey will mainly consider the data provided by the latter studies.

Synthesis of exopolysaccharide can by analogy with lipopolysaccharide and peptidoglycan synthesis be divided into several sections:

- a) the formation of sugar nucleotides
- b) transfer of sugar moieties to a carrier lipid
- c) polymerisation of the oligosaccharide repeating units
- d) modification and extrusion of the polymer.

(a) The sugar nucleotides such as UDP-glucose are requirements for other pathways in the cell and need not be considered exclusive to exopolysaccharide synthesis.

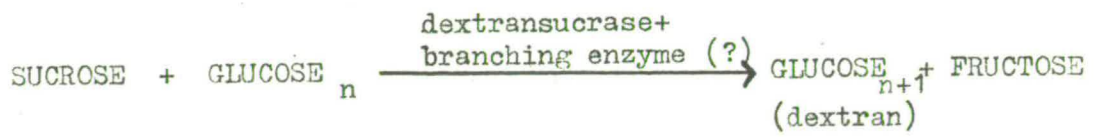


Figure 15 The extracellular formation of dextrans and levans.

No direct evidence exists for establishing the mode of release of the polymer from the isoprenoid lipid carrier.

REGULATION OF EXOPOLYSACCHARIDE PRODUCTION

Gross environmental conditions affect the production of exopolysaccharide. Thus, the ratio of carbon to nitrogen in the medium and the level of potassium and sulphate ions exercises control. Production in *Enterobacter* spp. is favoured by an excess of carbon in the presence of low levels of nitrogen, potassium and sulphate (Duguid and Wilkinson, 1954).

The oxygen tension is important. Dudman (1960) found that increased aeration of *Enterobacter* spp. increased polysaccharide synthesis.

Direct evidence for the genetic control of capsule production came from the studies of Markowitz and co-workers (Gayda *et al*, 1979). Cap R mutants of *E coli* K-12 overproduce capsular polysaccharide. This group showed that the CapR mutants are deficient in a 40K polypeptide of the outer membrane, which apparently acts in a regulatory capacity (See section on non-porin outer membrane proteins).

Several thorough reviews of exopolysaccharide biosynthesis and regulation are available (Sutherland, 1977a, b; Troy 1979).

LIPIDS OF THE GRAM-NEGATIVE CELL ENVELOPE

In the past decade an upsurge of interest has led to a large increase in the amount of literature on lipid content of the outer and inner membranes. Cronan (1978 and 1979) and Raetz (1978) have reviewed this area.

Several groups of workers have examined the lipid content of inner and outer membranes of the major enterobacteria including lipopolysaccharide and lipoprotein content (See relevant sections).

Phospholipids were shown to be distributed in differing proportions in the two membranes. Thus Osborn *et al* (1972); Joseleau-Petit and Kepes, (1975); Lugtenberg and Peters (1976); and Jones and Osborn (1977) reported that the outer membrane is enriched in phosphatidylethanolamine (PE), 80% as opposed to 60%. Nikaido *et al* (1977) showed that inner and outer membrane lipid lateral diffusion rates are identical.

The phospholipids in the outer membrane of *E coli* had a higher saturated fatty acid content (45%) 10% higher than in the inner membrane (Overath *et al*, 1975 and Lugtenberg and Peters, 1976).

The fatty acid content of the outer membrane is derived from three sources: lipoprotein, lipopolysaccharide and phospholipid. In the context of distribution of groups of molecules within the outer membrane it is indicated that LPS is found mainly in the outer leaflet (Funahara and Nikaido, 1980), that PE head groups are not cleaved by externally added phospholipase C (Kamio and Nikaido, 1976) and that the lateral diffusion constant for LPS is five orders of magnitude lower than that of phospholipids (Muhlradt *et al*, 1974). Overath *et al* (1975) found similar transition points for both inner and outer membranes. Such evidence tends to suggest that only a small percentage of phospholipids are engaged in the temperature-dependent transition and that phospholipids and LPS have separate domains within the outer membrane.

Further evidence for segregation of the phospholipids within the outer membrane comes from several groups. Smit *et al* (1975) calculated from results of wildtype *E coli* outer membrane phospholipid content that there was sufficient for a mono-layer only. Kamio and Nikaido (1976) reported that phospholipase activity and cyanogen bromide-activated dextran coupling did not occur at the outer leaflet of the outer membrane thereby leading to the hypothesis that all of the outer membrane phospholipid was segregated in the inner leaflet.

Van Alphen *et al* (1977) have shown similar results to those of Kamio and Nikaido (1976) but drew a somewhat different conclusion, postulating a role for other membrane components in shielding phospholipids from the environment.

Support for this hypothesis can be drawn from work on a galE strain of *S. typhimurium*, the lipid of which could not be labelled by dansyl chloride reagent unless membrane perturbants such as cationic antibiotics, EDTA, Tris-HCl and divalent ions were added (Schindler and Teuber, 1978).

Cronan (1979) proposed a model which would utilise all of the above findings. Phospholipids would be located in small bilayer patches closely allied to protein and LPS molecules thereby shielding the phospholipids from the environment. Such a model could also accommodate the results of the outer membrane freeze fractures which, in wild type *E coli* are more infrequent and less extensive than those of the inner membrane (Bayer *et al*, 1975).

As previously stated only a fraction of the outer membrane phospholipid contributes to the transition point and Rottem and Leive (1977) and Cheng *et al* (1974) adopt this fact to argue for decreased fluidity of phospholipid. Cronan (1979) argues the contrary and states that the exceptionally high protein content of the outer membrane would increase the fluidity of the phospholipids preventing more of the phospholipid from undergoing phase transition at lower temperatures. Thus the results of Overath *et al* (1975) would be in good agreement with the concept of boundary lipid.

BIOSYNTHESIS

The biosynthesis of fatty acid precursors is a prerequisite of phospholipid synthesis (Volpe and Vagelos, 1976; Bloch and Vance, 1977). sn-Glycerol-3-phosphate, L-serine and Cytidine 5' triphosphate are essential for synthesis. L-serine is required for the head group of phosphatidyl-ethanolamine, CTP for cytidinediglyceride while sn-glycerol-3-phosphate being the precursor which is acylated, is a key step in the biosynthetic pathway (Raetz and Kennedy, 1973; Kanfer and Kennedy, 1964).

A fuller understanding of the biosynthesis of phospholipids has been gained from the use of mutants of the phospholipid synthetic pathways.

gsA, plsB and revertants of plsB form the two main classes, the two former being auxotrophic for sn-glycerol-3-phosphate, having a defective glycerol phosphate acyltransferase. The revertants do not require an exogenous glycerol phosphate supply, possessing a mutant enzyme glycerol-phosphate dehydrogenase, altered at the gsA gene such that the enzyme is about twenty times less sensitive to glycerol phosphate thereby overcoming the plsB mutation (Edgar and Bell, 1977). The third mutant class, plsA, possesses a thermolabile glycerol phosphate acyltransferase and is defective in phospholipid synthesis at the non permissive temperature (Cronan and Godson, 1972; Glaser *et al*, 1975; Ray *et al*, 1976).

The site of synthesis of phospholipids is the cytoplasmic membrane. Evidence for this location is two fold: cell extracts harbour synthetic activity in the cytoplasmic membrane (Bell *et al*, 1971; Kito *et al*, 1972; White *et al*, 1972) and precursors such as CDP-diglyceride are bound to the cytoplasmic membrane (Kaufer and Kennedy, 1964; Raetz and Kennedy, 1973). One enzyme, phosphatidylserine synthetase was tightly bound to the ribosomes and not an integral molecule of the cytoplasmic membrane (Dutt and Dowhan, 1977).

The initial acylation reaction can be written thus:

- 1) glycerol-3-phosphate + fatty-acyl-CoA \rightarrow lysophosphatidic acid + CoA
- 2) lysophosphatidic acid + fatty acyl-CoA \rightarrow phosphatidic acid + CoA
(Finnerty, 1978)
- 3) phosphatidic acid + CTP \rightarrow CDP-diglyceride + PPi

In reaction (3) the presence of divalent cation is essential. The reaction was catalysed by phosphatidic acid cytidyl transferase (White *et al*, 1971; Bell *et al*, 1971).

CDP-diglyceride forms a branch point in phospholipid synthesis. This intermediate metabolite can function in phosphatidylglycerol or phosphatidylserine production.

- (4) CDP-diglyceride + sn-glycerol-3-phosphate \rightarrow phosphatidylglycerol phosphate + CMP.
- (5) phosphatidylglycerol phosphate \rightarrow phosphatidylglycerol + Pi

The last two reactions are membrane bound and required Mg²⁺, and a non-ionic detergent for maximal activity. It was shown that phosphatidylglycerol undergoes rapid turnover. (De Siervo and Salton, 1973) Synthesis of cardiolipin (diphosphatidyl glycerol) moieties from phosphatidylglycerol was demonstrated (De Siervo and Salton, 1971; Short and White, 1972; Hirschberg and Kennedy, 1972). Synthesis of phosphatidylserine and phosphatidylethanolamine from CDP-diglyceride and L-serine occurred in membrane preparations of *Acinetobacter* spp. (Finnerty, 1978). Ohta and co-workers (1974) demonstrated the dependence on phosphatidyl serine for phosphatidylethanolamine production in an *E coli* mutant defective in phosphatidyl serine synthesis.

A discussion of the enzymology of phospholipid synthesis is provided by Raetz (1978).

CONTROL AND REGULATION OF SYNTHESIS

Regulation of phospholipid head group composition and fatty acid chainlength and degree of saturation were reviewed by Raetz (1978) and Cronan (1978). Partial control of phospholipid synthesis may be achieved by regulation of fatty acid

synthesis. Mindich (1972) presented evidence for the coupling of fatty acid and phospholipid synthesis. Nunn *et al* (1977) and Cronan *et al* (1975) however, presented further evidence indicating the extensive limitations to this coupling.

The former using a *plsB* mutant, indicated that fatty acid although reduced 10-fold in abundance was still synthesised in a glycerol-3-phosphate starved culture while Cronan and co-workers showed that if β -oxidation and phospholipid synthesis were blocked, fatty acids accumulate.

The *rel* gene of *E coli* which controls phospholipid and RNA synthesis provided insight into a possible broad control. *rel*⁺, a stringent strain when starved of amino acids suffers a 2-4 fold inhibition of the rate of phospholipid synthesis (Sokawa *et al*, 1968; Sokawa *et al*, 1972; Golden and Powell, 1972) whereas *rel*⁻, a relaxed strain, produces phospholipid normally (Sokawa *et al*, 1968; Nunn and Cronan, 1974, 1976a, 1976b). Throughout amino acid starvation of *rel*⁺ strains there was accumulation of guanosine 5'-diphosphate-3' diphosphate (ppGpp) but this did not happen in *rel*⁻ strains. Experiments using a temperature shift conducted by Gallant and co-workers (Gallant *et al*, 1977) indicated that a rise in intracellular ppGpp did not correspond to reduced lipid synthesis and that previous work using amino acid starvation might wrongly have correlated ppGpp accumulation and reduction in phospholipid synthesis.

The interdependence of phospholipid synthesis and synthesis of outer membrane components is exemplified by the work of Lin and Wu (1976) using a glycerol deprived *S. typhimurium* glycerol auxotroph. This mutant synthesised lipoprotein lacking glycerol moieties after phospholipid synthesis had ceased.

This interdependence will be discussed further in the section on "Assembly of Polymers into the Outer Membrane."

MEMBRANE DERIVED OLIGOSACCHARIDES (MDO)

The discovery of the membrane derived oligosaccharides by van Golde *et al* (1973) and Kennedy *et al* (1976) places a second class of molecules, with lipoprotein, as being dependent on about 90% of the turnover of membrane phospholipids for their synthesis (Schulman and Kennedy, 1977).

This family of molecules all contain 9 to 10 glucose units derived from UDP-glucose. The glucose molecule may be substituted at C⁶ position with *sn*-glycerol-1-phosphate and phosphoethanolamine residues. The latter substitutions have been shown to be from turnover of polar head groups of membrane phospholipids (Kennedy *et al*, 1976; Schulman and Kennedy, 1977).

Schulman and Kennedy (1979) estimated the molecular weights of *E coli* MDO in a range between 2000D and 2600D, the most abundant species carrying 3 to 6 net negative charges per mole.

On the basis of calculations from known *E coli* MDO structures, Schulman and Kennedy (1979) estimated the glucose content at 81% and proposed that the MDO content of an unidentified strain of *Enterobacter aerogenes* comprised 1.1% of total cell dry weight, against a value of 0.86% for *E coli* K-12.

These molecules may be located in the periplasmic space or loosely associated with the inner face of the outer membrane. Schulman and Kennedy (1979) indicated that a location on the outer face of the outer membrane seems unlikely since less anionic MDO species must be substituted *in situ* and the synthetic machinery for this is unlikely to be present on the outer face of the outer membrane.

THE OUTER MEMBRANE PROTEIN OF THE GRAM-NEGATIVE BACTERIA

Early studies on the protein composition of isolated outer membrane fractions indicated the presence of a number of major polypeptides (Schnaitman 1970; 1974a, b; Bragg and Hou, 1972; Wu, 1972). Subsequently these proteins were subdivided into groups on the basis of gross distinctions such as peptidoglycan association (porins) or non-association, and ability to be modified by heat.

There are many minor proteins and inducible polypeptides. The number of molecules of each protein varies quite markedly: from a few hundred copies of a minor protein to 10^5 copies of the major constituents OmpA, matrix protein and lipoprotein.

As a consequence of the number of separate groups working on outer membrane proteins of different strains of *E coli* and *Salmonella typhimurium* there now exist several nomenclatures for identical proteins. A uniform nomenclature based on structural gene loci is now recommended (Reeves, 1979) and is shown in Table 2.

Under such a scheme OmpA protein is the product of structural gene OmpA while the new membrane protein, NmpA, is presently proposed to be the product of NmpA gene. It is not yet clear whether such genes are structural or regulatory (Lee *et al*, 1979).

OmpF protein or matrix protein would thus fall into a category called *matrix porins* to include OmpC and D of *E coli* and *S. typhimurium* (Reeves, 1979).

As can be visualised from Table 3, differences in the strain of *E coli* used in experimentation has shown that differences in membrane composition occur within strains of the *same* species (See also Schnaitman, 1974b).

Cultural differences including incubation temperature, salt and ion concentration and conditions of catabolite repression may have a profound influence on the outer membrane protein (Schnaitman, 1974b; Darveau *et al*, 1980; Overbeeke and Lugtenberg, 1980; Loeb and Kilner, 1979). The inclusion of maltose as sole carbohydrate source for *S. typhimurium* or *E coli* has been reported to induce a new outer membrane protein, involved in maltodextrin uptake, the product of lamB gene (Palva, 1978; Nakae and Ishii, 1980).

**Existing and Recommended Nomenclature
for Outer Membrane Proteins.**

TABLE 2

<i>Gene</i>	<i>Previous designation E coli</i>							<i>Salmonella typhimurium</i>	<i>Recommended norm data</i>
	<i>B</i>	<i>F</i>	<i>H</i>	<i>I</i>	<i>L</i>	<i>M</i>	<i>S</i>		
omp A	B	TolG	II*	7	D	0-11	3a	33K	OmpA
omp B									
omp C	A ²		Ib	4	c	0-8	1b	36K	OmpC
omp D								34K	OmpD
omp F	A ¹	TolF	Ia		b	0-9	1a	35K	OmpF
nmp A		E	Ic	4	e		NmpA		
nmp B							NmpB		NmpA-B
nmp C							NmpC		NmpC
lam B								44K	LamB
lpp	F		IV	11		0-18			Murein lipoprotein

B Bragg
F Foulds
H Henning
I Inouye
L Lugtenberg
M Mizushima
S Schnaitman

**Major Outer Membrane Proteins of
Several Gram-Negative Bacteria ^(a)**

TABLE 3

<i>MW</i>	<i>E coli</i> K12 ^(b, c, d)	<i>E coli</i> O111 ^(e)	<i>E coli</i> B/r ^(f)	<i>E coli</i> MX 74 ⁱ
40,000	a			
38,500 (36,500)	b Ia	1	1a, A matrix protein	e
38,000 (36,000)	c Ib	2		
33,000	d II*	3a + 3b	II*, B	g, h
17,000	III		III	
7,500	IV	(h)	IV	j

a) modified from Nikaido (1978) *f)* Bragg and Hou (1972)
b) Lugtenberg *et al* (1975)
c) Garten *et al* (1975) *h)* Torti (1977)
d) Rosenbusch (1974) *i)* Inoué and Lee (1973)
e) Schnaitman (1970)

After table cited by Wright and Tipper (1979)

MAJOR NON-PORIN PROTEINS

OmpA Protein (Protein II*)

OmpA protein of *E coli* K-12 was first shown to have the anomalous heat-modifiable characteristic thought to be due to increasing unfolding of the molecule in the presence of sodium dodecyl sulphate (SDS) in which it can be easily extracted from the envelope peptidoglycan at 37°C (Henning *et al*, 1973a). It was also found to be sensitive to protease cleavage in envelope fractions (Henning *et al*, 1973b).

It is a multifunctional protein acting as a phage receptor, a prerequisite for colicin action, serves as a mediator in F-pilus dependent conjugation and with lipoprotein is functional in generating and maintaining cell shape and membrane integrity (Skurray *et al*, 1974; Chai and Foulds, 1974; Braun, 1975; Schweizer and Henning, 1977; Van Alphen *et al*, 1977; Datta *et al*, 1977; Sonntag *et al*, 1978). Ferrichrome-mediated ion-transport and amino acid uptake have been attributed to OmpA as a result of deficiencies in mutants lacking OmpA (Manning, *et al*, 1977). However, there remains the possibility that these deficiencies occur because the membrane is reorganised as a result of OmpA possessing an important role in maintaining membrane integrity.

Endermann *et al* (1978) demonstrated that OmpA protein of *E coli* K12 is transmembrane and recent work has focussed on determining the primary structure (Chen *et al*, 1980). The polypeptide consists of 325 amino acid residues. Residues at 1-177 constitute the transmembrane section, having a predominately lipophilic 27 residue segment which may span the membrane as an α -helix or as a linear segment 19 residues in length. The section contained within the outer membrane shows an Ala-Pro-Ala-Pro-Ala-Pro-Ala-Pro sequence analogous to the polyproline hinge sequence of immunoglobulins.

Calculated from the number of amino acid residues the molecular weight of the molecule is 35 159.

The structural gene, OmpA, has been cloned by Henning and co-workers (Henning *et al*, 1979). This combined with knowledge of the primary structure may permit elucidation of the mechanism regulating synthesis. At present, a regulating gene equivalent to OmpB for structural genes OmpC and F is unknown.

The mechanism of biosynthesis of OmpA protein is unclear but a possible precursor has been shown to accumulate in minicells expressing plasmid pSC101-ompA⁺ and amplification of the OmpA gene was shown to result in higher levels of OmpA protein while lowering OmpC and F levels in the envelope (Henning *et al*, 1979).

PROTEIN a

Protein a, according to the nomenclature of Lugtenberg *et al* (1975) and widely adapted, is a 40 kilodalton protein whose synthesis is temperature dependent.

Until recently, little other than the molecular weight was known. Work by two groups has now suggested two possible and apparently widely varying functions. No obvious relationship between the functions has been shown.

Fiss *et al* (1979) showed that Protein a catalyses the conversion of an 81K polypeptide to 74K, the receptor for ferro-enterobactin. The second function is in the regulation of capsular polysaccharide synthesis. Markovitz and co-workers succeeded in cloning a 2 Megadalton DNA fragment which coded for Protein a and inhibited capsular polysaccharide synthesis in capR (lon) mutants which overproduce capsular polysaccharide. Reduced Protein a production correlates with low temperature-dependent increased mucoidness of *E coli* K12 (Markovitz, 1964; Gayda *et al*, 1979; Zehnbaauer, 1980).

MATRIX PROTEINS (Porins)

The major outer membrane proteins in the 33K to 38K molecular weight range (excluding lipoprotein) which remain in association with peptidoglycan after heating to 70°C in the presence of SDS are the peptidoglycan-associated matrix proteins. They are some of the most abundant species of protein (10⁵ molecules in the outer membrane) and can be solubilised by heating at 100°C in the presence of 2% SDS. Presently, the solubility properties form a convenient method of identifying matrix proteins.

Rosenbusch (1974) purified the matrix protein of *E coli* by treating with 0.5M NaCl/SDS. Matrix protein, which is arranged on the outer face of the peptidoglycan in a hexagonal lattice, is mainly found in a β -configuration and tends not to bind SDS strongly unless heat denatured and on removal of SDS will reassociate with peptidoglycan. It will also remain preferentially as oligomers. (Nakae, 1976a and b; Hasegawa *et al*, 1976; Palva and Randall, 1978; Nakamura and Mizushima, 1976).

Rosenbusch (1974) reported that matrix protein of *E coli* BE was homogeneous and had a mass of 36.5K. Subsequently, the production of more than a single matrix protein was detected in *S. typhimurium* using SDS polyacrylamide gel electrophoresis (Ames, 1974). These proteins had masses of 36K, 35K and 34K (OmpC, F, and D respectively) and the general properties of matrix proteins.

E coli K12 produces two matrix proteins, OmpF and OmpC (1a and 1b respectively) one of which, 1a, corresponds to the single matrix protein of *E coli* B (Schmitges and Henning, 1976).

A third unique protein, Protein 2, may be detected in *E coli* K12 lysogenised by phage PA-2.

The matrix proteins have clearly been implicated in pore formation. Hydrophilic pores in the membrane allow rapid diffusion of small, uncharged molecules up to a molecular weight of 600 (Nakae, 1976a, b; Decad and Nikaido, 1976).

Analysis of porins for hydrophobic amino acid residues has indicated low levels (Rosenbusch, 1974). Cross-linking analysis of Protein 1a and 1b in 0.1% SDS suggests that they form separate oligomers (Palva and Randall, 1979). Nakae and co-workers (cited in Nikaido and Nakae, 1979) found *S. typhimurium* 34K and 36K porins existing as stable trimers in 0.25% SDS. β -configuration appears to be a function of oligomeric structure (Nakamura and Mizushima, 1976).

Recently, Lee and Schnaitman (1980) found OmpF and OmpC pairs of proteins of both *S. typhimurium* and *E coli* K12 had very limited similarity on peptide maps. Ichihara and Mizushima (1978) showed that Proteins 1a (OmpF) and 1b (OmpC) of *E coli* K12 had significantly differing amino acid compositions as had the 34K and 36K proteins of *S. typhimurium* (Tokunago and co-workers cited in Nikaido and Nakae, 1979).

Until this evidence was presented, OmpB gene was proposed as the structural gene coding for a precursor which was altered by postranslational modification by OmpC and F genes products. Currently a hypothesis involving OmpB as the regulatory gene seems more promising.

REGULATION OF PORIN EXPRESSION

Analysis of *E coli* and *S. typhimurium* mutants lacking OmpB, OmpC, OmpF and OmpD porins and construction of lac⁺ operon fusion strains has contributed to a greater understanding of the regulation mechanism. The insertion of *E coli* OmpF region into the *S. typhimurium* chromosome resulted in the production of this protein with 34K, 35K and 36K salmonella proteins. In addition, OmpC gene mutants producing altered OmpC protein has been construed as evidence for the structural nature of these loci (Sato and Yura, 1979; Van Alphen *et al*, 1977). By constructing OmpC-lac operon fusions Silhavy and co-workers⁽¹⁹⁷⁹⁾ demonstrated that β -galactosidase exhibited regulatory properties of matrix protein 1b and consequently in these strains an OmpB mutation reduced β -galactosidase levels in a manner analogous to that of matrix protein 1b regulation, indicating the strong probability that OmpC and OmpF are structural genes.

Constitutive synthesis of OmpC and OmpF proteins has been achieved in two mutants of an OmpB strain of *E coli* K12 lacking OmpC and F proteins. Under conditions which would lead to repression of these proteins in the wildtype (lack of OmpB gene function, restrictive growth conditions, lysogenisation with phage PA-2) the mutants, OmpCpl and OmpFp9 synthesised their respective gene product constitutively. The mutations were located in the region of genes OmpC and OmpF and their expression was cis-dominant. From these data, Sato and Yura (1981) suggest that the mutations have occurred at the promoter region of the structural genes OmpC and OmpF.

OmpB gene maps at 73.7 min and mutations at this locus affect both 1a and 1b proteins. Evidence from Wanner *et al* (1979) suggest that it is an extremely complex locus; Per A mutation leading to overproduction of OmpC, approximately a 10-fold reduction in levels of alkaline phosphatase, six other periplasmic proteins and three major outer membrane proteins including OmpF.

P1 transduction of Per A indicated 97-98% linkage to OmpB.

Per A is proposed as a separate gene, a mutation in which leads to a block in the secretory mechanism. A similar group of mutants is discussed by Osborn and Wu (1980). Discovered by Schwartz and co-workers⁽¹⁹⁶⁹⁾ these mutants are in the OmpB region and result in the loss of OmpF protein and a severe reduction in the expression of lamB gene in uninduced cells. Transcription of lamB is affected.

Given the range of effects of mutations in the OmpB region, it seems probable that the OmpB locus exercises control over expression of a number of outer membrane and periplasmic proteins.

BIOSYNTHESIS

Messenger RNAs of the porins had a longer half-life than cytoplasmic mRNA, ranging between 5.5 and 11.5 minutes as does lipoprotein mRNA (Levy, 1975).

Binding of mRNA to membrane bound polysomes and a resulting shielding effect is proposed as an explanation for mRNA longevity.

Synthesis of matrix protein in *E coli* B/r sphaeroplasts indicated that the processed porin is inserted directly into the surface envelope on translation. An intermediate form of the porin is extractable by sarkosyl NL97, prior to assembly into the outer membrane, prompting the investigators to suggest that the porin is first inserted into the inner membrane, processed and immediately translocated to the outer membrane. They also put forward an alternative strategy to incorporate the finding of Bayer (1975). By inserting the porin directly into the outer membrane at a specific junction point (Bayer's adhesion sites) between outer and inner membranes, the porin would remain sarkosyl extractable until the porin formed non-covalent linkages with other surface components (Metcalf and Holland, (1980). In agreement with this possible mechanism, Begg (1978) demonstrated, by autoradiography of cell envelope ghosts of *E coli*, that porins are inserted at random locations on the cell surface and once inserted are not conserved in any spatial configuration.

STRUCTURE AND FUNCTION

Matrix proteins are arranged as a hexagonal lattice with a periodicity of 7.7 nm covering in excess of 60% of the outer surface of the peptidoglycan layer. The protein-protein interactions appear to be the most important factor in structural integrity of the lattice. As lysozyme-treated sphaeroplasts produce the same lattice Rosenbusch and co-workers postulated that the triplet indentations, which readily stained in each hexagonal unit, were channels spanning the outer membrane (Rosenbusch, 1974; Steven *et al*, 1977). Since 1974 much evidence has been produced as to their supramolecular structure and function.

The aggregation of porins as oligomers was indicated by Rosenbusch and co-workers (Tokunaga *et al*, 1979); and the smallest multimer size found by Nakae *et al* (1979) was a trimer. Ishii and Nakae (1980) suggested that the trimer is probably composed of homologous monomers. Nakae and Nikaido established that OmpC and OmpF proteins function as hydrophilic pores *in vivo* and in reconstitution studies *in vitro* (Nakae, 1976a, b; Nikaido *et al*, 1976).

OmpD of *S. typhimurium* was likewise implicated in pore formation (Nikaido *et al*, 1976).

PORIN LEVELS

Porin protein levels in the membrane can be radically altered by mutation or growth conditions. Thus 'deep rough' Rd₁, Rd₂, Re lipopolysaccharide mutants of *E coli* and *S. typhimurium* show drastically reduced levels of porin protein (Ames *et al*, 1974; Koplow and Goldfine, 1974).

Phage or colicin resistance results in similar protein deficiencies. Davies and Reeves (1975) isolated colicin tolerant *E coli* strains lacking both OmpC and OmpF protein. Numerous phage resistant *E coli* strains are lacking protein; bacteriophage Tu II* of strains P530 and P692 lack proteins OmpC, OmpF and OmpA (Henning and Haller, 1975). Resistance to toxic elements such as copper (Cu²⁺) appears to be mediated by OmpF (Lutkenhaus, 1977).

Schnaitman (1974) found the levels of porin proteins to fluctuate according to growth conditions. Further work by Lugtenberg *et al* (1976) has shown that the levels of OmpC and F vary according to growth phase but, interestingly, that the total amount of matrix protein remains constant.

NEW MEMBRANE PROTEINS (Nmp)

In mutants of *E coli* deficient in both OmpC and OmpF proteins, pseudoreversion (extragenic mutations) result in the constitutive production of a new outer membrane Protein (e) which possesses porin functions. These mutations have been localised in genes NmpA or NmpB at 82 and 8 min. respectively on *E coli* K12 genetic map (Pugsley and Schnaitman, 1978).

Overbeeke and Lugtenberg (1980) later found Protein e synthesis in wildtype cells of *E coli* K12 to be depressed by phosphate limitation. Argast and Boos (1980) and Tommassen and Lugtenberg (1980) found co-regulation of Protein e with alkaline phosphatase. The two groups concluded from genetic map positions that NmpA and NmpB mutants may contain mutations in *pho a* gene (phosphate regulation locus) and provide evidence to show that the Nmp genes are identical to some of the known *pho* genes.

The NmpC gene codes for a protein similar to that coded for by the lysogen PA-2, Protein 2. Lee *et al* (1979) showed that there exist almost identical peptide patterns and isoelectric focussing profiles for NmpC and Protein 2. Antiserum against Protein 2 showed partial cross-reactivity with NmpC protein. The mechanism by which regulation of new membrane proteins is achieved is unknown and subject to much speculation.

Lam B

Lam B protein, the receptor for lambda phage, has a molecular weight of 47K. On induction it becomes a major porin for maltodextrin diffusion, similar in abundance to the major matrix porins. Lam B protein associates with peptidoglycan and will form stable oligomers in SDS (Braun and Krieger-Brauer, 1977; Palva and Randall, 1978).

In a comparison between Lambda (λ) receptor protein and porins for saccharide permeation, Nakae and Ishii (1980) proved the λ receptor role in maltodextrin diffusion and raised the question of pore size, suggesting λ receptor pores were larger than the matrix porins. Boehler-Kohler *et al* (1979) determined the diameter of pore as 1.6 nm compared with 1.2 nm for matrix porin. Wandersman *et al* (1979) reported that *E coli* K12 can grow on maltodextrins up to Glc₇. However Nakae (1979) obtained ambiguous results for uptake of a variety of solutes. This could be explained as a molecular sieving of saccharides (limit molecular weight, approximately 600) and an exclusion mechanism (unknown) for certain solutes.

COMMON ANTIGENS IN THE ENTEROBACTERIACEAE

In an examination of antigenic cross-reactivity of major outer membrane proteins from several members of the family *Enterobacteriaceae*, Hofstra and Dankert (1979, 1980) found that antigenic cross-reactivity was a general phenomenon apparently independent of molecular weight variation in corresponding proteins. No reactivity was noted between enterobacterial species and species of distinct families.

Overbeeke *et al* (1980) examined antigenic cross-reactivity between OmpC, OmpF protein and Protein e of a single *E coli* strain and between OmpA protein and homologous protein from other strains. They demonstrated a structural relationship between porins of *E coli* K12 and other members of the Enterobacteriaceae, thereby confirming the results of Hofstra and Dankert.

Hofstra and Dankert (1979), Hofstra *et al* (1980) speculate that the genes coding for the major proteins of the outer membrane have been well conserved during evolution and that the synthesis of such common surface antigens may serve as a determinant for classification of member species of Families.

MUREIN LIPOPROTEIN

Braun and Rehn (1969) isolated a low molecular weight polypeptide from *E coli*. The molecule was covalently bound at the C-terminus to peptidoglycan, 1 molecule every 10 muramic acid residues and possessed lipid substituents at the N-terminus. The number of molecules of lipoprotein bound to peptidoglycan in cells growing exponentially was estimated at approximately 250,000 molecules per cell (Braun *et al*, 1970). However, the total number of molecules present included those which were not bound but were found in the free-form almost exclusively in the outer membrane (Bosch *et al*, 1973, Lee *et al*, 1974). Inouye *et al* (1972) indicated that two-thirds of the molecules were not bound, suggesting approximately 750,000 molecules of lipoprotein might be present in the cell.

Lipoprotein played a significant role in stabilising and maintaining the integrity of the outer membrane. Hirota *et al* (1977) isolated a lipoprotein deletion mutant, thought to lack the lipoprotein structural gene. The mutant produced no free or bound lipoprotein or lipoprotein mRNA. This lipoprotein negative mutant (lpo) grew and divided normally but displayed increased sensitivity to EDTA, cationic dyes and detergents and leaked periplasmic enzymes. While indicating a structural function for the molecule it is clearly not essential for cell viability.

Members of the *Enterobacteriaceae* possess lipoprotein in a highly conserved form (Nakamura *et al*, 1979) Possession of lipoprotein is not confined to *E coli*, *Proteus mirabilis* and other members of the *Enterobacteriaceae* but has also been isolated from *Pseudomonas aeruginosa* (Katz *et al*, 1978 and Mizuno *et al*, 1979).

CHEMICAL STRUCTURE

The lipoprotein, covalently linked to peptidoglycan had a molecular weight of approximately 7000. It lacked glycine, histidine, proline, phenylalanine and tryptophan (Braun and Rehn, 1969). Braun and co-workers later elucidated the structure of the lipoprotein (Braun *et al*, 1970, 1972a, 1972b and Hantke and Braun, 1973).

It had 58 amino acid residues with cysteine at the N-terminus and lysine at the C-terminus and contained only 15 amino acids. The ϵ -amino groups of lysine were linked to the carboxyl group of meso-diaminopimelic acid (DAP) of peptidoglycan while the cysteine was linked to one fatty acid by an amide bond and to a diacyl glycerol by a thioether linkage.

The fatty acyl residues had a similar composition to those of the cell. Recently Lai and Wu (1980) using phospholipid vesicle fusion obtained strong evidence for their hypothesis that the acyl moieties in membrane phospholipids

are the precursors for the fatty acids in murein lipoprotein of *E coli*. However, they did not observe specificity among any of the major glycerophosphates in *E coli* regarding the efficacy of the donor.

The primary structure showed a repetitive amino acid sequence: from amino acids 4 to 17 and almost identically repeated 18-31 followed by shorter sequence repetitions (Braun, 1975). Braun and co-workers (1973, 1974) showed that lipoprotein had a very high α -helical content. Two 3-dimensional models have been proposed.

Inouye (1974) proposed a model which utilises all of the amino sequence suggesting that the lack of molecule proline precludes any bends in the α -helical configuration. Noting the free lipoprotein in the membrane the model was described as a tubular, hydrophilic channel through the outer membrane which could serve as a passive diffusion pore (See figure 16).

As in the Inouye model where only small segments at both N- and C-terminals are not α -helical the Braun model is largely α -helical and has similar N- and C-terminals but possesses a folded region between amino acids 25-29 inclusive (Braun, 1975) (See figure 17).

Determination of the α -helical content of lipoprotein in 0.015% SDS indicated 88% α -helical tending to support the Inouye model (Lee *et al*, 1977).

Inouye (1975) further proposed a cylindrical assembly consisting of six α -helices (3.6 amino acids per turn) with regular hydrophobic residues orientated to the exterior of the cylinder, arranged to form a super helix, the angle between the axis of the superhelix and the axis of the α -helix was about 25°.

According to this model, pore or channel size is determined by the number (n) of lipoprotein molecules per assembly and Inouye has estimated the channel diameter for values of n ranging from 1 to 12.

Estimations of hydrophilic pore size involved in passive diffusion in *S. typhimurium* using oligosaccharides as solute gave an exclusion limit of molecular weight 800-900 (Decad *et al*, 1974).

Such an exclusion limit corresponds closely to that which Inouye expected from a 6 α -helice super helix with a pore diameter of 12.5A. In a recent reassessment of the likely configuration of lipoprotein in the membrane Nikaido and Nakae (1979) suggested a super helix lacking a central channel, rather like the tropomyosin molecule. Citing electron micrographic results from *E coli*, obtained by De Petris, Murray and co-workers, Nikaido and Nakae (1979) presented a cogent argument in favour of Braun and Bosch's (1972) model for lipoprotein molecules with only the fatty acyl groups inserted into the membrane.

As a consequence of the large degree of latitude in interpretation of possible tertiary and quaternary structures for the lipoprotein molecule a greater volume

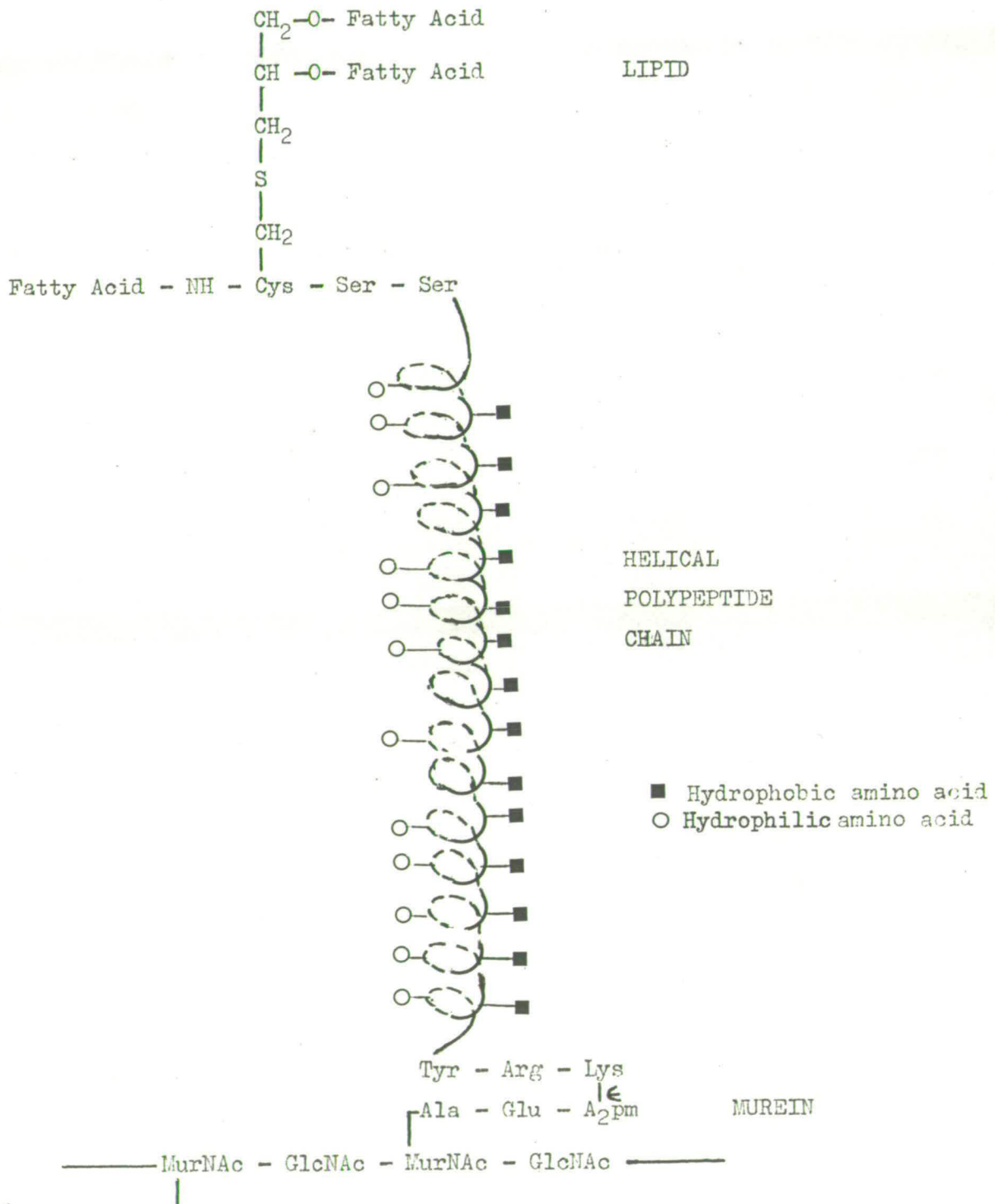


Figure 16 Model for the configuration of murein lipoprotein *in vivo*.

From Inouye, 1974.

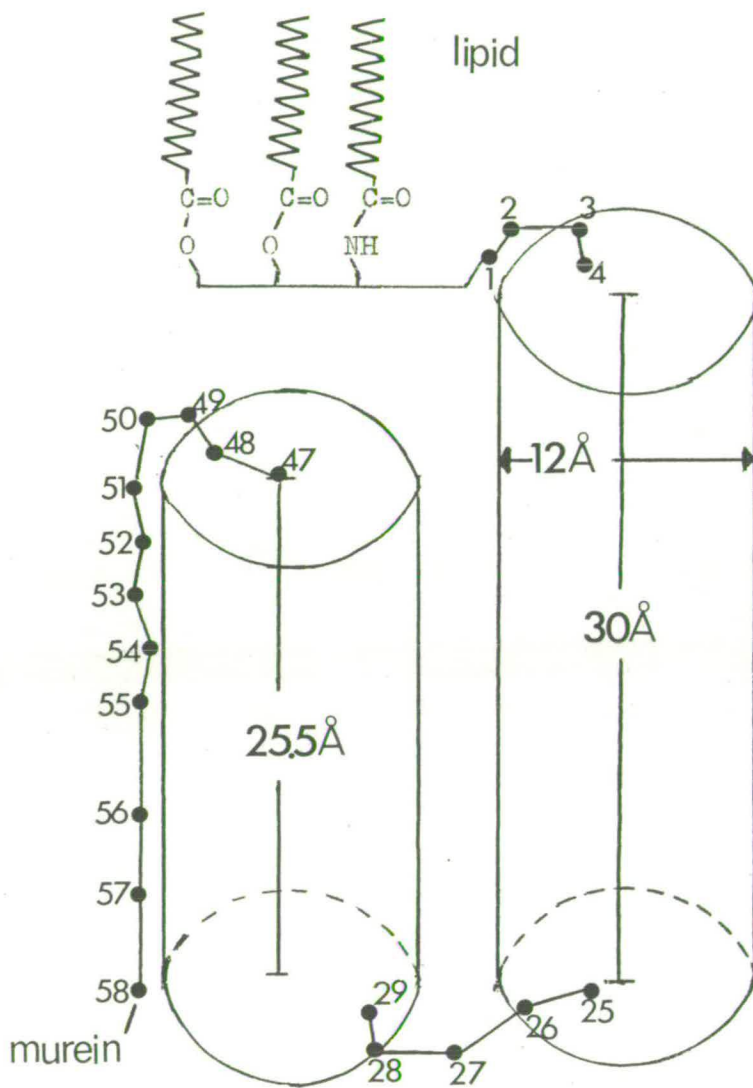


Figure 17 Model for the configuration of murein lipoprotein *in vivo*. From Braun, 1975.

of evidence for proposed models must be amassed before either can be accepted or dismissed.

BIOSYNTHESIS

The biosynthesis of lipoprotein has been examined in detail, largely as a result of the unusual nature of the protein and because it lacks several amino acids for which auxotrophic mutants of *E coli* are available.

Histidine starvation of a histidine auxotroph resulted in the production of a single envelope lipoprotein, whose synthesis continued normally for 4h. after which it fell to 30%, remaining constant at this level (Hirashima and Inouye, 1973).

Lipoprotein and other outer membrane protein biosynthesis is more resistant than cytoplasmic membrane protein synthesis to antibiotics such as chloramphenicol and rifampicin (Lee and Inouye, 1974). Lipoprotein biosynthesis in *E coli* is extremely resistant to the action of puromycin using both isolated polyribosomes invitro and in vivo (Hirashima, Childs and Inouye, 1973; Haleboua *et al*, 1974; Hirashima and Inouye, 1975). Half-life values for cytoplasmic proteins, envelope proteins and lipoprotein mRNA have been determined as 2min., 5.5min. and 11.5min., respectively (Lee and Inouye, 1974). Levy (1975) has shown lipoprotein mRNA to have a half-life of 1h. in *E coli* minicells. Both *et al* (1972) reported the existence of a long half-life mRNA for a *Bacillus amyloliquefasciens* extracellular protease. It, therefore seems probable that a common mechanism exists for stabilising mRNA coding for proteins excreted outside the cytoplasmic membrane. In 1976, Takeishi and co-workers isolated lipoprotein mRNA and estimated its size at 360 ± 10 nucleotides. The minimum number of nucleotides required to code for lipoprotein is $(58 \text{ amino acid} + 1 \text{ initiator} + 1 \text{ termination codon}) \times 3 = 180$ nucleotides. Inouye *et al* (1977) found that the mRNA coded for a single protein of larger molecular weight than lipoprotein. It contained the lipoprotein amino acid sequence plus an additional 20 amino acids at the N-terminus.

Inouye and co-workers suggested that this peptide extension possessing a non random arrangement of hydrophobic residues had a vital role in transport of the molecule to its final location. Analogy may be sought in the biosynthesis of other exported or secretory proteins such as alkaline phosphatase, λ receptor, maltose and arabinose binding proteins of *E coli* and penicillinase of *Bacillus licheniformis* (Inouye and Beckwith, 1977; Randall and Hardy, 1977; Randall *et al*, 1978; Yamamoto and Lampen, 1975, 1976) where the proteins are found in precursor form and have final locations exterior to the site of synthesis, the cytoplasmic membrane.

In eukaryotes the addition of a sequence of amino acids at the N-terminus has been found in several nascent secretory proteins. These sequences have been called signal peptides and are attributed with specifically directing the polypeptide across membranes, being cleaved during the transport process (Campbell and Blobell, 1976; Blobell and Sabitini, 1971; Blobell and Dobberstein, 1975) (See section on "Assembly").

Many signal peptides also exhibit a high content of hydrophobic amino acids (Devillers-Thiery *et al*, 1975; Schechter *et al*, 1975).

Ribosomes responsible for synthesising outer membrane polypeptides appear similar to those synthesising cytoplasmic polypeptides (Randall and Hardy, 1975).

Inouye, 1975, suggests that the unusual stability of the mRNA may be due to structural resistance to nucleases or compartmentalisation and consequently give protection from nuclease attack. Long half-life mRNA may result from structural differences in the mRNA which could lead to a greater affinity for ribosomes and greater stability of the initiation complex for protein synthesis.

Post-translational modification of the prolipoprotein molecule involves several steps. The prolipoprotein precursor polypeptide synthesised on cytoplasmic membrane-bound polysomes must first be translocated and cleaved (Randall and Hardy, 1977). A short pulse-labelling experiment was used by Kanazawa and Wu (1979) to show that the probable site of processing was at the cytoplasmic membrane and that the translocation step occurred very rapidly. Yem and Wu (1977) using mlpa mutants, mutations which prevent processing, showed that the accumulated precursors lacked glycerol, suggesting that glycerol was added after the apolipoprotein is cleaved.

Globomycin, a cyclic oligopeptide (See figure 18) rich in hydrophobic amino acids, is a relatively recently discovered antibiotic which has facilitated the study of lipoprotein biosynthesis and assembly. It apparently acts at the level of processing, inhibiting this step and resulting in the accumulation of prolipoprotein covalently linked to a glyceride moiety at the inner, cytoplasmic membrane.

Lai *et al* (1981) recently suggested that globomycin might bind to the diglyceride moiety of the prolipoprotein and thereby interfere with the maturation of the molecule.

After cleaving the cysteine residue now present at the N-terminus can be chemically modified. The free amino group must be acylated and a diglyceride group attached to the -SH group of the amino terminal cysteine residue. Hartke and Braun (1973) showed that the former fatty acid consisted of palmitic acid



Globomycin

----- N-MeIeu - I-leu - ser - thr - gly -----

----- - gly - ser - thr - -----

Prolipoprotein

Cited by Inouye, M., A.S.M. 79th.
Annual Meeting, May, 1979, Los Angeles

Figure 18 The similarity between globomycin and murein prolipoprotein peptides.

(65%), palmitoleic acid (11%) and cis-vaccenic acid (11%) while the ester linked fatty acids resembled those of the phospholipids of the same cell (Lai and Wu, 1980). Studies by Lin and Wu (1976) and Schulman and Kennedy (1977) indicated a probable role for phospholipids as donors of the diglyceride moiety. Subsequently, Chattopadhyay and Wu (1977) showed that the non acylated glycerol residue of phosphatidyl glycerol is the likely donor of the glycerol moiety and that the C carbon rather than C of *sn*-glycerol was involved in the thioether linkage.

The discovery of trypsin sensitive periplasmic lipoprotein (while outer membrane lipoprotein is resistant) calls into question the exact timing of fatty acid modifications since the presence of all the acyl moieties is responsible for trypsin resistance (Halegoua and Inouye, 1979).

The final stage of the modification is the conversion of one third of the free form molecules to the bound form.

Inouye *et al* (1972) using a ^3H and ^{14}C pulse-chase showed that about 40% of lipoprotein is bound after one generation. This value does not rise after a longer chase suggesting compartmentalising of the lipoprotein or an equilibrium between free and bound forms. Since these workers found the reaction to occur in the absence of amino acids, presence of chloramphenicol or when energy production was inhibited they postulated a transpeptidase reaction (See figure 19) by an enzyme, carboxy-peptidase II and that the reaction occurs after the free form is transferred to the outer membrane (Braun and Bosch, 1973).

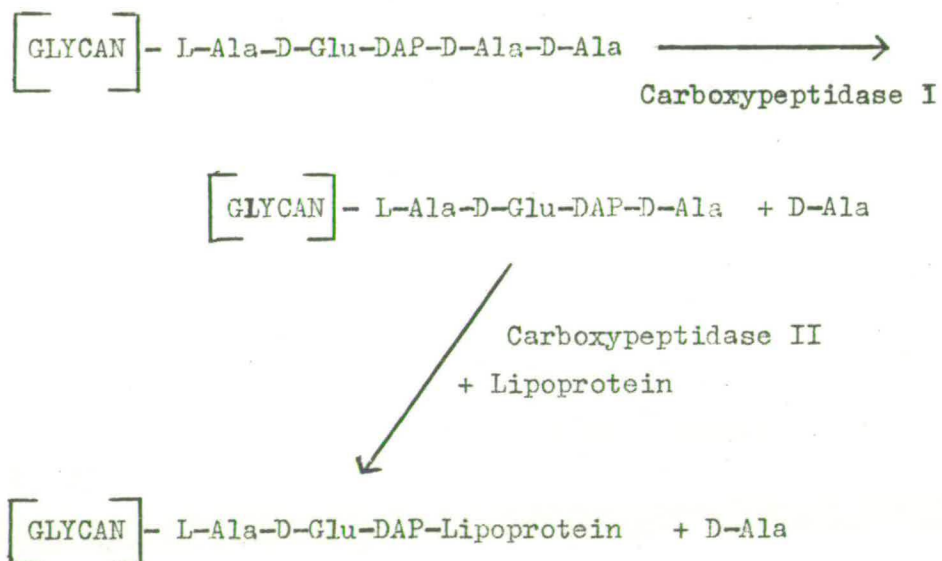


Figure 19 Transpeptidase reaction forming the bond between murein lipoprotein and peptidoglycan.

A NEW MUREIN-ASSOCIATED LIPOPROTEIN

The existence of a new murein-associated lipoprotein has been reported for several Gram negative organisms during the past few years (Mizuno, 1979, Gmeiner, 1981).

Mizuno isolated a novel lipoprotein, independent of the well-documented Braun lipoprotein, in *E coli* and *Pseudomonas aeruginosa*. These molecules both exhibit higher molecular weights than Braun lipoprotein, 21K as against 7.3K respectively. Hazumi *et al* (1978) identified a murein-bound protein in *E coli*, which they designated as protein O-14. It had a molecular weight of 22K and appeared to be synonymous with that of Mizuno (Gmeiner, 1981).

Gmeiner found an analogous lipoprotein, molecular weight 15K, in *Proteus mirabilis*. It is composed of a protein possessing both ester and amide-linked fatty acids. The hexadecanoic acid comprised 75% of the fatty acyl groups, as in Braun lipoprotein. However, amino acid composition bore no relationship to that of Braun lipoprotein.

Whether the structural configuration and biosynthesis of the two lipoproteins are similar is unknown.

MINOR PROTEINS

Two thorough reviews of the role of minor proteins are available: Braun and Hantke (1977), Braun (1975). Dependent on the growth conditions the Gram-negative cell outer membrane can incorporate a significant number of so-called "minor proteins". These include colicin and phage receptor sites. Through isolation of phage resistant and colicin tolerant or resistant strains, the physiological functions and regulatory mechanisms of minor proteins continue to be determined (See Table 4).

RECEPTOR DEPENDENT UPTAKE SYSTEMS

Iron Uptake Systems

E coli possesses three high affinity iron uptake systems: Fe³⁺-enterochelin, Fe³⁺ ferrichrome and Fe³⁺-citrate.

1. Ferric-enterochelin.

This uptake system employs the iron ligand 2, 3 dihydroxy-N-benzoyl-L-serine in the cyclic triester form. The complex can chelate one atom of ferric ion which is held by electronegative donor oxygen atoms. After excretion of the compound and chelation of a ferric ion the complex is taken into the cell and hydrolysed to release iron.

This mechanism was outlined by Rosenberg and co-workers from results obtained with two types of mutant *fepA* and *fesB* (Cox *et al*, 1970; Langman *et al*, 1972), where *fepA* lacked the receptor and *fesB* the hydrolytic activity.

Guterman and co-workers identified enterochelin as a substance capable of blocking the receptor for colicin B (Guterman and Dann, 1973, Guterman and Luria, 1969).

Subsequently the colicin B/enterochelin receptor was confirmed as mapping at 13 min. on the *E coli* chromosome (Hollifield and Neiland, 1978; Rosenberg and Young, 1974). Iron limitation was the critical factor in derepressing the receptor synthesis (McIntosh *et al*, 1979).

Guterman and Dann (1973) mapped colicin B insensitivity to the same region as *ton B* mutants. The role proposed for *ton B* was of a "couple" between the cytoplasmic and outer membranes, active in energy dependent translocation (Frost and Rosenberg, 1975; Bassford *et al*, 1976). Hantke and Braun (1975a) isolated colicin 1 and colicin B resistant mutants which they called *feuA* and *feuB*, respectively. *feuA* lacked a 74K outer membrane protein and *feuB* an 81K protein. However unlike *feuB*, *feuA* were not defective in enterochelin uptake.

Table 4

Proteins of the Outer Membrane involved in substrate uptake

<i>Designation</i>	<i>MW (KD)</i>	<i>Function</i>	<i>Receptor for</i>
83K	83	Inducible by low iron	—
feuB	81	Ferric enterochelin uptake	Colicin B
cit	80.5	Ferricitrate uptake	—
tonA	78	Ferrichrome uptake	T5, T1, O80, Colicin M
cir	74	Inducible by low iron	Colicins I & V
bfe	60	Vitamin B ₁₂ transport	BF23, E colicins
tsx	27	Nucleoside transport	T6, colicin K

after Braun and Hantke (1977)

Consequently Braun and Hantke (1977) favoured the term *cir* (colicin 1 resistant) instead of *feuA*.

The expression of the receptor dependent on iron limitation was recently investigated by Worsham and Konisky (1981). Using *cir-lac* operon fusions they obtained evidence for iron regulation of the *cir* gene at the level of transcriptional control.

2 Ferrichrome uptake

After isolating *S. typhimurium* mutants resistant to albomycin⁹², a structural analogue of ferrichrome the iron supplying hydroxamate-form siderophore, and determining their resistance patterns to colicins and phage, a common receptor for these agents was proposed (Fredericq and Smarda, 1970; Davies and Reeves, 1975).

This common receptor was the attachment site for phage T1, T5, 080 and colicin M. By mapping phage and colicin mutants it was discovered that the *sid* (siderophore) gene of *S. typhimurium* was coincident with *ton A* gene of *E. coli*. To substantiate this claim Wayne and Neilands (1975) showed that ferrichrome could protect the cells against 080 plaque formation. Hantke and Braun (1975b) showed protection against colicin M and that *ton A* protein was absent in SDS-polyacrylamide gels of the outer membranes of resistant cells. *Ton A* protein was identified by Braun and Wolff (1973) as a 78K protein localised in the outer membrane.

3 Ferric-citrate uptake

Citrate uptake of iron is inducible, reflected by the fact that iron uptake by this system in *E. coli* will not occur until after the medium is supplemented with citrate. The ferric-citrate system is definitely not an adjunct of the two previously described iron uptake systems since mutations in the gene specifying ferric-citrate receptor do not affect either enterochelin or ferrichrome uptake and vice versa (Cox *et al*, 1970).

Hancock *et al* (1976) revealed the ferric citrate receptor (coded for by gene '*cit*') as a 80.5K protein of the outer membrane, synthesised with or without the presence of citrate but in the latter case in extremely low quantities.

Two classes of ferric citrate transport mutants *fecA* and *fecB* have been demonstrated. Wagegg and Braun (1981) characterised several of these mutants from which it was concluded that *fecA* protein participates in the transport of ferric citrate.

Braun and Hantke (1977) indicated the similar mobilities of ferric citrate receptor protein and the distinct *feuB* protein on SDS-polyacrylamide gels.

TON B

Ton B gene product has not yet been localised in the cell envelope. Evidence exists for both outer and inner membrane sites (Konisky, 1979; Braun and Hantke, 1977). The Ton B gene has been cloned and apparently codes for a 36K protein (Konisky, 1979).

Both site and function of this protein may now be determined. Presently, two alternative functions are proposed.

1. To maintain contact between cytoplasmic and outer membranes and to link components of the uptake systems present in both.
2. To stabilise sites remote from adhesion sites where colicin and phage killing action can occur. Ton B product would migrate from the adhesion site and enter into a configuration which would facilitate activity in uptake (e.g. VitB₁₂).

These arguments are discussed by Konisky (1979).

VitB₁₂ UPTAKE (BF23, COLICIN E₃ RECEPTOR)

The uptake of Vitamin B₁₂ is a requirement of auxotrophic mutants deficient in methionine biosynthesis. It is a relatively large molecule (1327D) and consequently unlikely to be taken up through the generalised porin pores. It is thus sequestered by a specialised system.

Di Masi *et al* (1973) found that colicin E inhibits both binding and energy-dependent coupling of VitB₁₂. Bradbeer *et al* (1976) determined that the VitB₁₂ binding site was also a receptor (60K) for colicins E and E₃ and phage BF23. Sabet and Schnaitman (1971) showed that colicin E₃ binding site (and therefore VitB₁₂) was located in the outer membrane while Buxton (1971) and Jasper *et al* (1972) mapped the colicin E and phage BF23 receptor gene *bfe* as identical to that of VitB₁₂.

A feature distinguishing VitB₁₂ uptake from colicin or phage infection is dependence of the former on the function of a protein ton B, product of ton B locus. By using ton B temperature sensitive mutants the dependence was noted (Bassford *et al*, 1977).

Konisky (1979) reports that isolated receptor protein is sensitive to periodate and may possess a glycosyl moiety. Additionally, the purified receptor possess no activity toward VitB₁₂ but activity can be fully restored by adding lipopolysaccharide.

NUCLEOSIDE UPTAKE (T₆, COLICIN K RECEPTOR)

Combined sensitivity to colicin K and bacteriophage T₆ was first seen by Fredericq (1949). He also showed that resistant mutants to one lethal agent were also

resistant to the other agent and proposed a common receptor.

The receptor (27K) is vulnerable to the action of trypsin losing its ability to neutralise colicin K, although leaving T₆ phage receptors intact. Tryptophan specific reagents destroyed receptor activity for both T₆ and colicin K (Weltzien and Jesaitis, 1971; Braun and Hantke, 1975). Immunological labelling confirmed the proteinaceous nature of the receptor (Michael, 1968).

This receptor protein is called tsx protein after the gene determining T₆ receptor, tsx. Hantke (1976) showed that the protein is involved in nucleoside uptake, and deficient mutants show reduced uptake of thymidine, uridine, adenosine and deoxyadenosine.

ENZYMES:

Phospholipase A

Phospholipase A catalyses the deacylation of phospholipids. This 28K protein is localised in the outer membrane and requires calcium ions for activity (Scandella and Kornberg, 1971; Nishijima *et al*, 1977).

Phospholipase A is activated by the attachment of phage ghosts to the host (Buller *et al*, 1975). In a more recent paper, Hardaway and Buller (1979) suggest that EDTA induced loss of outer membrane barrier function was mediated through activation of a phospholipase A, deacylating phospholipids, thereby reducing the hydrophobic interactions between phospholipids and Lipid A of lipopolysaccharide. Under such circumstances LPS would be released, undermining the barrier function.

Although relatively few enzyme activities have been reported for outer membrane proteins a tetramethyl diamine oxidase was found in *Neisseria meningitidis* (Devoe and Gilchrist, 1976) while Nikaido and Nakae (1979) cite the finding of a protease in the outer membrane of *E coli* K-12.

SURFACE EXCLUSION PROTEIN (F¹, Tra locus)

For a thorough review see Achtman (1979)

The surface exclusion protein coded for by the F¹ plasmid locus tra (specifically gene Tra T) decreases the ability of cells carrying a specific sex factor to act as recipients for the same or related sex factors (Willems and Maule, 1974). The mechanism by which this phenomenon operates is unclear but lowered levels of Tra T protein, a 25K outer membrane protein, correlated with reduced recipient ability (Achtman *et al*, 1977).

An analogous protein was found in R222 plasmid-bearing *E coli*. Ferrazza and Levy (1980) noted the size (29K), abundance, location (outer membrane) and

solubility properties of this protein, MRB, which are very similar to those of the major matrix proteins. Using antisera it was possible to demonstrate immunological identity between MRB and Tra T protein. Ferrazza and Levy speculate that this protein may also have a diffusion function.

CELL CYCLE DEPENDENT MINOR PROTEINS

An 80K protein of the outer membrane is synthesised approximately halfway through the cycle before initiation of DNA replication in glucose-grown cells. This protein, D, was thought to act as a mediator in linking metabolism of protein and peptidoglycan to DNA initiation by acting as a site for DNA attachment on the cell envelope. However Boyd and Holland (1977) showed the protein implicated in DNA replication was identified on the basis of a procedural artifact and that a 76K protein (the ferric-enterochelin receptor) was identical to protein D (see iron-uptake systems).

As stated by Ohki (1979) further research on the role(s) of this protein is required. Another report of a protein G. of molecular weight 15K incorporated into the outer membrane, associated it with cell elongation (James, 1975).

ASSEMBLY OF POLYMERS INTO THE OUTER MEMBRANE

Thus far it can be seen that all of the known outer membrane polymers are synthesised in or on the cytoplasmic membrane. The major step in the assembly of the outer membrane is, therefore, the translocation of these polymers from the site of synthesis to their functional or structural location.

In the case of the outer membrane proteins, work on several aspects of protein synthesis and assembly are revealing the exact nature of the translocation.

The precursor proteins can be accumulated by use of inhibitors. These precursors are larger than mature proteins, and the mRNA (in vitro) codes for a larger protein than the mature protein (in vivo). By sequencing DNA and determining amino acid structures it can be shown that the N-terminal possesses a peptide extension. A method by which translocation may be achieved can thus be deduced (Inouye *et al*, 1977; Sutcliffe, 1978; Gayda *et al*, 1979; Lin *et al*, 1980).

SITE OF SYNTHESIS AND SYNTHESIS OF PRECURSORS

The differential inhibition shown by antibiotics such as puromycin and globomycin towards cytoplasmic, periplasmic and outer membrane protein synthesis has been interpreted as distinguishing between different biosynthetic mechanisms (Nakae, 1976). See section on murein lipoproteins.

Extensive literature now exists detailing the sites of synthesis of many proteins: outer membrane proteins (the periplasmic maltose binding protein) by membrane-bound polysomes, in the work carried out by Randall and Hardy (1977) and alkaline phosphatase by Varenne *et al*, (1978).

Conflicting evidence for the synthesis of small proteins (lipoprotein and phage coded M13 coat protein) on soluble polysomes was presented by Konings *et al* (1975). Osborn and Wu (1980) indicate this may be a procedural artifact resulting from the size of the protein leading to more ready release of polysomes.

However, Smith *et al* (1978) reports that puromycin releases membrane bound polysomes translating secretory protein. The resistance of lipoprotein synthesis to puromycin could thus be explained by synthesis on soluble polysomes.

By use of toluene to inhibit processing, Inouye and co-workers demonstrated the accumulation of precursors of lipoprotein, OmpA protein and matrix protein (Sekizawa *et al*, 1977). Randall and Hardy (1977) synthesised the precursor of λ receptor protein on an *in vitro* membrane-bound polysome system. All the precursor proteins were larger than the mature protein.

CO-TRANSLATIONAL TRANSLOCATION OF POLYPEPTIDES

The signal hypothesis of Blobell and co-workers demands that as the polypeptide is translated it traverses the membrane simultaneously. By labelling *E coli* and *B. subtilis* growing polypeptides (Smith *et al*, 1977, 1978) this requirement was shown to be fulfilled *in vitro*.

Silhavy *et al* (1979) point out that there are exceptions including colicin synthesis where translation does not have to take place and that the presence of a presumed signal sequence does not imply translation and transfer simultaneously.

SIGNAL SEQUENCE AND LOCATION OF PROTEIN

The essential role of the signal sequence in at least determining the gross location (inner membrane, periplasm or outer membrane) was elucidated in a series of elegant experiments by Silhavy, Beckwith and co-workers, designed to identify the region of the protein which determined location.

By fusing the soluble enzyme, β -galactosidase gene LacZ variously with the N-terminal regions of Mal E, Mal F and Lam B genes, hybrid genes could be produced. The latter group of genes code for Maltose and Maltodextrin transport and are coupled to a single locus, MalB, comprising two operons which transcribe in opposite directions. MalE codes for a periplasmic maltose binding protein, MalF for a cytoplasmic membrane protein and LamB for an outer membrane protein, which acts as a receptor for λ bacteriophage (See Silhavy *et al*, 1979). The products of gene fusions are hybrid genes coding for hybrid proteins termed protein fusions.

The following fusion genes were constructed: LamB-lacZ, MalE-lacZ, MalF-lacZ. From these fusion genes the following was found:

- a) LamB-lacZ fusion product was largely isolated in the outer membrane.
- b) MalE-lacZ fusion product was associated with the cytoplasmic membrane, not the periplasm.
- c) MalF-lacZ fusion product was associated with the cytoplasmic membrane.
- d) Gene fusions in which less than 10% of the N-terminus of the MalB region was incorporated result in the product locating at the cytoplasmic membrane. At 20% or more the product of the fusion could be translocated to a non-cytoplasmic location. Consequently, it can be inferred that a protein can be transferred to a non-cytoplasmic location if the correct genetic sequence is present, i.e. a segment of the gene located at the N-terminus.

COMPONENTS REQUIRED FOR PROTEIN EXPORT (RIBOSOMAL)

The signal sequence is a prerequisite for translocation of a protein. However, cellular factors probably have a specific role in the export process.

Ribosomal control of the process, using special initiation factors (Inouye, 1975) is contentious. Recently, Silhavy and co-workers (1979), studied a class of mutants which are revertants of a LamB point mutation, LamB569. Revertants were selected for the return of a Dex⁺ phenotype, the reversion allowing resumption of a degree of λ receptor export to the outer membrane.

These appeared to be true revertants, mapping at the original mutation site.

A second class of mutants which were revertants but did not map at the original mutational site were also described. They were mapped at the "ribosome cluster" site of the *E coli* linkage map. A mutation in a ribosomal protein is likely but ribosomal ambiguity in the translation process appeared unlikely since the phenotype of these (emr) mutants differs from that of 'ambiguity' mutants. Bassford and co-workers suggested that the ribosomal mutation resulted in alteration of the localisation process (cited in Silhavy *et al*, 1979).

SIGNAL SEQUENCE

The requirements which must be met by the signal sequence for correct export of a protein are extensive.

1. The signal sequence must be intact.

A mutant prolipoprotein, containing a single point mutation at the fourteenth amino acid residue (glycine to aspartic acid) was not modified by the addition of glyceryl and acyl moieties nor attached to peptidoglycan. Neither did processing occur. Export to the outer membrane continued, but at a reduced rate. Spontaneous revertants possess normal lipoprotein molecules correctly located in the membrane indicating that the deficiencies outlined were a direct result of the changed signal sequence (Wu *et al*, 1977; Lin *et al*, 1978, 1980a, 1980b).

2. The signal sequence must possess a hydrophobic segment.

Analysis of the DNA sequence of several LamB gene mutants at the signal sequence showed point mutations which resulted in similar changes, from hydrophobic to weakly hydrophilic amino acid residues, in the segment of the signal peptide which is hydrophobic (Emr *et al*, 1980).

This provides evidence for (2). The actual mechanism by which such a change in hydrophobicity alters the export ability of the protein is conjectural but may reasonably involve the alteration of the signal peptide configuration.

PROCESSING OF PRECURSORS

The site of processing and the mechanism by which it is achieved are uncertain. Processing of precursors might not be necessary for export to occur. This evidence will be discussed.

The discovery of several outer membrane proteins in precursor form in the outer membrane (for example lipoprotein, arabinose binding protein) after inhibition of processing has led to the conclusion that processing is unnecessary for export to occur (Randall *et al*, 1978, Lin *et al*, 1978). Confirmation comes from Markovitz and co-workers who have found piperocaine, procaine and cocaine as well as the amines atropine and neostigmine cause accumulation of Protein a precursor in the outer membrane of *E coli* minicells. They ascribed this to competitive inhibition of protease activity (trypsin) (Gayda *et al*, 1979^b).

Inouye and Beckwith (1977) found a proteolytic activity of the outer membrane which could convert a precursor synthesised in the Zubay system to mature form molecular weight.

Silhavy *et al* (1979) speculated on the nature of this proteolytic activity and proposed that due to the nature of the precursor signal peptide, it might be susceptible to a range of proteolytic activities at the outer membrane. The question of signal peptidase activity and the number of proteases which would participate were discussed by Osborn and Wu (1980). Different tolerances of outer membrane protein precursors to inhibitors of processing would infer a number of different proteolytic activities (peptidases) are required. Evidence from Lin *et al* (1980^a) demonstrates that the original peptidases are probably endopeptidases. The location of the endopeptidases in the envelope is unknown, though the outer membrane has been shown to possess some proteolytic activity (citations in Nikaido and Nakae, 1979; Osborn and Wu, 1980). In the processing of the periplasmic protein alkaline phosphatase or λ receptor (cited in Osborn and Wu, 1980) the outer surface of the cytoplasmic membrane appears a more likely site. Such a location would argue for processing as translocation began and not after completion of the event. Exceptions to this sequence of events exist: The *tra* locus outer membrane proteins coded for by the sex-plasmid, F, apparently are not processed on transport from the cytoplasmic to outer membranes (Kennedy *et al*, 1977; Achtman *et al*, 1979).

REGULATION OF ASSEMBLY

The rate of synthesis of lipoprotein and the age of the molecules found in specific regions of the envelope were examined by Lin *et al* (1980). They found that the inner membrane contained younger (more newly synthesised) molecules than the

outer membrane. They also noted that translocation from inner to outer membrane was rapid and irreversible.

The regulation of assembly of the outer membrane proteins into that membrane appears to have several sequential controls.

Regulation of transcription has been demonstrated by OmpB gene positive regulation of the OmpC gene. Translation and post-translation control may be exercised in the maintenance of a balance in the amount of a single protein with regard to the total protein or a number of proteins, e.g. relative levels of OmpC and F protein vary with growth conditions and cell cycle (See section on matrix protein).

The overproduction of matrix proteins and OmpA protein does not result in the accumulation of other normal outer membrane proteins in the cytoplasmic membrane. Noting this Henning and co-workers (Datta *et al*, 1976) suggest that membrane protein synthesis is under negative feedback control by the major polymers.

ENERGY REQUIREMENT FOR ASSEMBLY

The proton motive force does not appear to function as the energy source for co-translational assembly of periplasmic and outer membrane proteins. Abolition of this energy source by inhibitors does not affect the processing or translocation of lipoprotein (Lin *et al*, 1980).

The energy requirement may in part be serviced by energy from chain elongation on the ribosome (Osborn and Wu, 1980) although no firm evidence exists to substantiate this mechanism (Smith *et al*, 1978).

ASSEMBLY OF PHOSPHOLIPIDS AND LIPOPOLYSACCHARIDE

Translocation of phospholipids from inner to outer membrane was demonstrated by Jones and Osborn (1977a, b). Entry via the adhesion site has been regarded as the most probable insertion site into the outer membrane. However, Nikaido and Nakae (1979) report protein catalysis of phospholipid exchange. It should be noted that phospholipid translocation is both bidirectional and reversible.

Translocation of LPS has been studied by Leive and co-workers. Using a galE mutant of LPS synthesis it was possible to isolate outer membranes (with newly synthesised LPS) of higher density after pulsing with galactose. This result was construed to mean that a small number of discrete sites existed through which LPS was translocated. The result was subsequently confirmed using ferritin-labelled antibody (Kulpa and Leive, 1972, 1976; Muhlrardt *et al*, 1973).

MODELS FOR THE ASSEMBLY OF OUTER MEMBRANE PROTEINS INTO THE MEMBRANE

Currently two main models are favoured for the insertion of protein into the outer membrane. Both incorporate features to take account of other proposed models and for present knowledge of outer membrane assembly. Both models suffer drawbacks which will be outlined. (See figures 20 and 21).

Model I allows for the blebbing off of proteins and transport by vesicle formations to the outer membrane. Silhavy *et al* (1979) point out that such a model is consistent with knowledge gleaned from the gene fusion technique, being capable of transporting extremely large fusion proteins. In respect of translocation of the matrix and other proteins it appears suspect. Vesicle blebbing and transfer would seem unlikely to transport proteins quickly enough to account for export rates such as reported for lipoprotein (Lin and Wu, 1980). Osborn and Wu (1980) indicated that the orientation of proteins by this model would be inconsistent with the known orientation of OmpA protein and lipoprotein in the membrane by presenting COO H groups at the cell surface. As a negative feedback mechanism probably exists as proposed by Datta *et al* (1976) the vesicle system would be singularly unattractive as part of a model designed to enforce negative feedback by outer membrane proteins.

Model II incorporates as its main feature the adhesion sites proposed by Bayer (1968, 1975). These sites can be visualised in plasmolysed cells as points of adhesion between the cytoplasmic and outer membrane (See figure 22). Bayer has reported that many bacteriophages infect the host cell via these sites. There are a small number of adhesion sites, situated randomly over the cell surface. The various functions of these sites were exhaustively discussed in a recent review by Bayer (1979).

Model II is consistent with much of the known data on membrane assembly.

The insertion of patches of OmpC and OmpF and other matrix proteins could be accommodated by translocation through membrane junctions thereby agreeing with reported observations (Begg, 1978; Metcalfe and Holland, 1980).

This model could also account for the findings of Lin and Wu (1980) on the rapid translocation of protein and those of Datta *et al* (1976) for a negative feedback mechanism. The translocation of proteins such as λ receptor unlike that of matrix proteins appears to be by a different mechanism and would not be consistent with a number of random insertion sites such as this model proposes. In this respect the Silhavy model poses a more probable method of assembly: non-random sites capable of orientating the protein such that carboxyl groups are outermost.

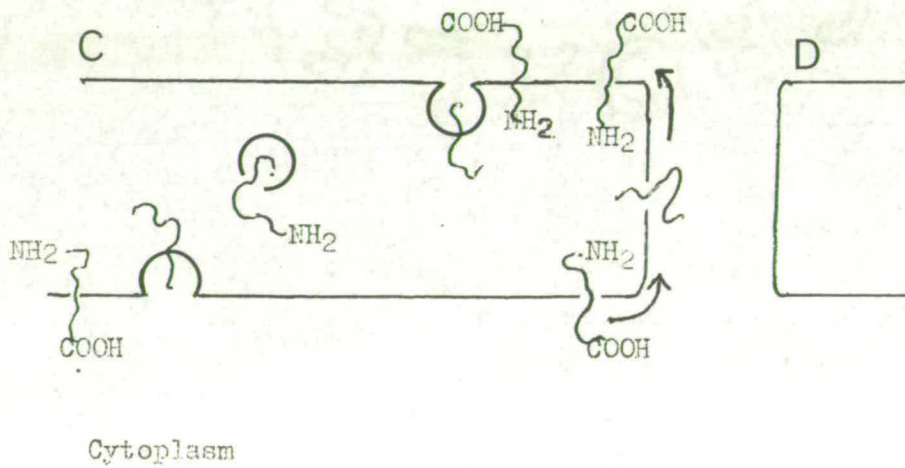
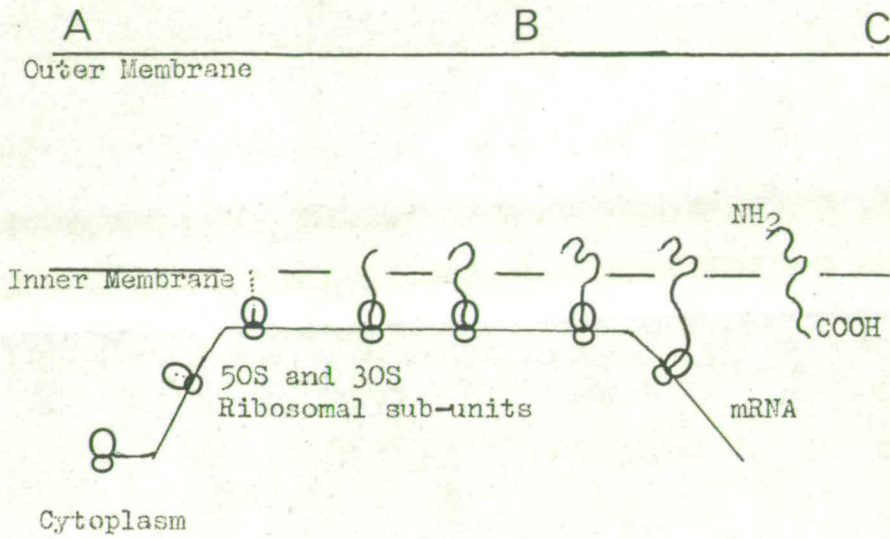


Figure 20 Model 1 for assembly of the outer membrane.

From Silhavy, 1979

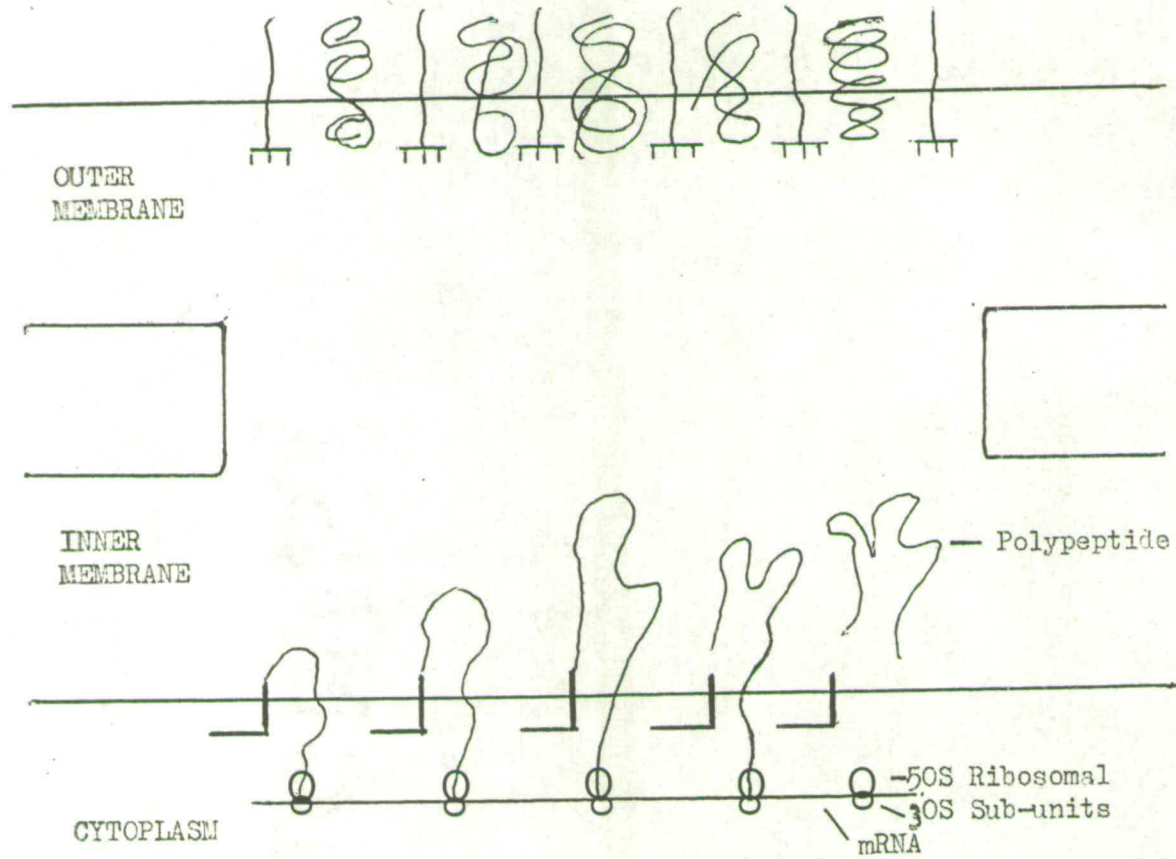


Figure 21 Model 2 for assembly of the outer membrane.

From Osborn and Wu, 1980

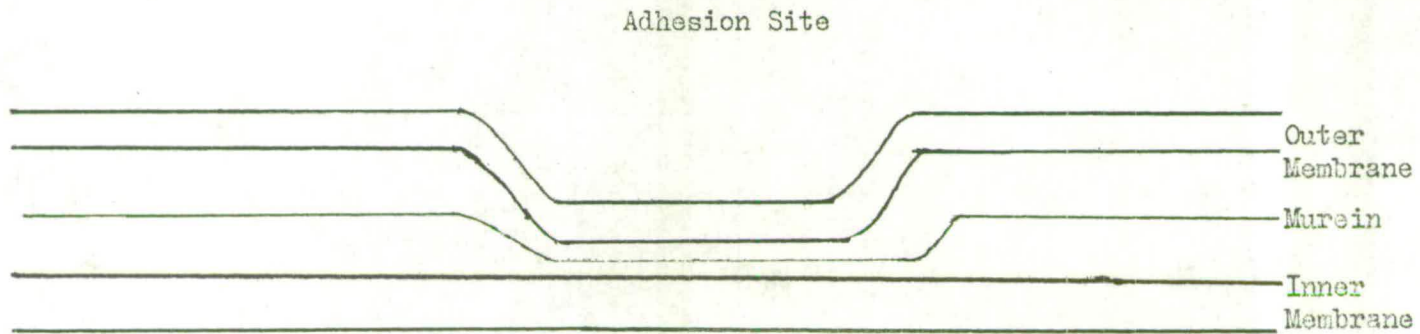


Figure 22: Diagrammatic sketch of postulated Adhesion Sites.

MOLECULAR ORGANISATION OF THE OUTER MEMBRANE

Adhesion Sites provide a mechanism for transfer of many of the outer membrane polymers from their sites of synthesis. But how is the membrane organised? What roles do interactions between similar *or* dissimilar molecules perform?

These answers may best be given by considering the present knowledge of molecular interactions obtained both *in vivo* and *invitro* and outlining a basic model to incorporate these features.

INTERACTIONS BETWEEN PROTEINS

Palva and Randall (1979) determined that porins OmpC and OmpF form trimers with homologous molecules. These workers have consistently shown this form of association contrary to the hypothesis of Henning *et al* (1973) who suggested association of all major outer membrane proteins in "stoichiometric aggregates."

OmpA protein was successfully crosslinked by short cross-linking agents to other OmpA molecules and also to lipoprotein (Reithmeier and Bragg, 1977). No association between lipoprotein and the matrix porins was indicated by results from this group.

Lipoprotein was found to affect the association of porins with peptidoglycan (See "porin" section) (Yamada and Mizushima, 1978, 1980).

INTERACTIONS BETWEEN PROTEINS AND PEPTIDOGLYCAN

The attachment of the "bound" form of lipoprotein to peptidoglycan is well documented (Braun, 1975; Inouye, 1975). The overall effect of these numerous covalent bonds between the polymers is likely to be that of holding the outer membrane against the peptidoglycan and preventing lethal amounts of blebbing.

The role of lipoprotein in porin interaction with peptidoglycan was suggested by Endermann *et al* (1978) to be indirect linkage. *lpp* deficient mutants of *E coli* were shown to lack the normal wild-type association between OmpC or F and peptidoglycan. Mutants lacking OmpC and F as well as lipoprotein did not appear to suffer from greater structural defects than would be seen in *lpp* mutants, thereby reaffirming a role for lipoprotein in porin-peptidoglycan association (Sonntag *et al*, 1978).

OmpA protein is known to associate with peptidoglycan (Endermann *et al*, 1978). The importance of this association is considerable. Mutants lacking lipoprotein and OmpA protein have a detached outer membrane. Under natural conditions such a double mutation would be lethal, unlike the loss of lipoprotein alone (Sonntag *et al*, 1978).

INTERACTIONS BETWEEN PROTEINS AND LIPOPOLYSACCHARIDE

Both in vivo and in vitro methods have been used to show direct binding of LPS molecules to OmpA, C and F proteins of *E coli*.

Yamada and Mizushima (1978) in a reconstitution study found *E coli* K-12 porin and LPS in 1% SDS formed vesicles bound by membranes composed of a hexagonal arrangement of porin-LPS aggregates.

Protection of OmpA protein from denaturation and proteolytic degradation by LPS has been demonstrated (Schweizer *et al*, 1978).

LipidA could replace the LPS function in reconstitution studies (Yamada and Mizushima, 1980). The same authors also proposed a role for the polysaccharide portion of the molecule in the binding of OmpC protein; suggesting the entire molecule functions in molecular organisation of the membrane.

INTERACTION OF PROTEINS WITH PERIPLASMIC PROTEINS

Osborn and Wu (1980) cited the results of Wandersman *et al* (1979) and suggested co-operation between mal E protein of the periplasm with the outer membrane porin.

ORIENTATION OF PROTEINS IN THE MEMBRANE

The orientation of the lipoprotein molecule has been described in the section dealing with lipoprotein biosynthesis.

The transmembrane position of the OmpA protein was established by its ability to attach bacteriophage at the cell surface while being subject to proteolytic cleavage from the periplasmic zone. The C' terminus was cleaved indicating the orientation of the molecule in the membrane with the C' terminus internal (Henning *et al*, 1978).

OmpC and OmpF proteins have an unknown orientation in the membrane.

A MODEL FOR MOLECULAR ORGANISATION

Osborn and Wu (1980) proposed a model (See figure 23) to take account of the major polymer interactions and the likely asymmetric distribution of LPS and phospholipid, although evidence for the latter is not conclusive (See LPS section).



The object of the work presented is to investigate the relationship between the cell membranes (in particular the outer membrane) and exopolysaccharide production by studying the effect of both mutation and environmental change on the cell surface composition of *Enterobacter aerogenes*.

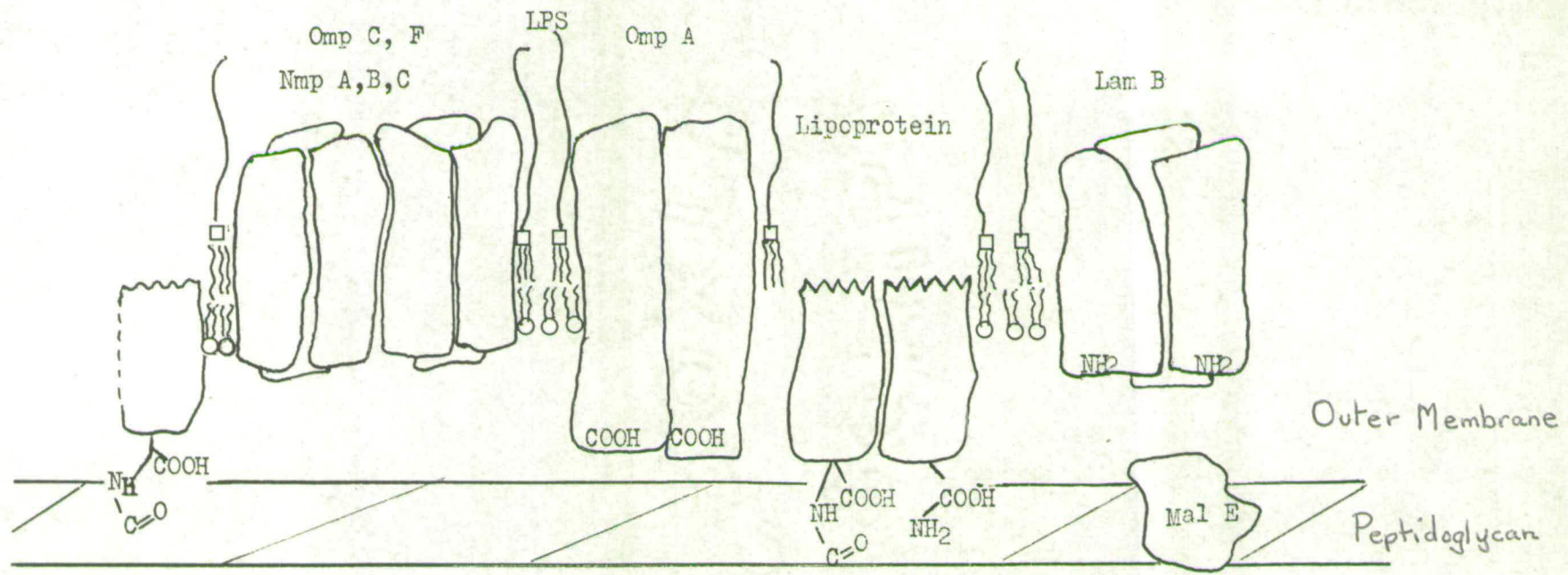


Figure 23 Diagrammatic sketch to show the molecular organisation of major outer membrane components of Gram-negative bacteria.

MATERIALS & METHODS

MATERIALS AND METHODS

BACTERIAL STRAINS

The principal strains used were: *Enterobacter aerogenes*, serotype 54, strains 54 and A1, and serotype 30, strain 7824, obtained from Dr. I. W. Sutherland. N.C.I.B. 8805, 8806 and 9261 were obtained directly from N.C.I.B.*. Mutants derived from these parental strains are listed in the 'Results.'

MAINTENANCE OF STOCK CULTURES

Stock cultures were routinely maintained on a Semi-synthetic medium (YE Agar) slope in 1/4oz. vials at room temperature, following 16h growth at 30°C.

To ensure maintenance of the genotype all strains were lyophilised.

BACTERIOPHAGE STRAINS

Strains F34 and F37 were obtained from Dr. I. W. Sutherland. Further strains were isolated from sewage water and are listed in the 'Results.'

* N.C.I.B.: Torry Research Station, P.O.Box 31, 135 Abbey Rd., Aberdeen.

YEAST EXTRACT MEDIUM (YE medium)

Glucose	20g
Casamino Acids	1g
Na ₂ HPO ₄	10g
KH ₂ PO ₄	3g
K ₂ SO ₄	1g
NaCl	1g
MgSO ₄ 7H ₂ O	0.2g
CaCl ₂	1ml/1% (w/v) Solution
FeSO ₄	0.1ml/1% (w/v) Solution
Yeast Extract	1g
H ₂ O (distilled)	1 litre, pH to 7.2

CaCl₂ and FeSO₄ were added separately, before sterilising; glucose was sterilised and added separately.

Yeast Extract Agar (YE Agar) was prepared as above but with the addition of 2% (w/v) 'Oxoid' Agar No. 3.

MODIFIED YEAST EXTRACT MEDIUM

Cultures were grown in YE Broth supplemented with:

sucrose (500mM); EDTA, disodium salt (0.5% w/v); NaCl (100mM or 300mM); 2-phenylethanol (10mM); CuSO₄ (1mM or 2.5mM), sodium dodecyl sulphate (0.01% w/v); sodium Lauryl sarcosinate (0.01% w/v); benzyl penicillin (100IU/ml).

Each agent was added at the concentration indicated to YE Broth and the pH adjusted to 7.2.

Ethanol, 2-phenylethanol and penicillin were filter sterilised. Glucose and sucrose were autoclaved and added separately. The remaining agents were added to the YE medium prior to sterilisation.

NUTRIENT LIMITED MEDIA

Mg²⁺, PO₄³⁻, SO₄²⁻, K⁺, Fe³⁺.

Sodium

* For all limitations the following compounds were present (g/litre): Citrate, 2.1; CaCO₃, 0.02; ZnO, 0.004; FeCl₃ 6H₂O, 0.0024; H₃BO₃, 0.006; NH₄Cl, 5.0.

Additionally, the media contained:

SALT	LIMITATION (g/L)				
	Mg ⁺⁺	PO ₄ ³⁻	SO ₄ ²⁻	K ⁺	Fe ³⁺ *
NaH ₂ PO ₄ H ₂ O				1.56	
KCl				0.15	
MgCl ₂				0.12	
CaCl ₂				0.0022	
Na ₂ SO ₄ 10H ₂ O	1.5	1.5	0.3	0.65	1.5
KH ₂ PO ₄	5.0	0.33	5.0		5.0
Mg SO ₄ 7H ₂ O	0.05	0.2	0.2		0.2

* Iron-limited media were prepared in two forms: with or without the citrate supplement. In both media no iron salt was added.

Glucose : 5g/l

MINIMAL MEDIUM

K ₂ HPO ₄	1.4g
KH ₂ PO ₄	0.6g
MgSO ₄ .7H ₂ O	0.02g
(NH ₄) ₂ SO ₄	0.2g
H ₂ O (distilled)	1 litre

Glucose [0.05% (w/v)] was sterilised and added separately.

TRYPTICASE SOY BROTH

A 'BBL' preparation was made up as per instructions (BBL, Md, USA).

GROWTH OF BACTERIA

Growth Curves

All wildtype and mutant strains were routinely grown in 250ml volumes of ^{YE} broth contained in 1l Erlenmeyer Flasks, on an orbital shaker set at 120 rpm. Growth curves were conducted over a period of 72h at 20°, 30°, or 37°C. Samples for exopolysaccharide production, cell weight, supernatant protein, pH and residual glucose were removed at appropriate times. Optical density ^(600nm) measurements were also taken regularly during the period 0 to 8 and at 24, 32, 48, 56 and 72 hours. **The growth temperature was 30° C unless otherwise stated.**

ASSAY OF GROWTH CURVE EXOPOLYSACCHARIDE SAMPLES

Samples (10ml) of culture fluid were treated with formaldehyde and boiled for ten minutes to strip off capsule (if present). The samples were centrifuged at 27,000g for 30 min. to deposit cells. The cell pellet was subsequently freeze-dried and weighed.

The exopolysaccharide-containing supernate was dialysed against running tap water for 3 days to remove formaldehyde and residual glucose. The amount of exopolysaccharide present was determined by the Phenol-Sulphuric acid assay of Dubois *et al* (1956).

CELL PRODUCTION FOR MEMBRANES

Liquid cultures were routinely grown in 1.2 litre volumes of Yeast Extract Broth in 2l Erlenmeyer flasks. A 20% ^(v/v) inoculum was used (2 x 100ml) of a 16 hour culture. Incubation with aeration (as above) took place at 20°, 30° or 37°C until an optical density of 0.8 units was achieved (approximately 3 hours). Cells were also grown in limited media and in the presence of membrane active compounds. The cells were deposited by centrifugation, resuspended in 15% (w/v) glycerol and frozen in 20ml aliquots at -80°C. Cultures grown at 10°C were not shaken.

MUTAGENESIS

U.V. irradiation

A 4h culture was centrifuged on a benchcentrifuge, the supernate discarded and the pellet washed twice in sterile physiological saline. The final pellet was re-suspended in 5ml of saline, and transferred aseptically to a sterile Petri dish. This was placed 25cm below a 260nm U.V. light, the lid removed and the cells irradiated for 4 minutes.

On completion of irradiation the cell suspension was aseptically transferred to an equal volume of double strength Yeast Extract Broth and incubated overnight.

Serial dilutions of the overnight culture were prepared and plated on to YE Agar plates.

N-METHYL-N-NITROSO-N-NITROGUANADINE (NTG)

Plates were flooded with the wildtype strain, dried and a single crystal of N.T.G. was carefully placed in the centre of the plate, and the plate incubated at 30°C for 24h. The zone of clearing around the crystal was examined for single colonies close to the edge of the confluent growth. Each was stab inoculated into 100ml of nutrient broth and incubated for 16h. The broth was diluted and plated for single colonies.

URANYL SALTS

Strains of *Enterobacter aerogenes* were inoculated into Trypticase Soy Broth containing uranyl salts^(2g/l) and incubated at 30°C for 4 days.

The culture was diluted to 10^{-6} in physiological saline and 0.1ml aliquots plated on Yeast Extract Agar. Plates were incubated at either 20°C or 30°C.

HEAT ATTENUATION

Aliquots (100ml) of YE Broth were inoculated with 1ml of log phase bacteria and placed in a shaking water bath at 42°C for 7 days. Subsequently the cultures were plated as above and possible mutants with altered colonial morphology were selected.

PHAGE ISOLATION

Samples (500ml) of sewage water obtained from local works were clarified by filtration through 3MM Whatman filter paper and subsequently through a 0.22 μ m membrane to remove protists.

A volume of filtrate was inoculated into equal volumes of young (4h) cultures of host bacteria.

After 3h growth the culture was filtered (as previously) to remove bacteria.

PREPARATION OF HIGH TITRE PHAGE STOCKS

A sample (0.1ml) of the 3h culture filtrate was added to 0.1ml of each host strain in 2ml of sloppy agar and 10^{-3} and 10^{-4} dilutions plated on YE Agar Plates. After 8h all plates were examined and material from different plaques transferred with a straight wire to 2ml of sterile Nutrient Broth No. 2 (Oxoid). These presumed phage suspensions were used to repeat the previous step. The suspension (0.1ml) was added to 0.1ml of log phase bacteria in 2.5ml of sloppy agar to give confluent lysis on a YE Agar Plate. Ten plates of each phage/host mixture were made.

Following 16h growth the lysed plates were flooded with 2ml of sterile nutrient broth and stored at 4°C overnight. This step was repeated with fresh broth.

The decanted broth was centrifuged in a bench centrifuge (MSE) at 3,000 rpm for 10 min. to sediment bacteria and agar.

The supernate was decanted and filtered through a 0.22 μm membrane (Millipore).

The phage suspensions, with an average of 10^{10} plaque forming units per ml., were stored at 4°C.

SELECTION OF PHAGE RESISTANT MUTANTS

YE Agar plates were flooded with culture of UV irradiated strains and one drop of each phage preparation spotted to to each plate.

The plates were incubated at 30°C overnight and screened for resistant colonies within the zone of lysis. All such colonies were inoculated into 100ml of Yeast Extract Broth and incubated for 3h. This log phase culture was used to make further lawns which were spotted with the same phage to check resistance.

MINIMUM INHIBITORY CONCENTRATION

Aliquots (1ml) of Yeast Extract Broth were dispensed in ¼oz. vials, sterilised and doubling dilutions of filter-sterilised antibiotic were added aseptically.

The above method was also used to determine sensitivity of stock cultures and mutants to surface active agents such as EDTA and SDS.

SELECTION FOR ANTIBIOTIC RESISTANCE

Based on the information obtained above antibiotic concentrations for selection of mutants were fixed above the minimum inhibitory concentration. Mutagenised cells were inoculated into Yeast Extract Broth containing an inhibitory concentration of antibiotics.

PREPARATION AND PURIFICATION OF LIPOPOLYSACCHARIDE

Cells were harvested and freeze-dried. The freeze-dried cells were extracted by a modification of the aqueous-phenol protocol of Westphal and Luderitz (1954).

An equal volume of 90% (w/v) phenol in water at 65°C was stirred into a 10% (w/v) suspension of cells in distilled water at 65°C. Stirring was maintained for 10 min. The mixture was cooled (in ice) and centrifuged at 5000g at 10°C for 20 min. in a swing-out rotor. The upper, aqueous phase was dialysed for 16h against running water to remove phenol. The non-dialysable material contained a mixture of lipopolysaccharide, glycogen and nucleic acids. Lipopolysaccharide was purified from this mixture by concentrating the solution under reduced pressure and ultracentrifugation at 100,000g for 4h. Any residual contaminating nucleic acids were removed by resuspending the LPS pellet in distilled water and treating with DNase and RNase (0.1mg/ml) in a dialysis sac against running water for 16h, then freeze-drying.

PARTIAL HYDROLYSIS OF LIPOPOLYSACCHARIDE

To obtain polysaccharide free of lipid-A the method of Davies (1955) was followed.

A 1% (w/v) solution of Lipopolysaccharide in 1% (v/v) acetic acid was hydrolysed in a sealed glass ampoule at 100°C for 1h. Lipid-A was deposited by centrifugation and the supernatant fluid, after overnight dialysis against running water, was freeze-dried.

PREPARATION OF HIGH AND LOW MOLECULAR WEIGHT EXOPOLYSACCHARIDE

Following centrifugation of a liquid culture to remove cells, the supernatant culture fluid was diluted with 2 volumes of acetone, stirred and the gelatinous (high molecular weight) exopolysaccharide precipitate wound onto a glass stirring rod. The solution was left for 16h at 4°C to allow any remaining high molecular weight exopolysaccharide to sediment.

The solution was then reduced in volume by rotary evaporation and re-precipitated with acetone to remove any remaining high molecular weight material. The supernatant fluid containing low molecular weight exopolysaccharide was dialysed for 16h against running water to remove the acetone, and freeze-dried.

CHROMATOGRAPHY OF POLYSACCHARIDES

a) *Paper*. Chromatography of hydrolysed polysaccharides was performed on Whatman No. 1 paper, irrigating with butan-1-ol: pyridine: water (6:4:3) for 24h.

b) *Column*. Low molecular weight exopolysaccharide (0.1ml at a concentration of 10mg/ml) was analysed on a Bio-Gel ACA54 column (40cm x 2.5cm) (Pharmacia, Uppsala, Sweden) by eluting with 300ml of pyridine : acetic acid : water (4:10:986) and collecting 3ml fractions.

All fractions were assayed for total unhydrolysed carbohydrate by the Phenol-Sulphuric Acid assay.

PREPARATION OF MEMBRANES

This was accomplished using the procedure of Osborn, Gander, Parisi and Carson (1972).

E.D.T.A.-Lysozyme Sphaeroplast Method

Cells pelleted from 1l of 3h culture were resuspended without previous washing in 100ml of 0.75M sucrose, 10mM Tris-HCl buffer, pH7.8. Following 2 min. exposure to lysozyme (100ug/ml) 2 vols of 1.5mM EDTA (disodium salt), pH7.5 was added over a period of 12-15 min, swirling the suspension gently. Sphaeroplasting was checked by phase contrast microscopy. Unlysed cells and debris were removed by centrifugation at 5000g for 10 min. The sphaeroplasts were sedimented by high speed centrifugation and the pellet resuspended, by repeated expulsion from a syringe, in 25ml of distilled water.

The suspension was sonicated for 1 min (2 x 30 second exposures) at a setting of 7 microns (amplitude) in a MSE 100W Ultrasonicator (60c/s). To sediment the membranes from the suspension the 25ml sample was centrifuged at 100,000g for 45 min. Subsequently the membranes were washed with distilled water.

All steps were performed on ice or at 4°C.

SEPARATION OF OUTER AND INNER MEMBRANES

a) Sucrose Gradient Density Centrifugation.

The procedure of Osborn, Gander, Parisi and Carson (1972) was adopted.

Stepped gradients of sucrose were constructed in 23ml polycarbonate tubes by layering 4.2ml each of 50, 45, 40, 35 and 30% (w/w) sucrose in a 5mM EDTA solution (pH7.5) over a cushion of 1ml 55% sucrose solution.

Sphaeroplast membranes obtained from 1l of cells (prepared as above) were suspended in 1ml of 25% sucrose solution and carefully layered on top of the gradient.

The membrane fractions were centrifuged in a 3 x 25ml swing-out rotor at 100,000g for 20h at 0°C.

Two bands were visible after centrifugation. The bottom band represented outer membrane, the top band, inner membrane. Both bands were removed separately using a long syringe needle and a peristaltic pump.

b) Sarkosyl Separation Of Outer And Inner Membrane

Sphaeroplast membranes derived from 1l of cells (as described above) were suspended in 10ml of 1.0% (w/v) Sarkosyl by repeated expulsion from a hypodermic syringe. The suspension was agitated in a shaking waterbath at room temperature for 20 min. and decanted into a 25ml polycarbonate tube, then centrifuged at 100,000g for 45 min. The pellet obtained was washed once in distilled water and labelled "outer membrane", the Sarkosyl-soluble fraction was labelled "inner membrane", and freeze-dried.

Sarkosyl was removed from the inner membrane preparation according to the method of Frasch (1976):-

Three volumes of 10% ethanol, 30% ethylene-glycol in 0.01M Tris buffer (pH8.5) were added to the freeze-dried pellet.

Four or five-fold concentration by ultrafiltration with a membrane was performed, thereby removing most of the excess detergent from the rejected protein. This cycle may be repeated to achieve further purification.

c) "Lithium Acetate" Method Of Outer Membrane Isolation

Modified from the Method of Heckels (1981)

Cells pelleted from 2l of 3h culture were suspended in 0.2M Lithium acetate (pH6.0, 10g wet weight of cells per 100ml) and shaken at 45°C for 2.5h. The mixture was then homogenised for 2 min (4×30 secs). The cells were removed by repeated centrifugation at 15,000g for 20 min. The supernatant fluid, containing the crude outer membrane, was pelleted at 100,000g for 2h. The pellet was suspended in 6M Urea-0.2M sodium acetate buffer (pH6.0) at a protein concentration of 10mg/ml, incubated at 25°C for 30 min and diluted with an equal volume of buffer. The outer membrane was pelleted at 100,000g for 3h and the supernate containing contaminating non-outer membrane proteins was discarded. After two washes in distilled water the pellet was freeze-dried.

BREAKAGE OF CELLS BY FRENCH PRESS

Cells suspended in cold physiological saline were broken by repeated passage through a French Pressure cell (Aminco, American Instrument Co., Inc., Silver Spring, Md., U.S.A.) at 4,000 p.s.i.

Unbroken cells and debris were removed by centrifuging at 10,000 rpm for 10 min, at 4°C. The membranes were harvested from the supernate by pelleting at 100,000g for 1h at 4°C in a MSE "Superspeed 65" centrifuge.

PREPARATION OF BUTAN-1-OL SOLUBLE MEMBRANES

This procedure was adapted from that described by Sandermann and Strominger (1972).

Cells were broken by passage through a French pressure cell. Membranes were prepared by centrifuging at 5000g for 10 min. to remove unbroken cells and debris and deposited at 100,000g for 1h at 0°C in an MSE "Superspeed 65" centrifuge. The membranes were resuspended to a final volume of 500ml in 50mM Tris-HCl buffer, pH 7.3 and 1mM MgCl₂ below and extracted with acid butan-1-ol (120ml 6M pyridinium acetate, pH 4.2 + 320 ml butan-1-ol) at room temperature for 40 min.

To separate the phases, the suspension was decanted into 250ml centrifuge bottles and centrifuged at 8,000 rpm for 20 min at 4°C in an MSE "Hi-Speed 18" centrifuge.

The upper aqueous phase was pipetted into a separating funnel containing 250ml of butan-1-ol saturated water. The remainder was re-extracted with 300ml of butan-1-ol, twice, and the layers pooled.

The butan-1-ol/water azeotrope of the pooled layers was drawn off by evaporation under reduced pressure at a temperature less than 30°C. The concentrated butan-1-ol was cooled to 0°C for 24-72h and the heavy precipitate removed by centrifugation.

The butan-1-ol was then held at -20°C in 50ml centrifuge tubes for 72h. This precipitate was collected by rapidly centrifuging the suspension in a rotor pre-cooled to -20°C to 15,000 rpm and bringing it to rest immediately.

The precipitate suspended in butan-1-ol and labelled " crude butanol soluble extract " was stored at -20°C.

PROCEDURE FOR THE EXTRACTION OF PROTEIN K IN *E COLI*

Extraction method as described by Paakkanen, Gotschlich and Makela (1979)
Cell paste from growth in 6l of YE Broth for 3h was suspended in 500ml of 0.05M Tris-HCl buffer (pH 8.0) containing 0.01% (w/v) disodium EDTA and 2% (w/v) hexadecyltrimethyl-ammonium bromide (Cetavlon; BDH, Poole, England) and placed in a boiling waterbath for 10 minutes. After centrifugation at 20,000g for 20 minutes, 258g of ammonium sulphate was slowly added with stirring and the mixture allowed to ^{stand} at room temperature overnight.

The solution was centrifuged at 5000g for 20 minutes and the floating oily layer removed with a spatula, dissolved in 0.02M potassium phosphate buffer, and dialysed against 0.02M potassium phosphate buffer (pH 8.0) containing 2% (w/v) Cetavlon.

After the removal of insoluble debris, the solution was applied to a

hydroxylapatite column (1.6cm × 30cm; Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The column was eluted by a 300ml linear gradient with an initial composition of 0.02M potassium phosphate (pH 8.0) and 2% (w/v) Cetavlon and a final concentration of 1M potassium phosphate buffer (pH 8.0) and 1% (w/v) Cetavlon. The 2.5ml fractions were monitored by 280nm absorbance and SDS—PAGE.

PROTEOLYTIC CLEAVAGE OF 34K OUTER MEMBRANE PROTEIN

A sphaeroplast suspension was prepared (as above) and Pronase (Calbiochem, Bishops Stortford, England) added to a final concentration of 100 μ g/ml. Cleavage was allowed to proceed for five minutes and the reaction was stopped by addition of haemoglobin (1 mg/ml) and rapid centrifugation of the sphaeroplasts from suspension (repeated twice) before continuing with membrane preparation (as above).

AMMONIUM SULPHATE PRECIPITATION OF SUPERNATANT PROTEIN

Cell-free supernate (20.0ml) was placed in a 500ml beaker and slow stirring commenced. 13.2g of ammonium sulphate, previously finely ground in a mortar and pestle, was added slowly over a period of several hours. The precipitation was conducted at room temperature.

The protein precipitated was deposited at 18,000 rpm, washed once in distilled water and freeze-dried.

PREPARATION OF 1-DIMENSION POLYACRYLAMIDE GELS

From Davis (1964), Maizel (1971)

Stock acrylamide solution of 29.2% (w/v) acrylamide (Sigma, Poole, England) and 0.8% (w/v) bis-acrylamide was filtered through glass-wool and held in a dark bottle at 4°C.

Chromic acid-washed glass slab cassettes (17cm x 18cm) were constructed and sealed with soft paraffin wax. The gel was carefully pipetted into the cassette after de-gassing (2 minutes, under reduced pressure), immediately on the addition of Ammonium persulphate ^{and} NNN'N' tetramethylenediamine (TEMED); (catalyst) and (accelerator) as detailed. It was then covered by a layer of distilled water to allow polymerisation in the absence of oxygen. One hour was allowed for complete polymerisation at room temperature.

NON-GRADIENT GEL

		Final Concentration
Stock acrylamide	0.43 vol.	13%
1.5M Tris-HCl (pH8.8) 0.4% SDS	0.25 vol.	0.375M Tris, 0.1% SDS
Distilled Water	0.32 vol.	
Catalyst: 10% Ammonium persulphate (Analar quality, BDH, Poole, England) 0.005 vol.		
Accelerator: TEMED (BDH, Poole, England) 0.001 vol.		

GRADIENT GEL

		Final Concentration
High Concentration		
Stock Acrylamide	0.75 vol.	22.5%
1.5M Tris-HCl (pH8.8) 0.4% SDS	0.25 vol.	0.375M Tris, 0.1% SDS

LOW CONCENTRATION

Stock Acrylamide	0.25 vol.	7.5%
1.5M Tris-HCl (pH8.8) 0.4% SDS	0.25 vol.	0.375M Tris, 0.1% SDS
Distilled Water	0.5 vol.	

Equal volumes of both high acrylamide concentration and low acrylamide concentration solutions were pipetted, after degassing, into the respective wells of a gradient maker. Catalyst and accelerator (as detailed above) were added and slowly pumped by peristaltic pump (LKB Produkter, Bromma, Sweden) into the cassette over a 10 min. period. When the gradient had been formed a thin layer of distilled water was carefully applied onto the surface of the gradient gel with a pipette to ensure the exclusion of oxygen.

For both types of gel the stacking gel comprised:

		Final Concentration
Stock Acrylamide	0.16 vol.	5%
0.5M Tris-HCl (pH6.8) 0.4% SDS		
	0.25 vol.	0.125M Tris
Distilled Water	0.59 vol.	0.1% SDS
Catalyst: 10% Ammonium persulphate, 0.01 vol.		
Accelerator: TEMED, 0.01 vol.		

The stacking gel was applied with a pipette onto the resolving gel with the well-forming comb having been inserted such that the bases of the teeth are fractionally above the lip of the front slide. Care was taken to ensure that air bubbles did not form under the comb.

The stacking gel polymerised within 1h and should comprise approximately 5% of the total slab volume.

PREPARATION OF SAMPLES FOR GEL ELECTROPHORESIS

Samples of membrane fractions were prepared in concentrated sample buffer such that each solution had a final concentration:

0.01M Tris-HCl (pH6.8)

1% SDS

0.1% β -mercaptoethanol

10% Glycerol

0.005% Bromophenol Blue

Approximately 50 μ g protein/50 μ l sample

Before application, sample solutions were heated in a boiling water-bath for 10 min. and cooled. In addition half of each outer membrane sample was heated for 30 min. (at 37°C) with shaking. Insoluble membrane debris was removed from the sample by centrifugation according to the method of Hofstra and Dankert (1979).

ELECTROPHORESIS

Electrophoresis was initially performed at 15mA (constant current) until the dye front entered the resolving gel and subsequently at 20mA until the dye front was 1cm from the base of the gel.

STAINING AND DESTAINING

The gel was immersed for 1½-2h. in stain solution with gentle shaking.

*Coomassie Brilliant Blue R250	1.25g
Page Blue G90	
Methanol	227 ml
Glacial Acetic Acid	46 ml
Distilled Water	500 ml

(* In later gels, Page G90 dye was used as it was found to give less background staining, better staining of minor bands and faster destaining).

followed by destaining in:

Methanol	50 ml
Glacial Acetic Acid	75 ml
Distilled Water	1000 ml

with several changes of destaining solution over several days.

To facilitate destaining, 1g of Dowex 1 x 8 20-50 Mesh (C.I.) in dialysis tubing was added.

PROTEIN STANDARDS FOR PAGE

Bovine serum albumin (66K), ovalbumin (45K), lysozyme (14K) (egg white) haemoglobin (16K) and myoglobin (17K). Each protein was present at a concentration of 1 μ g/1 μ l in sample buffer.

CALCULATION OF MOLECULAR WEIGHTS

The migration distance of the standard proteins in relation to the dye front (i.e. Rf) were calculated and a standard curve constructed. From this the molecular weights of unknown polypeptides could be calculated by plotting their Rf values on the standard curve.

To take account of swelling of the gels during staining and destaining the following calculation was adopted.

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length before destaining}}{\text{Distance of dye migration}}$$

POLYACRYLAMIDE GEL DENSITOMETRY OF 1-DIMENSION GELS

Slab polyacrylamide gels after thorough destaining were cut into 1cm wide longitudinal tracks. Each slice was washed gently with distilled water to remove extraneous material, placed in distilled water in a cuvette, and scanned (25mm per min) in a Joyce-Loebl Scan 400 (Joyce-Loebl, Gateshead, England) using a 280nm filter.

The trace was recorded on a Servoscribe IS Potentiometer with a paper speed of 30 or 60cm per min.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF LPS

Modified from the method of Di Rienzo *et al* (1978).

A 5% non-gradient gel was made (see above) by reducing the quantity of acrylamide stock solution and increasing the volume of distilled water.

The electrophoresis buffer contained 0.05M sodium phosphate, 0.05M sodium molybdate and 1% (w/v) SDS adjusted to pH7.0 with HCl.

Samples of LPS (5mg/ml of sample buffer) were solubilised for 10 min. at 100°C. The sample buffer was normal PAGE sample buffer supplemented with 0.05M sodium molybdate. The gel was pre-run in electrophoresis buffer for 30 min. at 10mA.

The samples (100µl) were applied to the gel and electrophoresed at 5mA for 5h.

The gels were stained using the Periodic Acid-Schiff stain.

PERIODIC ACID-SCHIFF (PAS) STAIN FOR GLYCOPROTEINS IN UNIDIMENSIONAL GELS

To determine whether proteins (polypeptides) in (SDS) polyacrylamide gels contain a carbohydrate moiety the Periodic acid-Schiff stain was applied to half of an unstained gel (electrophoresed as above). The other identical half was stained by the orthodox Page blue stain detecting only protein.

Each gel was fixed overnight in 100-200ml of PAS fixative solution of composition:

- 40% (w/v) ethanol
 - 5% (v/v) glacial acetic acid
 - 55% (v/v) distilled water
- (Stored at room temperature)

The gels were treated with 0.7% periodic acid solution (1.4g periodic acid dissolved in 200ml of 5% acetic acid) for 2-3h. followed by treatment with 0.2% sodium metabisulphite (0.4mg sodium metabisulphite dissolved in 200ml of 5% acetic acid) for 2-3h. with one solution change after 30 min.

The gels, after clearing, were put in large beakers filled with Schiff reagent.

Schiff Reagent: Basic fuchsin (10g) was dissolved in 2l of distilled water with heating. After cooling, 200ml of 1N HCl was added, followed by 17g of sodium metabisulphite; the solution was mixed until decolourised. It was stirred with acid washed charcoal and the charcoal removed by centrifugation to avoid contact with filter paper. The supernatant was filtered through glass wool to remove any remaining charcoal; the filtrate should be clear and colourless. The solution was stored in a dark bottle at 4°C. Colour developed in 12-18h. at room temperature.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

(Adapted from O'Farrell, 1975)

Proteins (freeze-dried) for analysis were resuspended in buffer at high concentration (1.5mg/ml).

Solutions:

- 1) 9.0M Urea
2% Tergitol (NP40)
2% Ampholines pH2.5-10 Stored as frozen aliquots
5% Mercaptoethanol

- 2) As above but with 8.0M Urea

3) Acrylamide Stock (I)

19.67g acrylamide
0.33g bis-acrylamide
to 100ml with distilled water

4) NP40 Stock 10% (w/v) in distilled water

5) Dialysis Buffer:

62.5mM Tris-HCl pH6.8
2.3% SDS
5% β -mercaptoethanol (v/v)
10% glycerol (v/v)

Acrylamide Stock (II)

29.8g acrylamide
0.2g bis-acrylamide
to 100ml with distilled water

7) Resolving Gel Buffer

1.5M Tris-HCl pH8.8
0.4% SDS

8) Stacking Gel Buffer

0.5M Tris-HCl pH6.8
0.4% SDS

9) Ammonium persulphate

10% (w/v) in distilled water

10) Electrode Buffer

25mM Tris 6.05g
192mM glycine 29.44g
0.1% SDS 2.00g
to 2 litres

Separation in the first dimension was achieved by re-suspending lyophilised protein to 1-3mg/ml in solution(1).

(1) 10-30 μ l of sample were loaded onto iso-electric focussing tube gels containing

0.54g urea
0.20ml Solution (3)
0.20ml Solution (4)
0.05ml Ampholine (pH3.5-10; 4% solution)
made up to 1ml

After thorough mixing, polymerisation was initiated with $1\mu\text{l/ml}$ TEMED and $2\mu\text{l/ml}$ ammonium persulphate (9). The temperature during polymerisation was maintained at 25°C .

The gel surface was initially overlaid with water and subsequently with 8M Urea. After 60 min. the tubes were loaded into the I.D. gel apparatus. $10\mu\text{l}$ of 8M Urea was loaded onto the gel surface and the tubes and the top electrode tank (-ve) filled with degassed 0.1M NaOH. The bottom electrode tank (+ve) was filled with 0.01M H_3PO_4 and the tubes prefocussed at 200V and a constant current of 0.3-0.5mA/tube for 1.5-2h.

The tops of the gels were then washed with fresh NaOH and the tubes loaded with 10-30 μl of sample overlaid by $10\mu\text{l}$ of 8M Urea. The upper tank was filled with fresh 0.1M NaOH and a voltage of 400V applied for 19h. at 25°C .

On removing the gels one end was labelled with cotton and the gel washed twice (1h. per wash) in dialysis buffer, before freezing for storage.

The second dimensional separation was achieved in a gradient gel (15%), composed of:

25% Buffer (7)
50% acrylamide II (6)
20% glycerol
5% distilled water (30ml)

(to 5%) : 25% Buffer (7)
17% acrylamide II (6)
58% water (25ml)

The stacking gel on which the tube was overlaid, was

Buffer (8) 2.5ml
acrylamide II (6) 1.5ml
water 6.0ml

The tube gel was sealed in position with molten 1% Agar in electrode buffer (10) containing 0.001% Bromophenol Blue. Constant current was applied at 10mA (30 min.) and 18-20mA for 5-6h. When the dye front reached the bottom of the gel, the gel was removed from the cassette and fixed in 10% Acetic Acid.

SILVER STAIN FOR DETECTING PROTEINS IN POLYACRYLAMIDE GELS

The method of Oakley *et al* (1980) was adopted

Initially, the gel was soaked in 10% unbuffered glutaraldehyde for 30 min. followed by 2 washes in 500-1000ml of distilled water, for at least 2h. (preferably

12h.). Having drained off the distilled water the gel was soaked in sufficient ammoniacal silver solution (1) to allow the gel to float freely under gentle agitation for 15 min.

The gel was then removed from the container and washed in distilled water for 2 min. The water drained and the gel soaked in citric acid-formaldehyde solution (2). The gel was removed when the background began to develop and washed in distilled water for 1h.

Solution (1) 1.4ml fresh NH_4OH

21.0ml, 0.36% NaOH

4ml of 19.4% AgNO_3 was added with vigorous agitation. The solution was made up to 100ml, or multiples thereof, with distilled water.

Solution (2) 0.005% citric acid

0.019% formaldehyde [made by diluting a 38% formaldehyde solution containing 10-15% methanol (Fisher)].

CHEMICALS AND BIOCHEMICALS

Chemicals and biochemicals used were of the highest grade of purity available:

Penicillin, as Crystapen, benzyl penicillin, from Glaxo Ltd., Greenford, Middlesex.

Levallorphan, tartrate salt, was a gift from Dr. F. Scott, Hoffman-La Roche Inc., Nutley, N.J., U.S.A.

Polymyxin B sulphate, Novobiocin, Tetracaine, Lysozyme, Sodium lauryl sulphate, Sodium lauryl sarcosinate and Tergitol NP40 were obtained from Sigma Biochemicals, London, England.

Acrylamide, bis-acrylamide and Lithium acetate were obtained from BDH, Poole, England.

Hydroxylapatite from Bio-Rad, Richmond, Calif., U.S.A.

Glucose oxidase reagent as "Glucostat", from Boehringer GmbH., Mannheim, W. Germany.

ASSAYS:

Total Carbohydrate in unhydrolysed polysaccharide or in culture supernatants was estimated by the Phenol-Sulphuric Acid method of Dubois, Gilles, Hamilton, Rebers and Smith (1956).

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

KDO was measured by the method of Weissbach and Hurwitz (1958) using the modification of Osborn (1963).

Heptose was determined by the cysteine-sulphuric acid method of Osborn (1963).

SPECTROPHOTOMETRY

All measurements were made using a Unicam (Pye-Unicam Instruments Ltd., Cambridge, England) SP600 or SP800, spectrophotometer.

RESULTS

MUTAGENESIS

Mutagenesis of the heavily encapsulated parental *Enterobacter aerogenes* strains A1, 54C, and 7824 was required to produce an array of strains affected in the mode or quantity of exopolysaccharide production or on other cell surface component (See Table 5). No selective tool existed for isolating A1, 54C or 7824 mutants differing in exopolysaccharide synthesis. Gross distinctions in colonial morphology (mucoid, non-mucoid) and consistency (non-viscous, viscous, friable) were used to distinguish non-mucoid (O) mutants and slime-forming or viscous mutants (SI or V) from wild-type mucoid strains. Concomitant with attempting to obtain exopolysaccharide mutants efforts were made to isolate mutants with altered membranes, in particular the outer membrane. Two major selective tools exist for this purpose: phage resistance and drug sensitivity/resistance, the latter being largely an effect of the outer membrane barrier function.

Although numerous mutagens were initially used; $MnCl_2$, γ -irradiation, acriflavine and ethylmethanesulphonate mutagens failed to yield the required mutants.

Spontaneous mutants of altered exopolysaccharide synthesising capability were extremely rare. One viscous slime-forming mutant of strain 7824 was isolated after UV irradiation, being readily distinguished on the basis of colony consistency. No spontaneous reversion or mutagen-induced back mutation to capsulation was found.

UV-irradiation mutagenesis generated phage-resistant mutants of strains 54C and 7824 and polymyxin B sulphate-resistant mutants of the three parental strains. These mutants were capsulate.

Uranyl salts incorporated into Trypticase Soy Broth, producing low level α -irradiation of the culture, gave rise to a phage resistant mutant of 54C. An (O) mutant of 7824 was selected by virtue of its phage resistance and altered (balloon-shape) cellular morphology from a heat attenuated culture of 7824.

The most efficient mutagen, NTG, was used to obtain mutants resistant to high levels of levallorphan, novobiocin and tetracaine.

The following strains were isolated by M. Norval (1969) who recorded their exopolysaccharide-synthesising properties: A1X, A1SI, A1SIBR, A1SINR, A1SIHis, A1.92, A1(O).

N.C.I.B. strains 8805, 8806 and 9261, serotype 54, were isolated and described by Wilkinson *et al* (1954). Drug resistant mutants were selected as described below.

TABLE 5 : List of Strains and their derivation

<i>Strain (Serotype 1)</i>	<i>Mutation</i>	<i>Method of Mutagenesis</i>
AIX	Transductant AI (O) AI	—— ★
AI	wild-type	—— ★
AISI	slime-former	UV ★
AISIBR	slime-former, bacitracin resistant	UV ★
AISINR	slime-former, novobiocin resistant	UV ★
AISI His ⁻	slime-former, histidine auxotroph	AminoPurine ★
AIX PB600	polymyxin B resistant (600U/ml)	UV
AIX Pb2000	polymyxin B resistant (2000U/ml)	UV
AI (O)	non-mucoid	AminoPurine ★
AI.92	'leaky' phenotype (for exopolysaccharide)	pFA ★
AIX TR	Tetracaine resistant	NTG
AIX NR	novobiocin resistant	NTG
AIX LR	levallorphan resistant	NTG
<i>(Serotype 54)</i>		
54C	wild-type	—— ★
54C F34	phage resistant	Uranyl salts
54C F37	phage resistant	UV
54C PB3000a	polymyxin B resistant (3000U/ml)	UV
54C PB3000b	polymyxin B resistant (3000U/ml)	UV
54C TR	Tetracaine resistant	NTG
54C NR	novobiocin resistant	NTG
54C LR	levallorphan resistant	NTG
8805	wild-type resistant	☆
8806	slime-former	☆
9261	non-mucoid	☆
<i>(Serotype 30)</i>		
7824	wild-type	——
7824V	slime-former	UV
7824 φ	phage resistant	UV
7824 PB300	polymyxin B resistant (300U/ml)	UV
7824 (O)	morphological, non mucoid	Heat Attenuation
7824TR	Tetracaine resistance	NTG
7824NR	novobiocin resistance	NTG
7824LR	levallorphan resistance	NTG
7824f	Filament forming	UV
<i>(Serotype 8)</i>		
A4	wild-type	★
A4CR	low exopolysaccharide production, possible defect in carrier lipid at 20°C; as wild-type at 37°C	★

☆ obtained from N.C.I.B.

The remaining strains were isolated in the course of this work.

★ Departmental Stock Cultures.

ANTIBIOTIC SENSITIVITY

In order to obtain an overall view of the changes in the membrane of mutant strains a variety of antibacterial compounds with different sites and modes of action and different methods of membrane penetration were used (See Table 6).

TABLE 6 : Action of Antibiotics

<i>Antibiotic</i>	<i>Action</i>
Penicillin D-Cycloserine	Affect polymerisation of peptidoglycan and cause weakening of the cell wall.
Novobiocin	Causes accumulation of UDP-muramyl pentapeptides, prevents incorporation of intermediates into DNA, RNA and protein. Used to select LPS mutants.
Polymyxin B sulphate	A peptide antibiotic which has a detergent activity perhaps mediated through action on phosphatidyl ethanolamine groups.
Streptomycin Kanamycin	Aminoglycoside antibiotics which cause mis-reading of mRNA and affect synthesis of protein.
Erythromycin	A macrolide antibiotic possessing a substituted lactone ring. Normally ineffective against Gram-negative organisms.
Levallorphan	A synthetic morphinan which interacts with the cytoplasmic membrane. Bactericidal action is unclear.
Tetracaine	An ionophore and local anaesthetic.

The antibiotic concentrations set for the sensitivity test were chosen at less than inhibitory concentration for the wild-type strain since the test was not to be purely bactericidal but to differentiate strains with altered membrane properties.

Table 7 lists the growth patterns for a number of mutants on antibiotic-containing Y.E. Agar. Serotype 1 and 54 strains which were polymyxin B resistant were more sensitive to penicillin and Levallorphan than any of the other strains tested. All non-tetracaine resistant strains were extremely sensitive to tetracaine. The effect of novobiocin was non-specific as might have been expected from its wide ranging methods of action. Polymyxin B had a killing effect particularly with respect to Levallorphan and tetracaine resistant-strains and against most 7824 strains. D-Cycloserine was ineffective against most of the organisms. Phage and polymyxin B resistant strains and strains grown in the presence of Cu^{2+} were sensitive to erythromycin. Kanamycin was effective against 7824 strains. Streptomycin was highly bactericidal, the killing effect apparently being unrelated to serotype, strain or type of mutant.

TABLE 7 Antibiotic Sensitivities of *Enterobacter aerogenes* strains

Strain	ANTIBIOTIC (concentration $\mu\text{g/ml}$)											
	P		C		N	Poly B		S	K	E	L	T
	$\mu\text{g/ml}$ 100 200	$\mu\text{g/ml}$ 100 200	$\mu\text{g/ml}$ 500	$\mu\text{g/ml}$ 50 200	$\mu\text{g/ml}$ 100 200	$\mu\text{g/ml}$ 50 100	$\mu\text{g/ml}$ 50 100	$\mu\text{g/ml}$ 50 100	$\mu\text{g/ml}$ 500	$\mu\text{g/ml}$ 1000		
AI	++	++	+	--	--	+-	++	+/-	-			
AIX	++	++	+	--	++	+-	++	+	-			
AIXTR	++	++	-	--	--	++	++	+/-	+			
AIXNR	++	++	+	+/- +/-	+/- +/-	++	+/- +/-	+	-			
AIXLR	++	++	+/-	+/- +/-	--	--	+/-	+	-			
AIX PB2000	++	++	-	++	--	--	--	-	-			
AIX (2.5mMCu ₂₊)	++	++	+/-	++	--	--	+/- -	+/-	-			
AI(O)	++	+/- +/-	+	--	--	++	++	+	-			
54C	++	++	+/-	--	--	--	+/- +/-	+/-	-			
54C PB3000a	--	+/- +/-	-	++	--	--	+/- +/-	-	-			
54C PB3000b	--	--	-	++	--	--	--	-	-			
54C F37		++	+	+-	--	++	++		-			
54C TR	++/-	++		+/- +/-	--	+-	++	+	+			
54C NR	++	++	++	++	+/- +/-	++	++	+	+			
54C LR	+-	++/-	-	--	--	+/- -	+/- -	+	-			
8805	++	++	+/-	++	--	++	++	+/-	-			
8806	++	++/-	+/-	+-	--	++	++	+/-	-			
9261	--	+/- -	-	--	--	--	--	-	-			
7824	++	++	-	--	--	--	++	+/-	-			
7824V	++	+/- +/-	-	--	--	+/- -	++	+/-	-			
7824 \emptyset	+/- -	++	-	--	--	--	+/- -	-	-			
7824TR	++	++	+	--	--	--	++	+	+			
7824NR	++	++/-	+	+/- +/-	--	--	++	+/-	-			
7824LR	++	+/- +/-	-	--	--	++	++/-	+	-			
7824 PB300	++	+-	-	+-	--	+/- -	++	+/-	-			
7824 (O)	++	++	-	--	+/- -	+/- +/-	++	+/-	-			
A4CR	++	++	+/-	+/- +/-	--	+/- +/-	--	+/-	-			

+ growth, +/- poor growth, - no growth

P = Benzyl Penicillin, C = D-Cycloserine, N = Novobiocin, Poly B = Polymyxin B Sulphate
S = Streptomycin, K = Kanamycin, E = Erythromycin, L = Levallorphan, T = Tetracaine.

MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTICS (MIC)

Generally, *Enterobacter aerogenes* proved to have a high tolerance for most antibiotics. Therefore, as can be seen in Table 8 the minimum inhibitory concentrations of most antibiotics used (to obtain drug resistant mutants) were high.

Antibiotic Sensitivity of *Enterobacter aerogenes* strains

TABLE: 8 Minimum Inhibitory Concentrations

Strain	ANTIBIOTIC (per ml)				
	Penicillin	Polymyxin B	Novobiocin	Tetracaine	Levallorphan
AIX	150U	300U	800µg	600µg	1000µg
54C	150U	100U	1000µg	600µg	2800µg
7824	150U	* 50U	800µg	600µg	1500µg

* Failed to grow in YE Broth in presence of Polymyxin B MIC of 50U/ml in Nutrient Broth.

SELECTION FOR ANTIBIOTIC RESISTANCE

After determining the M.I.C. for each antibiotic a lethal concentration was chosen:

Novobiocin 2mg/ml

Tetracaine 1mg/ml

Levallorphan 3mg/ml

Several colonies mutagenised with NTG (See Methods) were inoculated into the lethal concentration of antibiotic in Y.E. Broth, incubated at 30°C and examined daily for growth.

Selection for Polymyxin B resistance was achieved by inoculating a large number of UV irradiated cells into various lethal concentrations (300U, 600U, 1000U, 2000U/ml) of the antibiotic in Y.E. Broth.

GROWTH OF STRAINS

Serotype 1

A1, the wild-type strain, yielded by various methods of mutagenesis a family of mutants of exopolysaccharide formation. Most of this group were thoroughly characterised with respect to exopolysaccharide synthesis (Norval, 1969).

A1 and mutants A1X and A1SI produced large mucoid colonies of Y.E. agar (See Plates 1-3).

A1 and A1X were highly capsulate especially in old cultures (48h). A1SI mutants were differentiated on the basis of the consistency of the colony: these were extremely viscous and consequently difficult to remove with an inoculating loop. The visual appearance of India Ink stained films of these mutants differed markedly from the wild-type as the cells were non-capsulate and the surrounding medium contained amorphous slime.

A1.92 was a "leaky" mutant, producing low levels of exopolysaccharide at 37°C only. A1(O) unlike the mucoid wild-type and mutants, produced a flat friable colony with an irregular outline. The other mutants of A1 or A1X, mucoid or non-mucoid could be categorised as above.

Serotype 54

54C, the wild-type strain of Serotype 54, gave rise to a number of mutants all of which were mucoid (to varying extent) and capsulate. Colonial appearance was variable from the extremely mucoid 54C to the much less mucoid 54CF34, a phage resistant mutant.

A series of Serotype 54 strains was obtained from N.C.I.B. These cultures were wild-type capsulate, slime-former and non-exopolysaccharide synthesising (O) mutants derived directly from the preceding organism (8805, 8806 and 9261, respectively).

Serotype 30, strain 7824 a mucoid and capsulate organism yielded slime forming (SI) and "O" mutants in addition to further capsulate mutants. By analogy with the colonial morphology and consistency reported for A1 and mutants, 7824 and its mutants fell into similar categories: wild-type, highly mucoid; SI mutant, mucoid and extremely viscous; "O" mutant, non-mucoid and friable.

Serotype 8, Strain A4CR, a "crenated" mutant possessed a phenotype which varied with incubation temperature. At 37°C on Y.E. Agar it grew identically to wild-type strain A4, producing large mucoid colonies but at 20°C appeared to be non-mucoid and had a rough, ridged appearance with lines radiating out from a central dome. In liquid minimal medium at 20°C the cells autoagglutinated and the culture was very granular. At 37°C it appeared normal (See Plate 4).

Table 9 lists the form of the exopolysaccharide (if present) in the aforementioned strains.

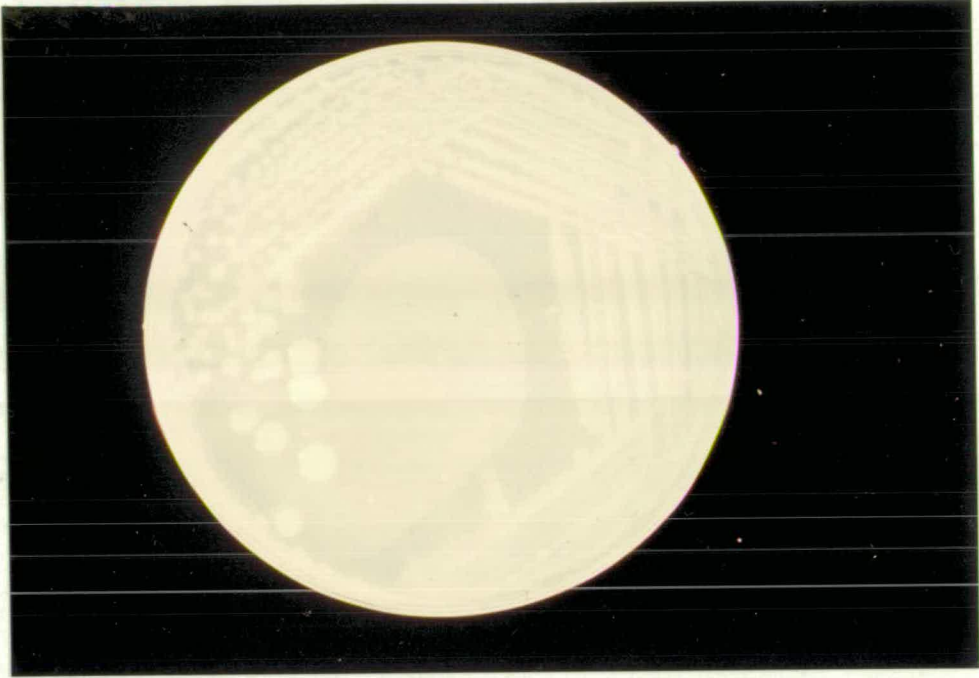


Plate 1 *Enterobacter aerogenes* A1 grown on Y.E. Agar at 30°C for 48h.

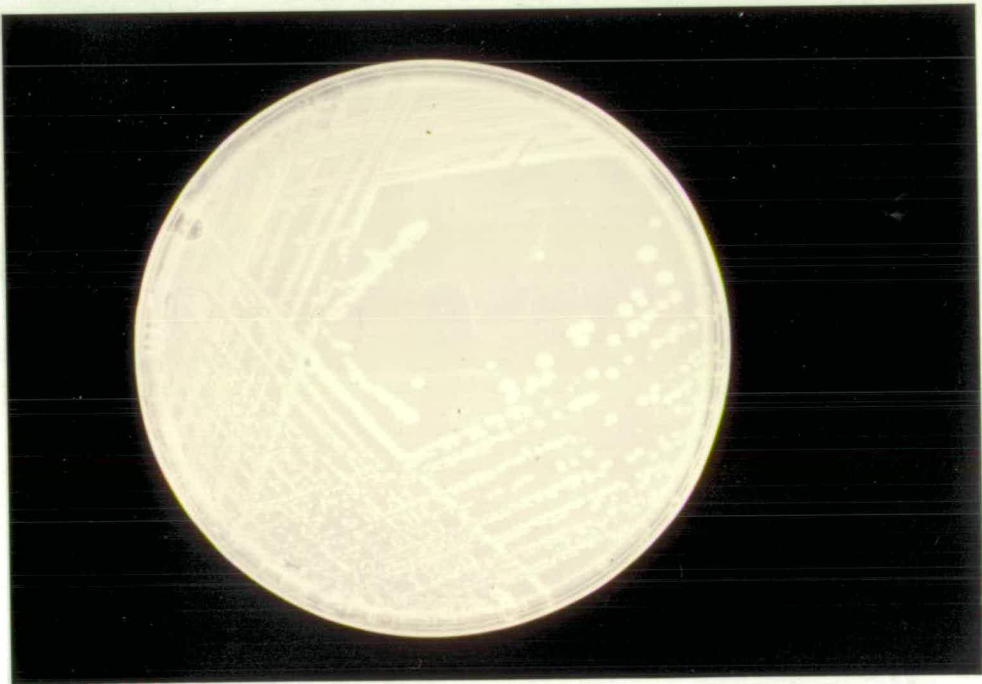


PLATE 2 : *Enterobacter aerogenes* A1X grown on YE Agar at 30°C for 48h.

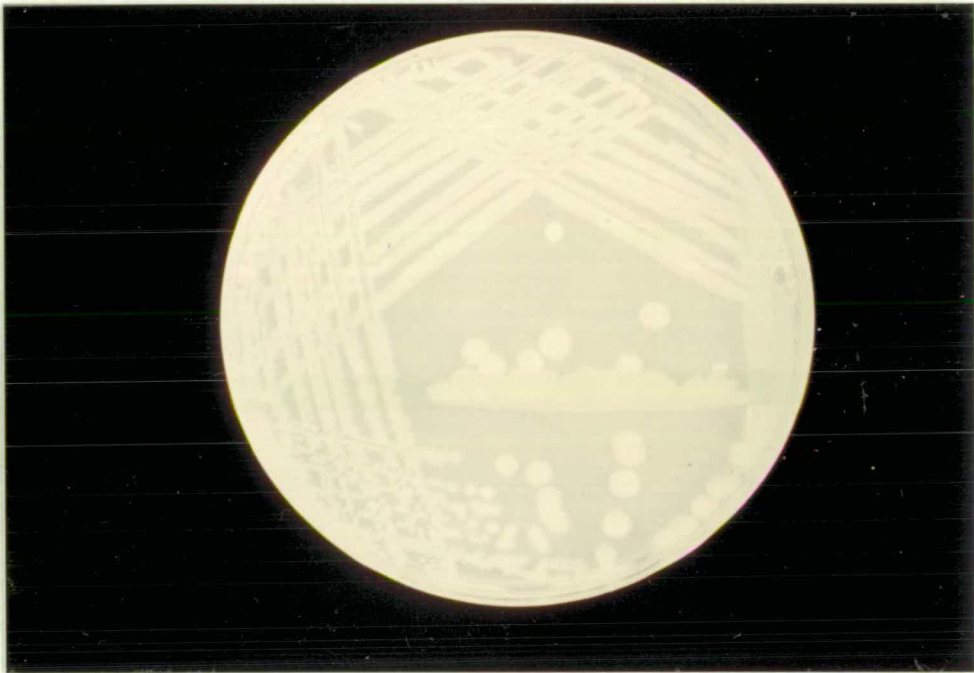
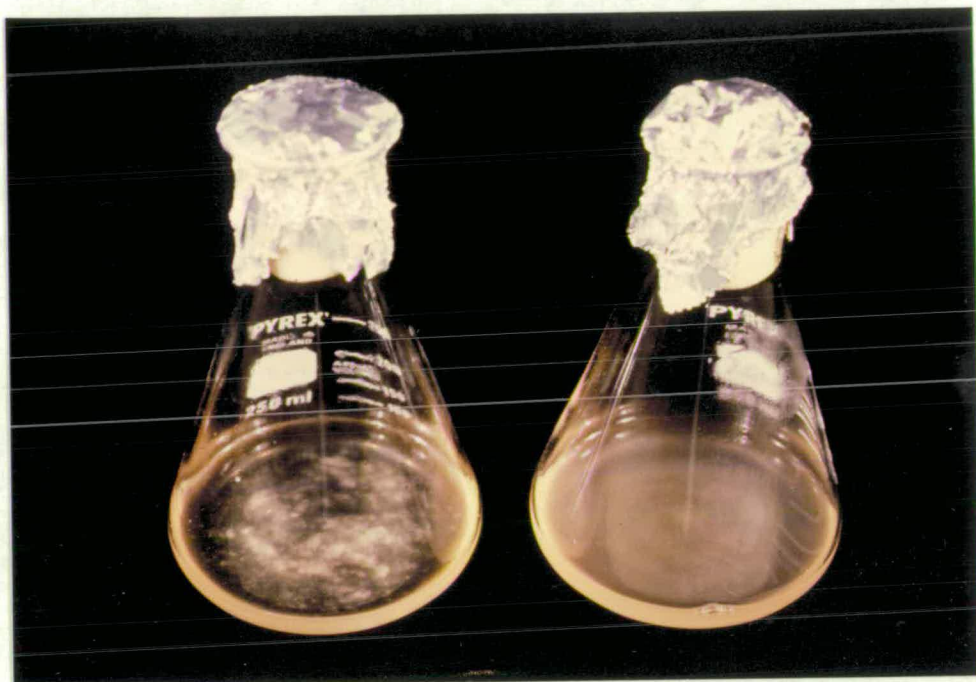


Plate 3 *Enterobacter aerogenes* A1SI grown on Y.E. Agar at 30°C for 48h.



1

2

PLATE :4 Growth of A4CR in Minimal Medium for 48h
at:

- 1.) 20°C
- 2.) 37°C

TABLE 9 : Form of Exopolysaccharide in *Enterobacter aerogenes*

<i>Strain</i>	<i>Form of Exopolysaccharide (IF PRESENT)</i>
AIX	Capsule + Slime (Cap + SI)
AI	Cap
AISI	SI
AISIBR	SI
AISINR	SI
AISIHIS	Cap + SI
AIX PB600	thinly Cap
AIX PB2000	None
AI (O)	None
AI92	None
AIXTR	Cap + SI
AIXNR	Cap + SI
AIXLR	Cap + SI
54C	Cap
54C F34	Cap
54C F37	Cap
54C PB3000a	Cap
54C PB3000b	Cap
54C TR	Cap
54C NR	Cap
54C LR	Cap
8805	Cap
8806	SI
9261	None
7824	Cap
7824V	SI
7824 \emptyset	Cap
7824f	None
7824 PB300	Cap
7824 (O)	None
7824 TR	Cap
7824 NR	Cap
7824 LR	Cap
A4	Cap
A4CR	Cap

GROWTH CURVES

Given the variation in the colonial morphology and probably the surface layer of the strains, there was a surprising continuity in their growth curves (See Figures 24-26).

At 30°C, the average division time for all mucoid strains in Y.E. Broth was approximately 45 min. Non-mucoid strains divided in 55 min. Under the various nutrient limitations growth of mucoid strains was slower, approximating that of a non-mucoid strain under normal conditions. The change in the pH of the medium over the 72h of the growth curve was characteristic for all strains. Beginning at pH7.2 it fell to pH5.0 at 24h and recovered to pH6.8 by 72h.

In all strains the quantity of free glucose remaining in the culture (residual glucose) fell to 10% of the original value (2g/l) by the beginning of the stationary phase, 12h.

EXOPOLYSACCHARIDE PRODUCTION

The yield of exopolysaccharide (as non-dialysable carbohydrate) for each strain was determined, at a variety of temperatures, by the Phenol-Sulphuric Acid assay of Dubois *et al* (1956) on culture supernates. This method was chosen in preference to precipitating exopolysaccharide with acetone since the latter only gave a measure of the high molecular weight material present, and in preference to the Anthrone method which is subject to a greater degree of interference from polymers such as protein.

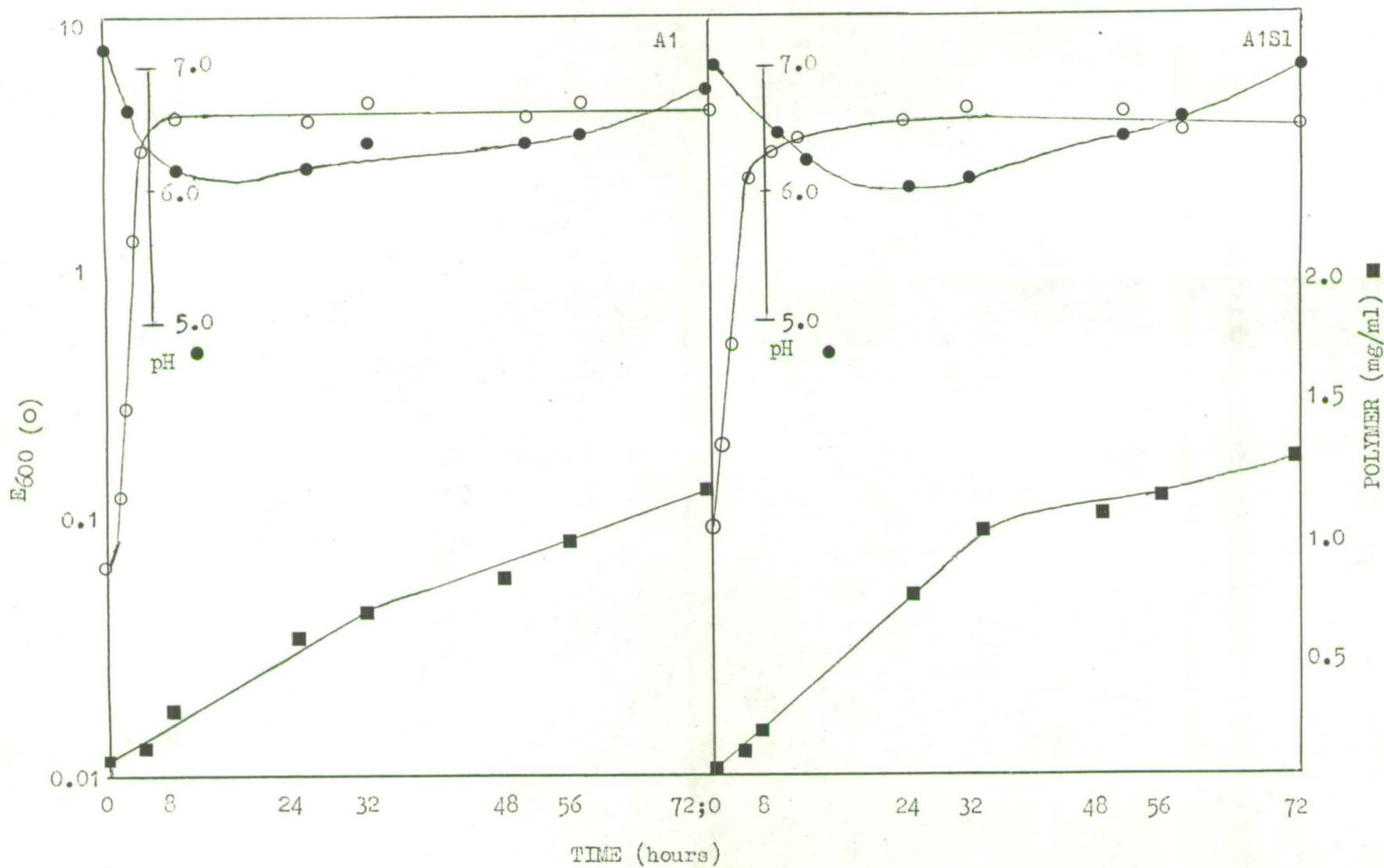
The results for yield of exopolysaccharide in Y.E. Broth are given in Table 10.

For mucoid strains the yield of exopolysaccharide was substantially higher than those determined for non-mucoid strains. From the combined visual observations in India Ink films and yields it seemed probable that yields of less than 400-500 μg ^{Hexose/mg dry wt. of} cells represented non-mucoid strains. Although such a value appears relatively high this could be a function of "turnover" of lipopolysaccharide and membrane-derived oligosaccharide which would give positive results in the assay used. Thus "O" mutants such as 7824(O), 9261 and A1(O) fell into this category.

The yield of capsulate mutant strains may be similar to or less than the capsulate wild-type strain as reflected in 7824PB300 and 7824 wild-type and A1XTR and A1, respectively.

No appreciable differences occurred between the yield from the capsulate wild-type strain and its slime-forming mutant.

Mutant A4CR showed a 44% increase in yield when growth temperature was



Figures 24,25 *Enterobacter aerogenes* A1 and A1S1 grown in batch culture in Y.E. Broth at 30°C.

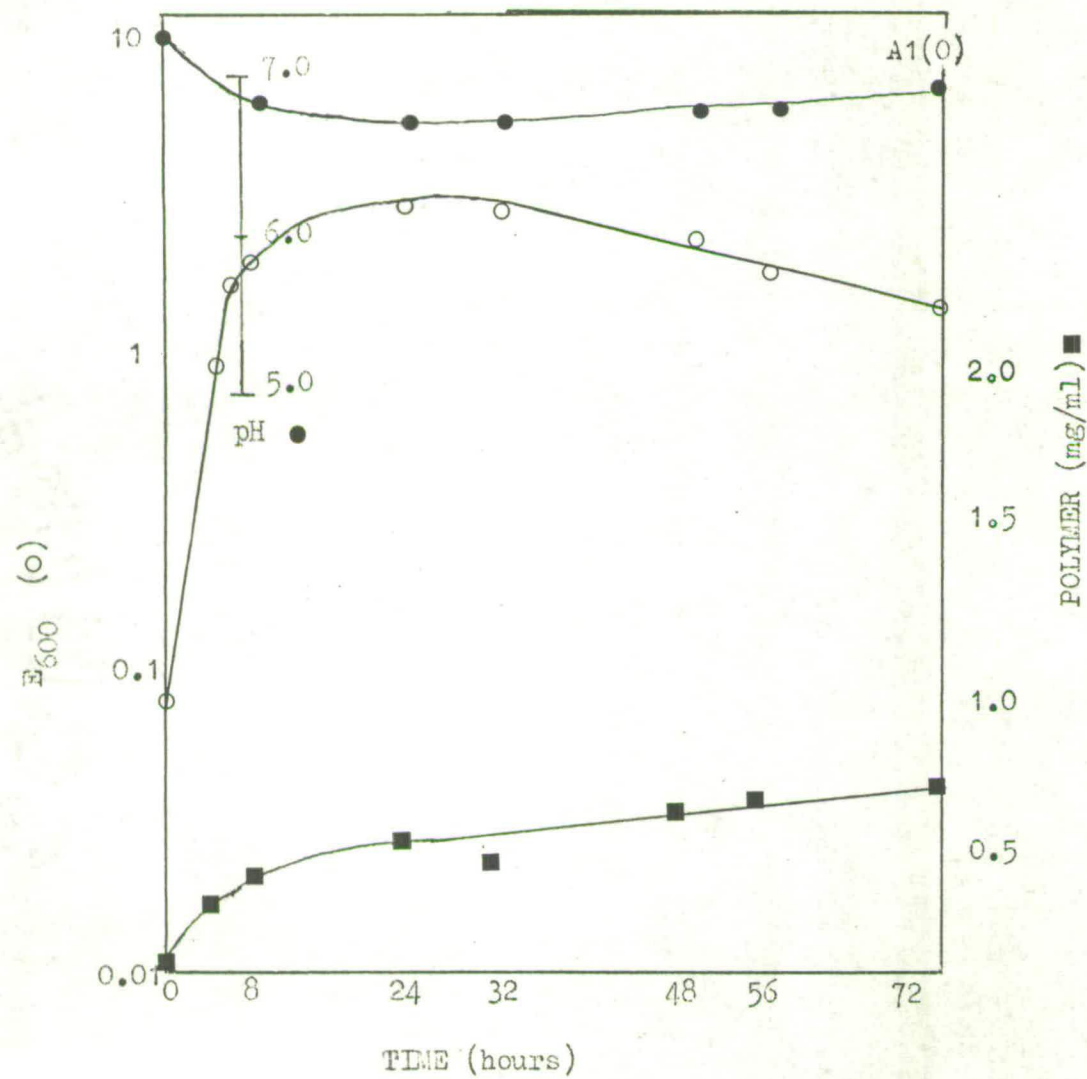


Figure 26 *Enterobacter aerogenes* A1(O) grown in batch culture in Y.E. Broth at 30°C.

TABLE 10 : Yields of Exopolysaccharide for *Enterobacter aerogenes*

Strain	Yield in YE Broth (μg Hexose/mg dry wt. cells)		
	20°C	30°C	37°C
AIX	1004	1601	2421
AI	1348	1250	1247
AISI	1684	1415	1169
AIX PB600	125	465	680
AIX PB2000	ND	394	ND
AI (O)	ND	516	493
AI.92	449	419	777
AIX TR	612	1248	1206
AIX NR	3248	3052	3830
AIX LR	1482	1603	2066
54C	819	ND	1247
54C F34	ND	1754	ND
54C F37	830	995	979
54C PB3000a	ND	1472	ND
54C PB3000b	1281	1302	1279
54C TR	615	785	1527
54C NR	875	1203	1295
54C L.R	542	1069	1577
8805	627	1204	1679
8806	856	1283	2249
9261	356	313	393
7824	2331	2193	2338
7824V	1353	1565	1706
7824 \emptyset	ND	805	ND
7824f	ND	351	ND
7824 PB300	2462	2671	2482
7824 (O)	146	203	513
7824 TR	3795	3001	3440
7824 NR	1956	2137	2536
7824 LR	2535	1986	2133
A4	2103	ND	2337
A4CR*	835	ND	1472

(* Similar in Minimal Medium)

shifted from 20°C to 37°C. This result was confirmed in both Y.E. Broth and Minimal Medium.

The values given for growth at 10°C are not comparable to those at other temperatures. Cultures were not shaken. However a valid comparison can be made *within* the 10°C group.

Strains A1 and A1SI produced a high yield compared to 8805 and 8806 at 10°C. This suggested differential abilities to grow at this temperature. Whereas A1 and A1SI gave similar yields, 8805 and 8806 were widely differing, the latter falling into the non-mucoid category ($\sim 500\mu\text{g}/\text{mg}$). Growth at 10°C progressed by colonisation of the base of the glass flasks as opposed to growth in suspension and the yields possibly reflected the ability to colonise the glass surface.

(See Table 11).

NUTRIENT LIMITATION

The yields of exopolysaccharide in nutrient-limited media were widely varying, dependent on the limitation imposed (Table 12-14)

Sulphate limitation reduced the yield between 30 and 56% in 7824 and A1X. In 54C the reduction was closer to 80% but this strain gave poorer growth overall.

Phosphate and magnesium limitation yielded large quantities of exopolysaccharide, close to the value for cells grown in limited medium with all nutrients at normal levels.

The most severe limitations, Fe^{3+} or K^+ reduced the yield to "non-mucoid" level or lower.

Iron-limited medium supplemented with citrate produced a 3-fold rise in yield over solely iron-limited media, probably due to the cells scavenging extra iron.

SUPPLEMENTS TO Y.E. BROTH

Table 15 shows the effect of a variety of membrane active agents on the yield of exopolysaccharide of strain A1X grown at 30°C.

Inclusion of 0.01%(w/v) SDS or sodium lauryl sarcosinate in the medium did not affect the yield of exopolysaccharide. The cells were heavily capsulate. The addition of 10mM phenylethanol resulted in approximately 50% reduction in exopolysaccharide synthesis while the presence of 1M ethanol abolished exopolysaccharide synthesis.

The osmotic agents sucrose and sodium chloride affected yields. NaCl caused a reduction similar to that of phenylethanol but sucrose effected a 70-80% reduction. The results for strain 7824 exposed to the same agents resembled those of strain A1X.

The metabolically toxic heavy metal Cu^{2+} (as CuSO_4) at a concentration of

Table 11 : Exopolysaccharide Yields from cultures grown at 10°C

Strain	Yield of Exopolysaccharide µg Hexose/mg dry wt. cells.
A1	2105
A1SI	2390
8805	816
8806	313

TABLE:12 : Effect of Nutrient Limitation on Exopolysaccharide Yield

Strain	Yield (μg Hexose/mg dry wt cells)				
	None*	PO_4^{3-}	Mg^{2+}	SO_4^{2-}	K^+
AIX	5803	5944	5220	3992	456
7824	6065	5420	5258	2677	nd
54C	1228	1234	789	268	nd

* All nutrients present at normal levels, nd—not determined

TABLE13 : Effect of Iron (Fe^{3+}) limitation on Yield

Strain	Yield (μg Hexose/mg dry wt cells)
AIX	190

TABLE14: Effect of Iron (Fe^{3+}) limitation + citrate supplement on Yield

Strain	Yield (μg Hexose/mg dry wt cells)
AIX	567

TABLE:15 :Effect of Supplements to YE Broth on the Yield of Exopolysaccharide from strain AIX

Supplement	Yield (μg Hexose/mg dry wt cells)
1mM CuSO_4	651
2.5mM CuSO_4	114
1M EtOH	67
10mM Phenylethanol	794
300mM NaCl	832
500mM Sucrose	764
100U Penicillin/ml	1505
0.01% (w/v) S.D.S.	1425
0.01% (w/v) Sarcosinate (Sodium lauryl)	1771
Unsupplemented	1601

TABLE:16:Effect of E.D.T.A. (disodium salt) supplement to YE Broth on the Yield of Exopolysaccharide

Strain	Yield (μg Hexose/mg dry wt cells)
AIX	312.2
54C	200.0
7824	186.0

1mM reduced the yield by 59%; at 2.5mM the yield fell by 93% and the colonies appeared non-mucoid (See Plate 5).

The chelating agent E.D.T.A. (disodium salt) affected the yield, inhibiting production of exopolysaccharide by 84%. Again strain 7824 gave comparable results.(Table 16).

Incorporation of penicillin into the medium at a level sufficient to cause slight filamentation and blebbing of cells had no significant effect on polymer synthesising capacity, the yield remained high.

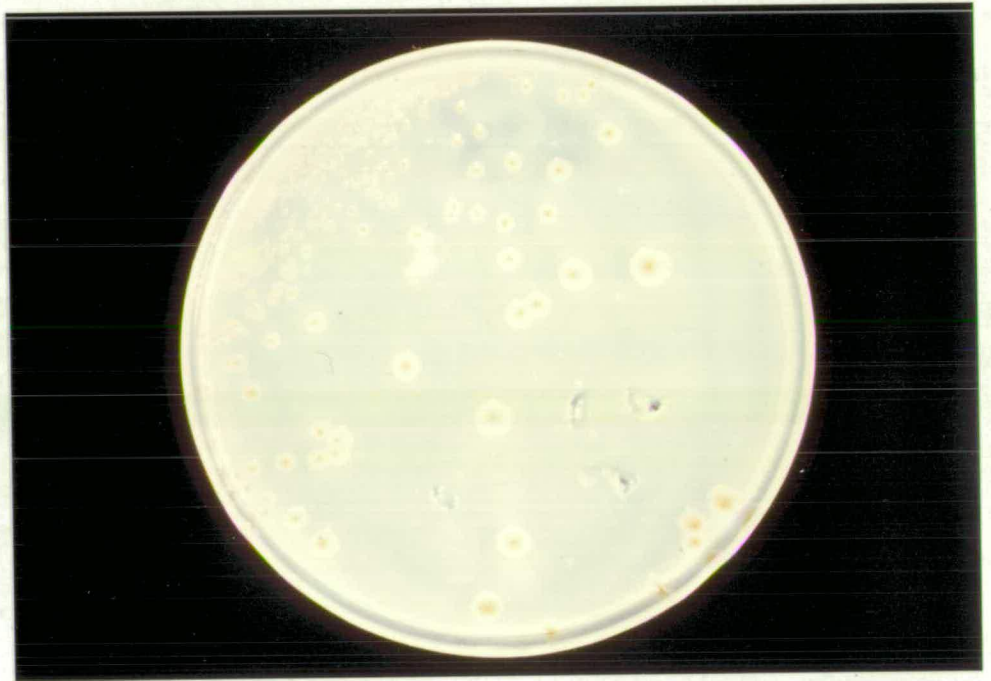


Plate **5** : Production of non-mucoid colonies by *Enterobacter aerogenes* strain A1X grown on 2.5mM CuSO₄ supplemented Y.E. Agar at 30°C for 14 days.

SUPERNATANT PROTEIN FROM GROWTH CULTURES

Normal growth of the bacterial cell envelope requires the "turnover" of components of the envelope. These are lost to the environment. As a readily quantifiable component, protein was used to estimate the degree of stability of the cell envelope by comparing values for wild-type strains with those of their mutants.

Thus a sharp increase in the supernatant protein (in cells growing at the same rate) was construed as indicating an alteration in the molecular organisation or assembly of protein into the cell envelope (See Table 17).

The "O" mutants of exopolysaccharide synthesis A1 (0), 7824 (0) and 9261, the "leaky" mutant A1.92 and 7824 \emptyset and 54CF34, phage resistant mutants released significantly more protein than the wild-types.

The other mutants (slime-forming and drug resistant) show increased or decreased output of protein but generally were more stable. An examination of the wild-type capsulate, slime former and "O" mutant of each strain (A1, A1SI, A1 (0); 8805, 8806, 9261; 7824, 7224V, 7824 (0)) indicated a common pattern. The amount of protein in the supernatant increased sequentially from capsulate to slime-former to "O" mutant.

TABLE:17 : Release of Protein to the Culture Fluid.

Strain	Supernatant Protein * (μg protein/mg dry wt. of cells)	Strain	Supernatant Protein * (μg protein/mg dry wt. of cells)
A1X	100.3	54C NR	95.5
A1	73.72	54C LR	99.8
A1SI	110.00	8805	43.02
A1.92	488.09	8806	111.2
A1 (O)	396.4	9261	139.35
A1XTR	67.47	7824	45.9
A1XNR	63.58	7824V	183.8
A1XLR	41.16	7824 \emptyset	324.48
54C	97.72	7824 PB300	81.40
54C	142.67	7824 (O)	229.0
54C	90.18	7824 TR	102.48
54C PB3000a	42.18	7824 NR	101.04
54C PB3000b	61.22	7824 LR	96.74
54C TR	132.73		

(* Protein found in the Supernatant after 72h growth. All samples were dialysed against tap-water for two days and analysed by the Lowry method (1951)).*

* Lowry et al (1951)

YIELD OF LIPOPOLYSACCHARIDE

The yields of LPS obtained are listed in Table **18**. Several yields were higher than expected. Although nuclease treatment was performed for an extensive period (See 'Materials and Methods') it remains possible that the crude LPS was contaminated by nucleic acids.

However A4CR which was characterised as having reduced (50% less) LPS when grown at 20°C did show a 47% decrease in LPS yield at 20°C when prepared this way.

SDS-POLYACRYLAMIDE GEL ANALYSIS OF LIPOPOLYSACCHARIDE

SDS-PAGE was successfully used by Di Rienzo *et al* (1978) to differentiate species of LPS molecule from the same organism. In their paper the authors demonstrated the heterogeneity of the LPS as determined by this method was *not* an artifact resulting from the extraction procedure.

From the selection of capsulate, slime-forming and 'O' mutants in Plates **6,7** it was concluded that no specific changes in LPS profile could be correlated with one of the aforementioned strains.

However, A4CR gave less intense staining in Section I (Species I) in 20°C grown cells than seen in 37°C grown cells.

Table 18: YIELD OF LIPOPOLYSACCHARIDE

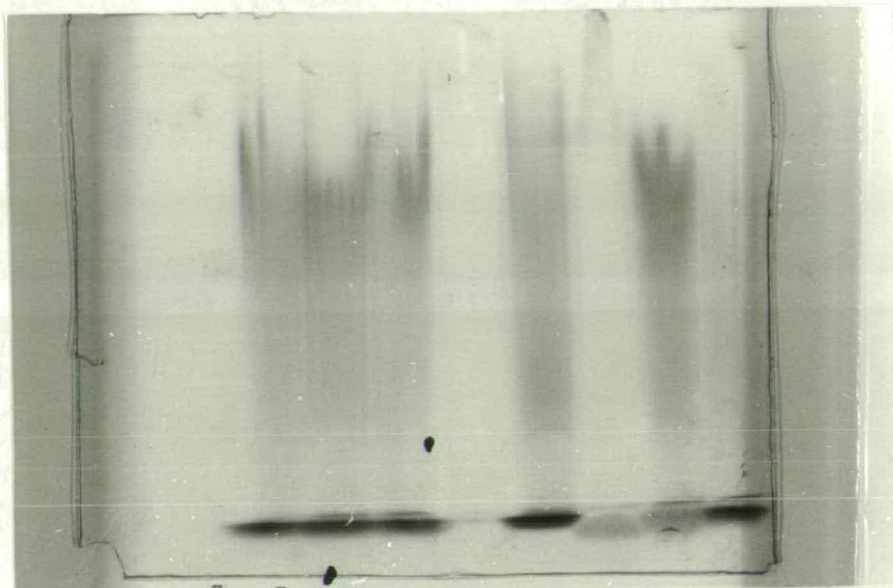
Strain	Yield of Crude LPS as % (w/w) of freeze-dried cells (at 30°C unless otherwise indicated)
A1	1.66
A1SI	8.3
A1X600	3.13
8805	6.0
8806	5.23
9261	4.14
54C	4.7
54CF34	6.3
54CF37	5.9
7824	2.1
7824V	2.83
7824(O)	2.7
7824PB300	7.2
7824TR	6.0
7824NR	6.8
7824LR	7.4
A4CR (20°C)	1.7
A4CR (37°C)	3.2

Section

I

II

III



a b c d e f g

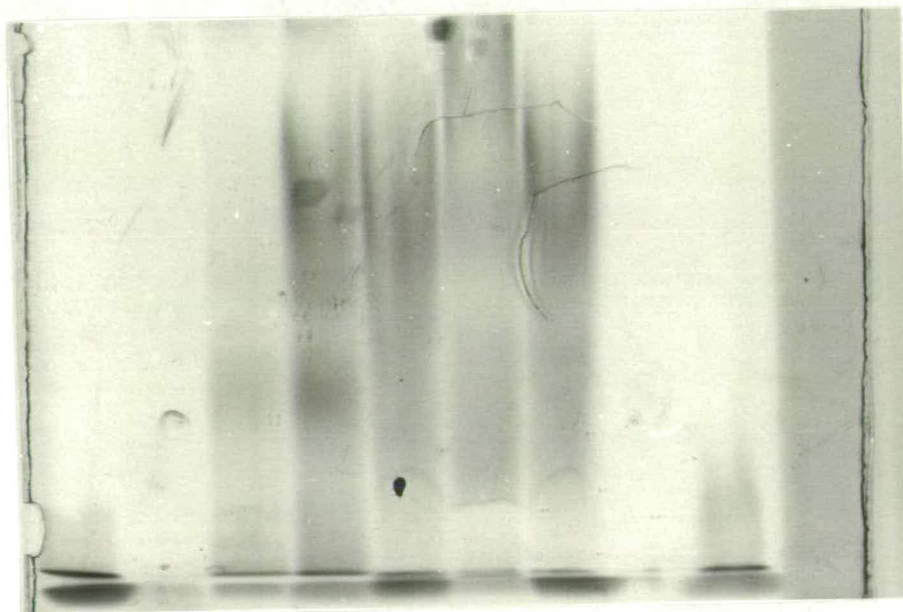
a, 7824LR; b, 7824NR; c, 7824TR; d, 7824PB300;
e, 7824 (C); f, 7824V; g, 7824.

Section

I

II

III



a b c d e f g

a, A1S1; b, A4CR 20°C; c, A4CR37°C; d, 8805;
e, 8806; f, 9261; g, 54CF34

Plates: 6-7 : Gel Electrophoresis of lipopolysaccharide from *Enterobacter aerogenes* strains.

LECTIN AGGLUTINABILITY OF STRAINS

Three lectins were used in an attempt to distinguish strains with different exposed glycosidic surface components. Concanavalin A (ConA) from the jack-bean *Canavalia ensiformis* which binds specifically to carbohydrates with the D-arabinopyranoside configuration at C₃, C₄, or C₆ (e.g. D-glucose, D-mannose, fructose). Wheat Germ Lectin (WGL) from *Triticum vulgare* is specific for D-GlcNAc residues and Soy Bean Lectin (SYL) from *Glycine max* binds specifically with D-GalNAc or D-galactose.

All strains were grown in Y.E. Broth and Y.E. Broth supplemented by 0.05% (w/v) EDTA (disodium salt) and tested for agglutination with ConA, WGL and SYL by spotting four drops of cells on to a clean glass slide (the fourth spot as a control) and adding an equal quantity of each lectin.

In both media, A1.92 and 7824(O) gave slight agglutination with all lectins. The remaining strains failed to agglutinate irrespective of the growth medium or lectin used in the test.

GROWTH OF STRAINS IN MINIMAL MEDIUM

All strains grew normally at 30°C and 37°C. However at 20°C variations in growth were detected. A4CR autoagglutinated and produced extremely 'bitty' growth (See Plate 4).

Two of the tetracaine-resistant mutants A1XTR and 54CTR grew in similar manner although autoagglutination was less pronounced than in A4CR.

PHAGE SENSITIVITY PATTERNS

The sensitivity of many strains to a number of phage preparations was tested by overlaying a lawn of the strain of bacteria with phage suspended in 2.5ml sloppy agar. Sufficient phage particles were present to cause confluent lysis.

Marginally greater sensitivity was detected at 37°C compared to 20°C thereby suggesting that synthesis of certain receptors was adversely affected by the lower growth temperature. Strain A4 was sensitive to phage F41 at 20°C or 37°C. Strain A4CR was sensitive at 37°C but not at 20°C.

This latter result would tend to indicate a change associated with LPS or exopolysaccharide. At 20°C the yield of both polymers was approximately 50% of that at 37°C. Possibly LPS or capsule forms the receptor or part of the receptor (in conjunction with an outer membrane protein) and a reduction would give the mutant protection (Table 19)

TABLE: 19 *Phage Sensitivity Patterns*

Strain	Phage	Growth temperature													
		20°C						37°C							
		34	37	40	45	S	W	O	34	37	40	45	S	W	O
A1		-	-	+	+	-	+	-	-	-	+	+	-	+	-
A1X		-	-	-	-	-	-	-	-	-	-	-	-	-	-
A1SI		-	-	+	+	-	-	-	-	-	+	+	-	-	-
A1XPB600		-	-	+	+	-	-	-	-	-	+	+	-	-	-
A1.92		-	-	-	-	-	-	-	-	-	-	-	+	-	-
A1(O)		-	-	+	-	+	-	-	-	-	-	+	+	-	-
A1XLR		-	+	-	+	-	-	-	-	+	-	+	-	-	-
A1XNR		-	-	-	+	+	-	-	-	-	-	+	+	-	-
A1XTR		+	+	-	-	-	-	-	+	+	-	-	-	-	-
A1X(Copper) 54C grown		-	-	+	+	-	-	-	-	-	+	+	-	-	-
54CF37		+	-	-	+	-	+	-	+	-	-	+	-	+	-
54CPB3000a		+	-	-	+	-	-	-	+	+	-	-	-	-	-
54CPB3000b		-	+	-	-	-	-	-	+	+	-	-	-	-	-
54CLR		+	+	-	-	+	-	-	+	+	-	-	-	-	-
54CNR		+	-	-	-	-	-	-	+	+	-	-	-	-	-
54CTR		+	-	-	-	-	-	-	-	-	+	-	-	+	-
8805		+	-	-	-	-	-	-	+	-	-	-	-	-	-
8806		+	-	+	+	-	-	-	+	-	+	+	-	+	-
9261		-	-	-	+	-	-	-	-	-	+	+	-	+	-
7824		-	-	-	-	-	-	+	-	-	-	-	-	-	+
7824V		-	-	-	-	-	-	+	-	-	-	-	-	-	+
7824Ø		-	-	-	+	-	-	-	-	-	-	+	-	-	-
7824PB300		-	-	-	+	-	-	+	-	-	+	-	-	-	+
7824(O)		-	-	-	-	-	-	-	-	-	+	-	-	-	+
7824LR		-	-	-	-	-	-	+	-	-	-	-	-	-	+
7824NR		-	-	-	+	-	-	+	-	-	-	-	-	-	+
7824TR		-	-	-	-	-	-	+	-	-	-	-	-	-	+
Original Host		Type 54		Type 1		Type 54		Type 30							

Phage Preparations S, W, O isolated during the course of this work.

EFFECT OF SURFACE ACTIVE AGENTS ON CELL SURVIVAL

Table 20 relates the effect of growth of *Enterobacter aerogenes* strains in high concentrations of membrane active agents. SDS completely inhibited growth and killed the cells (No growth when plated on YE Agar after 16h exposure to SDS).

EDTA acted more selectively. At 1%(w/v) EDTA, Polymyxin resistant mutants and 'O' mutants were inhibited. At 2%(w/v) EDTA the majority of 7824 strains were affected, possibly intimating an altered arrangement of lipopolysaccharide molecules in 7824. Interestingly, this strain differed in outer membrane protein profile also. Combined, these features may have reduced the resistance of these strains to agents affecting molecular organisation.

A clear indication of the role of LPS in the protection of the cell integrity was obtained in the A4CR mutant. By growing it at 20°C, the lipopolysaccharide and exopolysaccharide yields were halved. When such cells were exposed to lethal levels of membrane active agents Polymyxin B and SDS, for a short period, survivor curves could be determined (See Table 21).

Polymyxin B was least effective against A4CR grown at 20°C while SDS was more effective against A4CR at either temperature but not significantly more effective against A4CR grown at 20°C than at 37°C.

TABLE:20 Resistance to Surface Active Agents in Y.E. Broth

Growth in 1% EDTA in Y.E.B. (at 30°C)

All grew except as follows:

A1XPB600, poor growth.

A1(O), A1.92, A1XPB2000, 7824PB300 , 7824(O), no growth.

Growth in 2% EDTA in Y.E.B.

All grew except as follows:

7824TR ,7824LR , 7824NR

A1X600 , A1.92, A1X2000 , A1(O) , 7824PB300 , 7824(O)

7824V.

No strains grew in 1 or 2% (w/v) SDS in Y.E. Broth.

TABLE:21 Survival Rate after 15 mins exposure to surface active agents in physiological saline. (Survival determined at 30°C).

<i>Surface Active Agent</i>	<i>% Survivors</i>			
	<i>A4</i>		<i>A4CR</i>	
	<i>37°C</i>	<i>20°C</i>	<i>37°C</i>	<i>20°C</i>
Polymyxin B (8000 USP units/ml)	0.095	0.063	0.348	4.464
SDS [5% (w/v)]	100	30	22.35	11.75
Control (physiological saline only)	100	100	100	100

ELUTION PROFILE OF LOW MOLECULAR WEIGHT EXOPOLYSACCHARIDE

From a BioGel ACA54 column two distinct major peaks were eluted with acetic acid/pyridine/water from low molecular weight exopolysaccharide of 7824 and 7824V. The molecular weights of these peaks were approximately 60000 and 30000.

While sharing peaks at the same molecular weight the two strains differed in the proportion of sample within the peaks. Thus the capsulate strain (7824) was distributed mainly into the 30000 peak and the slimeformer (7824V) into the 60000 peak (See Figure 27 and Table 22).

A few minor peaks appeared at under 10000 but probably represent degradation products resulting from the preparative method. These peaks comprised a small fraction of the total sample.

ANALYSIS OF THE CHEMICAL COMPOSITION OF HIGH AND LOW MOLECULAR WEIGHT EXOPOLYSACCHARIDE FROM STRAINS 7824 AND 7824V

Polysaccharide (2mg) was hydrolysed in a sealed glass ampoule containing 400 μ l 1M Trifluoroacetic acid at 100°C for 16h. The tube was then opened and the contents dried by rotary evaporation at reduced pressure and washed once. The hydrolysed polysaccharide was then dissolved in water to a known concentration and applied to 3MM Whatman paper.

Neutral sugars were detected by irrigating the paper (during chromatography) with butanol/pyridine/H₂O (6:4:3) and acidic sugars with ethyl acetate/acetic acid/formic acid/H₂O (18:3:1:4) for 18h and 40h respectively.

Sugars were detected by immersion in alkaline silver nitrate reagents (Trevelyan, *et al*, 1950).

Figures 28-30 represent the chromatograms obtained from the above procedures. Low molecular weight material was qualitatively distinct possessing only glucose while high molecular weight material possessed glucose, mannose and galactose together with glucuronic acid as the sole acidic residue. Capsulate and slime material were qualitatively indistinguishable.

A partial acid-hydrolysis (0.1M sulphuric acid for 1h) of the low molecular weight exopolysaccharide preparation was performed to determine the likely structure of the glucan. The chromatographic results are shown in Figure 30.

Maltose, Maltotriose, nigerose, sophorose, kojibiose, gentibiose, and glucose standards were run with the 7824 and 7824V samples. Most of each of the 'unknown' samples was glucose; however a faint spot was detected. This spot had an R_f value of 0.86 against glucose. It did not coincide with any of the standards used

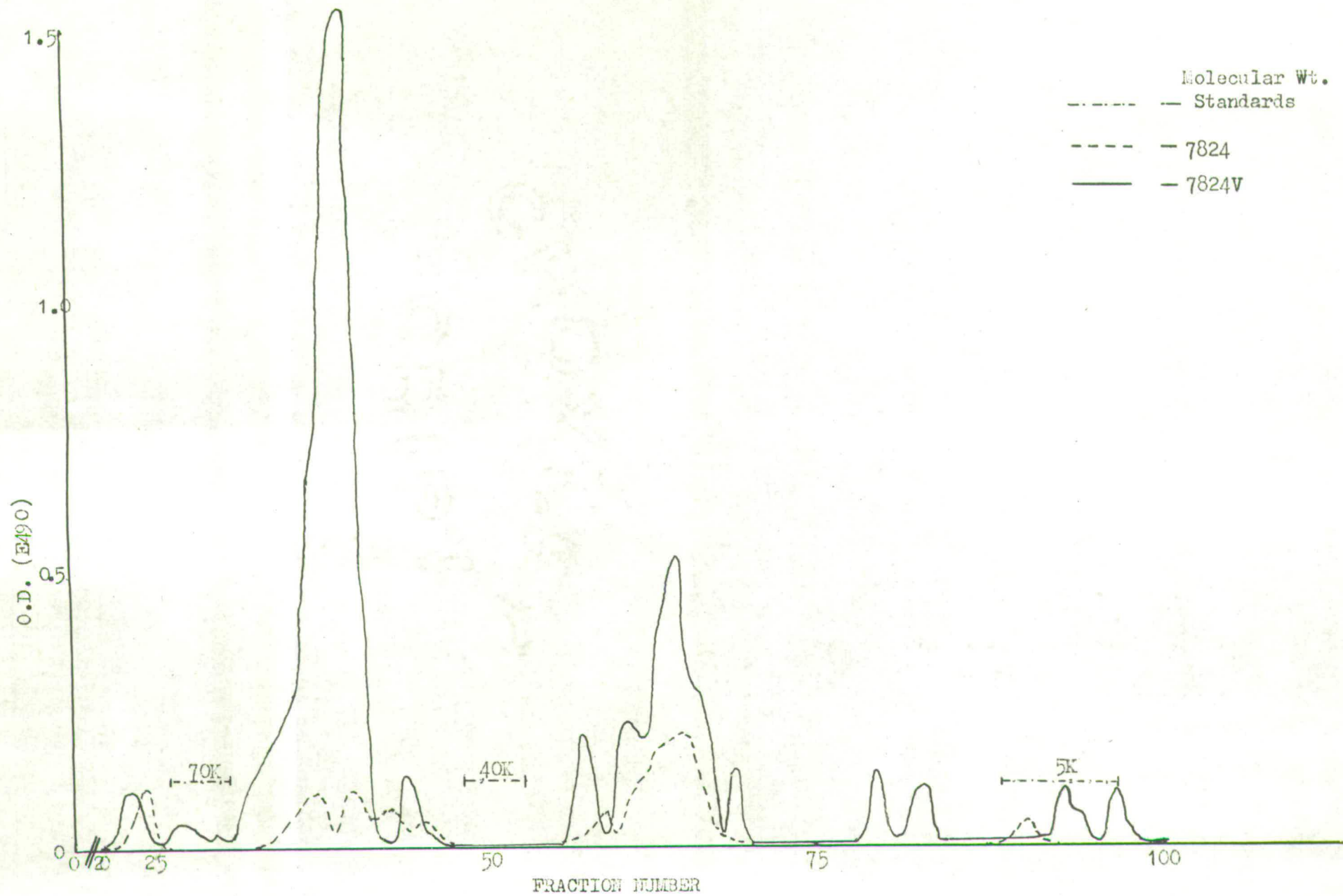


Figure 27 : Elution profiles of low molecular weight exopolysaccharide from

Table:22: Distribution of Molecular Weights in Low Molecular Weight Exopolysaccharide

Strain	% Distribution into Peaks (Molecular Weight)		
	60K	30K	10K
7824	30.4	51.0	18.6
7824V	60.4	32.9	6.7

Solvent: butanol / pyridine / H₂O

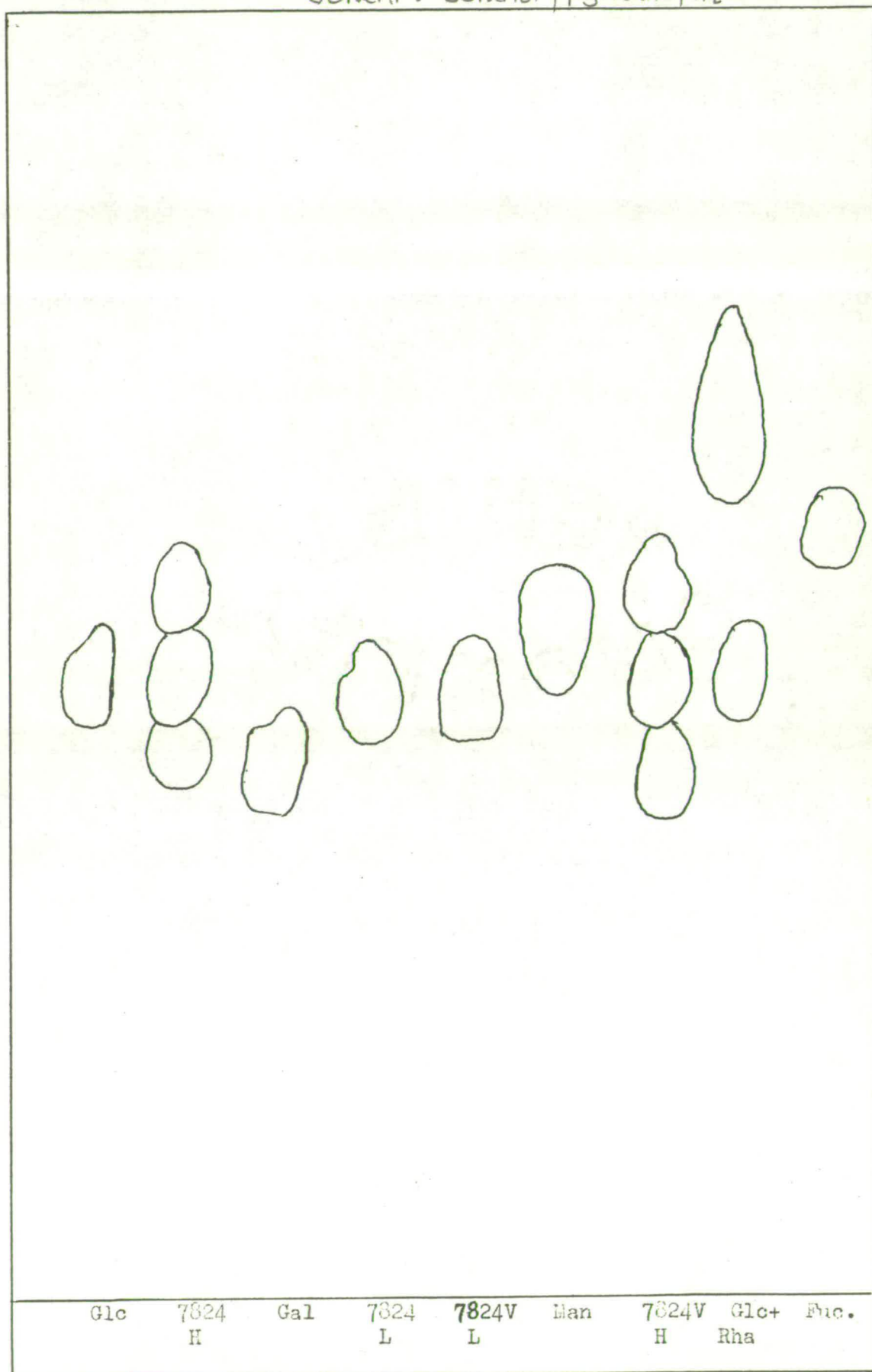


Figure 28: Diagrammatic sketch of a chromatogram showing the neutral sugar products of acid-hydrolysis of high and low molecular weight exopolysaccharide from strains 7824 and 7824V.

Solvent: ethyl acetate/acetic acid/formic acid/H₂O

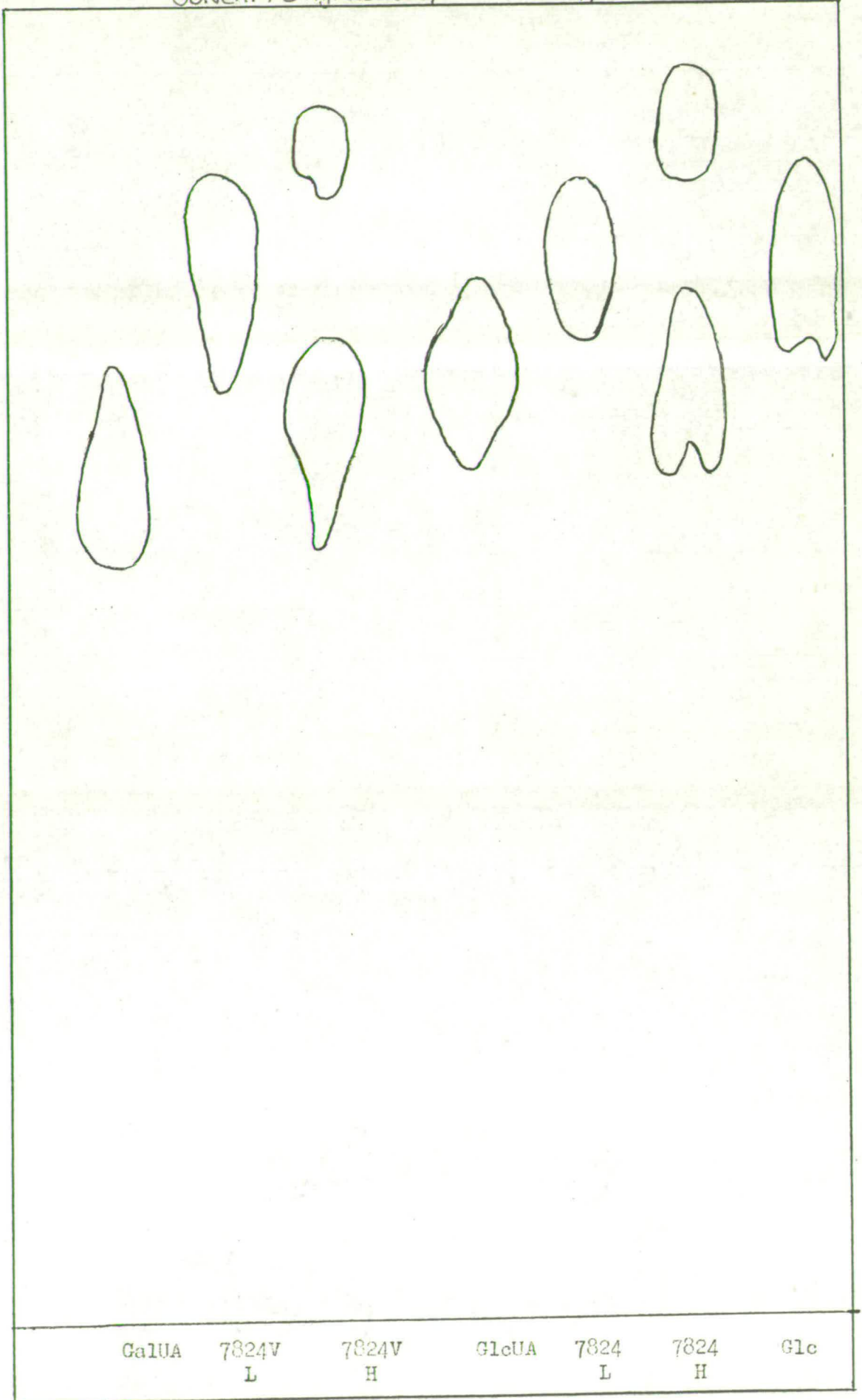


Figure 29: Diagrammatic sketch of a chromatogram showing the uronic acid products of acid-hydrolysis of high and low molecular weight exopolysaccharide from strains 7824 and 7824V.

Solvent: butanol/pyridine/H₂O

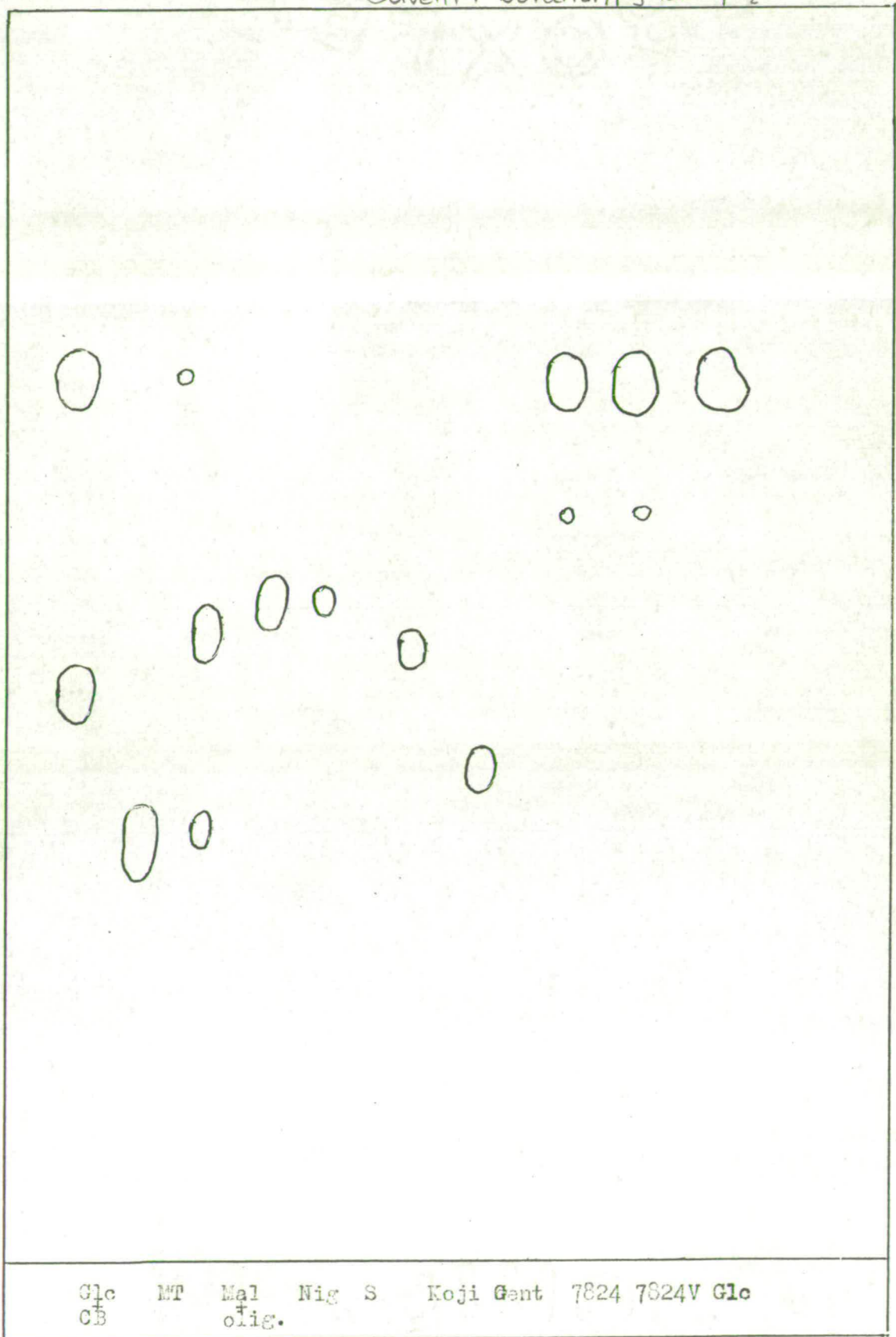


Figure 30: Diagrammatic sketch of a chromatogram of the partial acid-hydrolysis of low molecular weight exopolysaccharide from strains 7824 and 7824V.

CB - Cellobiose, Nig - Nigerose, S - Sophorose, Koji - Kojibiose

THE CELL ENVELOPE PROTEINS OF *ENTEROBACTER AEROGENES*

The profiles of whole cell envelopes conveyed the similarities between strains of the same serotype and distinguished between strains of different serotypes.

The large number of polypeptides visualised in stained SDS-polyacrylamide gels of *Enterobacter aerogenes* whole cell envelopes, as seen in Plate 8, indicated that detecting changes in the protein profile of cells either by direct observation or densitometric scanning of gels would be hindered by the close proximity of bands leading to overlapping.

Several methods were utilised in the attempt to examine changes in the protein profiles of wild-type strains and their mutants. These methods and the results obtained are examined in section .

THE SARKOSYL METHOD FOR PREPARATION OF OUTER MEMBRANE

Filip *et al* (1973) proposed a method for chemical separation of outer and inner membranes using the ionic detergent Sarkosyl (sodium lauryl sarcosinate). They reported that Mg^{2+} ions protected the inner membrane from solubilisation and that sarkosyl separation should be used in conjunction with the EDTA-lysozyme sphaeroplast method of Osborn *et al* (1972) thereby eliminating contaminating Mg^{2+} ions.

Cells grown at 10°, 20°, 30° and 37°C were sphaeroplasted and outer and inner membranes prepared by this method. Membranes were solubilised at 100°C unless otherwise indicated.

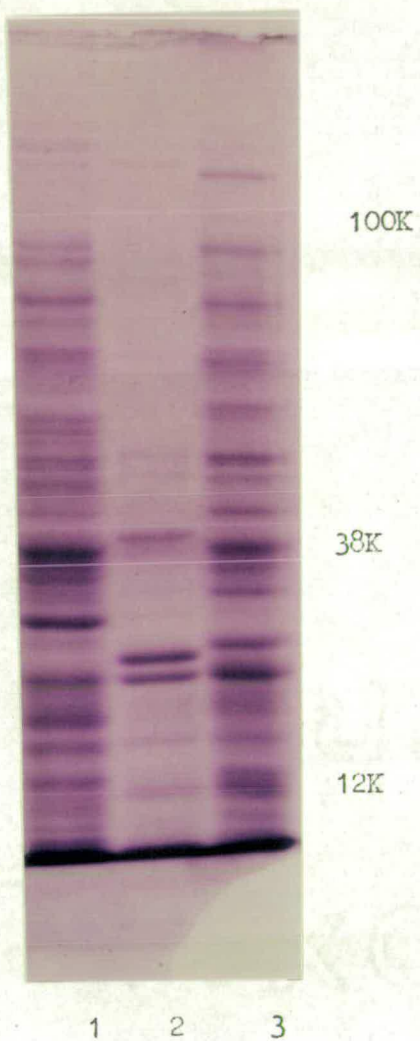
In order to obtain a thorough review of the changes noted in protein content of the outer membrane, the results for each family of strains at a given temperature and, subsequently, a comparison and contrast of results within all the families will be related. Figure 31 shows a standard curve used in the determination of protein molecular weights.

SEROTYPE 1, A1 FAMILY

10°C

At 10°C the differences in polypeptide composition of strains A1X and A1SI as detected by SDS-PAGE and densitometry were slight and concentrated in the region 40-60K. (Figure 32)

Compared to A1X, A1SI had greatly reduced levels of 41K polypeptide. The latter strain possessed a 58K polypeptide not present in A1X,



LANE: 1 Strain A1X
2 Strain 54C
3 Strain 7824

Plate **8** Cell envelope protein of *Enterobacter aerogenes*.

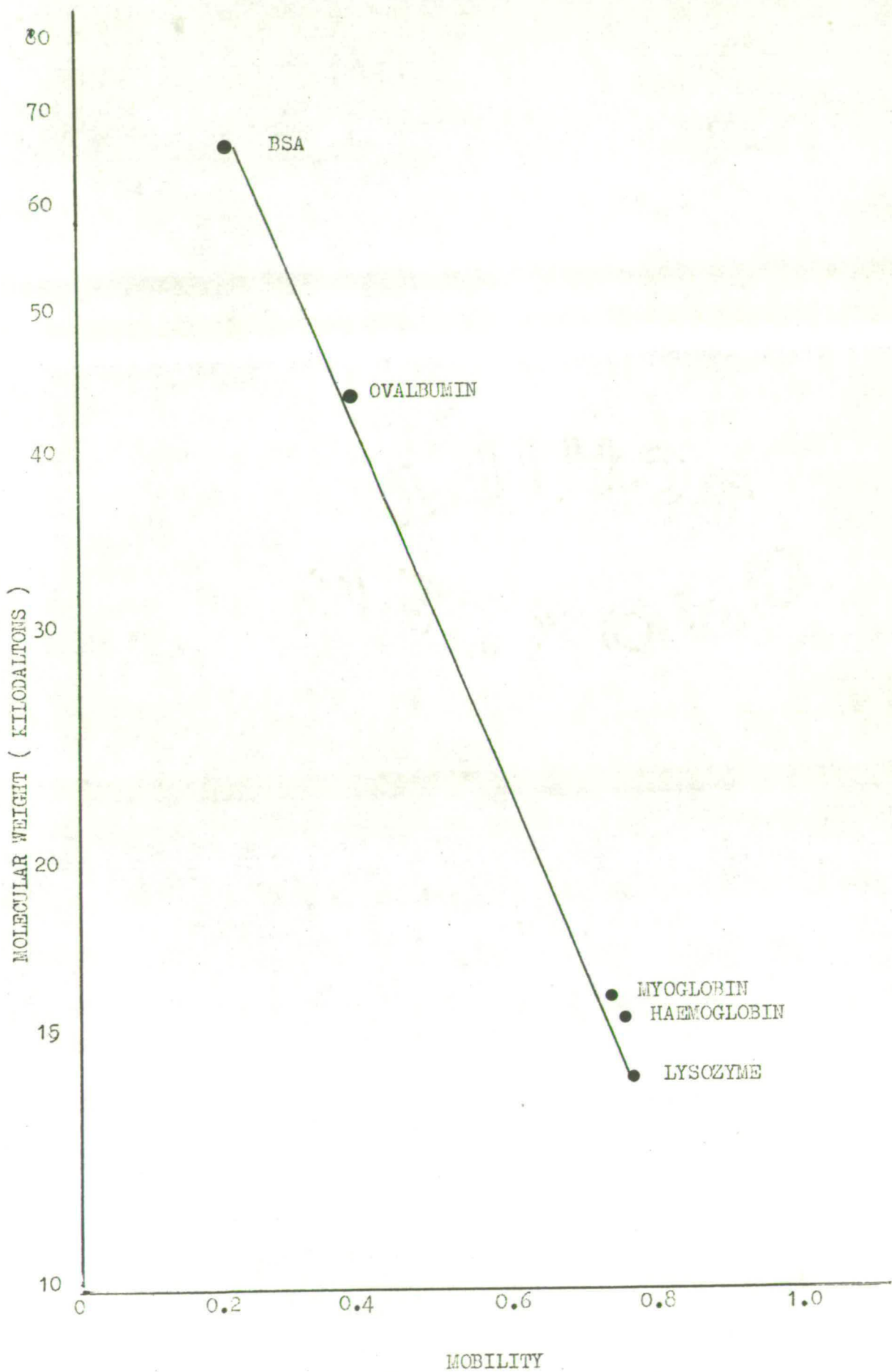


Figure 31 Calibration curve for the determination of protein molecular weights on SDS-PAGE.

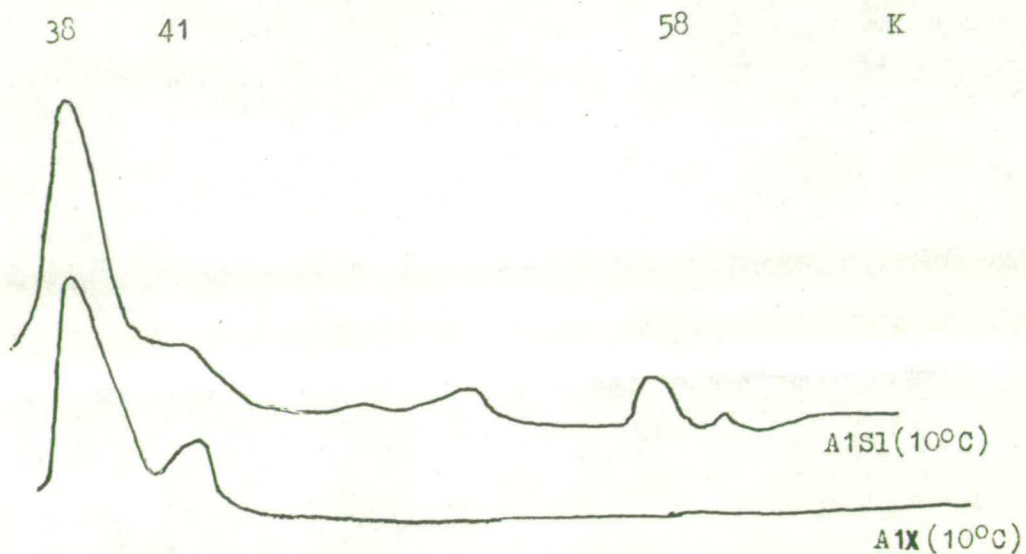


Figure 32 Effect of growth temperature on outer membrane proteins.

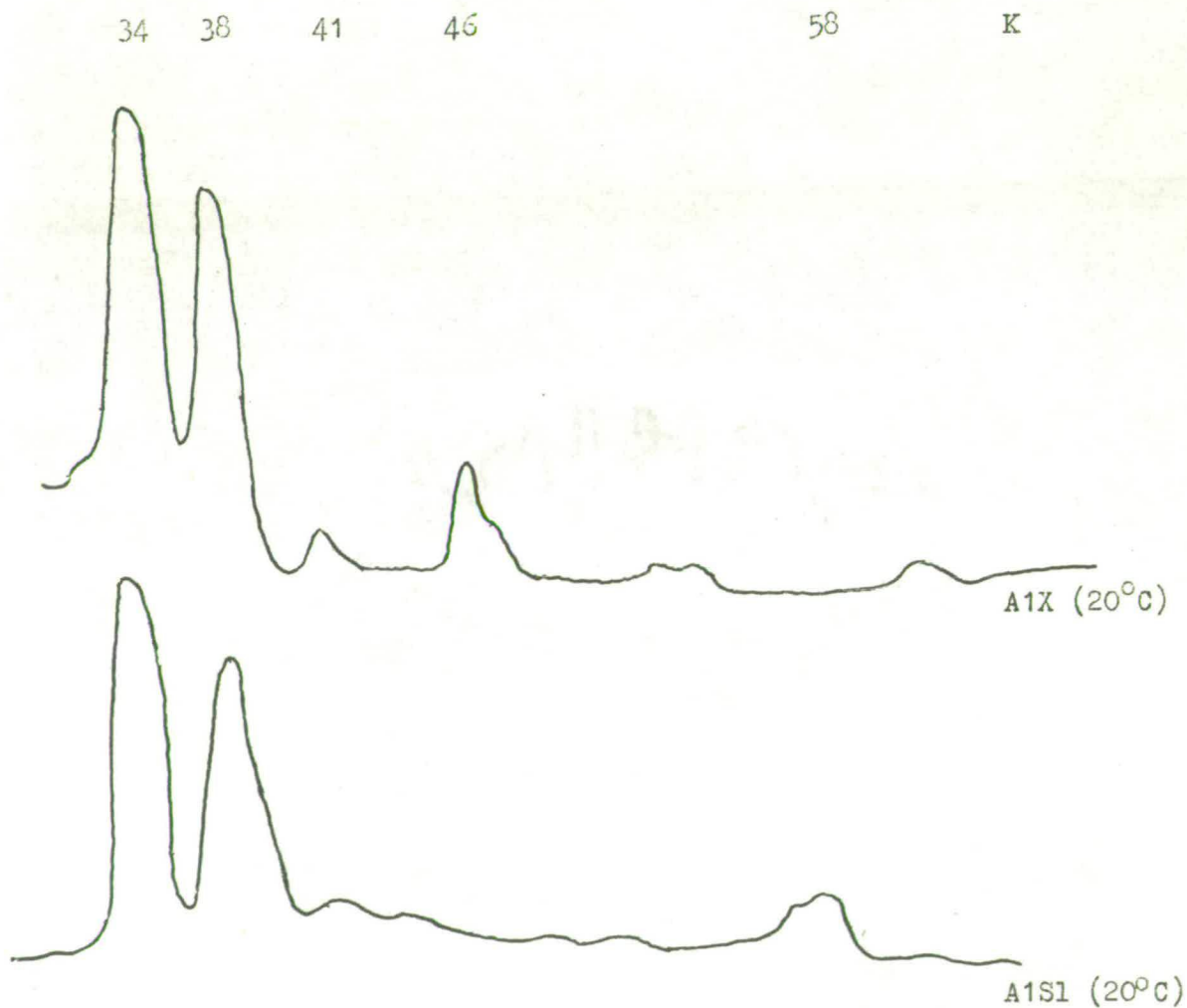


Figure 33 Effect of growth temperature on outer membrane proteins

20°C

At 20°C the normal densitometry profile of gels of strain A1 indicated more 38K than 34K, 30K and 46K bands: 38K > 34K > 30K and 46.5K.

By comparison with A1, A1X bands at 30K, 41K and 46K were of lower intensity while minor bands below 30K or above 46K were unaffected and expressed in their normal properties with respect to 38K and 34K. A1SI differed from both A1 and A1X in lacking the 46K band and possessing a band which migrated more slowly at 54K. (Figure 33).

The most severely affected mutant of the A1 family was A1.92. This strain apparently lacked virtually all of the outer membrane protein and possessed only bands of molecular weight 40K, 22.5K and 14K. As can be seen from Plate 9, none of these bands migrated at typical positions for the family. However, they could be assigned probable equivalents: 41K, 25K and 15K, respectively.

30°C

A1, A1X and A1SI possessed 34K, 38K and 41K in the same proportions. Additionally A1X possessed equal proportions of 50K and 58K bands. At this growth temperature strains A1SI, A1SIBR, A1SINR and A1SI His all possessed high levels of a minor band at 58K. A1SI had a minor band at 50K while A1SI His possessed two bands 50K and 52K. A1SINR and A1SIBR did not have equivalent polypeptides. (See Plate 10). Strain A1.92 displayed the same profile as for 20°C grown cells. Strain A1 (O) outer membrane showed a 50% reduction in the quantity of 34K protein present.

Plate 11 shows the configuration of polypeptides (in SDS-PAGE) of A1 and A1SI outer membranes from cells grown at 30°C.

37°C

The strains A1, A1X, A1SI (including BR, NR, His) or A1(O) showed gel patterns resembling those obtained at 30°C except that 41K was greatly reduced. Exceptionally, strain A1.92 recovered a "normal" polypeptide profile (Figure 34).

SEROTYPE 54, 54C FAMILY

As with the A1 Family the major proteins of the outer membrane of the 54C Family of strains were 34K and 38K. Of the 54C mutants examined (in this section) all were encapsulated: one strain was the wild-type, two others were phage resistant.

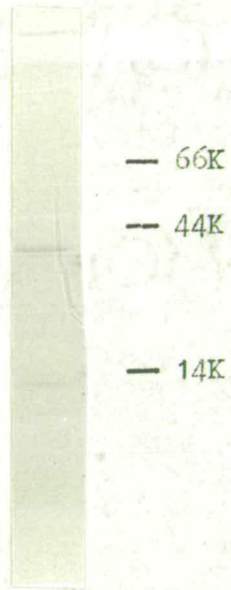
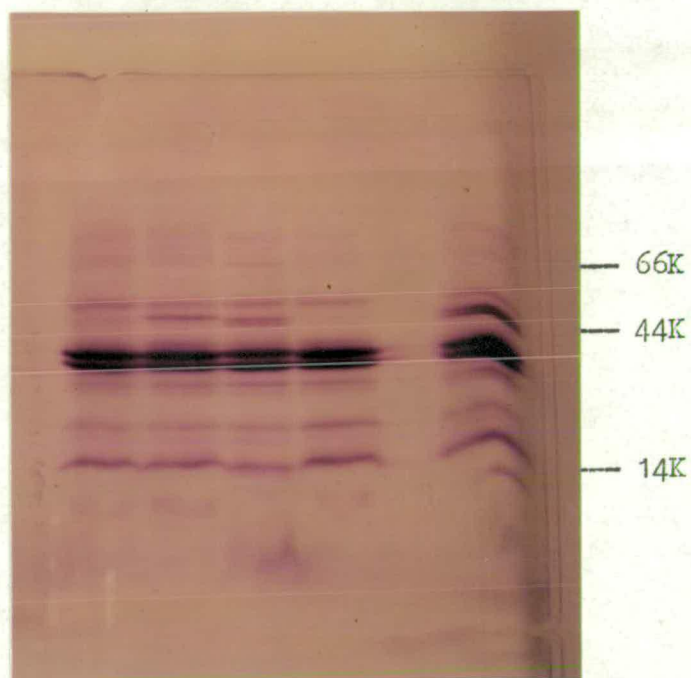


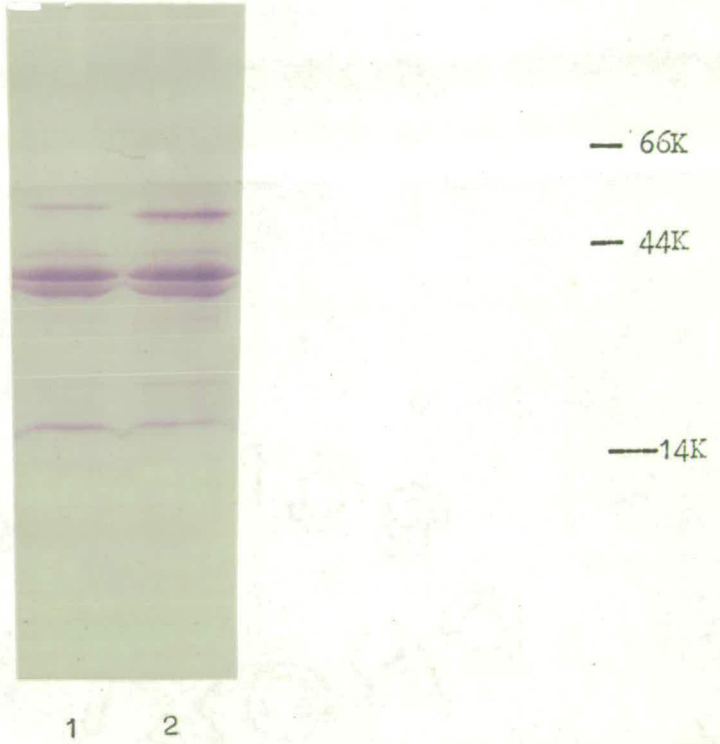
Plate 9 Outer membrane proteins of strain A1.92 grown at 20°C.



1 2 3 4

LANE 1: A1S1 NR
2: A1S1
3: A1S1 BR
4: A1S1 His⁻

Plate 10 : Outer membrane Proteins of Slime-forming mutants.



LANE 1: A1S1 (Slimeformer)
2: A1 (Capsulate)

Plate11 ; Capsulate and slime-forming strains of A1.

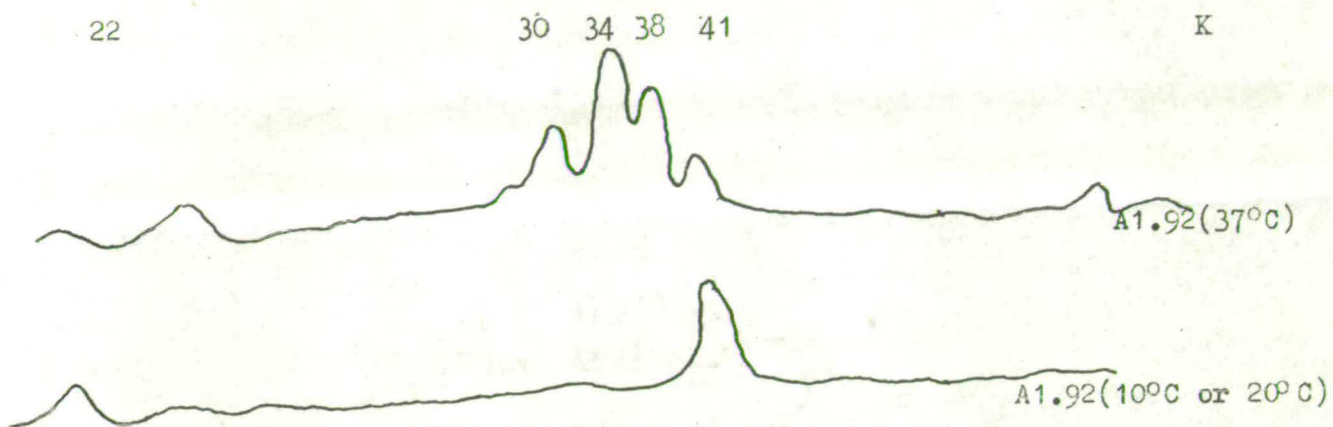


Figure 34: Strain A1.92 showed recovery of "normal" outer membrane profile at 37°C.

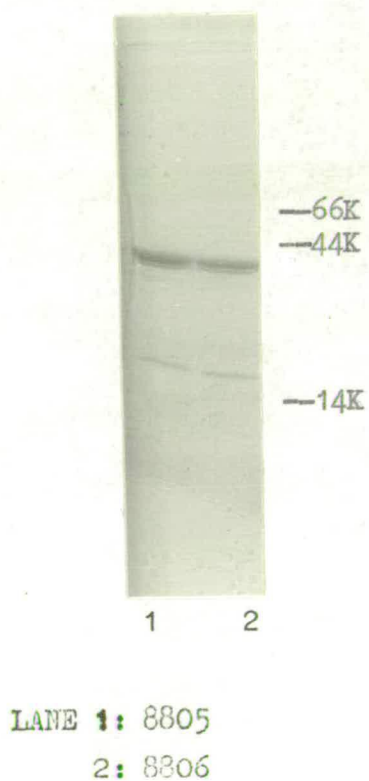


Plate 12 Comparison of strains 8805 and 8806 (capsulate and slime-former) protein profiles from growth at 10°C.

20°C and 30°C

54C and 54CF37 possessed, in addition to the major polypeptides, significant bands at 51K, 45K and 41K, 54CF34 strain had comparable proteins at 54K, 48K and 41K. At 30°C 54C produced a 19K protein not detected in the other strains.

37°C

54CF37 overproduced two lower molecular weight bands 26K and 19K and reduced expression of 41K.

SEROTYPE 54, N.C.I.B. 8805—8806—9261

10°C

In this family of strains it was strikingly obvious only 2% of the total outer membrane protein was present in polypeptides over 38K molecular weight at 10°C. In densitometry scans a single high molecular weight peak appeared at 94K. (Plate 12)

30°C

Strains 8806 and 9261 shared similar gel scan profiles. The latter featured two extra bands of 21.5K and 95K. In contrast to findings at 10°C 9261 and 8806 were distinguishable from 8805; they possessed a two-peaked band of 54-58K. Strain 8805 possessed only traces (up to 10% of 8806 value) of this band, where present (See Figure 35).

All strains exhibited bands at 46K and 64K. 37°C-solubilised membrane samples from strains 9261 and 8805 gave identical SDS-PAGE patterns (See Plate 13). Strain 8806 lacked a band of approximately 12K.

Neither A1SI or 7824V portrayed a similar deficiency when membrane samples were solubilised at 37°C (See section on solubilisation of membranes at 37°C below).

SEROTYPE 30, 7824 FAMILY

20°C

At this temperature the profiles of 7824 and 7824V were virtually identical: 30K, 34K, 38K, 40K were major bands. In 7824V two low molecular weight bands 11K and 15K were more emphasised: containing twice the proportion of protein of the same bands in 7824.

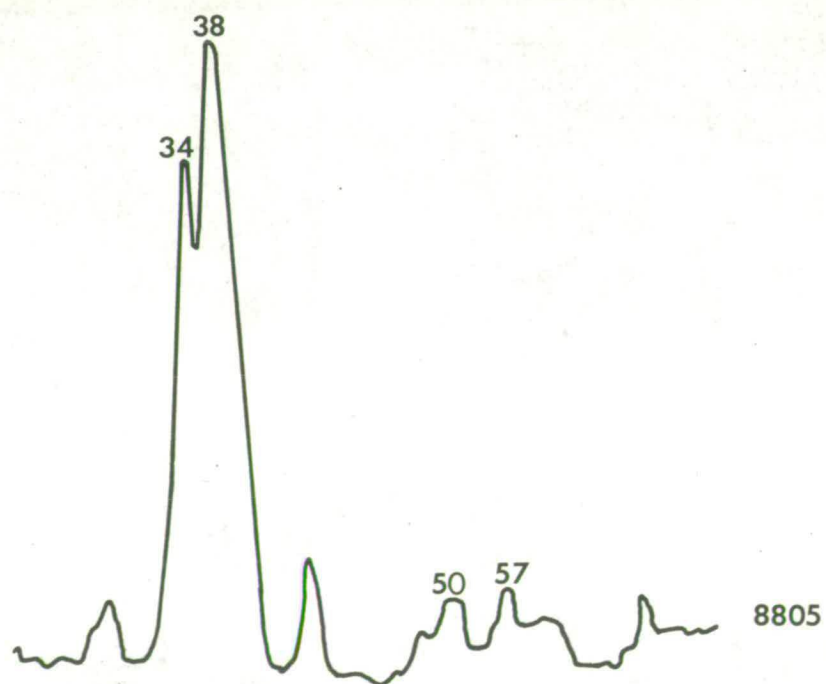
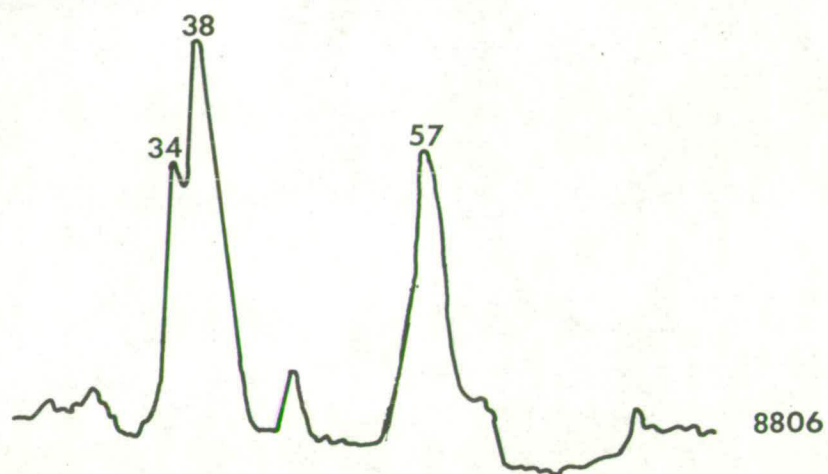
7824(O) overproduced 30K and 95K.

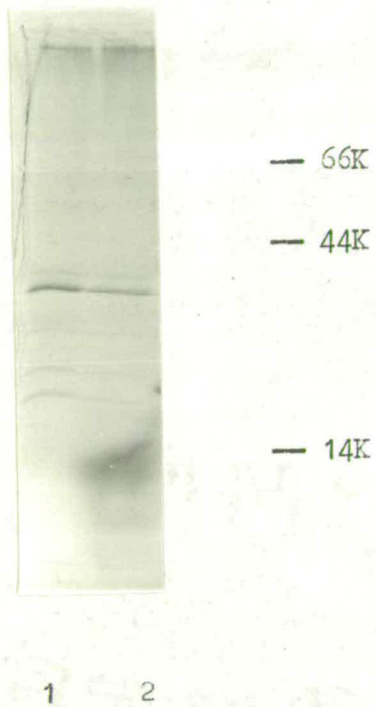
30°C

Strain 7824V differed from 7824 in possessing a large band of approximately 58K

Figure 35:

THE EFFECT OF MUTATION TO
SLIME - FORMING IN TYPE 54





LANE 1: 8805
2: 9261

Plate **13** 37°C-solubilised samples of outer membrane protein from capsulate and non-mucoid strains (8805, 9261).

(See Figure 36). Again 7824(O) overproduced a band of 30K. 7824S produced more 47K protein while 7824f lacked a 22.5K polypeptide.

37°C

All strains were identical with 30°C material. 7824(O) 41K band was reduced approximately 50%.

SEROTYPE 8, A4 FAMILY

Two strains were used: A4 and A4CR, the latter being a crenated mutant (See "Introduction"). Both were capsulate. Membranes were prepared from 20° and 37°C-grown cells and analysed.

A4CR at 20° or 37° gave similar profiles when analysed by SDS-PAGE and densitometry and these were, apart from possession of a 78K polypeptide band, identical to A1 grown at 30°C. Common to all membranes was the presence of 34K and 38K major bands and a large band at approximately 50K. In the case of A4CR this comprised two closely opposed bands of 48K and 49K forming a double peaked band. (Figure 37).

LEVALLORPHAN RESISTANCE AND OUTER MEMBRANE PROTEINS

A1XLR mutant expressed pleiotrophic alterations to the outer membrane dependent on growth temperature.

The most drastic alteration to the polypeptide constituents was detected at 37°C where the 38K polypeptide was absent. At 20°C, 41K and 48K polypeptides were reduced while a new band appeared at 21.5K.

54CLR mutant at 20°C lacked a 23.5K polypeptide and had a "substitute" 21.5K polypeptide of equal intensity. This result concurred with that above for the A1XLR mutant.

The levallorphan resistant mutant of 7824 did not fall into a similar pattern. This strain did not show any significant alteration to major or minor polypeptides under 70K. At all growth temperatures (20°, 30°, 37°C) 70K, 74K and 86K bands were produced.

NOVOBIOCIN RESISTANCE AND OUTER MEMBRANE PROTEINS

A1XNR and 7824NR presented a normal profile at all growth temperatures. However the level of 48K and 58K polypeptides in the membrane decreased compared to the wild-type. 54CNR produced a new band of 20K at 20°, 30° and 37°C.

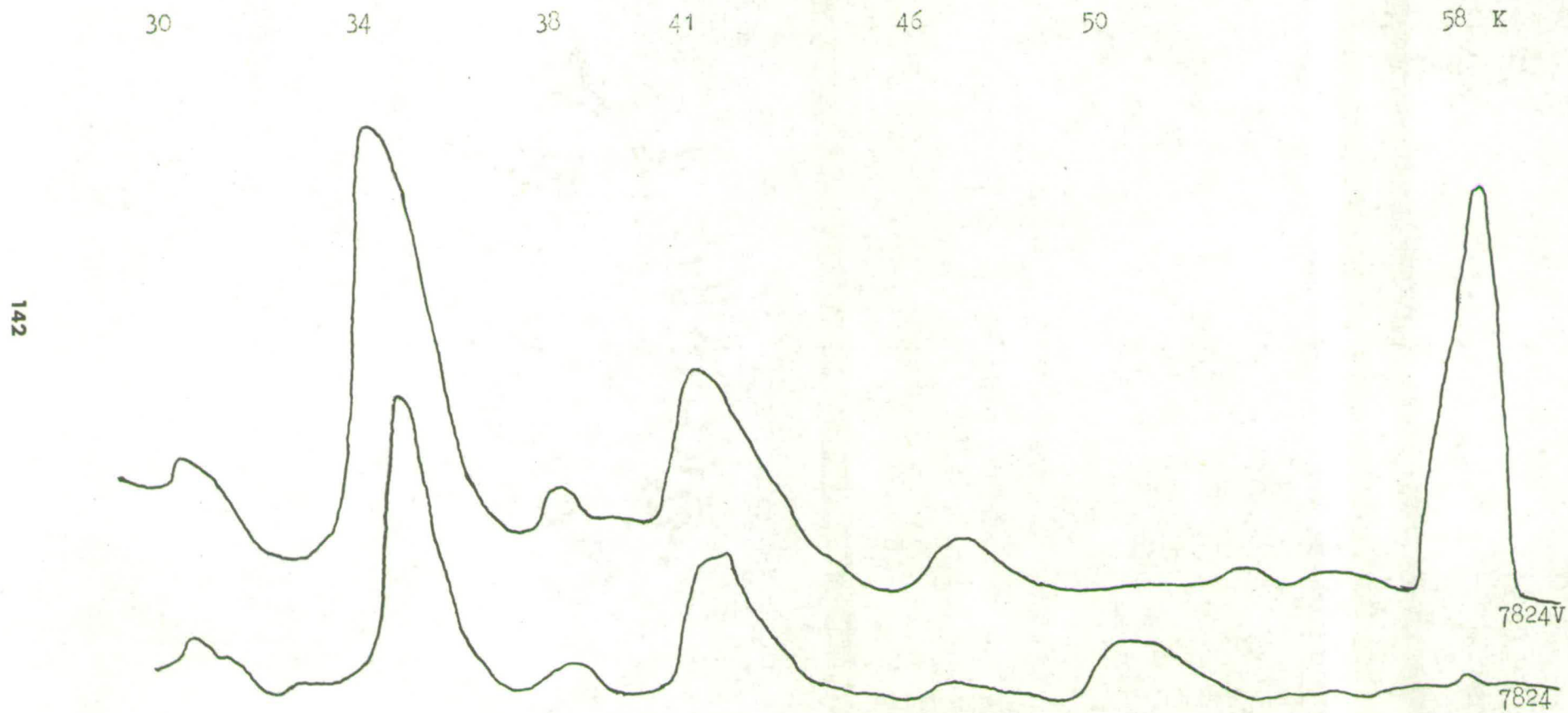


Figure 36 7824 and 7824V (30°C grown) outer membrane protein profile (30-58K region).

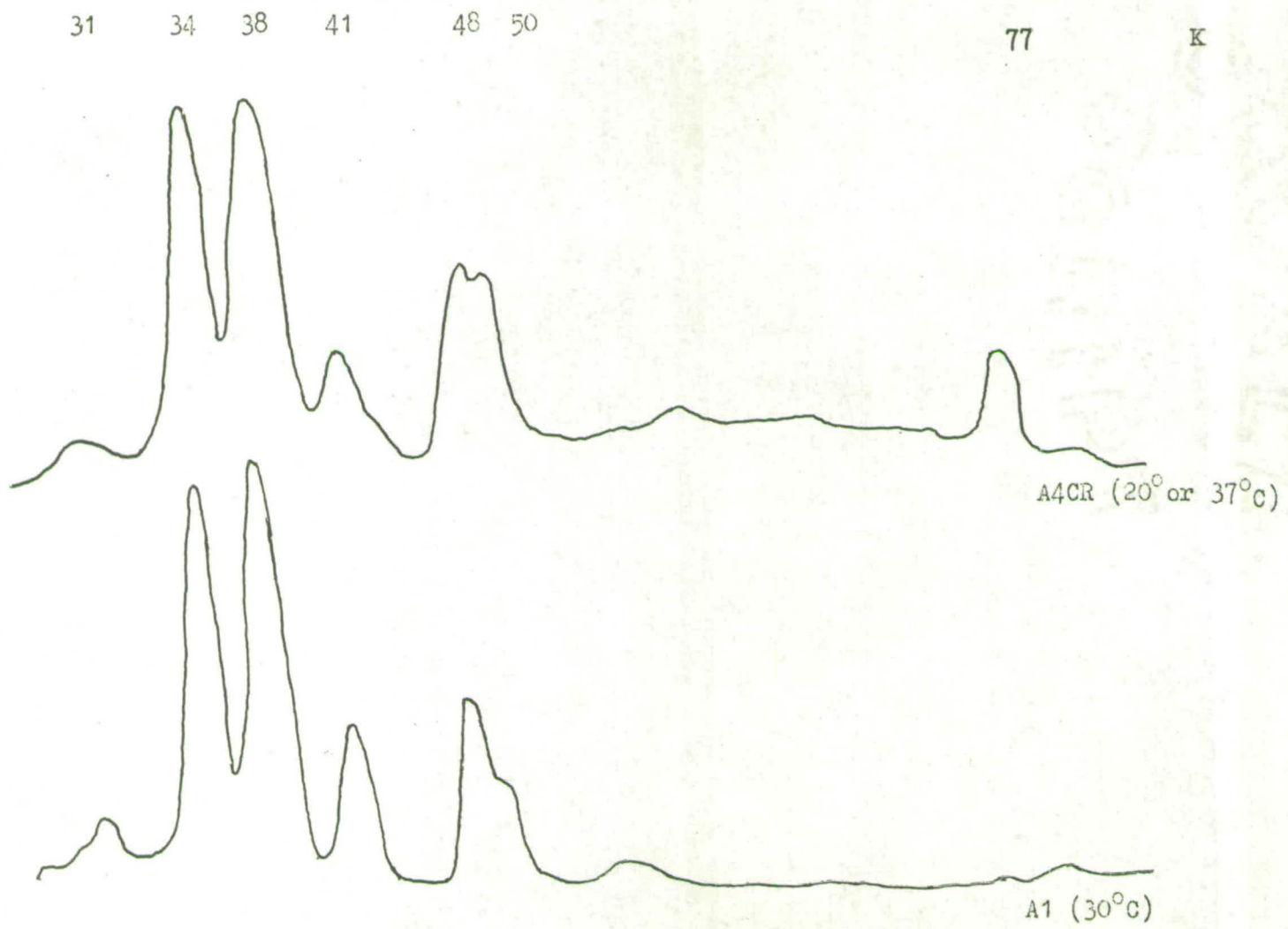


Figure 37 Similarity in outer membrane profiles of Serotype 8 and 1 (A4 and A1) capsulate strains.

TETRACAINE RESISTANCE AND OUTER MEMBRANE PROTEINS

AIXTR strain expressed a variety of changes in outer membrane protein dependent on growth temperature. At 37°C, 48K and 58K polypeptides were lacking. They were present although reduced in proportion at 20° and 30° compared to A1X. At 20°C there was a severe reduction in the 30K polypeptide.

54CTR produced a new band at 63K and increased the concentration of 20K when grown at 30° and 37°C. at 20°C a 23.5K band was overproduced. In 7824TR a single band was affected: 40K. At all temperatures 40K was depleted (when compared to the wildtype). The effect was temperature dependent and a sequential (20°, 30° and 37°C) reduction in the band was found. At 37°C it was absent. At all temperatures, 25K polypeptide was absent.

POLYMYXIN B RESISTANCE AND OUTER MEMBRANE PROTEINS

Serotype 1, A1X PB600

This strain presented a phenotypically normal complement of polypeptides on SDS-PAGE analysis of outer membrane 30°C and 37°C grown cells. At 20°C the profile of the organism was radically altered: 38K polypeptide was reduced by 50% (with respect to 34K) and a new polypeptide of 20K was incorporated.

(See Plate 14).

Serotype 54C PB3000b

The 45K polypeptide of the outer membrane of these strains (at 20°C) was almost absent and was discerned as a very minor peak by gel densitometry. 28K and 30K polypeptides were slightly increased.

Serotype 30 7824 PB300

At 30°C this strain overproduced on 18K minor polypeptide. On lowering the growth temperature to 20°C the overproduction of 18K increased (by 200% of the original value) and a second band of 30K was overproduced by a similar margin.

EFFECT OF NUTRIENT LIMITATION ON OUTER MEMBRANE PROTEINS

Strains A1X, 54C and 7824 were grown under Mg^{2+} and PO_4^{3-} limitation and in non-limited media.

The outer membrane proteins showed several variations and these were reflected in all of the strains for the limitation concerned. Minor changes which appeared to be specific to a strain were also noted.

Although the strains have variable amounts of, for example, the major bands, (See respective results A1X, 54C, 7824 at 30°C) the alteration in band density on

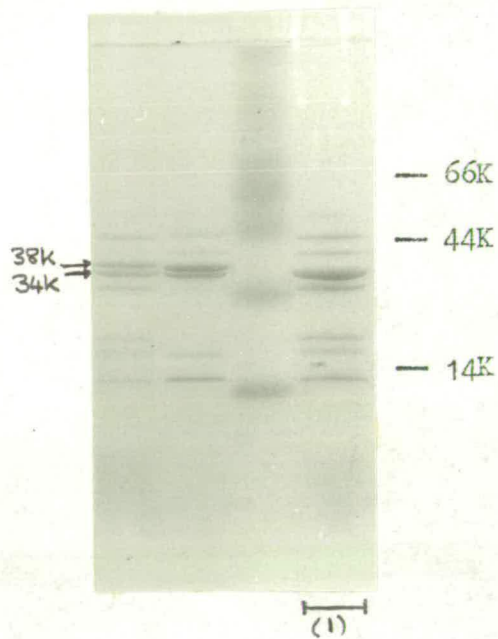


Plate **14** : Effect of Polymyxin resistance on a mutant of A1X.

(1); The gel slice shows strain A1XPB600 grown at 20°C:
38K polypeptide is absent.

scanning could be presented as a percentage increase or decrease over the strain in non-limiting media.

In non-limiting media the strains possessed normal levels of these polypeptides present when grown in YE Broth. However, all strains grown in non-limited or Mg^{2+} , SO_4^{2-} and PO_4^{3-} limited media possessed high molecular weight polypeptides (74K, 78K, 81K, 86K, 93K) not normally found in YE Broth grown cells. Table **23** relates the alterations to the protein profile of the outer membrane.

Strain A1X grown in K^+ -limited media did not produce polypeptides at 74K or 81K and totally lacked the 48-50K and 56-58K double peaks normally found in densitometry traces of outer membrane gels (Figure **38**).

TABLE: 23

*Alterations in the abundance of Outer Membrane
Proteins under nutrient limiting conditions*

Strain	Mg ²⁺	PO ₄ ³⁻	SO ₄ ²⁻
AIX	38K + 34K (30%)	38K + 34K normal	38K + 34K (20%)
	16K (30%)	16K (30%)	16K (170%)
			58K (400%)
	12K absent	12K absent	12K absent
54C	38K + 34K normal	38K + 34K normal	38K + 34K normal
	58K (50%)		
	16K (50%)	16K (40%)	
7824	38K + 34K (20%)	38K + 34K normal	38K + 34K (10%)
			30K (50%)
			16K produced
			41K (50%)

(K = 1000D)

(%) percentage change in presence of polypeptide compared to growth in non-limiting media.

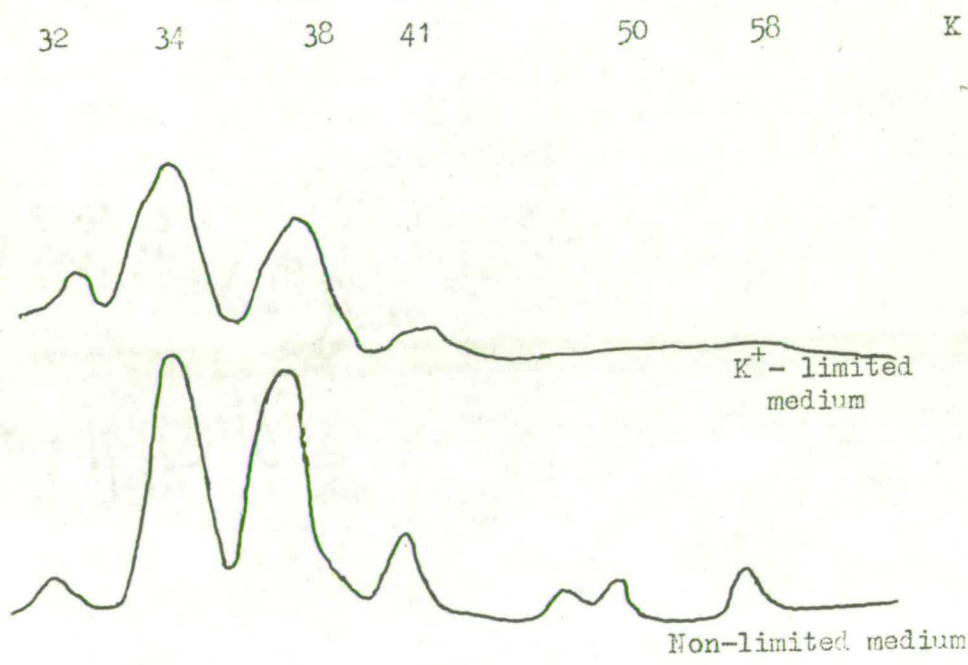


Figure 38 Effect of K^+ limitation on higher molecular weight proteins of the outer membrane of strain A1X.

EFFECT OF SUPPLEMENTS TO Y.E. BROTH ON OUTER MEMBRANE PROTEINS IN STRAIN A1X (and other Strains where indicated)

0.01%(w/v) Sodium Dodecyl Sulphate

A1X grown in the presence of SDS lacked a 41K polypeptide as evidenced by the clear gap between the major polypeptide 38K and 46K (Figure**39**).

0.01%(w/v) Sodium Lauryl Sarcosinate

In this broth cells lacked 41K polypeptide, and apparently, all bands in excess of 60K (Figure**39**)

10mM Phenylethanol (PeA)

Inclusion of PeA in the medium resulted in the overproduction of 32K polypeptide such that it was present in quantities equivalent to 34K. (Figure**39**)

1M Ethanol (EtOH)

Ethanol in high concentration produced cells lacking 41K and with increased levels of a 29K polypeptide. No bands were present in the region 30-33K unlike normal A1X profiles (Plate **15** , Figure**39**).

500mM Sucrose

High concentrations of sucrose reduced the quantity of 38K polypeptide without alterations in the relative amounts of the remaining bands. In strain 7824, which normally possesses low levels of 38K polypeptide, the polypeptide was absent (Figure**40**).

300mM NaCl

41K polypeptide was absent from all samples and the 31K polypeptide overproduced (Figure**39**).

1.0mM CuSO₄

The presence of the metabolically toxic heavy metal, copper, caused a total re-organisation of the major bands. 29K polypeptide was more plentiful than either 34K or 38K polypeptides taken singly. 41K was present only in low quantity (Figure**41**).

2.5mM CuSO₄

Higher concentrations of copper exaggerated the phenomenon seen above: 29K polypeptide became the dominant species, this time equivalent in concentration to both 34K and 38K combined. 41K was absent. Two bands at high molecular weight, approximately 88K and 95K were observed (Plate **15** , Figure **41**).

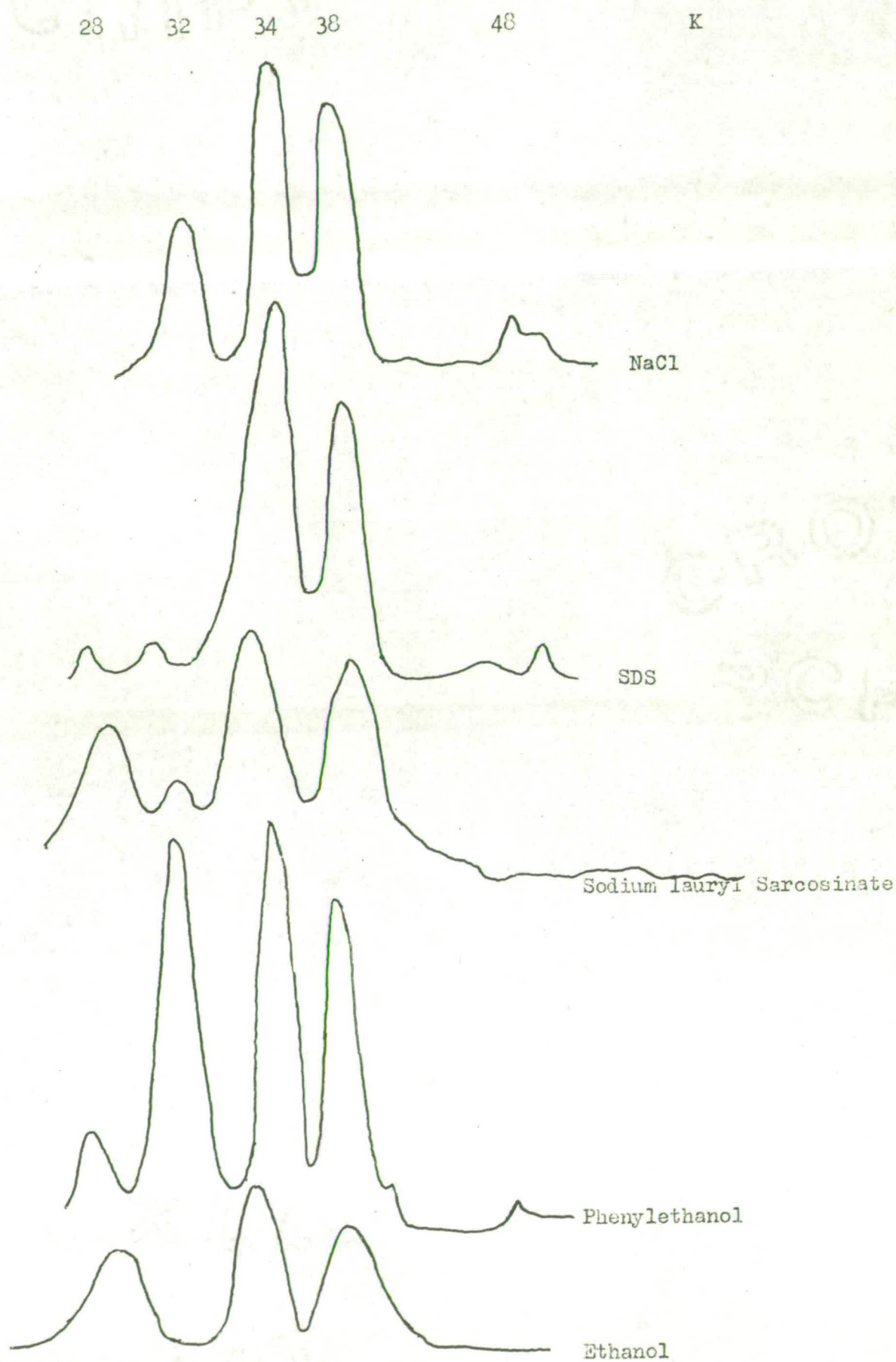


Figure 39 Alterations to the outer membrane profile in supplemented Y.E. Broth.

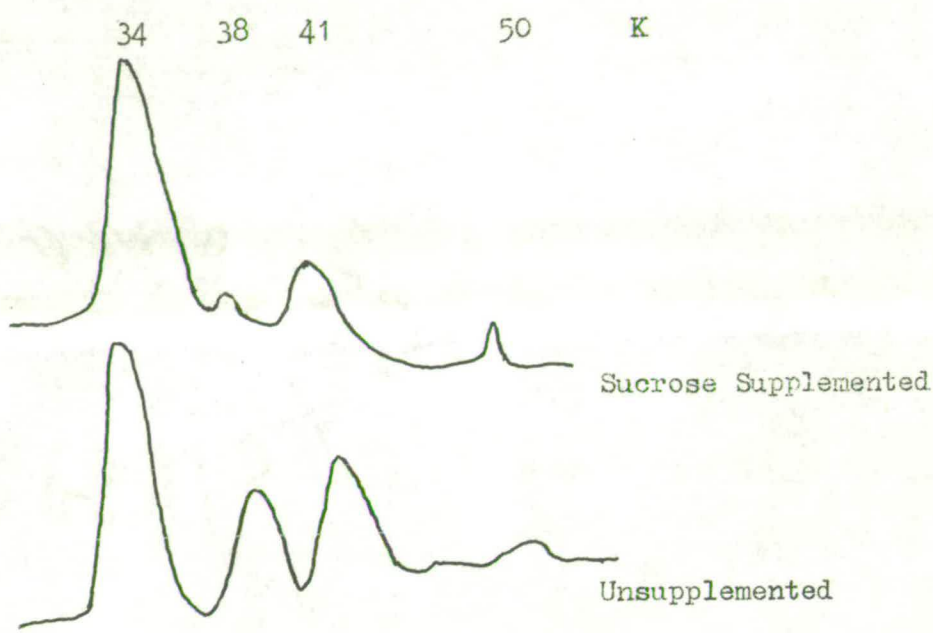


Figure 40 Effect of a Sucrose supplement on outer membrane proteins of 7824.

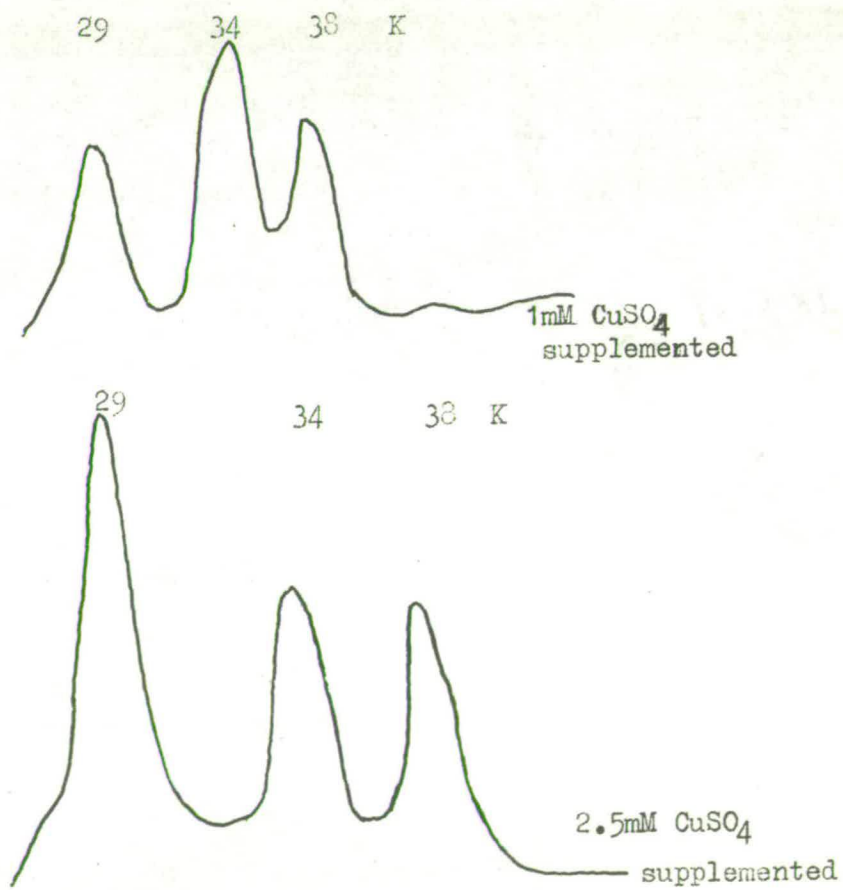
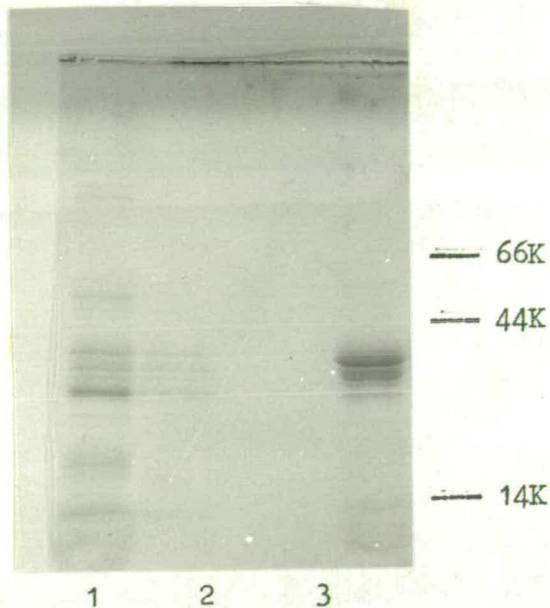
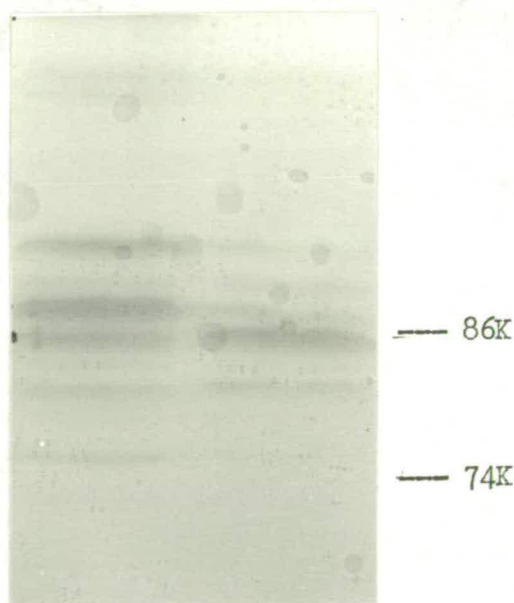


Figure 41 Effect of Cu^{2+} supplements (to Y.E. Broth) on the outer membrane protein profile of strain A1X.



Lane 1: 2.5mM CuSO_4 ; lane 2: 1M EtOH; lane 3: Normal

PLATE 15: Alterations to the outer membrane protein profile in cells grown in supplemented YE medium



Lane 1: Fe^{3+} Limited ; Lane 2: Fe^{3+} limited with citrate

Plate 16 Induced iron-uptake proteins of *Enterobacter aerogenes*.

Penicillin (100 μ /ml broth)

31K and 86K polypeptides were slightly increased .

0.5%(w/v) EDTA (disodium salt)

The presence of EDTA induced the production of high molecular polypeptides. These bands formed a large percentage of the total outer membrane protein (equivalent to the major 34K and 38K bands combined). Strain 7824 grown under the same conditions produced the same highmolecularweight polypeptides (See Figure**42**).

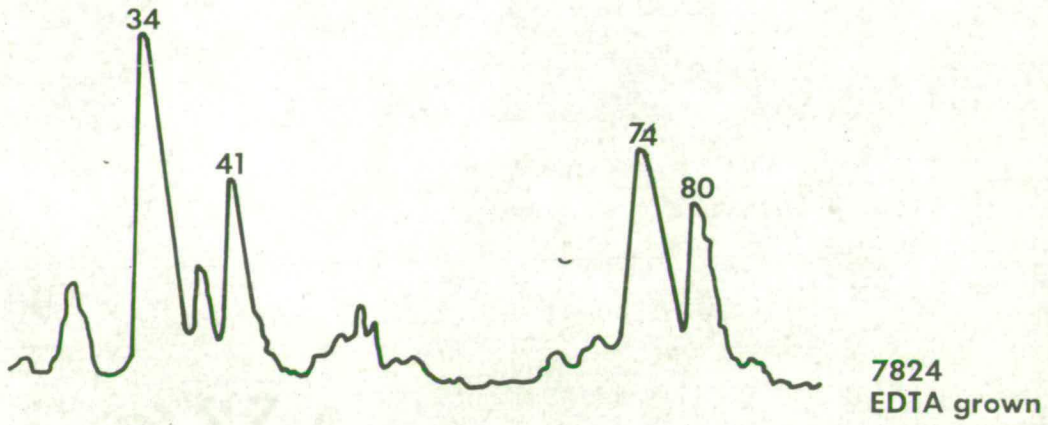
Fe³⁺-UPTAKE PROTEINS

By growing strain A1X under iron limiting conditions it was possible to induce five main bands at 74K, 79K, 83.5K, 86K and 93.5K. To differentiate between possible ferrichrome or enterochelin type uptake proteins and citrate-Fe³⁺ receptor protein A1X was grown under iron limitation with a citrate supplement to induce the citrate dependent uptake protein. This caused massive overproduction of the 86K band compared to the outer membrane of iron limited cells alone. 79K and 93K proteins were also produced, but in quantities similar to those in iron limitation alone. These limitations can be compared in Plate **16** of a gel of the 70-95K region and in Figure**43** of the same region scanned in a densitometer.

The existence of proteins induced by iron limitation is well documented for *E. coli* (See "Introduction").

Figure 42

**THE EFFECT OF EDTA ON OUTER MEMBRANE
PROTEINS IN STRAIN 7824**



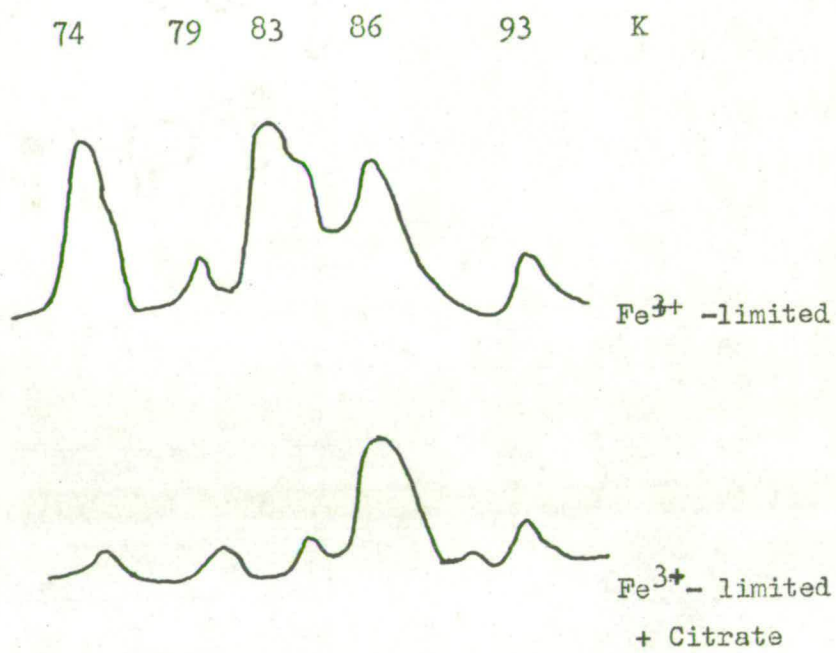


Figure 43 Induction of iron-uptake proteins.

EFFECT OF THE AGE OF A CULTURE ON OUTER MEMBRANE PROTEINS

Strains A1 and A1SI grown in batch culture were sampled at 8, 24 and 48h and outer membranes prepared by sphaeroplasting and sarkosyl extraction.

In a comparison of the A1 strain preparations (also of A1SI) it was evident that there were subtle alterations in the quantities of certain polypeptides. Thus A1 24h had reduced 16K and increased 19K polypeptide levels compared to A1 8h, and A1 48h had reduced 19K and increased 48K polypeptides and a new band at 22K compared to A1 8h.

A1SI 48h possessed identical amounts of 58K compared to A1SI 8h but in contrast produced virtually no protein in the 48-50K region while in A1SI 8h 50 and 58K polypeptides were present in equivalent amounts.

Examination of densitometric scans of A1 8h and A1SI 8h and A1 48h and A1SI 48h gels indicated that in the 8h samples A1 lacked the 58K polypeptide found in A1SI but that both possessed a 48-50K band. A1SI had an extra polypeptide in the 60-70K region.

At 48h strain A1 produced more 22K polypeptide than strain A1SI. Both strains produced 58K in equivalent amounts but A1SI produced only trace amounts of 48K protein. Identical quantities of a 78K polypeptide were present in both A1 and A1SI.

The most obvious effect of age of the culture on the polypeptides present was the change in the ratio of the quantities of 38K and 34K protein. At 3-4h 34K was in considerable excess over 38K. By 8h and entry to stationary phase the ratio was approximately 1:1 and at 24h and 48h (both a substantial period into stationary phase) the ratio favoured a slight excess of 38K over 34K. The ratios were calculated by weighing peaks from densitometry scans (Figure 44).



Figure 44: Changing ratio of 34 : 38K protein with the age of the culture (strain A1).

THE EFFECT OF DIFFERENT GEL SAMPLE-SOLUBILISING TEMPERATURES ON THE REPRESENTATION OF OUTER MEMBRANE PROTEINS IN SDS-PAGE

Major Proteins

In all membrane preparations the temperature used to solubilise the outer membrane sample in gel sample buffer determined which proteins would migrate in the gel and the speed at which they migrated.

Thus samples of A1X heated for 5 or 10min at 100°C and electrophoresed produced bands at 38K and 34K. If the sample were heated at 37°C for 30min and gel electrophoresed (or ultracentrifuged to remove non-solubilised debris before electrophoresing) a single "major" band appeared at 28K (See Plate **17**). By excising this band from the gel, macerating it in a small volume of sample buffer and heating to 100°C for 10min and subjecting the resolubilised sample to SDS-PAGE again the single band now migrated at 34K.

The 38K band, absent from 37°C solubilised samples, was solubilised by raising the temperature to approximately 70°C for 10min.

Minor Proteins

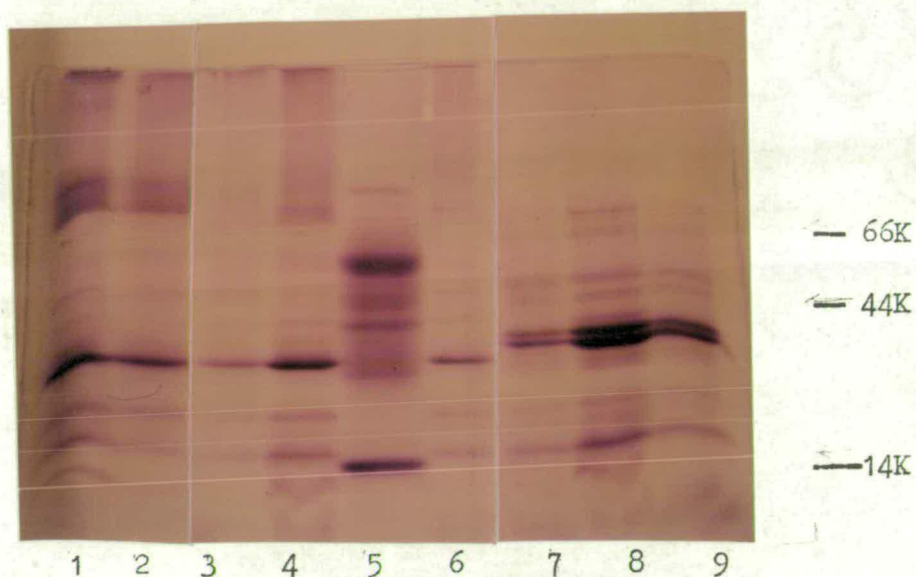
The total number of bands stained on a gel in a 37°C solubilised preparation was less than that found in a 100°C solubilised gel (apart from the absence of 38K from the former). Bands below 32K were not affected but those over 40K were affected both in number and intensity as shown in Plate **17**.

All 37°C solubilised membrane samples (unless centrifuged) gave rise to a number of high molecular weight bands when electrophoresed. Possibly these bands were composed of oligomers of lower molecular weight polypeptides since these bands were absent when the same sample was 100°C-solubilised, a more drastic treatment capable of reducing the multimer to its monomeric form.

37°C SOLUBILISED OUTER MEMBRANES (SARKOSYL)

Due to the appearance of fewer bands (as explained above) at least one of which migrated differently, the gel samples solubilised at 37°C yielded little information on SDS-polyacrylamide gels.

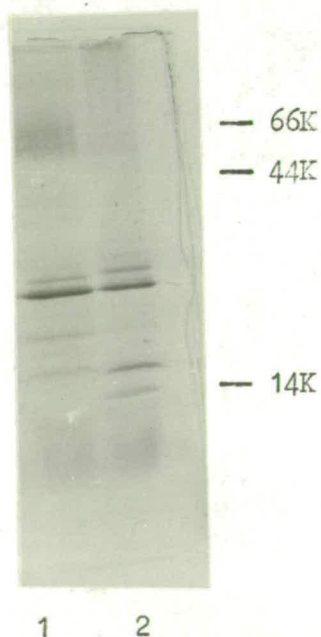
All samples contained a polypeptide of 11K which was not always detected in 100°C solubilised samples. 54C mutant 54CF37 lacked 11K while both 54CPB3000 mutants (a and b) overproduced 17K (See Plate **18**). The 11K might have represented free form murein lipoprotein which has been difficult to visualise in gels of numerous Gram-negative cells. Addition of MgCl₂ to enhance the murein lipoprotein in the gel was tried but had no effect.



Lanes : 1-6 : A1X, A1Sl, A1XNR, A1XBR, Standards, A1XTR, (37°C solubilised)

Lanes : 7-9 : A1X, A1Sl, A1XNR, (100°C solubilised)

Plate 17 Effect of Gel Sample solubilising temperature on the representation of polypeptides on SDS-PAGE.



Lane 1: 54CF37 ; Lane 2: 54CPB3000b

Plate 18 Alterations to the normal polypeptides profile of 37°C-solubilised samples.

PRONASE TREATMENT OF SPHAEROPLASTS

From the solubility properties and heat modifiable nature of the 34K polypeptide in the preceding results it was considered possible that this protein of *Enterobacter aerogenes* was the equivalent of 35K (OmpA) of *E. coli*. Studies by Hofstra and Dankert (1980) have shown an antigenic relationship for 34K protein of an unidentified *Enterobacter aerogenes* strain and *E. coli* 026K60 OmpA protein.

Pronase treatment of A1 sphaeroplasts resulted in the loss of 34K protein from the outer membrane, leaving a single major outer membrane protein band at 38K. Noticeably the band normally migrating at 28K was also absent. 28K represented the unmodified (37°Csolubilised) form, a small quantity of which appeared to be present in all preparations even after 5 min boiling in SDS containing sample buffer (Plate**19** and Figure**45**).

That the polypeptide has been cleaved to leave a fragment associated with the outer membrane as found in *E. coli* (Chen *et al*, 1980) was evident by the appearance of a heavy band at approximately 22K which is in the non-sarkosyl soluble, outer membrane (See Figure **46**).

This band was not the result of the addition of haemoglobin during the Pronase treatment (See Materials and Methods page) since they migrated at a substantially different point on the gel.

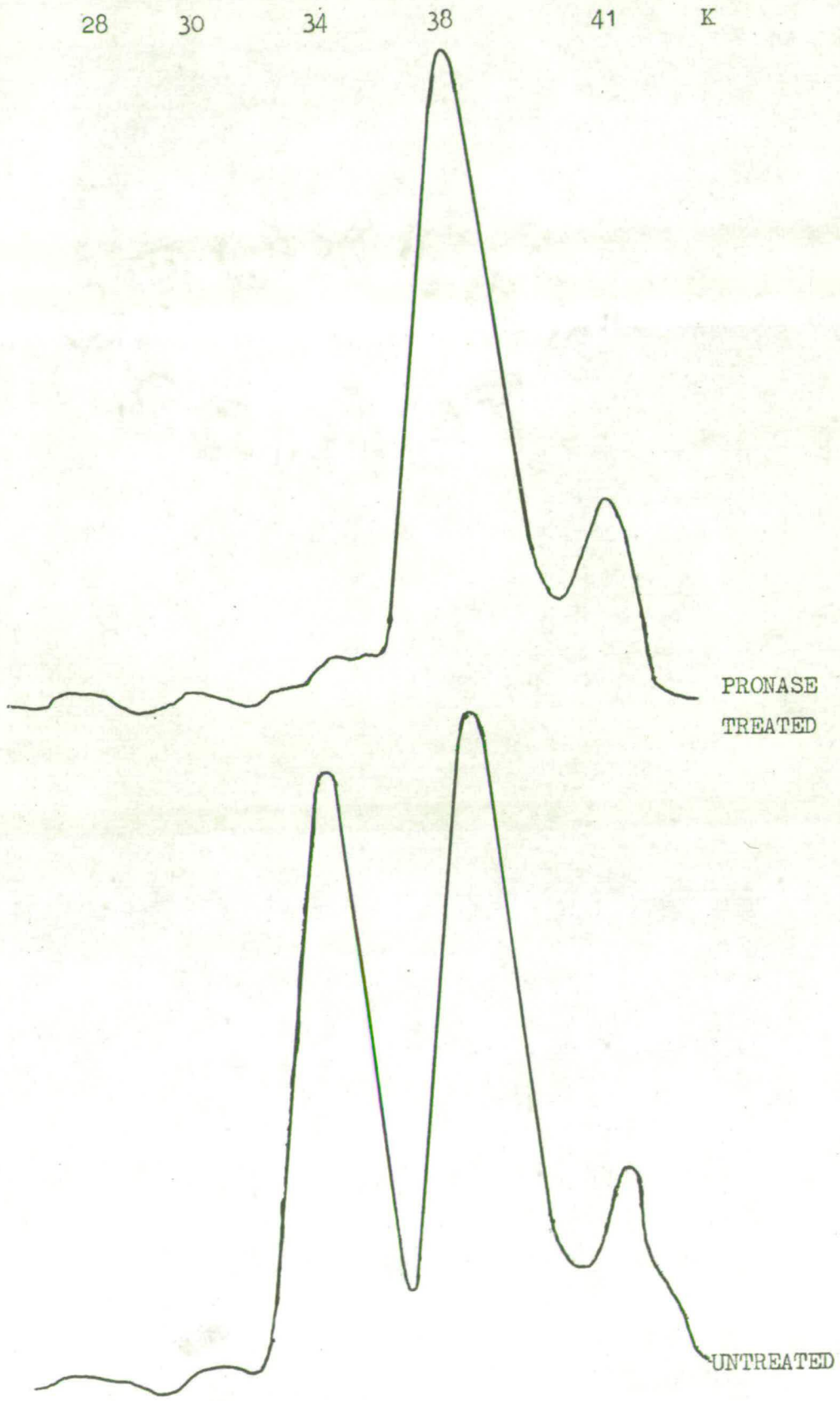


Figure 45: Effect of Pronase on 34K protein of *Enterobacter aerogenes* outer membrane.

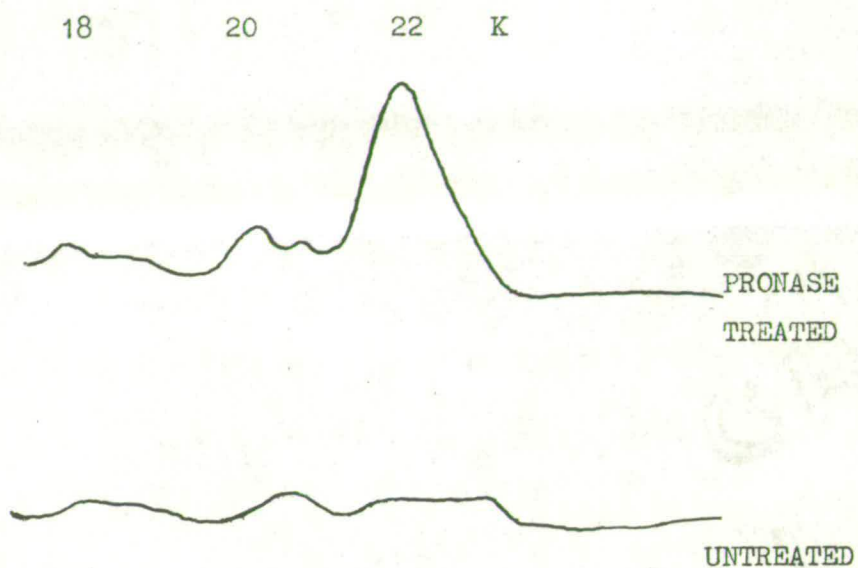
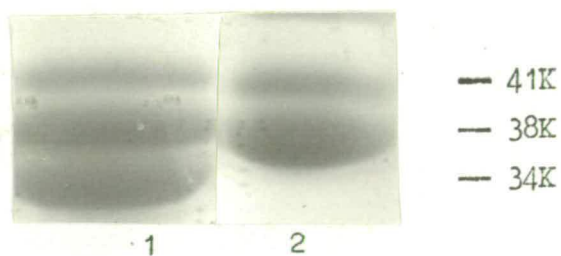


Figure 46: Densitometry scan of the 18-22K region of Pronase treated and untreated outer membranes.



LANE 1: Normal levels of 34K protein
LANE 2: Pronase action on 34K protein

Plate 19: The effect of Pronase on 34K protein.

SUCROSE GRADIENT DENSITY CENTRIFUGATION

The sucrose gradient is a useful tool for separating membranes on the basis of their buoyant densities and can therefore resolve the whole Gram-negative membrane into two fractions in a gradient. The uppermost fraction being inner membrane and the lower, outer membrane.

Analysis of outer membrane fractions of strains A1 and A1SI obtained in this manner was by SDS-PAGE (Plate**20**).

Compared to strain A1, A1SI lacks a 50K polypeptide but overproduces 46.5K polypeptide. Both strains possess low levels of a 59K polypeptide.

FRENCH PRESS PROCEDURE

Outer membrane preparations of strains A1 and A1SI were obtained by sarkosyl extraction of a cell envelope mixture produced by the French Press procedure.

To prevent divalent cations protecting the inner membrane from solubilisation the membranes were suspended in 15mM EDTA before sarkosyl solubilisation.

Outer membrane samples prepared by this method of cell disruption appeared to be enriched for major bands and lacked several minor bands (See Plate**21**), even when twice the normal amount of protein (100 μ g) was applied per well of the gradient gel.

A striking difference between the two strains was in the migration of bands in the 48-60K region. Each possessed a single band but A1 strain protein migrated considerably faster than that of A1SI (Plate**21**, Figure**47**), at 49K.

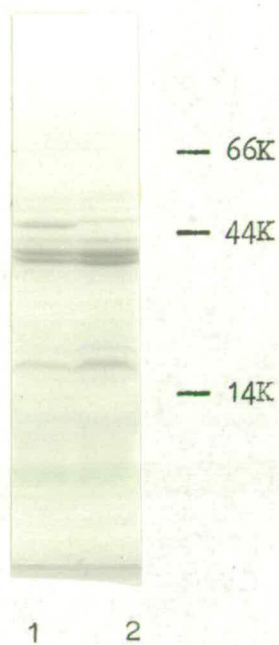
LITHIUM ACETATE ISOLATION OF OUTER MEMBRANE

The method of Heckels (1981) uses the biologically inert salt lithium acetate which appears to act by exchanging lithium for other metal ions whose role is to stabilise the outer membrane components *in situ*. This replacement results in a loss of stability and organisation of the membrane.

Two cultures, A1 and A1SI, were prepared by the Heckels Method and analysed by SDS-PAGE and densitometry.

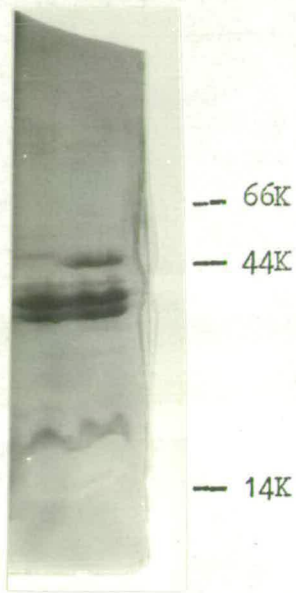
In a comparison of the two strains it was obvious that both produced equivalent amounts of the 34K, 38K and 42K bands; however between 50 and 60K the profiles were markedly different. A1 produced a massive band at 50K whereas this band in strain A1SI was replaced by a smaller band at 58K.

The representation of bands below 34K differed also: a 14K protein was



Lane 1: A1S1 ; Lane 2: A1

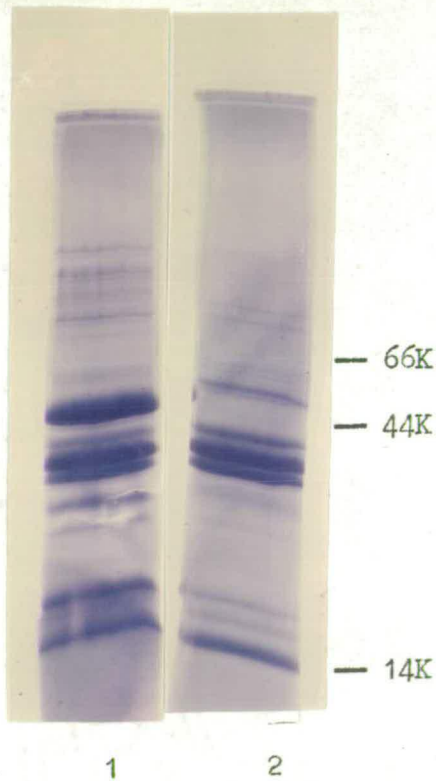
Plate **20**: Outer membrane protein samples prepared by sucrose gradient density centrifugation.



1 2

Lane 1: A1S1 ; Lane 2: A1

Plate **21** : Outer membrane proteins of strains prepared by the French Press method.



1

2

Lane 1: A 1 ; Lane 2: A1S1

Plate **22** : Lithium acetate extraction of outer membrane complex.

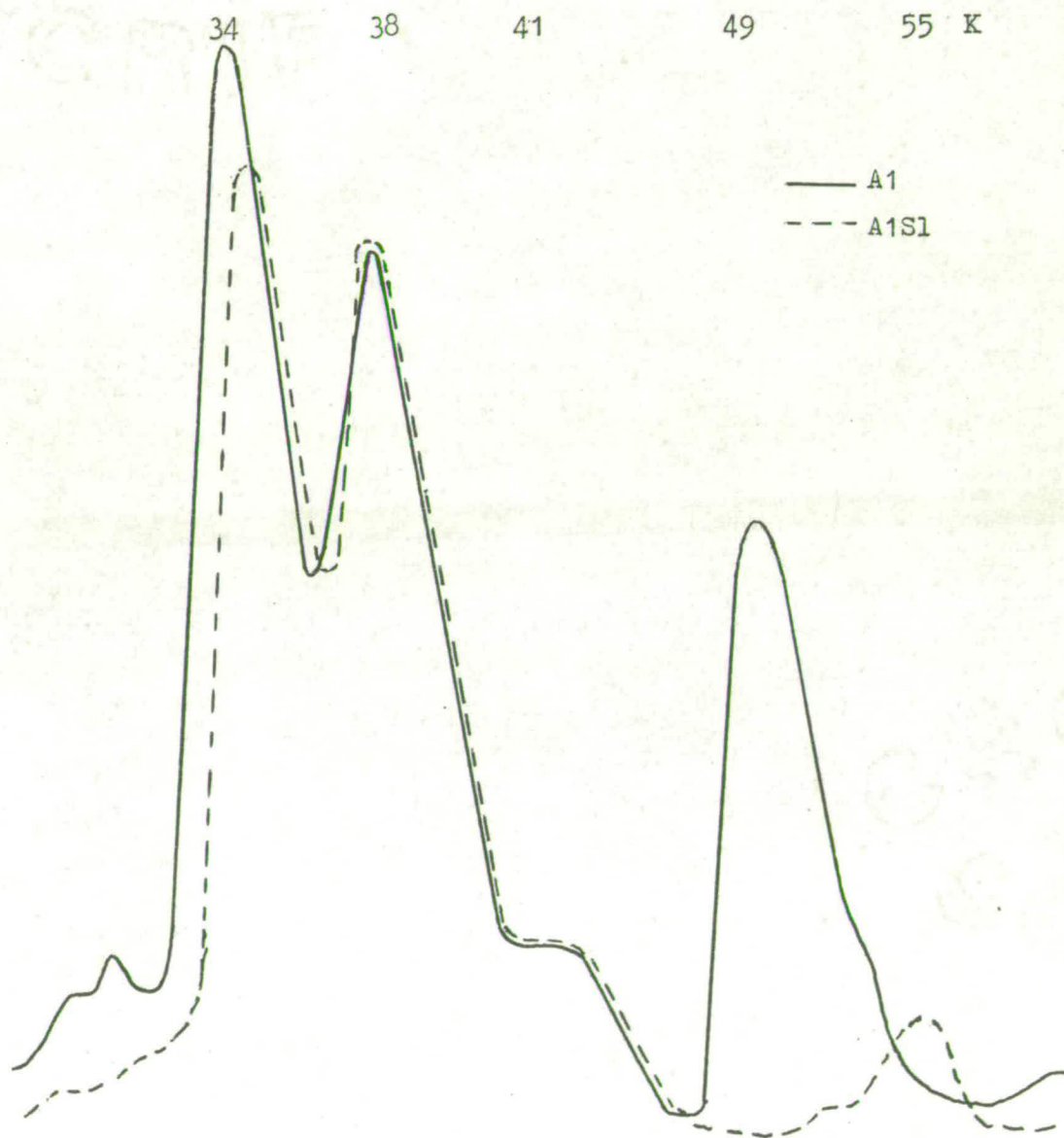


Figure 47: Outer membrane proteins of strains prepared by the French Press method. Densitometry scan of the 34-55K region.

found only in A1SI while this strain possessed reduced quantities of 16K, 26K and 31K compared to strain A1. (See Plate 22, Figure 48)

No gross distinctions (such as new bands) existed to distinguish the profile of lithium acetate prepared outer membrane of strain A1 from that of sarkosyl/sphaeroplast derived preparation. However, the ratios of certain polypeptides with respect to total outer membrane protein did appear to be increased in the former preparation: the major polypeptides 34K and 38K and large minor band 46.5K.

YIELD OF OUTER MEMBRANE

The yield of outer membrane of both French Press prepared membranes treated with sarkosyl, and lithium acetate prepared membranes was lower than that achieved by sphaeroplasting and solubilising the cytoplasmic membrane with sarkosyl. The lower yield by French Press may reflect a reduced amount of outer membrane in the total membrane preparation or an alteration in the representation of certain proteins in the outer membrane produced by pressure treatment of the cell. Certainly, evidence for the latter was presented by SDS-PAGE of outer membranes prepared by this method; similarly with lithium acetate preparation of outer membrane (Table 24).

The extraction was possibly not specific: certain regions of the outer membrane may be more sensitive to extraction by this chemical, resulting in the extraction of a non-representative fraction of the total outer membrane.

Sucrose gradient density centrifugation entailing the physical separation of the sphaeroplast can give rise to both inner and outer membrane fractions and at least two further fractions with a majority of either inner or outer membrane. Consequently it might be expected that such a method of separation would result in a lower yield of "pure" outer membrane.

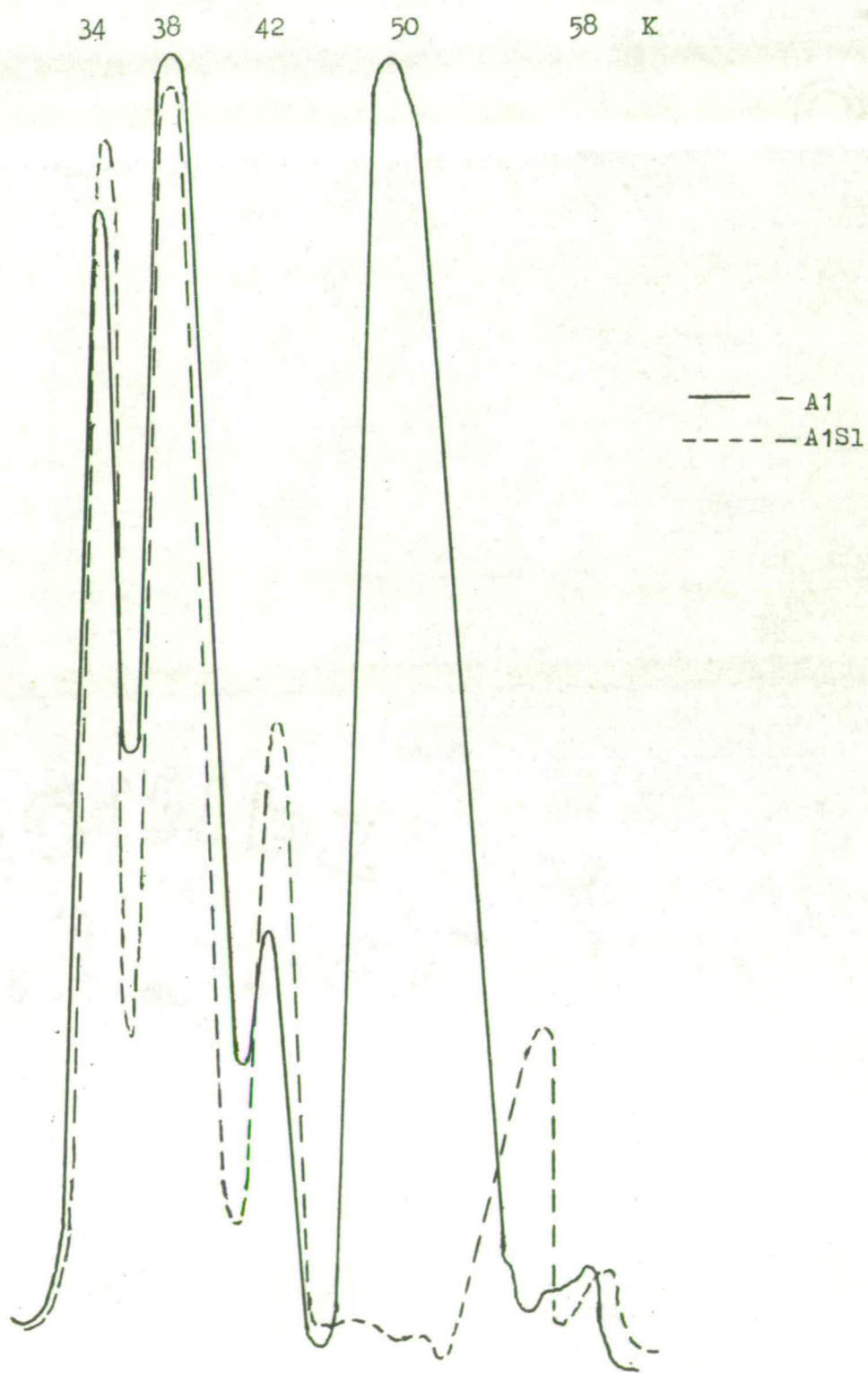


Figure 48: Densitometry trace of the 34-58K region of gels of Lithium acetate extracted strains.

Table 24

Comparison of yields of outer membrane dependent on the isolation method.

Preparation of Outer Membrane	% Yield* (as compared to Sarkosyl) treatment of sphaeroplast
Sphaeroplast + Sarkosyl	100
French Press + Sarkosyl	64.8
Lithium Acetate	43.5
Sphaeroplast + Sucrose Gradient density centrifugation	40.6

* calculated by membrane weight

GLYCOPROTEINS OF THE OUTER MEMBRANE

Coomassie Blue R250 and Page Blue G90 were routinely used to stain non-selectively all the proteins present in gels of membrane samples. To detect those proteins possessing a carbohydrate moiety the Periodic Acid-Schiff Stain (for glycoprotein) was adopted.

By using this procedure a single band of approximately 17K was stained. Since the staining was light the gels did not lend themselves to photography and a line drawing of the band compared to standards (which were counterstained with Page Blue G90) was made (Figure 49).

All strains possessed this polypeptide, differing only slightly in the intensity of staining (i.e. quantity of polymer).

METHOD OF PROTEIN K EXTRACTION FROM *E. COLI*

Paakkanen *et al* (1979) used this method to scrutinise a large number of clinical *E. coli* isolates and with the exception of a single strain correlated the possession of "Protein K" with encapsulation. Therefore, extracting 8805, 8806 and 9261 by this method could indicate any differences in extractable protein between the three strains.

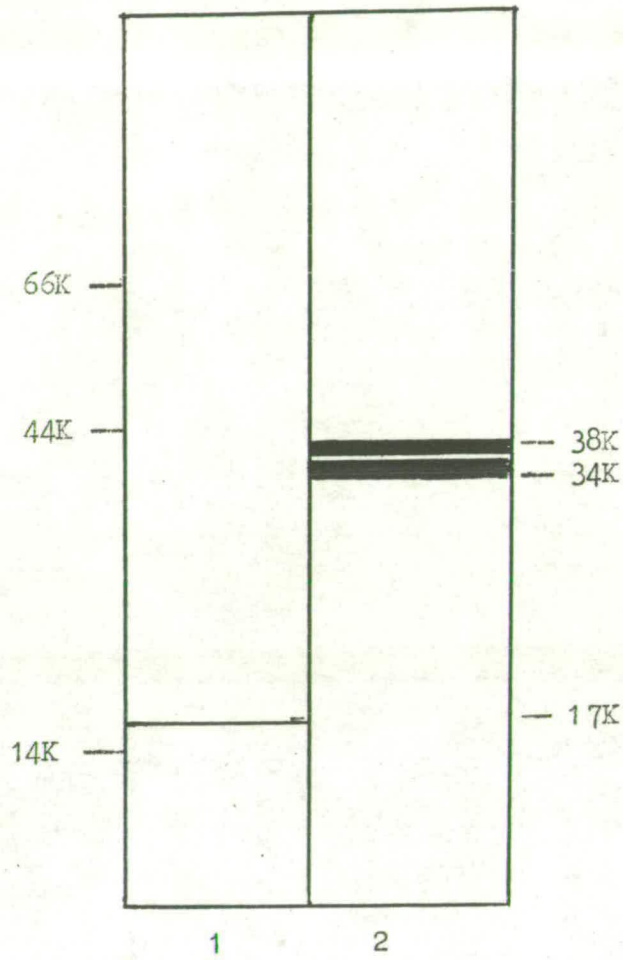
Monitoring of the eluate by SDS-PAGE revealed the presence of 3 polypeptide bands from the major peaks. Attempts to concentrate the protein from the minor peaks were unsuccessful due to the low concentration of protein and the high concentration of Cetavlon which produced interference on the gels even after dialysis.

Makela and coworkers (Paakkanen *et al*, 1979) found the bulk of the protein extract was eluted at 0.5M potassium phosphate concentrations.

In the capsulate strain 8805 no peaks were eluted over 0.5M phosphate buffer. A single peak at 0.4M phosphate was composed of a 43K polypeptide. The slime-former 8806, produced peaks at 0.75M and 0.85M phosphate comprising 43K, 28K and 23K, and 43K and 23K polypeptides, respectively. Strain 9261, the "O" mutant, produced a peak at 0.65M phosphate which was composed of 43K, 28K and 23K polypeptides. (Figure 50).

ACID-BUTAN-1-OL EXTRACTION OF MEMBRANES

Sandermann and Strominger (1971, 1972) successfully used an acid-butanol mixture to solubilise membrane proteins of Gram-positive bacterial cells. Poxton (1974) using the same technique demonstrated the presence of enzymic activities



LANE 1: Glycoprotein stained polypeptide

LANE 2: Relative position of 38K and 34K polypeptides

Figure 49 : The glycoprotein content of *Enterobacter aerogenes*.outer membrane

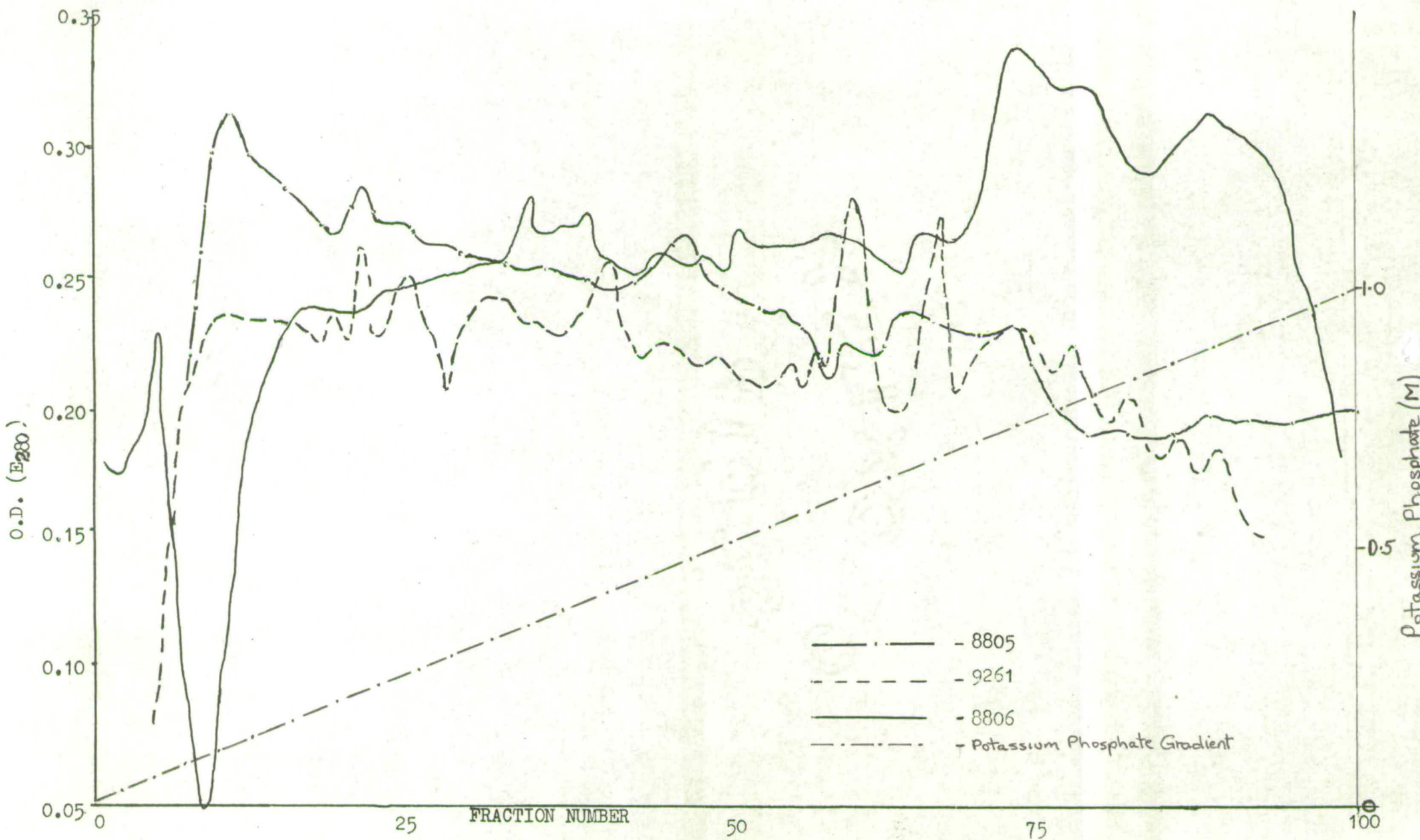
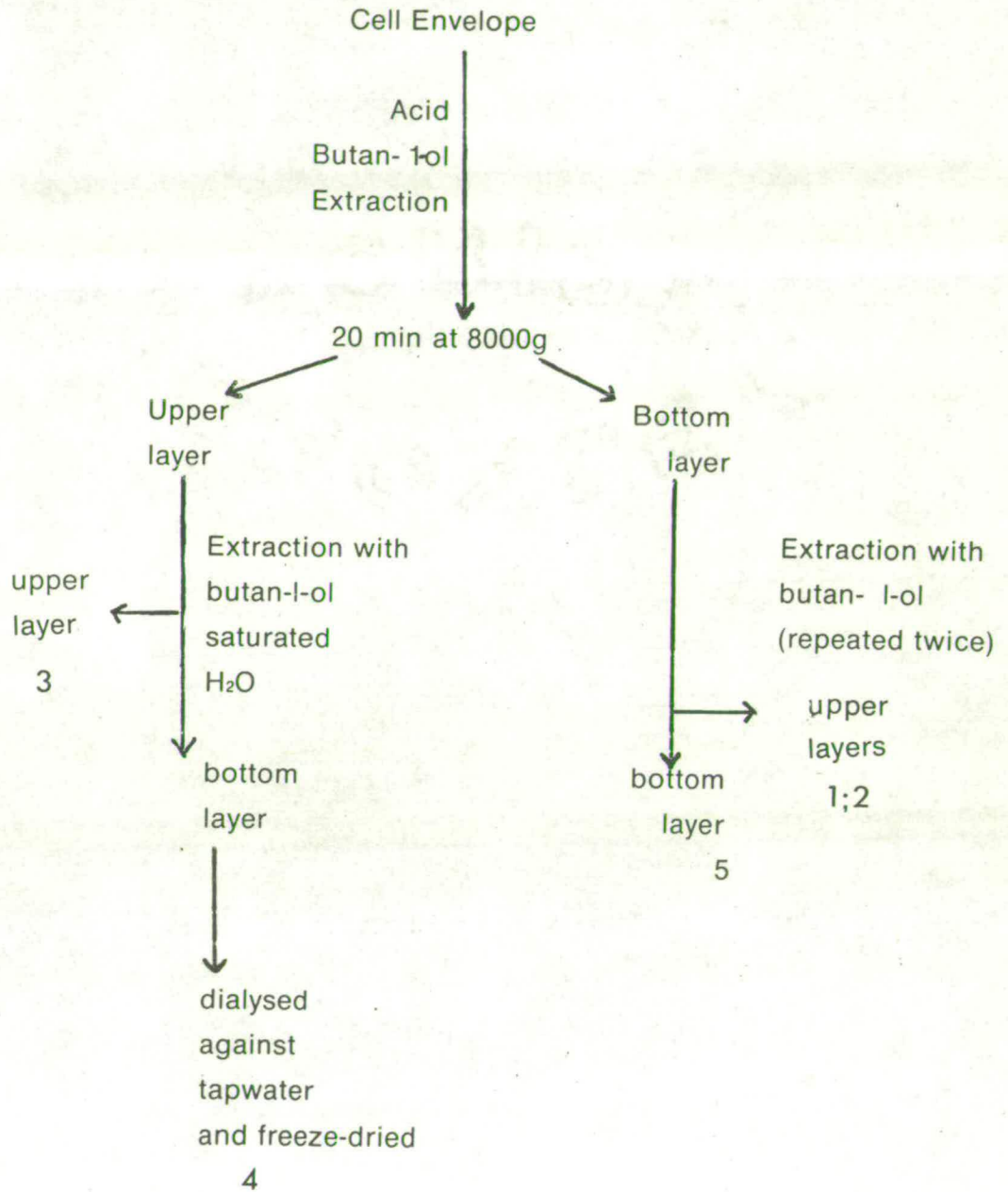


Figure 50: Eluate of "Protein K" Extraction. (For procedure see p 84-85.)

in the acid-butanol fraction of *Enterobacter aerogenes*. This method was adopted to allow comparison of extracts of capsulate and slime-forming strains.

The non-butanol solubilised protein from B (See Figure 51) contained several polypeptides as separated on SDS-PAGE. These samples possessed a larger number of polypeptides than could reasonably have had their origins in the outer membrane; a significant number of contaminatory cytoplasmic membrane polypeptides were assumed to be present. However this fact should not qualify the discovery of differences in profile between the two strains except that the origin of the difference may not be the outer membrane. A1SI had a greater percentage of high molecular weight (over 60K) protein than A1, and exhibited two extra bands at 14K and 13K (See Figure 52). A1SI and A1 pooled butanol soluble material A contained only two polypeptides 38K and 50K, in equal quantities (Data not presented).

Figure 51 : Scheme for the extraction of Acid-Butanol soluble protein.



Upper layers 1 2 and 3 were pooled:A
Bottom layers 4 and 5 were pooled:B

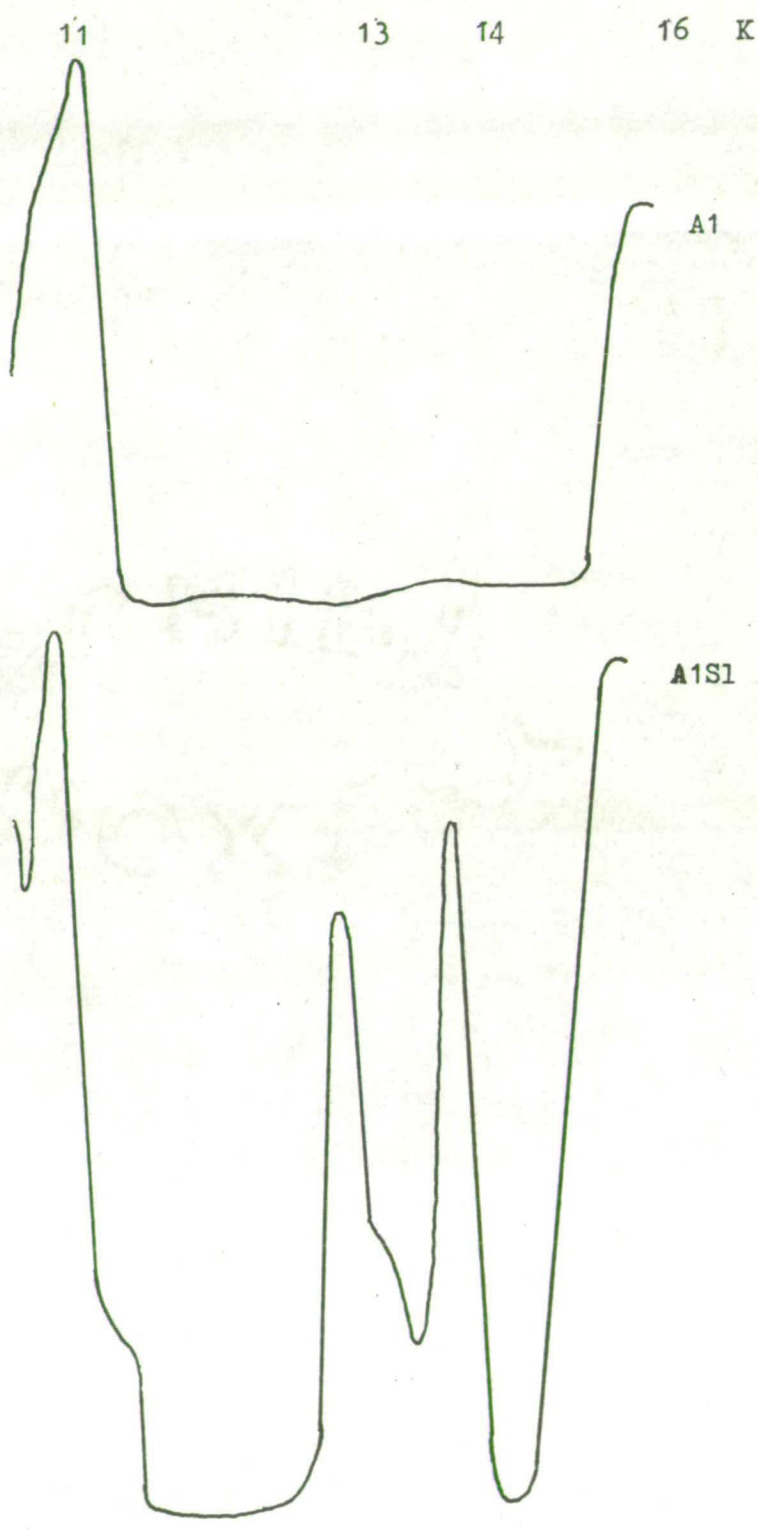


Figure 52: 11-16K region of gel scans from Acid-Butanol extracted membranes.

2-DIMENSION POLYACRYLAMIDE GEL ELECTROPHORESIS OF OUTER MEMBRANE SAMPLES

Attempts to separate A1 and A1SI outer membrane samples on 2-dimension gels, although separating the polypeptides did not give good resolution and showed fairly extensive smearing of 'spots'.

EFFECT OF MUTATION OR GROWTH IN SDS OR SARCOSINATE ^{ON} OUTER MEMBRANE PROTEIN CONTENT AND CULTURE SUPERNATE (AMMONIUM SULPHATE PRECIPITABLE) PROTEIN

Protein precipitated from the culture supernate was analysed by SDS-PAGE.

Strains A1, A1SI and A1X gave identical supernate protein profiles on acrylamide gels. However A1X grown in 0.01(w/v) SDS or Sarcosinate lacked a single band of 41K in the supernate. Previously (Page 149) it was shown that the outer membrane of this strain lacked the 41K when grown in SDS or Sarcosinate.

It would seem unlikely that this polypeptide could be a requirement for encapsulation since its synthesis was, apparently, repressed and yet exopolysaccharide production and encapsulation were unaffected. A second possible reason for lack of retrieval of this polypeptide from the supernate could be proteolysis. However, this seemed rather improbable since the other supernatant fractions contained the major polypeptides and 41K and these exhibited their normal mobilities on gels.

ANALYSIS OF THE PROTEIN PROFILE OF THE CYTOPLASMIC MEMBRANE

Inner membranes were prepared by sucrose gradient density centrifugation or sarkosyl solubilisation. The latter method required the removal of large quantities of detergent from the sample. Initially this was achieved by ultra-filtration (Frasch, 1976) but given the large number of samples to be filtered and the number of passages each required this method was discontinued in favour of dialysis against tapwater in the cold.

To obtain exact yields of inner or outer membrane it was necessary to ensure the total removal of sarcosinate since this altered yield in favour of inner membrane. Average yields* were:

Outer membrane 52.3%

Inner membrane 47.7%

The inner membrane samples when analysed by SDS-PAGE presented a more complex profile than the equivalent outer membrane. The inner membranes contained a larger number of polypeptides many of which shared or possessed very similar molecular weights and appeared in groups of bands which were difficult to distinguish by eye. Although the gels used were high concentration gradients this had little effect on the separation of lower molecular weight bands, presumably because of numbers and quantities. To have reduced the amount of protein per well would have risked losing more minor bands and portraying an extremely artificial profile.

The protein per well was 50µg/50µl sample buffer.

The differences detected in the inner membrane samples of several strains grown at a variety of temperatures were largely due to intensity of staining of bands: the relative amounts of polypeptide present. Intense staining of particular bands appeared to be independent of both strain and growth temperature.

Due to the multiplicity of bands, gel densitometry scans were unsuccessful and could *not* be used to assign percentage increases or decreases in a band (with respect to total protein) between strains. (See Figure 53).

* percentage of total membrane weight

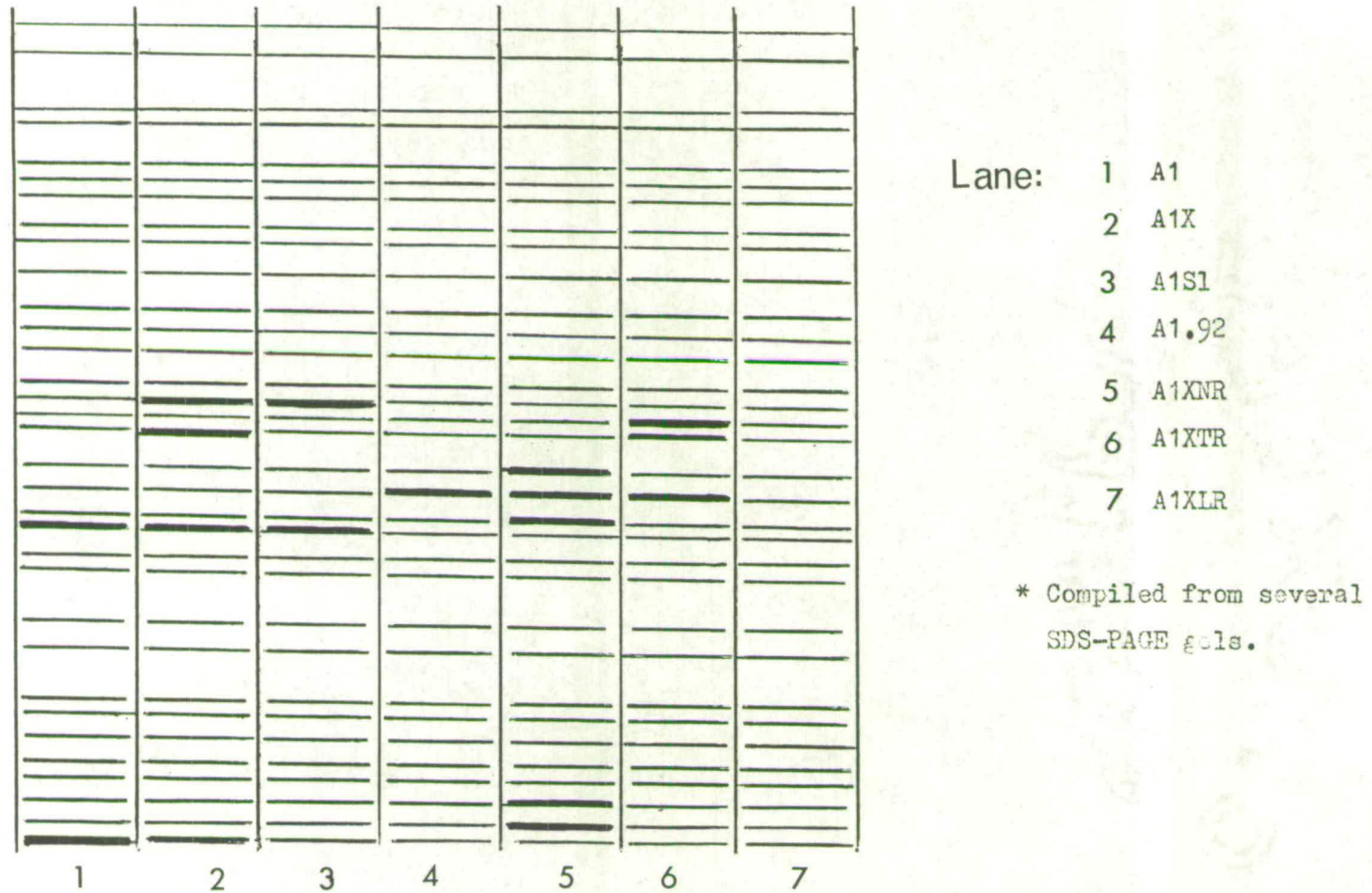


Figure 53 Cytoplasmic membrane proteins of *Enterobacter aerogenes*. *

DISCUSSION

OUTER MEMBRANE PROTEINS OF *ENTEROBACTER AEROGENES*

The possession of a number of polypeptides of intermediate weight (30-54K) is a common feature in the ^{outer membrane of} Gram-negative bacteria.

Table 25 lists the major ^{outer membrane} proteins of several enteric bacteria as visualised on SDS-gels at a sample solubilising temperature of 100°C.

Table 25: Major Outer Membrane Proteins of Enteric Bacteria.

Strain	Protein Molecular Weight (K)	
	Peptidoglycan-associated Cross reactive with protein I of <i>E. coli</i> 026K60	Non-peptidoglycan associated Cross reactive with OmpA of <i>E. coli</i> 026K60
<i>E. coli</i> 01K ⁻	37	35
<i>E. coli</i> 026K60	36	33
<i>Shigella flexneri</i>	37	34
<i>Styphimurium</i>	36.5	35
<i>Citrobacter freundii</i>	35	34
<i>Enterobacter aerogenes</i>	38	34

(After Hofstra and Dankert (1980))

The *Enterobacteriaceae* shared antigenically similar proteins as determined by the SDS-gel immunoperoxidase (SGIP) technique (Hofstra & Dankert, 1980). This antigenic cross-reactivity was not found for non-enteric species (such as *Pseudomonas aeruginosa*) and these strains produced outer membrane major protein profiles distinct from the enteric species (with the exception of *Serratia marcescens*).

Unlike other enteric species *Serratia marcescens* non-peptidoglycan bound protein failed to exhibit normal heat-modifiable characteristics. As a result the major bands seen on SDS-gels are found at 28 and 40K, a larger difference in molecular weights than seen in enteric bacteria normally but common in pseudomonads.

As shown in Table 25 and in this work, the lower molecular weight major outer membrane protein was not peptidoglycan-associated unlike the higher molecular weight protein.

Differences noted in wild-type strains, or their mutants which did not exhibit the general characteristics associated with a particular mutation, will be

discussed before considering the influence of cultural conditions on outer membrane protein and polysaccharide.

The wild-type strains of *Enterobacter aerogenes* examined in this work were heavily capsulate when grown in Y.E. medium and produced chemically distinct exopolysaccharides, the basis for serotyping.

A remarkable similarity in outer membrane protein profiles was detected irrespective of the capsular serotype. Strain 7824 (serotype 30) was an exception. While possessing the normal major polypeptides in the 32-45K region the relative amounts differed considerably from those expressed in other strains. Strain 7824 and mutants derived from it tended to be less resistant to surface active agents, possibly due to a less favourable arrangement of membrane components at the cell surface.

Mutants to drug or phage resistance were selected and examined. Polymyxin B and tetracaine resistance in serotype 1 generated mutants with unusual properties which were temperature dependent.

Polymyxin B acts against the Gram-negative cell wall by binding to lipopolysaccharide and phospholipids, penetrating the outer membrane and interacting with cytoplasmic membrane phospholipids. This promotes an imbalance in the osmotic equilibrium of the cell and leakage of cytoplasmic contents. Polymyxin resistance in *E. coli* was found to correlate with a decrease in the outer membrane protein-lipopolysaccharide complexes (van Alphen *et al*, 1978).

A1XPB600

The polymyxin resistant strain lacked the 38K protein at lower temperatures and had a reduced exopolysaccharide yield. The loss of the protein may have decreased the viability of the cells and reduced exopolysaccharide synthesis by decreasing solute uptake thereby reducing the amount of substrate available for exopolysaccharide synthesis since essential carbohydrate requirements of the cell would be met first.

Lutkenhaus (1977) selected Cu^{2+} resistant *E. coli* strains lacking a major protein, porin b. Glucose transport was unimpaired but other metabolites were not transported as effectively. Strains lacking both porins, b and c failed to transport glucose. An alternative porin to the 38K protein has not been demonstrated by kinetic studies of other *Enterobacter aerogenes* outer membrane proteins and the loss of the major porin could therefore seriously affect solute transport.

Dame and Shapiro (1979) demonstrated that tetracaine affected phospholipid metabolism in *E. coli*, increasing the level of cyclopropane fatty

acid. Tetracaine greatly altered the membrane protein in sensitive cells. However, unlike the change in fatty acid composition of drug-grown strains, resistant mutants possessed essentially normal phospholipid, fatty acid and lipopolysaccharide. A slight relative increase in the membrane protein was detected.

The tetracaine resistant mutants showed temperature dependent changes in exopolysaccharide yield and membrane protein, and slight autoagglutination occurred in minimal medium. The latter observation suggested possible perturbation in the protein-lipopolysaccharide complexes of the outer membrane.

The 38K protein of *Enterobacter aerogenes* was shown in this work to possess certain properties of a matrix protein (i.e. peptidoglycan association) and independently observed to be antigenically similar (cross-reactive) to *E. coli* 026K60 36K porin by Hofstra and Dankert (1980). Thus, lack of 38K protein might have affected carbohydrate uptake.

All Serotype 54 (wild type and drug resistant capsulate) strains showed reduced exopolysaccharide synthesis capability at 20°C and 10°C but normal quotas of the major outer membrane proteins. This phenomenon was not detected in the other serotypes. Such a result could be construed as evidence excluding a role for 38K protein in some facet of exopolysaccharide production, but not in solute uptake. At 20°C and lower temperatures the membrane lipid would be less fluid and might have affected the colonising ability of the cells. Since the phenomenon was peculiar to Type 54 species only and did not correlate with changes in the membranes it was not investigated further.

Common amongst the drug resistant 54C strains was the production of a new or overproduction of an existing low molecular weight polypeptide (20-25K region) apparently unrelated to exopolysaccharide yield.

The mechanism by which this polypeptide was generated was uncertain. It could have been synthesised in response to a double mutation by NTG (which can produce double mutations unlike the other mutagens used in this work which cause single point mutations) and be unrelated to the phenomenon of drug resistance.

As this polypeptide was in the outer membrane and, as such, part of the permeability barrier of the cell a generalised role in the prevention of uptake of lipophilic drugs cannot be excluded. It was, however, detected in 54C strains alone.

The three stable, phage resistant mutants selected were capsulate. Differences in the outer membrane composition which could account for mutation to phage resistance were detected. Loss of a wild-type receptor site (not

capsule) and exposure or gain of a different site may have occurred at the outer leaflet of the outer membrane and would involve at least one membrane component.

A large volume of documentary evidence exists for the role of major outer membrane proteins and lipopolysaccharide as phage receptor sites (Hantke, 1978; Hudson *et al*, 1978; Johansson *et al*, 1978 and Chai and Foulds, 1979). Many minor and inducible proteins also serve as receptors and several have multiple receptor activities (Braun and Hantke, 1977).

The effect of mutation to slime-forming from capsulate wild type was limited to changes in the outer membrane protein. Yield of exopolysaccharide was unaffected as was the chemical composition of high and low molecular weight polysaccharides isolated from the culture supernate.

Slime-forming strains possessed approximately a 10-fold increase in two outer membrane polypeptides of 54K and 58K which on gel electrophoresis migrated as a double-peaked band not found in their respective capsulate wild type strains.

Exopolysaccharide synthesis was thought to be affected by the availability of isoprenoid lipid and by the level of glycosyl transferase activity in crude membrane preparations from non-exopolysaccharide synthesising *Klebsiella aerogenes* (Sutherland, 1977; Sutherland and Norval, 1970).

Therefore 'O' strains need not be altered in membrane protein(s) attachment or transfer sites for exopolysaccharide. However an attachment mechanism is feasible. By analogy LPS appears to be intercalated in the membrane by its interaction with protein (Koplow and Goldfine, 1974; Ames *et al*, 1974; Parton, 1975; Schweizer *et al*, 1978). Both components are required as a complex before incorporation into the membrane can occur.

'O' strains did not differ significantly in outer membrane protein profiles visualised from slime-forming mutants. Lipopolysaccharide types as determined by gel electrophoresis were variable.

As 'O' mutants produced low levels of extracellular polymeric carbohydrate this may correspond to somatic antigen, extruded and exposed at the cell surface (Lankford *et al*, 1951; Wilkinson *et al*, 1954).

Norval and Sutherland (1969) reported the isolation of a new class of mutant which produced lipopolysaccharide and exopolysaccharide maximally at 37°C and to a lesser extent (50%) at 20°C. (See Tables 10 and 18). In these created (CR) mutants, of which A4CR is a representative, polysaccharide synthesis did not occur until stationary phase was reached. Sutherland (1977) speculated that this feature of their growth was related to the availability of isoprenoid lipid.

In mutants with otherwise unimpaired cellular functions it was thought that the protein composition of the outer membrane might be correlated with high and low levels of exopolysaccharide production. The protein profiles at 20°C and 37°C were identical and were similar to the wild-type A4, suggesting that protein changes in the membrane, if any, gave an 'all or none' response dependent only on *ability or inability* to synthesise exopolysaccharide and did not reflect the level of synthesis.

The insensitivity of A4CR to lytic phage at 20°C compared to 37°C may be related to the lower quantity of lipopolysaccharide or to the thick layer of exopolysaccharide in the capsule (which was large and diffuse in appearance under India Ink films) preventing binding of the phage in the correct alignment to other envelope components.

ION LIMITATIONS AND RELATED EFFECTS

Mg²⁺ and PO₄³⁻ ions are essential for basic processes of cellular metabolism. Webb (1948) found that reduced levels of Mg²⁺ in the growth medium allowed good cell growth but proportionately increased the exopolysaccharide yield. On addition of Mg²⁺ the production of exopolysaccharide fell. Numerous effects of reduced Mg²⁺ concentration have been recorded: loss of ribosomes, reduction in RNA content, and loss of barrier function as determined by assay for the normally cryptic enzyme alkaline phosphatase (Kennell and Magasanik, 1962; Tempest *et al*, 1965; Hassan, 1976). Similarly, PO₄³⁻ ions are essential. Glycolysis by the Embden-Meyerhof Pathway requires inorganic phosphate; many enzymes and polymers such as lipopolysaccharide are phosphorylated (Rogers *et al*, 1980).

A précis of the antimicrobial activity of EDTA was compiled by Russell (1971). Coliform organisms when treated with EDTA released surface bound enzymes and LPS and showed increased permeability to other antibacterial agents without effecting cell lysis.

By chelating divalent cations at the cell surface, the Mg²⁺ ions (which form ionic interactions between LPS molecules in the outer membrane of Gram-negatives) are extracted. Such a mechanism would cause a weakening of LPS-

protein interactions undermining the membrane barrier function resulting in the observations listed above.

The effect of nutrient limitation on outer membrane protein was varied and is summarised in Table 26.

Table 26: Effect of nutrient limitation on outer membrane protein

Protein	Effect of Nutrient limitation				EDTA Supplement
	Mg ²⁺	PO ₄ ³⁻	SO ₄ ²⁻	K ⁺	
70-90K	+	+	+	—	+
58K	V	—	increase	absent	—
41K	—	—	decrease	—	decrease
38K	decrease	—	decrease	—	decrease
34K	decrease	—	decrease	—	decrease
30K	—	—	—	—	—
16K	decrease	decrease	increase	—	—

+ = induced new polypeptide(s)

— = no effect. V = variable

The expression of new or different quantities of polypeptides in the outer membrane has been observed in a number of enteric species (Overbeeke and Lugtenberg, 1980; Darveau *et al*, 1980; Schnaitman, 1974; Loeb and Kilner, 1979).

In *E. coli* K12 wild-type alone, growth in a phosphate-limited medium induced a new protein e. Overbeeke and Lugtenberg (1980) postulated that this protein may serve as a non-specific diffusion pore for anionic compounds and thus be capable of allowing diffusion and uptake of phosphorus-containing compounds.

Phosphate limitation of *Enterobacter aerogenes* did not induce new protein.

Mg²⁺ and PO₄³⁻ limited cultures possessed decreased levels of a 16K protein, possibly reflecting conservation of limited nutrients by reduction in incorporation of less necessary membrane components. Magnesium ion - limitation of *Pseudomonas aeruginosa* which induces EDTA resistance led to the stimulated synthesis of a low molecular weight polypeptide, H1, which Nicas and Hancock (1980) postulated to have a role in stabilising lipopolysaccharide. Mg²⁺-limitation of *Enterobacter aerogenes* did not produce a new polypeptide and the substantial differences in the normal outer membrane protein profiles of the

Pseudomonadaceae and the *Enterobacteriaceae*, should be borne in mind. Sulphate-limitation produced extremely variable results dependent on the strain of *Enterobacter aerogenes*.

The total protein of the outer membrane was decreased (data not presented), largely mediated by a decrease in the 38K and 34K polypeptides. Robinson and Tempest (1973) reported a decrease in total protein of the envelopes of sulphate-limited *K. aerogenes* in which the major polypeptide was 30K. The solubilisation temperature of the gel samples was not indicated and consequently a direct comparison cannot be made with the present work.

In EDTA-grown cells alterations in the outer membrane molecular organisation were not suggested by examination of the protein complement. Two 'minor' proteins were induced and represented a large percentage of the total outer membrane protein. As the molecular weights coincided with those of iron-limited membranes the polypeptides may have had an iron-uptake function. These bands were found in several nutrient-limited cells and may reflect a low effective iron content in these defined media.

Nicas and Hancock (1980) elucidated the role of protein (H1) in stabilising lipopolysaccharide in EDTA-resistant *Pseudomonas aeruginosa* strains. The change in *Enterobacter aerogenes* was purely adaptive and the magnitude of the response would seem excessively large if the role of the protein was solely that of stabilising the membrane architecture.

The iron-scavenging systems developed by many micro-organisms were found to be present in *Enterobacter aerogenes*. Five polypeptides in the molecular weight range 74-93K were induced, and the citrate-ferric iron receptor identified as an 86K polypeptide. Multiple uptake systems are commonly found in enteric bacteria (Braun and Hantke, 1977).

Unlike the essential metal Fe^{3+} , the heavy metal ion Cu^{2+} , like most ions of this group, has been well documented as both bacteriostatic and bactericidal at low concentration. The composition of the growth medium was shown to be a critical factor in determining the inhibitory concentration of the Cu^{2+} ion (Kushner, 1971).

The outer membrane of *Enterobacter aerogenes* reacted to growth in Cu^{2+} -containing Y.E. medium by altering the ratios of three proteins: 29K, 34K and 38K. By raising the Cu^{2+} concentration of the medium the ratios of these proteins were shifted from 38K/34K to 29K dominance.

The putative role of 38K protein of *Enterobacter* as a porin allowing diffusion of low molecular weight solutes would render the cell liable to Cu^{2+} toxicity as shown by Lutkenhaus (1977) for *E. coli*. Stimulated production of 29K protein offers some evidence for this protein being a porin. In such a case the protein may exercise a greater control on solute uptake, unlike the presumably more generalised uptake porin, 38K.

MEMBRANE ACTIVE AGENTS

As might be expected, agents specifically affecting the molecular composition or organisation of the cell envelope altered the quantities of outer membrane proteins.

The agents chosen: SDS, sodium lauryl sarcosinate, phenylethanol, ethanol, sucrose, NaCl and Penicillin G act on the cell by a variety of mechanisms.

The action of anionic detergents sodium dodecyl sulphate and sodium lauryl sarcosinate on the cell envelope of Gram-negative bacteria was discussed by Harold (1970) and Filip *et al*, (1973).

These agents lysed protoplasts. Although the lipopolysaccharide surface layer was attacked, the primary target was the cytoplasmic membrane where breakdown of synthetic macromolecules and autolytic changes occurred.

The activity of anionic detergents was related to the chainlength and nature of the polar group.

Phenylethanol action in the bacterial cell was originally reported as bacteriostatic as opposed to bactericidal (Lilley and Brewer, 1953). Later reports indicated that static or lethal effects in *E. coli* were possibly dependent on the pH of the suspending medium and allelic forms of genes of the *acr* locus (Nakamura, 1967 and cited by Nakamura, 1968). Both DNA and RNA synthesis were affected.

Jackson and DeMoss (1965) demonstrated disruption of the permeability barrier of *E. coli* (to uptake of acriflavine and potassium) in the presence of phenylethanol.

A recent report attributed the bacteriolytic action of ethanol to the ability of this chaotropic agent to inhibit peptidoglycan cross-linking, possibly by disrupting the hydrophobic interactions between enzymes involved in the cross-linking reactions and the cytoplasmic membrane (Ingram and Vreeland, 1980). Addition of ethanol to a growing culture resulted in the synthesis of lipids enriched in unsaturated fatty acids (Buttke and Ingram, 1980). This may also result from an alteration in the hydrophobicity of the environment.

Sullivan *et al* (1979) postulated a role for n-alkanols in acting against the membrane-bound acyltransferase enzymes which regulate incorporation of saturated and unsaturated fatty acids into phospholipid by disorganising the lipid surrounding the enzymes. Several workers have reported that the presence of compounds impermeable to the cell envelope of *E. coli* influence the levels of OmpC and OmpF (porin) proteins. The manner in which transcription of the OmpC and OmpF genes is affected by the medium osmolarity is not known (van Alphen and Lugtenberg, 1977; Kawaji *et al*, 1979). Sato and Yura (1981) speculated that the proposed regulatory gene OmpB may be involved in the modulation of OmpC/OmpF in high osmotic concentration media.

Table 26 summarises the effects of several agents on the outer membrane protein of *Enterobacter aerogenes*.

Table 26: Effect of Membrane Active Agents on the outer membrane proteins of *Enterobacter aerogenes*.

Protein K	Effect of Membrane Active Agent						
	SDS	Sarcosinate	Pe A	Ethanol	Sucrose	NaCl	Penicillin G
80	—	—	—	—	—	—	increased
45-70	—	—	—	—	—	—	—
41	absent	absent	—	absent	—	absent	—
38	—	—	reduced	reduced	reduced	—	—
34	—	—	—	—	—	—	—
32	—	—	increased	—	—	—	—
31	—	—	—	—	—	increased	increased
29	—	—	—	increased	—	—	—

— = no effect.

Noticeably, proteins of higher molecular weight than 34K tend to be reduced in quantity and lower molecular weight proteins increased.

High concentrations (1%-2% w/v) of detergent in Y.E. medium were bactericidal, presumably, by the mechanism outlined above. A lower concentration (0.01% w/v) chosen for supplementing the growth medium, did not exhibit bacteriostatic or bactericidal effects and the cells grew normally without a lag phase or expressing pleiomorphic traits. All cells were capsulate and the outer membrane lacked a 41K protein. Ethanol and NaCl grown cells similarly lacked 41K.

As previously described (Page 40) the colanic acid synthetic system of *E. coli* was found by Markovitz and co-workers (Gayda *et al*, 1979a, Gayda *et al*, 1979b; Zehnbaauer and Markovitz, 1980) to possess several control systems (coded for by a 2 Md DNA fragment) one of which a 40K polypeptide, protein a, of the outer membrane acted as a repressor, preventing overproduction of capsular polysaccharide in *CapR(lon)* strains of *E. coli*.

Subsequently, Makela and co-workers (Paakkanen *et al*, 1979) correlated the possession of a 40K polypeptide (protein K) with capsulation in clinical *E. coli* isolates.

SDS grown strains lacked 41K in the membrane and supernate suggesting repression of synthesis. The question of a possible role in control of capsulation in a similar manner to the colanic acid system is left open by this finding.

The effect of solvents on the outer membrane proteins was similar: 38K protein decreased and a lower molecular weight protein increased in concentration.

Pugsley found that 10mM phenylethanol reduced the OmpF protein of *E. coli* to 5% of normal values and enhanced OmpC synthesis (Pugsley, A. P., FEMS Symposium on Microbial Envelopes, 1980).

Apart from its effect on fatty acids, ethanol can affect the formation of peptidoglycan. Whether this could inflict changes in the membrane organisation sufficient to reduce the incorporation of peptidoglycan-bound matrix protein is not known. Interestingly, Y.E. medium augmented by a non-lethal concentration of penicillin produced cells with a higher than normal concentration of 31K protein.

Sucrose and NaCl, compounds which possess an osmotic action, repressed synthesis of major proteins, as previously documented for *E. coli* (Kawaji *et al*, 1979; Sato and Yura, 1981).

POLYSACCHARIDE PRODUCTION

Mg²⁺, PO₄³⁻, SO₄²⁻ and K⁺ limitations exacted stimulating or inhibitory effects on synthesis of exopolysaccharide in batch cultures.

The effect of Mg²⁺ limitation on *Enterobacter aerogenes* exopolysaccharide yield was not as marked as that of sulphate. A slight reduction in yield was noted compared to the non-limited medium in batch culture. Tempest *et al* (1965) showed that Mg²⁺ limited cells grown at low dilution rates possessed reduced ability to synthesise polysaccharide and that this was probably due to lower enzyme biosynthesis and a lack of excess free Mg²⁺ in the medium. Markovitz and Sylvan (1962) showed that Mg²⁺ was essential for maximum synthesis of polysaccharide in a Gram-negative soil bacterium.

Williams and Wimpenny (1977) studied the effect of phosphate limitation on *Pseudomonas* NCIB11264 and found that polysaccharide yield varied little when the medium was buffered. Deprivation of phosphorus in washed cell suspensions of *Aerobacter aerogenes* increased polysaccharide production several-fold (Duguid and Wilkinson, 1953) although suspension of cells in buffers other than phosphate caused considerable reduction in polymer formation (Wilkinson and Stark, 1956).

When phosphate-limited, *Enterobacter aerogenes* gave exopolysaccharide yields equivalent to those of cells grown in non-phosphate limited medium. As predicted above, a higher yield might have been expected but the medium was not phosphate buffered and would reduce the synthetic ability of the cells. Williams and Wimpenny (1977) reported that *Pseudomonas* NCIB 11264 did not increase exopolysaccharide yield under phosphate limiting conditions.

The exopolysaccharide yield of batch cultures of sulphate-limited *Enterobacter aerogenes* was lower than that of phosphate-limited cultures. This finding was in agreement with yields determined for several members of the *Enterobacteriaceae* (Duguid and Wilkinson, 1953). Strain 54C was effectively non-mucoid; whether this indicated a greater strain dependence on an exogenous sulphate supply is unclear. Potassium-limitation unlike the former limitations exerted a strong effect on exopolysaccharide yield. Duguid and Wilkinson (Wilkinson *et al*, 1954) found that in potassium-limited lactose containing media cell growth was reduced and exopolysaccharide yields slightly increased. From this result the authors concluded that deprivation of K^+ ions was specifically inhibitory to polysaccharide synthesis.

Dicks and Tempest (1967) demonstrated K^+ and NH_4^+ antagonism in K^+ -limited media and reported that K^+ -limitation had little effect on polysaccharide production by *Aerobacter aerogenes*. Given the role of K^+ in carbohydrate uptake and probable antagonism between K^+ and NH_4^+ ions in K^+ -limited media the exopolysaccharide yield of *Enterobacter aerogenes* might be expected to reflect the reduction in synthetic ability.

Lin and Tseng (1979) using an ammonium-salt buffered K^+ -limited medium observed a 93% reduction in the exopolysaccharide yield of *Xanthomonas oryzae*. In growth experiments with *Enterobacter aerogenes* it was found that EDTA supplements to Y. E. medium reduced exopolysaccharide yield probably by chelation of a variety of essential ions such as Ca^{2+} , Mg^{2+} , Fe^{3+} affecting enzymic activity and membrane integrity causing an efflux of solutes from the cell.

Enterobacter aerogenes grown under iron deprivation with and without a citrate supplement produced normal cell growth but decreased levels of exopolysaccharide. Citrate supplemented cultures gave a higher yield than iron deprived cells alone and it seems probable that this was not due to the increased carbohydrate source (since cell growth was unaffected) but a result of the stimulation of a citrate-dependent iron uptake system giving the cells greater ability to scavenge iron present in the form of contamination from culture vessel and basal medium chemicals.

Lin and Tseng (1979) deprived cultures of *X.oryzae* of iron and exopolysaccharide yield fell to 75% while cell growth was largely unaffected. *X.oryzae* may have a highly developed iron uptake mechanism.

Unlike Fe^{2+} , Cu^{2+} is not an essential ion and has a highly toxic action on the cell. Increasing Cu^{2+} concentration in the medium was reflected in decreasing exopolysaccharide yield. Such a result could be mediated by an increasing concentration of copper traversing the membrane by matrix protein pores (as

demonstrated in *E. coli* by Lutkenhaus, 1977) and inhibiting exopolysaccharide synthesising enzymes at the cytoplasmic membrane. Lin and Tseng (1979) using CuSO_4 as a Cu^{2+} source reduced exopolysaccharide production in *X. oryzae*. Zinc salts had the same effect.

Increasingly, continuous culture has been used to determine the effect of nutrient limitations on exopolysaccharide production.

Xanthan production in continuous culture was favoured by a high carbon: nitrogen ratio and controlled pH (at pH7) (Moraine and Rogovin, 1973) as seen in batch culture. The required concentration of substrate in the medium was significantly lower in continuous culture than in batch culture (Silman and Rogovin, 1972). The effect of phosphate-limitation of both batch and continuous culture cells on *Pseudomonas* NCIB11264 exopolysaccharide production was identical, reducing the amount of polymer formed (Williams and Wimpenny, 1978).

Evans *et al* (1979) using *Xanthomonas juglandis* in continuous culture studies obtained similar results to those reported here for ion-limitation. Interestingly, under sulphate limitation in broth, different strains of *Xanthomonas juglandis* gave a variety of yields, several at variance with those obtained in solid medium.

SDS, sodium lauryl sarcosinate and penicillin at non-lethal concentrations did not significantly alter the exopolysaccharide yield in Y.E. medium, indicating that at the concentrations employed the former agents did not affect the enzymes synthesising exopolysaccharide. Penicillin is not known to antagonise this cellular function directly, but its multiplicity of effects at higher levels might be expected to influence polysaccharide production.

In *Enterobacter aerogenes* the lowering of the exopolysaccharide yield by phenylethanol possibly reflected changes in the permeability barrier (i.e. membrane organisation) and a minor effect on the localisation of synthetic enzymes. The alteration in synthetic ability was only fractionally reduced compared to ethanol, a reflection of the wide-ranging action of ethanol.

The high concentration of ethanol used to supplement Y.E. medium, although not inhibitory to growth, diminished exopolysaccharide yields to a negligible level. The ethanol probably acted directly on the exopolysaccharide synthetic enzymes at the cytoplasmic membrane. The glycoside transferases which Lomax *et al* (1973) suggested to be highly hydrophobic molecules could then be detached from the membrane. Interruption of the polysaccharide synthetic capability of the cell at such an early stage could substantially reduce the amount of polymer produced.

Sucrose and NaCl both reduced the exopolysaccharide yield by similar margins. Since neither agent would affect the enzyme-synthetic ability of the cells directly, a more indirect mechanism may be through reduced solute uptake resulting from a change in the molecular composition of the outer membrane: reduction in the porin protein.

The low molecular weight species of polysaccharide isolated from the supernatant fluid of cultures 7824 and 7824V was chromatographically identical. Both were comprised of glucose but on partial hydrolysis did not yield any information on possible glucose oligomeric structures. A slow migrating spot (Fig. 30 ; Rf 0.86) may have represented heptose derived from lipopolysaccharide. R-form lipopolysaccharides have reduced water solubility (Wilkinson, 1977) but would not precipitate from solution on treatment of the culture supernate with acetone during exopolysaccharide precipitation because of the short polysaccharide chain length.

If the low molecular weight polysaccharide was a rough lipopolysaccharide present in both 7824 and 7824V it was noteworthy that the elution profiles of the two polysaccharides differed in the percentage of 60K and 30K chains present but that these species represented the major part of the non-acetone precipitable material.

Low molecular weight polysaccharides have been detected in *Rhizobia* spp. Zevenhuizen *et al*, (1979) reported a β 1-2 linked glucan.

OUTER MEMBRANE PROTEIN AND EXOPOLYSACCHARIDE

Basic to most of the methods of examining the membrane protein used in this work were two procedures. 1, Production of sphaeroplasts by exposing the cells to lysozyme in the presence of EDTA and Tris buffer. 2, Mechanical breakage of the cell and subsequent extraction of membrane proteins. Both procedures may cause differences in the final membrane preparation.

In 1967, Voss elucidated the role of organic cations such as Tris (hydroxymethyl) aminomethane in modifying the permeability of the cell envelope (Voss, 1967). Irvin *et al* (1981a) confirmed these findings and in an accompanying paper (Irvin *et al*, 1981b) indicated that a deep-rough mutant of *E. coli* 08 released sections of the outer membrane when pre-treated with EDTA or citrate.

A large fund of literature exists on the effect of EDTA on the cell envelope (Leive, 1965 and 1968; Schnaitman, 1971; Hardaway and Buller, 1979).

Thus both compounds can affect the membrane preparation produced by sphaeroplasting.

Mechanical breakage of the cells by French Press or shearing (part of the Lithium acetate Method) produced membrane preparations with increased levels of 50K and 58K protein, particularly the latter. The 58K protein was absent from outer membrane prepared by sucrose gradient density centrifugation of sphaeroplast membranes but present in sarkosyl prepared outer membrane.

Lugtenberg *et al* (1976) found an *E. coli* K12 strain possessed two outer membrane bands 58K and 50K corresponding to flagellin and pilin, respectively.

Direct observation of the *Enterobacter* strains by electron microscopy and by standard staining procedures did not indicate flagella. The strains were non-motile.

Parton (1975) identified a 54K band in cell envelopes of *S. minnesota* which was partly due to flagellin protein. The presence of a co-migratory protein in the cell envelope could not be ruled out.

Capsulate cells apparently lacked the 58K band, whereas in slime-forming mutants it could be enhanced as a percentage of the outer membrane protein dependent on the method of preparation, Similar treatment of capsulate cultures did not affect the membrane protein profile.

Protection of the 58K band by capsule, from extraction (by shearing) in the Lithium acetate procedure seems improbable as the 50K band was extracted at an increased level in capsulate cells.

Possibly these proteins effect a role in exopolysaccharide production. In cells possessing 50K protein, exopolysaccharide is bound to the cell surface. Mutation to slime-forming leads to a loss of 50K protein and the appearance of 58K. This suggests a receptor role for 50K in binding exopolysaccharide. No evidence for a protein such as the 40K protein of *E. coli*, which was implicated in the control of the colanic acid synthetic system, was found. Although 41K protein of *Enterobacter aerogenes* outer membrane varied slightly in capsulate, SI and 'O' strains, total loss of the protein did not affect exopolysaccharide production or capsulation.

The work presented here indicates the extent to which the composition of *Enterobacter aerogenes* cell surface can vary in response to physiological and genetic changes and the difficulties encountered when attempting to relate a *cause* to a single *effect* in such an organelle.

REFERENCES

- Achtman, M. (1979) in "Bacterial Outer Membranes" (ed. M. Inouye). chapter 11, Wiley, N.Y.
- Achtman, M., Kennedy, N., and Skurray, R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5104-5108.
- Achtman, M. Manning, P. A., Edelbluth, C. and Herrlich, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 4837-41.
- Ames, G. F-L. (1974) J. Biol. Chem. 249, 634.
- Ames, G. F-L., Spudich, E.N. and Nikaido, H. (1974). J. Bact. 117, 406-416.
- Argast, M. & Boos, W. (1980). J. Bact. 143, 142-150.
- Ballou, C. (1976) in Adv. Microbial Physiol. 14, 93-158.
- Bassford, P. J., Bradbeer, C., Kadner, R. J. and Schnaitman, C. A. (1976), J. Bact. 128, 242-247.
- Bassford, P. J., Schaitman, C.A., and Kadner, R. J. (1977). J. Bact. 130, 750-758.
- Bayer, M. (1968) J. Virol 2, 346-356.
- Bayer, M. (1968) J. Virol 2, 346-356.
- Bayer, M. E. (1975) in "Membrane Biogenesis" (ed. Tzagoloff), Plenum Press, N.Y., pp 393-427.
- Bayer, M. (1979) in "Bacterial Outer Membranes" (ed M. Inouye) Chapter 6 Wiley, N.Y.
- Bayer, M. E., and Thurow, H. (1977) J. Bact. 130, 911-936.
- Bayer, M. E., Koplw, J. and Goldfine, H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 5145.
- Barker, S. A., Bourne, E. J. and Theander, O. (1957) J. Chem. Soc., 2064.
- Begg, K. J. (1978) J. Bact. 135, 307-310.
- Bell, R. M., Mavis, R. D., Osborn, M. J. and Vagelos, P. R. (1971) Biochim. biophys. Acta. 249, 628-635.
- Bjorndal, H., Lindberg, B., Lönngren, J., Nilsson, K., Nimmich, W. (1972) Acta. Chem. Scand. 26, 1269-1273.
- Bjorndal, H., Lindberg, B. and Nimmich, W. (1970) Acta. Chem. Scand. 24, 3414-3415.
- Bjorndal, H., Lindberg, B., and Nimmich, W. (1971) Acta. Chem. Scand. 25, 750-752.

- Blobell, G., and Dobberstein, D. (1975) . J. Cell Biol 67, 835-851.
- Blobell, G. and Sabatini, D. D. (1971) in "Biomembranes" (ed. L. E. Manson) Vol. 2, pp 193-195, Plenum Press, N.Y.
- Bloch, K. and Vance, D. (1977) Ann. Rev. Biochem 46, 263-298.
- Boehler-Kohler, B. A., Boos, W., Dieterle, R., Benz, R. (1979) J. Bact. 138, 33-39.
- Bosch, V. and Braun, V. (1973) FEBS Letts. 34, 307-310.
- Both, G. W., McInnes, J. C., Hanlon, J. E., May, B. K., Elliott, W. H. (1972) J. Molec. Biol. 67, 199-217.
- Botta, G. A., and Park, J. T. (1981) J. Bact. 145, 33-340.
- Boyd, A., and Holland, I. B. (1977) FEBS Letts 76, 20-42.
- Boylen, C. W. and Ensign, J. C. (1968) J. Bact. 96, 421.
- Bradbeer, C., Woodrow, M. L., and Khalifah, L. I. (1976), J. Bact. 125, 1032-1039.
- Bragg, P. D., and Hou, C. (1972) Biochim. biophys. Acta 274, 478-488.
- Braun, V. (1973) J. Infect. Dis. 128, 9-15.
- Braun, V. (1975) Biochem. biophys. acta 415, 335-377.
- Braun, V. and Bosch, V. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 970-974.
- Braun, V. and Bosch, V. (1972b) Eur. J. Biochem 28, 51-69.
- Braun, V., and Bosch, V. (1973) FEBS Letts. 34, 302.
- Braun, V., Bosch, V., Hantke, K., and Schaller, K. (1974) Annals N.Y. Acad. Sci. 235, 66-82.
- Braun, V., and Hantke, K. (1977) in "Microbial Interactions", Vol 3 (ed. J. L. Reissig) pp 99-138, Chapman and Hall, London.
- Braun, V., and Krieger-Brauer, H.-J. (1977) Biochim. biophys. acta. 469, 89-98.
- Braun, V. and Rehn, K. (1969) Eur. J. Biochem. 10, 426-438.
- Braun, V. Rehn, K. and Wolff, H. (1970) Biochemistry, 9, 5041.
- Braun, V. and Sieglin, U. (1970) Eur. J. Biochem 13, 336-346.
- Braun, V., and Wolff, H. (1973) FEBS Letts. 34, 77-80.
- Buller, C. S., Vander Maten, M., Faurot, D. and Nelson, E. T. (1975). J. Virol 15, 1141-1147.
- Burdett, I. D. J. and Murray, R. G. E. (1974). J. Bact. 119, 1039-56.
- Burdett, I. D. J. and Rogers, H. J. (1970) J. Ultrastructure Res. 30, 354-367.
- Burnett, J. H. (1979) in "Fungal Walls and Hyphal Growth" (ed. J. H. Burnett and A. P. J. Trinci) pp1-25, Camb. Univ. Press.
- Buttke, T. M. and Ingram, L. O. (1980) Arch. Biochem. Biophys. 203, 565-71.
- Buxton, R. S. (1971) Molec. Gen. Genet. 113, 154-156.

- Campbell, J. N. and Blobell, G. (1976) *FEBS Letts* 72, 215-224.
- Chai, T.-J. and Foulds, J. (1974) *J. Molec. Biol.* 85, 465-474.
- Chai, T.-J. and Foulds, J. (1979) *J. Bact.* 137, 226-233.
- Chatterjee, A. N. and Young, F. E. (1972). *J. Bact.* 111, 220-230.
- Chattopadhyay, P. K. and Wu, H. C. (1977). *Proc. Natl. Acad. Sci., U.S.A.*, 74, 5318-5322.
- Chen, R., Schmidmayer, W., Kramer, C., Chen-Schmeisser, U., and Henning, U. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 4592-96.
- Cheng, E. and Inouye, M. (1977) *Biochim. biophys. acta.* 465, 650-656.
- Cheng, S., Thomas, J. K. and Kulpa, C. F. (1974). *Biochemistry*, 13, 1135-1139.
- Conrad, H. E., Bamburg, J. R., Epsley, J. D. and Kindt, T. J. (1966) *Biochemistry* 5, 2808-2817.
- Costerton, J. W., Ingram, J. M., and Cheng, K.-J. (1974) *Bact. Rev.* 38, 87-110.
- Cox, G. B., Gibson, F., Luke, R. K. J., Newton, N. A., O'Brien, I. G. and Rosenberg, H. (1970) *J. Bact.* 104, 219-226.
- Cronan, J. E. Jr. (1978) *Ann. Rev. Biochem.* 47, 163-189.
- Cronan, J. E. Jr. (1979) in "Bacterial Outer Membranes" (ed. M. Inouye) Wiley, N.Y. PP 35-66
- Cronan, J. E. Jr., and Godson, G. N. (1972) *Mol. Gen. Genet* 116, 199-210.
- Cronan, J. E. Jr., Weisberg, L. J. and Allen, R. G. (1975) *J. Biol Chem.* 250, 5835-5840.
- Curvall, M., Lindberg, B., Lönnngren, J., Ruden, U., and Nimmich, W. (1973) *Acta. Chem. Scand.* 27, 4019-4021.
- Dame, J. B. and Shapiro, B. M. (1979) *J. Bact.* 137, 1043-1047.
- Darveau, R. P., Charnetzky, W. T. and Hulbert, R. E. (1980) *J. Bact.* 143, 942-949.
- Datta, D. B., Arden, B. and Henning, U. (1977) *J. Bact.* 131, 821-829.
- Datta, D. B., Kramer, C., and Henning, U. (1976) *J. Bact.* 128, 834-841.
- Davies, D. A. L. (1955). *Biochem, J.* 59, 696-704.
- Davies, J. K. and Reeves, P. (1975) *J. Bact.* 123, 102-117.
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404.
- Decad, G., Nakae, T. and Nikaido, H. (1974) *Fedn. Proc.* 33, 1240.
- Decad, G. and Nikaido, H. (1976). *J. Bact.* 128, 325-336.
- De Siervo, A. J. and Salton, M. R. J. (1971). *Biochim. biophys. acta.* 239, 280-292.
- De Siervo, A. J. and Salton, M. R. J. (1973) *Microbios* 8, 73.
- Devillers-Thiery, A., Kindt, T., Scheele, G. and Blobell, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.*, 72, 5016-5020.
- DeVoe, I. W. and Gilchrist, J. E. (1976) *J. Bact.* 128, 144.

- Dicks, J. W. and Tempest, D. W. (1967) *Biochim. biophys. acta* *136*, 176-179.
- Di Masi, D. R., White, J. C., Schnaitman, C. A. and Bradbeer, C. (1973) *J. Bact.* *115*, 506-513.
- Di Rienzo, J. M. and MacLeod, R. A. (1978) *J. Bact.* *136*, 158-167.
- Di Rienzo, J. M., Deneke, C. F., MacLeod, R. A. (1978). *J. Bact.* *136*, 148-157.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* *28*, 350-356.
- Dudman, W. F. (1960). *J. gen. Microbiol*, *22*, 25-39.
- Duguid, J. P. (1951) *J. Path. Bact.* *63*, 673-685.
- Duguid, J. P. and Wilkinson, J.F. (1953) *J. gen. Microbiol* *9*, 174-189.
- Duguid, J. P. and Wilkinson, J. F. (1954) *J. gen. Microbiol* *11*, 71-72.
- Dutt, A. and Dowhan, W. (1977) *J. Bact.* *132*, 159-165.
- Dutton, G. G.S., Stephen, A. M. and Churms, S. C. (1974). *Carbohyd. Res.* *38*, 225-237.
- Edgar, J.R., and Bell, R.M. (1977) *Fed. Proc.* *36*, 857.
- Ellwood, D. C. (1970) *Biochem. J.*, *118*, 367.
- Emr, S. D., Clement, J. M., Silhavy, T. J., Hedgpeth, J. and Hofnung, M. (1980). *Nature* *285*, 82-85.
- Endermann, R., Kramer, C., and Henning, U. (1978) *FEBS Letts* *86*, 20-24.
- Erbing, C., Kenne, L., Lindberg, B., Löngren, J. and Sutherland, I. W. (1976) *Carbohyd. Res.* *50*, 115-120.
- Erbing, C., Lindberg, B., Löngren, J., and Nimmich, W. (1977) *Carbohyd. Res.* *56*, 377-381.
- Evans, C. G. T., Yeo, R. G., and Ellwood, D. C. (1979) in "Microbial Polysaccharides and Polysaccharases" pp 51-64 (ed. Berkeley, R. C. W., Gooday, G. W. and Ellwood, D. C.) Academic Press, London.
- Ferrazza, D. and Levy, S. B. (1980). *J. Bact.* *144*, 149-158.
- Filip, C., Fletcher, G., Wulff, J. L., and Earhart, C. F. (1973) *J. Bact.* *115*, 717-722.
- Finnerty, W. R. (1978) in *Adv. Microbial Physiol.* *18*, 177-233.
- Fiss, E. H., Hollifield, W. C. Jr., and Neilands, J. B. (1979) *Biochem. Biophys. Res. Comm.* *91*, 29-34.
- Fontana, R., Canepari, P. and Satta, G. (1979) *J. Bact.* *139*, 1028-1038.
- Fordham, W. D. and Gilvarg, C. (1974) *J. Biol. Chem.* *249*, 2478-2482.
- Frasch, C. (1976) *Dialog.* published by Amicon Corp., Lexington, Mass., U.S.A.
- Fredericq, P. (1949) *C.r. Soc. Biol.* *143*, 1014-1017.
- Fredericq, P. and Smarda, J. (1970) *Ann. Inst. Pasteur* *118*, 767-774.
- Frost, G. E. and Rosenberg, H. (1975) *J. Bact.* *124*, 704-712.
- Funahara, Y., and Nikaido, H. (1980). *J. Bact.* *141*, 1463-1465.

- Gallant, J., Palmer, L. and Pao, C. C. (1977). *Cell* 11, 181-185.
- Gallin, J. I., and O'Leary, W. M. (1968) *J. Bact.* 96, 660-664.
- Garegg, P. J., Lindberg, B., Onn, T., Holme, T. (1971). *Acta. Chem. Scand.* 25, 1185-1194.
- Gayda, R. C., Auni, H., Berg, P. F. & Markovitz, A. (1979a) *Mol. Gen. Genet.* 175, 325-332.
- Gayda, R. C., Henderson, G. W. and Markovitz, A. (1979b) *Proc. Natl. Acad. Sc., U.S.A.* 76, 2138-2142.
- Ghuysen, J.-M. (1968) *Bact. Rev.* 32, 425-464.
- Giesbrecht, P., Wecke, J., Reinicke, B., and Tesche, B. (1977) *Arch. Microbiol* 115, 25-35.
- Glaser, L. (1958) *J. Biol. Chem.* 232, 627.
- Glaser, M., Nulty, W., and Vagelos, P. R. (1975) *J. Bact.* 123, 128-136.
- Glauert, A. M., and Thornley, M. J. (1969) *Ann. Rev. Microbiol.* 23, 159-198.
- Gmeiner, J. (1981) *Arch. Microbiol.* 128, 299-302.
- Golden, N. G., and Powell, G. L. (1972) *J. Biol. Chem.* 247, 6651-6658.
- Gooday, G. W., and Trinci, A. P. J. (1980) in "The Eukaryotic Microbial Cell" (ed. Gooday, Lloyd and Trinci). *Soc. gen. Microbiol. C.U.P.*
- Grant, W. D., Sutherland, I. W., and Wilkinson, J. F. (1969) *J. Bact.* 100, 1187-1193.
- Gudas, L. J., James, R., and Pardie, A. B. (1976) *J. Biol. Chem.* 251, 3470-3479.
- Guterman, S. K. and Dann, L. (1973) *J. Bact.* 114, 1225-1230.
- Guterman, S. K. and Luria, S. E. (1969) *Science* 164, 1414.
-
- Halegoua, S., Hirashima, A. and Inouye, M. (1974) *J. Bact.* 120, 1204-1208.
- Halegoua, S., and Inouye, M. (1979) *J. Molec. Biol.* 130, 39-61.
- Hancock, R. E. W., Hantke, K., and Braun, V. (1976) *J. Bact.* 127, 1370-1375.
- Hantke, K. (1976) *FEBS Letts.* 70, 109-112.
- Hantke, K. (1978) *Mol. Gen. Genet.* 164, 131-135.
- Hantke, K., and Braun, V. (1973) *Eur. J. Biochem.* 34, 284-296,
- Hantke, K., and Braun, V. (1975a) *FEBS Letts.* 59, 277-281.
- Hantke, K., and Braun, V. (1975b) *FEBS Letts.* 49, 301-305.
- Hardaway, K. L. and Buller, C. S. (1979) *J. Bact* 137, 62-68.
- Harold, F. M. (1970) *Adv. Microbial. Physiol.* 4, 45-104.
- Hasegawa, Y., Yamada, H., and Mizushima, S. (1976) *J. Biochem, Tokyo*, 80, 1401.
- Hassan, H. M. (1976) *J. Bact.* 125, 910-915.
- Hazumi, N., Yamada, H., and Mizushima, S. (1978) *FEMS. Microbiol Letts.* 4, 275-277.
- Heckels, J. E. (1981) *J. Bact.* 145, 736-742.

- Henning, U. and Haller, I. (1975) *FEBS Letts.* **55**, 161-164.
- Henning, U., Hoehn, B., and Sonntag, I. (1973b) *Eur. J. Biochem* **39**, 27-36.
- Henning, U., Rehn, K., and Hohn, B. (1973a) *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2033.
- Henning, U., Royer, H., Teather, R. M., Hindennach, I., and Hollenberg, C. P. (1979) *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4360-4364.
- Henning, U., Sonntag, I., and Hindennach, I. (1978) *Eur. J. Biochem.*, **92**, 491-498.
- Hirashima, A., Childs, G., and Inouye, M. (1973) *J. Molec. Biol.* **79**, 373-389.
- Hirashima, A. and Inouye, M. (1973) *Nature* **242**, 405-407.
- Hirashima, A. and Inouye, M. (1975) *Eur. J. Biochem* **60**, 395-398.
- Hirota, Y., Suzuki, H., Nishimura, Y. and Yasuda, S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1417-1420.
- Hirschberg, C. B. and Kennedy, E. P. (1972). *Proc. Natl. Acad. Sci., U.S.A.*, **69**, 648-651.
- Hofstra, H., and Dankert, J. (1978) *J. gen Microbiol* **104**, 311-320.
- Hofstra, H., and Dankert, J. (1979) *J. gen. Microbiol.* **111**, 293-302.
- Hofstra, H., and Dankert, J. (1980) *J. gen Microbiol.* **119**, 123-131.
- Hofstra, H., van Tol, M. J. D., and Dankert, J. (1980) *J. Bact.* **143**, 328-337.
- Hollifield W. C. Jr., and Neilands, J. B. (1978) *Biochemistry* **17**, 1922-1928.
- Hudson, H. P., Lindberg, A. A. and Stocker, B. A. D. (1978) *J. gen Microbiol* **109**, 97-112.
- Ichihara, S. and Mizushima, S. (1978) *J. Biochem., Tokyo* **83**, 1095.
- Ingram, L. O. and Vreeland, N. S. (1980) *J. Bact.* **144**, 481-488.
- Inouye, M. (1974). *Proc. Natl. Acad. Sci., U.S.A.*, **71**, 2396-2400.
- Inouye, M. (1975) in "Membrane Biogenesis" (ed. A. Tzagoloff) pp. 351-391, Plenum Press, N.Y.
- Inouye, M., and Beckwith, J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1440-44.
- Inouye, M., Shaw, J., and Shen, C. (1972) *J. Biol. Chem.* **247**, 8154-8159.
- Inouye, S., Wang, S., Sekizawa, J., Halegoua, S., and Inouye, M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1004-1008.
- Irvin, R. T., MacAlister, T. J., Chan, R., and Costerton, J. W. (1981b) *J. Bact.* **145**, 1386-1396.
- Irvin, R. T., MacAlister, T. J., Costerton, J. W. (1981a) *J. Bact.* **145**, 1397-1403.
- Ishii, J., and Nakae, T. (1980) *J. Bact.* **142**, 27-31.
- Jackson, R. W., and De Moss, J. A. (1965) *J. Bact.* **90**, 1420-1425.

- James, R. (1975) *J. Bact.* **124**, 918.
- Jann, K., and Jann, B. (1977) in "Surface Carbohydrates of the Prokaryotic Cell" pp 247-288 (ed. I. W. Sutherland) Academic Press, London.
- Jasper, P. E., Whitney, E., and Silver, S. (1972) *Genet. Res.* **19**, 305-312.
- Johansson, V., Aarti, A., Nurminen, M., and Makela, P. H. (1978) *J. gen. Microbiol.* **107**, 183-187.
- Jones, N. C., and Osborn, M. J. (1977a) *J. Biol. Chem.* **252**, 7398-7044.
- Jones, N. C., and Osborn, M. J. (1977b) *J. Biol. Chem.* **252**, 7405-7412.
- Joseleau-Petit, D., and Kepes, A. (1975) *Biochim. biophys. acta.* **406**, 36-39.
- Kamio, Y., and Nikaido, H. (1976) *Biochemistry* **15**, 2561.
- Kanazawa, H., and Wu, H. C. (1979) *J. Bact.* **137**, 818-823.
- Kanfer, J. and Kennedy, E. P. (1964) *J. Biol. Chem.* **239**, 1720.
- Katz, E., Loring, D., Inouye, S., and Inouye, M. (1978) *J. Bact.* **134**, 674-676.
- Kawaji, H., Mizuno, T. and Mizushima, S. (1979) *J. Bact.* **140**, 843-847.
- Kennedy, N., Beutin, L., Achtman, M., Skurray, R., Rahmsdorf, U. and Herrlich, P. (1977) *Nature* **270**, 580-585.
- Kennedy, E. P., Rumley, M. K., Schulman, H., van Golde, L. M. G. (1976) *J. Biol. Chem.* **257**, 4208-13
- Kennell, D., and Magasanik, B. (1962) *Biochim. biophys. acta.* **55**, 139.
- Kito, M., Aihara, S., Kato, S., and Hata, T. (1972) *Agric. Biol. Chem.* **36**, 837-844.
- Konings, R. N. A., Huselbos, T. van den Hondel, L. A. (1975) *J. Virol.* **15**, 570-584.
- Konisky, J. (1979) in "Bacterial Outer Membranes" (ed. M. Inouye) pp. 319-60 Wiley, N.Y.
- Koplow, J., and Golfine, H. (1974) *J. Bact.* **117**, 527-543.
- Kulpa, C. F. Jr., and Leive, L. (1972) in "Membrane Research" (ed. C. F. Fox) p155, Academic Press, London.
- Kulpa, C. F. Jr., and Leive, L. (1976) *J. Bact.* **126**, 467.
- Kushner, D. J. (1971) in "Inhibition and Destruction of the Microbial Cell" (ed. W. B. Hugo) Academic Press, London.
- Lai, J-S., Philbrick, W. A., Hayashi, S., Inukai, M., Arai, M., Hirota, Y., and Wu, H. C. (1981) *J. Bact.* **145**, 657-660.
- Lai, J-S., Wu, H. C. (1980) *J. Bact.* **144**, 451-453.

- Langman, L., Young, I. G., Frost, G. E., Rosenberg, H. and Gibson, F. (1972) *J. Bact.* **112**, 1142-1149.
- Lankford, C.E., Hoyo, H. & Lutteringer, J.F. (1951) *J. Bact.* **62**, 621
- Lazdunski, C. and Shapiro, B. M. (1972). *J. Bact.* **111**, 499-509.
- Le Dur, A., Chaby, R. & Szabo, L. (1980) *J. Bact.* **143**, 78-88.
- Lee, N., Cheng, E., and Inouye, M. (1977) *Biochim. biophys. acta.* **465**, 650-656.
- Lee, N., & Inouye, M. (1974) *FEBS Letts.* **39**, 167-170.
- Lee, N., Scandella, C., & Inouye, M. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 127-30.
- Lee, D. R. and Schnaitman, C. A. (1980) *J. Bact.* **142**, 1019-1022.
- Lee, D. R., Schnaitman, C. A., and Pugsley, A. P. (1979) *J. Bact.* **138**, 861-870.
- Lehmann, V. (1977) *Eur. J. Biochem* **75**, 257-266.
- Lehmann, V. Redmond, J. Egan, A. and Minner, I. (1978). *Eur. J. Biochem*, **86**, 487-496.
- Leive, L. (1965) *Biochem. Biophys. Res. Commun.* **21**, 290-296.
- Leive, L. (1968) *J. Biol. Chem.* **243**, 2373-2380.
- Leive, L., Showlin, V. K., Mergenhagen, S. E. (1968) *J. Biol. Chem.* **243**, 6384.
- Levy, S. B. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2900-2904.
- Lilley, B. O., and Brewer, J. H. (1953) *J. Am. Pharm. Assoc. Sci. Ed.* **43**, 6.
- Lin, H-M and Tseng, Y-H (1979). *Proceedings of the National Science Council Vol. 3*, 279-284 (National Science Council, Taipei, Taiwan, Republic of China).
- Lin, J. J-C., Giam, C. Z., and Wu, H. C. (1980) *J. Biol. Chem.* **255**, 807-811.
- Lin, J. J-C., Kanazawa, H., Ozols, J., Wu, H. C. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4891-95.
- Lin, J. J-C., Kanazawa, H., and Wu, H. C. (1980a) *J. Biol. Chem.* **225**, 1160-1163.
- Lin, J. J-C., Kanazawa, H., and Wu, H. C. (1980b) *J. Bact.* **141**, 550-557.
- Lin, J. J-C., and Wu, H. C. (1976) *J. Bact.* **125**, 892-904.
- Lin, J. J. C. and Wu, H. C. (1980). *J. Biol. Chem.*, **255**, 802-806.
- Lindberg, B., Lönngren, J. and Nimmich, W. (1972a) *Carbohydr. Res.* **23**, 47-55.
- Lindberg, B., Lönngren, J. and Nimmich, W. (1972b) *Acta. Chem. Scand.* **26**, 2231-2236.
- Lindberg, B., Lönngren, J. Nimmich, W. and Ruden, U. (1973) *Acta. Chem. Scand.* **27**, 3787-3790.
- Lindberg, B., Lönngren, J., Ruden, U. & Nimmich, W. (1975) *Carbohydr. Res.* **42**, 83-93.
- Loeb, M., and Kilner, J. (1979) *J. Bact.* **137**, 1031-1034.
- Lomax, J. A., Poxton, I. R. and Sutherland, I. W. (1973) *FEBS Letts.* **34**, 232-234.

- Nakae, T. (1976a) *J. Biol. Chem.* **251**, 2176-78.
- Nakae, T. (1976b) *Biochem. Biophys. Res. Comm.* **71**, 877-884.
- Nakae, T. (1979) *Biochem. Biophys. Res. Comm.* **88**, 774-781.
- Nakae, T., and Ishii, J. (1980) *J. Bact.* **142**, 735-740.
- Nakae, T., Ishii, J., and Tokunaga, M. (1979) *J. Biol. Chem.* **254**, 1457-1461.
- Nakamura, H. (1967) *J. Bact.* **93**, 1183-1184.
- Nakamura, H. (1968) *J. Bact.* **96**, 987-996.
- Nakamura, K., and Mizushima, S. (1976) *J. Biochem. Tokyo* **80**, 1411-1422.
- Nakamura, K., Pirtle, R. M., and Inouye, M. (1979) *J. Bact.* **137**, 595-604.
- Nicas, T. I. and Hancock, R. E. W. (1980) *J. Bact.* **143**, 872-878.
- Nikaido, H. (1973) in "Bacterial Walls and Membranes" (L. Leive, ed) pp 131-208, Marcel Dekker, N.Y.
- Nikaido, H., and Nakae, T. (1979) *Adv. Microbial Physiol* **20**, 163-250.
- Nikaido, H., Song, S. A., Shaltiel, L., and Nurminen, M. (1976) *J. Bact.* **132**, 324-330.
- Nikaido, H., Takeuchi, Y., Ohnishi, S-I., and Nakae, T. (1977) *Biochim. biophys. acta.* **465**, 152.
- Nimmich, W., and Korten, G. (1970) *Pathologia et Microbiologia* **36**, 179-190.
- Nishijima, M., Nakaike, S., Tamori, Y., and Nijima, S. (1977) *Eur. J. Biochem.* **73**, 115.
- Norval, M. (1969) Ph.D. Thesis, University of Edinburgh.
- Norval, M., and Sutherland, I. W. (1969) *J. gen. Microbiol.* **57**, 369-377.
- Nunn, W. D., and Cronan, J. E. Jr. (1974) *J. Biol. Chem.* **249**, 3994-3996.
- Nunn, W. D. and Cronan, J. E. Jr. (1976a) *J. Molec. Biol.* **102**, 167-172.
- Nunn, W. D., and Cronan, J. E. Jr. (1976b) *Biochemistry* **15**, 2546-2550.
- Nunn, W. D., Kelly, D. L., and Stumfall, M. Y. (1977) *J. Bact.* **132**, 526-531.
- Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) *Analytical Biochemistry* **105**, 361-363.
- Ohki, M. (1979) in "Bacterial Outer Membranes: Biogenesis and Functions" (ed. M. Inouye). Wiley, N.Y.
- Ohta, A., Shibuya, I., Maruo, B., Ishinaga, M. and Kita, M. (1974) *Biochim. biophys. acta.* **348**, 449-454.
- Oldmixon, E. H., Dezétée, P., Ziskin, M. and Shockman, G. D. (1976) *Eur. J. Biochem* **68**, 271-280.
- Osborn, M. J. (1963). *Proc. Natl. Acad. Sci., U.S.A.*, **50**, 499-506.
- Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247**, 3962-3972.
- Osborn, M. J., and Wu, H. C. (1980) *Ann. Rev. Microbial* **34**, 369-422.
- Overath, P., Brenner, M., Gulik-Krzywicki, T., Shechter, E., and Letellier, I. (1975) *Biochim. biophys. acta.* **389**, 358.
- Overbeeke, N., and Lugtenberg, B. (1980) *FEBS Letts.* **112**, 229-232.
- Overbeeke, N., Van Scharrenburg, G., and Lugtenberg, B. (1980) *Eur. J. Biochem* **110**, 247-254.

- Paakkanen, J., and Gotschlich, E. C. and Makela, P-H. (1979) *J. Bact.* 139, 835-841.
- Palva, E. T. (1978) *J. Bact.* 136, 286-294.
- Palva, E. T. and Randall, L. L. (1978) *J. Bact.* 133, 279-286.
- Palva, E. T., and Randall, L. L. (1979) *J. Bact.* 138, 254-256.
- Parton, R. (1975) *J. gen. Microbiol.* 89, 113-123.
- Pate, J. L., and Ordal, J. E. (1967) *J. Cell Biol.* 35, 37-44.
- Poxton, I. R. (1974) *Ph.D. Thesis, University of Edinburgh.*
- Pugsley, A. P. and Schnaitman, C. A. (1978). *J. Bact.* 135, 1118-1129.
-
- Raetz, C. R. H. (1978) *Microbiol. Rev.* 42, 614-659.
- Raetz, C. R. H., Kennedy, E. P. (1973) *J. Biol. Chem.* 248, 1098.
- Randall, L. L., and Hardy, S. J. S. (1975) *Molec. Gen. Genet.* 137, 151-160.
- Randall, L. L., and Hardy, S. J. S. (1977) *Eur. J. Biochem* 75, 43-53.
- Randall, L. L., Hardy, S. J. S. and Josefsson, L. G. (1978) *Proc. Natl. Acad. Sci.* 75, 1209-1212.
- Ray, T. K., Godson, G. N., and Cronan, J. E. Jr. (1976) *J. Bact.* 125, 136-141.
- Reeves, P. (1979) in "Bacterial Outer Membranes: Biogenesis and Functions." (ed. M. Inouye) Wiley, N.Y.
- Reithmeier, R. A. F. and Bragg, P. G. (1977) *Biochim. biophys. acta.* 466, 245-256.
- Robinson, A. and Tempest, D. W. (1973). *J. gen Microbiol*, 78, 361-370.
- Rogers, H. J., McConnell M., and Hughes, R. C. (1971) *J. gen Microbiol.* 66, 297-308.
- Rogers, H. J., Perkins, H. R., Ward, J. B. (1980) Chapter 8 in "Microbial Cell Walls and Membranes." Chapman & Hall, London.
- Rogers, H. J. and Garrett, A. J. (1965) *Biochem J.* 96, 231.
- Romeo, D., Girard, A. Rothfield, L. (1970) *J. Molec. Biol.* 53, 475-490.
- Rose, A. H. (1978) in "Biochemistry and Genetics of Yeast." (ed. M. Bacila, B. L. Horecker, A. O. M. Stoppani). Academic Press, N.Y.
- Rosenberg, H., and Young, I. G. (1974) in "Microbial Iron Metabolism" (Neiland, J. B. ed) pp 67-82, Academic Press, N.Y.
- Rosenbusch, J. P. (1974) *J. Biol. Chem.* 249, 8019-8029.
- Rosner, M. R., and Khorana, G. H. (1979) *J. Biol. Chem.* 254, 5926-5933.
- Rosner, M. R., Tang, J., Barzilay, I., and Khorana, H. G. (1979) *J. Biol. Chem.* 254, 5906-5917.
- Rottem, S., and Leive, L. (1977) *J. Biol. Chem.* 252, 2077-2081.
- Russell, A. D. (1971) in "Inhibition and Destruction of the Microbial Cell" (ed. W. B. Hugo). Academic Press, London.

- Sabet, S. F., and Schnaitman, C. A. (1971) *J. Bact.* 108, 422-403.
- Sandermann, H., and Strominger, J. L. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 2441-2443.
- Sandermann, H., and Strominger, J. L. (1972) *J. Biol. Chem.* 247, 5123-5131.
- Satta, G., Fontana, R., Canepari, P., and Botta, G. (1979) *J. Bact.* 137, 727-34.
- Satta, G., Canepari, P., Botta, G., and Fontana, R. (1980) *J. Bact.* 142, 43-57.
- Sato, T., and Yura, T. (1979) *J. Bact.* 139, 468-477.
- Sato, T., and Yura, T. (1981). *J. Bact.* 145, 88-96.
- Scandella, C. J. and Kornberg, A. (1971) *Biochemistry*, 10, 4447.
- Schechter, I., McKean, D. J., Guyer, R., and Terry, W. (1975) *Science* 188, 100-2.
- Schindler, P. R. G. and Teuber, M. (1978). *J. Bact.*, 135, 198-206.
- Schleifer, K. H., Huss, L., and Kandler, O. (1969) *Arch. Mikrobiol* 68, 387.
- Schleifer, K. H., and Kandler, O. (1972) *Bact. Rev.* 36, 407-477.
- Schmitges, C. J. and Henning, U. (1976). *Eur. J. Biochem*, 63, 47-52.
- Schnaitman, C. A. (1970) *J. Bact.* 104, 890-901.
- Schnaitman, C. A. (1971) *J. Bact.* 108, 553-563.
- Schnaitman, C. A. (1974a) *J. Bact.* 118, 442-453.
- Schnaitman, C. A. (1974b) *J. Bact.* 118, 454-464.
- Schnaitman, C. A. and Pugsley, A. P. (1978) *J. Bact.* 135, 1118-1129.
- Schulman, H., and Kennedy, E. P. (1977) *J. Biol. Chem.* 252, 4250-4255.
- Schulman, H. and Kennedy, E. P. (1979). *J. Bact.* 137, 686-688.
- Schwarz, U., Asmus, A. and Frank, H. (1969) *J. Molec. Biol.* 41, 419-429.
- Schweizer, W. L. and Henning, U. (1977) *J. Bact.* 139, 1651-1652.
- Schweizer, M., Hindennach, I. Garten, W. and Henning, U. (1978) *Eur. J. Biochem.* 82, 211-217.
- Sekizawa, J., Inouye, S., Halegou, S., Inouye, M. (1977). *Biochem. Biophys. Res. Comm.* 77, 1126-1133.
- Sharon, N., Osawa, T., Flowers, H. M. and Jeanloz, R. W. (1966) *J. Biol. Chem.* 241, 223-230.
- Short, S. A. and White, D. C. (1972). *J. Bact.* 109, 820-826.
- Silhavy, T. J., Bassford, P. J. Jr., and Beckwith, J. R. (1979) in "Bacterial Outer Membranes: Biogenesis and Functions" (ed. M. Inouye) pp 203-54, Wiley.
- Silman, R. W., and Rogovin, S. P. (1972) *Biotechnology and Bioengineering* 14, 23-31.
- Skurray, R. A., Hancock, R. E. W. and Reeves, P. (1974) *J. Bact.* 119, 726-735.
- Smit, J., Kamio, Y., and Nikaido, H. (1975) *J. Bact.* 124, 942-958.
- Smith, W. P., Tai, P. C., Thompson, R. C. and Davis, B. B. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 2830-2834.
- Smith, W. P., Tai, P. C., and Davis, B. D. (1978) *Biochemistry* 18, 198-202.
- Sokawa, J., Nakao, E., ✕ Kaziro, Y. (1968) *Biochem. Biophys. Res. Comm.* 33, 198- 212.
- Sokawa, J., Sokawa, Y., ✕ Kaziro, Y. (1972) *Nature New Biology* 240, 242-245.

- Sonntag, I., Schwarz, H., Hirota, Y., and Henning, U. (1978) *J. Bact.* *136*, 280-285.
- Stan-Lotter, H., and Sanderson, K. E. (1981) *J. Bact.* *146*, 542-551.
- Steven, A. C., Ten Heggeler, B., Muller, R., Kistler, J., Rosenbusch, J. P. (1977) *J. Cell. Biol.* *72*, 292-301.
- Stocker, B. A. D., & Makela, P. H. (1978) *Proc. Roy. Soc. Lond.* *202*, 5-30.
- Sullivan, K. H., Hegeman, G. D., & Cordes, E. H. (1979) *J. Bact.* *138*, 133-138.
- Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci., U.S.A.* *75*, 3737-41.
- Sutherland, I. W. and Norval, M. (1970) *Biochem. J.* *120*, 567-576.
- Sutherland, I. W. (1972) *Adv. Microbial Physiol.* *8*, 143-213.
- Sutherland, I. W. (1977a) (ed) "Surface Carbohydrates of the Prokaryotic Cell." Academic Press, London.
- Sutherland, I. W. (1977b) in "Extracellular Microbial Polysaccharides" (ed. P. A. Sandford & A. Laskin) pp 40-57. A.C.S. Symposium Series, Washington, D.C.
- Takeishi, K., Yasumura, M., Pirtle, R. and Inouye, M. (1976). *J. Biol. Chem.* *251*, 6259-6266.
- Tempest, D. W., Hunter, J. R., and Sykes, J. (1965) *J. gen Microbiol.* *39*, 355-366.
- Tokunaga, M., Tokunaga, H., Okajima, Y., Nakae, T. (1979) *Eur. J. Biochem.* *95*, 441-448.
- Tommassen, J., & Lugtenberg, B. (1980) *J. Bact.* *143*, 151-157.
- Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950) *Nature* *166*, 444-445.
- Troy, F. A. (1979) *Ann. Rev. Microbiol.* *33*, 519-560.
- Troy, F. A., Frerman, F. A. and Heath, E. C. (1971) *J. Biol. Chem.* *246*, 118-133.
- van Alphen, L., Havekes, L. & Lugtenberg, B. (1977) *FEBS Letts.* *75*, 285-290.
- van Alphen, W., and Lugtenberg, B. (1977) *J. Bact.* *131*, 623-630.
- van Alphen, L., Lugtenberg, B., van Boxtel, R. & Verhoef, K. (1977) *Biochim. biophys. acta.* *466*, 257-266.
- van Alphen, L., Verkleij, A., Leunissen-Bijvelt, J., Lugtenberg, B. (1978) *J. Bact.* *134*, 1089-1098.
- van Golde, L. M. G., Schulman, H. and Kennedy, E. P. (1973) *Proc. Natl. Acad. Sci. U.S.A.* *70*, 1368-1372.
- Varenne, S., Piovant, M., Pages, J. M. & Lazdunski, C. (1978) *Eur. J. Biochem.* *86*, 603-606.
- Volpe, J. J. and Vagelos, P. R. (1976) *Physiol. Rev.* *56*, 339-417.
- Voss, J. G. (1967) *J. gen. Microbiol.* *48*, 391-400.

- Wagegg, W., & Braun, V. (1981) *J. Bact.* 145, 156-163.
- Wandersman, C., Schwartz, M. & Ferenci, T. (1979) *J. Bact.* 140, 1-13.
- Wanner, B. L., Sarthy, A. & Beckwith, J. (1979) *J. Bact.* 140, 229-239.
- Ward, J. B. (1973) *Biochem. J.* 133, 395-398.
- Wayne, R. and Neilands, J. B. (1975). *J. Bact.* 121, 497-503.
- Webb, M. (1948) *J. gen Microbiol.* 2, 275-287.
- Weibull, C. (1973) *J. Ultrastruct. Res.* 43, 150-159.
- Weidel, W., & Pelzer, H. (1964). *Adv. Enzymol.* 26, 193-232.
- Weltzien, H. U. and Jesaitis, M. A. (1971). *J. Exp. Med.*, 133, 534-553.
- Westphal, O., and Lüderitz, O. (1954) *Angew Chem.* 66, 407-417.
- White, D. A., Albright, F. R., Lennarz, W. J. and Schnaitman, C. A. (1971) *Biochim. biophys. acta.* 249, 636.
- White, D., Lennarz, W., and Schnaitman, C. A. (1972) *J Bact.* 109, 686-690.
- Wilkinson, S. G. (1977) in "Surfacecarbohydrates of the prokaryotic Cell" (ed. I. W. Sutherland). Academic Press. London.
- Wilkinson, J. F., Duguid, J. P., and Edmunds, P. N. (1954) *J. gen Microbiol.* 11, 59-73.
- Wilkinson, J. F., Dudman, W.F., Aspinall, G. O. (1955) *Biochem J.* 59, 446.
- Wilkinson, J. F. and Stark, G. H. (1956) *Proc. Royal. Phys. Soc., Edinburgh* 25, 35-38.
- Willets, N. S. and Maule, J. (1974) *Genet. Res. Camb.* 24, 81-89.
- Williams, A. G., and Wimpenny, J. W. T. (1977) *J. gen. Microbiol.* 102, 13-21.
- Williams, A. G., and Wimpenny, J. W. T. (1978) *J. gen Microbiol* 104, 47-57.
- Worsham, P. L., Konisky, J. (1981) *J. Bact.* 145, 647-650.
- Wright, A., & Tipper, D.J. (1979) in "The Bacteria" Vol. VIII, Chap. 7
(Ed. Sokatch, J.R., & Orston, L.N.) Academic Press, NY,
- Wu, H. C. (1972) *Biochim. biophys. acta.* 290, 274-289.
- Wu, H. C., Hou, C., Lin, J. J.-C., Yem, D. W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 1388-1392.
- Yamada, H. & Mizushima, S. (1978) *J. Bact.* 135, 1024-1031.
- Yamada, H. & Mizushima, S. (1980) *Eur. J. Biochem* 103, 209-218.
- Yamamoto, S. & Lampen, J. O. (1975) *J. Biol. Chem.* 250, 3212-3213.
- Yamamoto, S., & Lampen, J. O. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 1457-1461.
- Yem, D. W. Wu, H. C. (1977) *J. Bact.* 131, 759-764.

Zaar, K. (1977) *Cytobiologie* 16, 1-15.

Zehnbauer, B. A. and Markovitz, A. (1980) *J. Bact.* 143, 852-863.

Zevenhuizen, L. P. T. M., Scholten, I and Koerselman, H. J. (1979)
Antonie van Leeuwenhoek. 45, 165-175.

Zwiebel, L. J., Inukai, M., Nakamura, K., & Inouye, M. (1981) *J. Bact.* 145, 654-656.