Periplasmic Enzymes and the Cell Envelope of Gram Negative Bacteria

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SUMMARY

In wild type Escherichia coli the periplasmic enzyme alkaline phosphatase is closely associated with the cell, since it cannot be released by altering the pH or cation concentration of the medium.

Mutants which release periplasmic enzymes or have altered cell envelopes have been isolated and their properties studied. Two mutants that released periplasmic enzymes were studied in detail. One released large amounts of the periplasmic enzymes alkaline phosphatase, ribonuclease I and asparaginase II, but little β galactosidase, a cytoplasmic enzyme, and was more sensitive to deoxycholate than the wild type. The mutation responsible for this phenotype omd (outer membrane defect) was mapped at approximately 6 minutes on the E. coli chromosome close to proA. The mutant had altered levels of several outer membrane proteins but in particular had reduced amounts of the outer membrane lipoprotein.

The other mutant was a temperature-sensitive lysis mutant, which at semi-permissive temperatures released large amounts of ribonuclease I but no alkaline phosphatase, was sensitive to deoxycholate and gentian violet and was mucoid. This mutant is probably temperature-sensitive for cell wall synthesis.

In mutants of E. coli and Salmonella typhimurium defective in lipopolysaccharide core biosynthesis the amount of ribonuclease I released increased as the core defect became greater. In E. coli these mutants did not release alkaline phosphatase. Mutations causing resistance to T1 or T6 did not cause periplasmic enzyme release.

Ribonuclease I is more easily released from the cell envelope than alkaline phosphatase which may be due to a difference

between them in size or a difference in location. Increasing the permeability of the outer membrane does not necessarily destroy the integrity of the periplasm. The maintenance of periplasmic enzymes in the cell envelope cannot be explained by a simple permeability model. However, no evidence was found for the specific association of periplasmic enzymes with cell envelope structures.

INTRODUCTION

SECTION 1

The Periplasm

Mitchell (1961) working with Escherichia coli showed that glucose-6-phosphatase (E.c. 3.1.3.9) behaved as if it were located at the cell surface, although it was also present in the soluble fraction following cell homogenisation. He proposed that there was an area outside the cytoplasmic membrane which could contain enzymes, and which would be separated from the media by a molecular sieve layer. He termed this area the periplasm. Since Mitchell's first observation many workers have found enzymes and proteins which behave as if they are located in this area (for reviews see Heppel, 1971, and Rosen & Heppel, 1973).

The periplasm is confined to gram-negative bacteria (Heppel, 1971) although wall associated enzymes are present in other organisms such as yeasts, fungi and gram-positive bacteria (Demis, Rothstein & Meier, 1954; Trevithick & Metzenberg, 1966; Sargent, Gosh & Lampen, 1968). As defined by Mitchell (1961), the periplasm is peculiar to the gram-negative bacteria, probably as a result of the complexity of its cell envelope, and the presence of the outer membrane (see Section 2 for an account of the gram negative cell envelope).

Periplasmic enzymes differ from other enzymes in that their activities can be measured in whole cells using substrates known not to penetrate the cytoplasmic membrane (Brockman & Heppel, 1968; Torriani, 1968). They are also found in the supernate following cell homogenisation and ultra-centrifugation (Heppel, 1971).

Osmotic shock (Nossal & Heppel, 1966) and sphaeroplast formation using lysozyme (Malamy & Horecker, 1961) causes the selective release of these proteins, but not the enzymes known to be

cytoplasmic in location. Sphaeroplast formation using penicillin or lysine starvation of a lysine auxotroph does not cause selective release of periplasmic enzymes (Heppel, 1971). Mutants isolated for cell envelope defects, often show specific release of periplasmic enzymes into the medium during growth, (Mangiarotti, Apirion & Schlessinger, 1966) which is taken as an indication of periplasmic localisation.

Periplasmic proteins may be split into two functional groups; the binding proteins and the periplasmic enzymes.

Binding Proteins

These form a group of small molecular weight proteins (less than 50,000 M.W.) which specifically and reversibly bind small ligands and substrates, but have no known enzymatic function (Rosen & Heppel, 1973). Binding proteins are present in animal tissues, bacteria and fungi, but in gram negative bacteria some of them behave as if they are periplasmic, being released by osmotic shock and by lysozyme sphaeroplast formation (Nossal & Heppel, 1966). Pardee and Watanabe (1968) demonstrated the periplasmic localisation of the sulphate binding protein using an inhibitory dye, which can penetrate the outer membrane but not the cytoplasmic membrane. The sulphate-binding activity of cell extracts, but not of whole cells, was inhibited by the addition of specific antisera, showing that the sulphate-binding protein was not exposed on the surface of the cell. Nakane, Nichoalds and Oxender (1968) using a histochemical technique showed that the leucine/valine/isoleucine binding protein was present in the periplasm.

Binding proteins are thought to be involved in transport across the cytoplasmic membrane (for a review see Rosen & Heppel,

1973). A mutant of E. coli, the result of a single mutation, which lacks sulphate-binding protein was also deficient in sulphate transport (Ohta, Galsworthy & Pardee, 1971). Osmotic shock not only causes release of the periplasmic binding proteins (see Table 1) but also a substantial decrease in the active transport of several substrates (Rosen & Heppel, 1973). Active transport of substrates for which no binding protein was found in shock fluid was unaffected by this treatment. The loss of phosphate transport in shocked cells can be partially restored by the addition of phosphate-binding protein to the cells (Medreczky & Rosenberg, 1970).

Singer (1974) proposed a model for substrate transport which involved periplasmic binding proteins (Fig. 1). The binding protein recognises and binds the substrate, causing an allosteric change in an integral membrane protein, which opens a water-filled pore for the substrate to enter the cell. He also suggested that the periplasmic binding proteins are lightly attached to integral proteins of the cell membrane and are peripheral membrane proteins. Rosen and Heppel (1973) also discuss this possibility, but point out that membrane vesicles, made by the method of Kaback (1971) did not contain periplasmic binding proteins. They do not dismiss the possibility that these binding proteins are free in the periplasm.

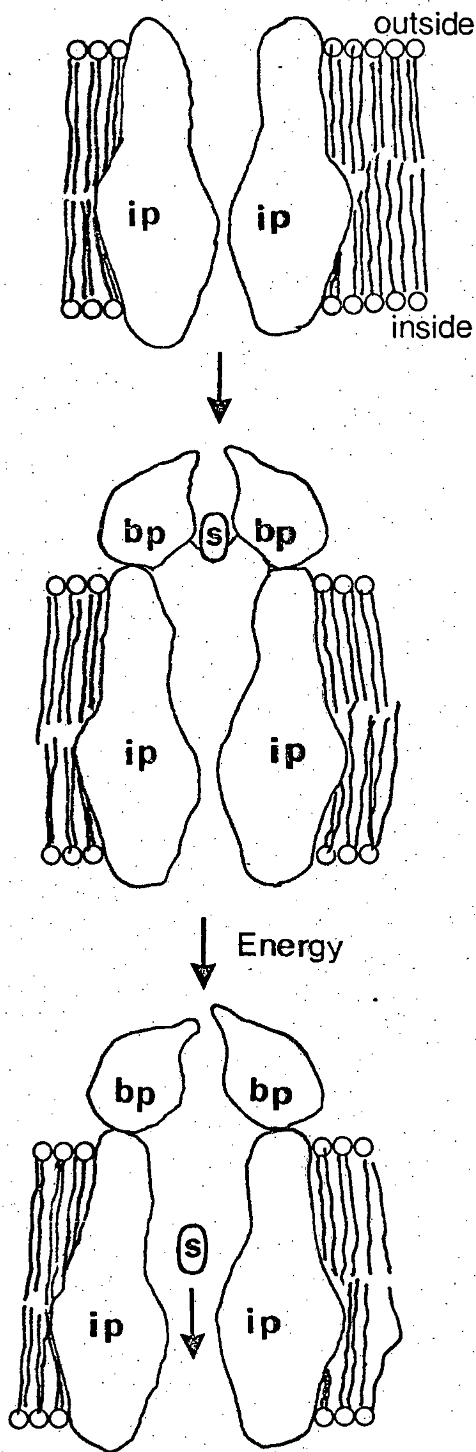
Kaback (1970) proposed that periplasmic binding proteins might have a role in chemotaxis, which is thought to occur by non-metabolic means (Adler, 1970). He also pointed out that the evidence for involvement of periplasmic binding proteins in active transport is indirect, and that osmotic shock would also release the acid-soluble nucleoside pool from the cytoplasm, which

Table 1

Enzymes Released by Osmotic Shock	
Enzyme	**Molecular Weight**
Alkaline phosphatase	80,000
Acid hexose phosphatase	-
Cyclic phosphodiesterase	60,000
5'-Nucleotidase	52,000
Ribonuclease 1	11,500
Deoxyribonuclease 1	32,000
Aspariginase 2	-
Penicillinase	Various
Streptomycin adenylating enzyme	-
Thymidine phosphorylase) Deoxyriboaldolase) Deoxyribomutase)	-
ADP-Glucose pyrophosphatase	-

Proteins Released by Osmotic Shock	
Binding Protein	**Molecular Weight**
Sulphate	32,000
Phosphate	42,000
Galactose	-
L-Arabinose	32,000
Ribose	36,000
Leucine/Isoleucine/Valine	35,000
Arginine	27,700
Lysine	26,200
Phenylalanine	24,000
Histidine	25-26,000
Glutamine	-
Cystine/DAP	-
Glutamic acid	-

Fig. 1. Possible Role of Periplasmic Binding Protein in Transport Across the Cytoplasmic Membrane



bp = periplasmic binding protein

ip = integral membrane protein

s = substrate

(from Singer, Ann. Rev. Biochem., 1974)

could affect active transport by depletion of high energy reserves. However, many transport systems which do not have periplasmic binding proteins are unaffected by osmotic shock.

The R2 protein, a regulator of alkaline phosphatase, (E.c. 3.1.3.1), is also released by osmotic shock. It binds phosphate but is not involved in phosphate transport, and is distinct from the phosphate-binding protein (Medreczky & Rosenberg, 1970). Mutants deficient in R2 protein produce large amounts of alkaline phosphatase.

Periplasmic enzymes

This is the term given to a group of hydrolytic enzymes which are located at the cell surface of gram negative bacteria. They are mainly sugar phosphatases, nucleosidases or nucleases, which are thought to modify substrates so they can be transported into the cell. All these enzymes can be released by osmotic shock or sphaeroplast formation. A list is given in Table 1.

Sugar Phosphatases

Alkaline phosphomonoesterase (E.c. 3.1.3.1) is probably the best studied example. This enzyme is produced by E. coli when growing under phosphate-limiting conditions, (Echols, Garen, Garen & Torriani, 1961) and is also necessary for growth on glycerol phosphate as the sole carbon source (Torriani & Rothman, 1961). The enzyme is repressed by high levels of inorganic phosphate. Localisation of alkaline phosphatase has been studied using many techniques. Malamy and Horecker (1961) were the first to demonstrate release of this enzyme by lysozyme sphaeroplast formation. It has also been shown to be located in the cell envelope by both ferritin antibody labelling and reaction product

deposition under the electron microscope (Wetzel, Spicer, Dverak & Heppel, 1970). Alkaline phosphatase (APase) is a dimer of approximately 80,000 molecular weight. Monomers of APase are excreted across the cytoplasmic membrane and dimerise in the periplasmic space (Schlessinger, 1968).

E. coli also contains several periplasmic acid phosphatases (Neu & Heppel, 1964). These include acid hexose phosphatase specific for certain sugar phosphate esters (Dvorak, Brockman & Heppel, 1967), a non specific acid phosphatase (E.c. 3.1.3.2) (Dvorak et al., 1967) and an acid phosphatase with a pH optimum of 2.5 (Hafkenscheid, 1968).

Nucleosidases and Nucleotidases

5' Nucleotidase (E.c. 3.1.3.5) is inhibited by a cytoplasmic protein (Melo & Glaser, 1966) which has to be destroyed by heating before its activity can be measured in cell homogenate. The inhibitor protein is not released by osmotic shock. The enzyme has hydrolytic activity against UDPG as well as 5' nucleotides (Glaser, Melo & Paul, 1967). A cyclic phosphodiesterase which hydrolyses ribonucleoside 2'-3'-cyclic phosphates to nucleosides and phosphates was found by Anraku (1964a & b). Three enzymes which are involved in thymidine breakdown were released by osmotic shock as an organised complex. Toluenization or sonication caused inhibition of this function in shock fluid (Munch-Petersen, 1968).

Nucleases

The periplasm of E. coli contains two nucleases. One of them, deoxyribonuclease I produces "nicks" in one strand of the DNA duplex. It is unusual in that it is partially released by EDTA/Tris treatment. The other nuclease is the latent

ribonuclease, ribonuclease 1 (Spahr & Hollingworth, 1961). In cell homogenates it is bound to the 30S ribosomal sub-unit, but this is an artifact of homogenisation (Neu & Heppel, 1964b). The enzyme is fully active when released by osmotic shock.

Other Periplasmic Enzymes

E. coli also contains a periplasmic asparaginase, asparaginase II (E.c. 3.5.1.1) which is active against mouse lymphomas (Mashburn & Gordon, 1968). This enzyme is only produced at low oxygen tensions (Jeffries, 1974; Cedar & Schwartz, 1967). Several penicillinases are periplasmic (Datta & Richmond, 1966; Burman, Nordstrom & Boman, 1968) although there is doubt as to whether all of them are. Smith (1974) showed that β-lactamases above a certain molecular weight were not released by osmotic shock. Strains of *E. coli* which carry an R factor conferring resistance to streptomycin, contain an enzyme which is periplasmic and adenylates streptomycin (Harwood & Smith, 1969). This enzyme requires ATP, which implies that ATP must become available in the periplasm if the enzyme is truly periplasmic.

Enzyme Localisation in Bacteria

The study of enzyme localisation is often difficult. Attempts to fractionate cells following homogenisation do not preclude the possibility that the association of an enzyme with a particular cell fraction, may be caused by the homogenisation procedure (Neu & Heppel, 1964; Hardy & Karland, 1966). Many enzymes are known to be located in the bacterial cell membrane and many, if not all, of the particulate enzymes are likely to be membrane bound (Rothfield & Romeo, 1971). The cytoplasmic membrane of bacteria has to be responsible for many of the functions

of the cell organelles of eukaryotic organisms, e.g. replication and segregation of the chromosome (Jacob, Brenner & Cuzin, 1963) and the photosynthetic apparatus of bacteria (Tuttle & Gest, 1959).

It is possible that periplasmic enzymes are closely associated with one of the two cell envelope membranes of gram negative bacteria (Costerton, Ingram & Cheng, 1974). Singer's model (1974) of ligand transport by periplasmic binding protein supposes an attachment of protein to cytoplasmic membrane (see Fig. 1) but this remains to be demonstrated. Heppel (1971) points out that the association of periplasmic enzymes within the cell envelope is not clear, and he emphasises that it is not know if the enzymes occupy sites in the cell envelope, or are free, sandwiched between the two cell envelope membranes.

SECTION 2

The Bacterial Cell Envelope

Bacteria are classified into two groups according to the composition and organisation of their outer layers. These are the gram positive and the gram negative bacteria. The cell envelopes of both these groups conform to a basic pattern; consisting of the osmotic barrier of the cell, the cytoplasmic membrane, and a rigid cell wall, in which peptidoglycan is the main structural component (Fig. 2). The gram negative cell envelope contains much less peptidoglycan and more lipid than the gram positive envelope.

Gram Negative Cell Envelope

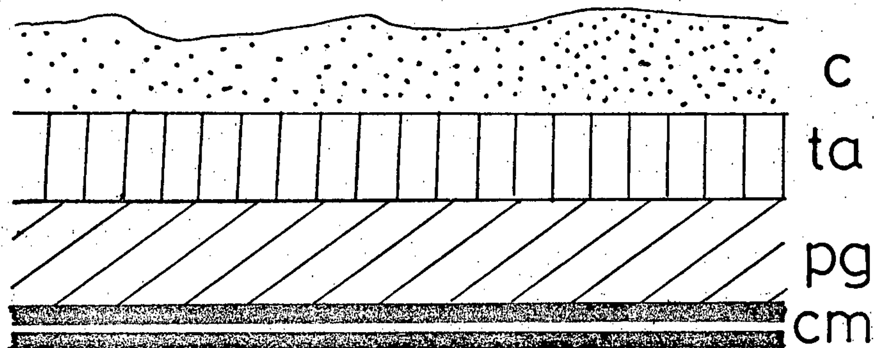
The cell envelope of gram negative bacteria appears as a multilayered structure in thin sections examined by electron microscopy. Five definite layers can be seen (Fig. 2) (Glauert & Thornley, 1969). These correspond to the two membranes of the cell envelope, the cytoplasmic membrane and the outer membrane and a densely staining intermediate layer, the cell wall. The cell envelope is made up of peptidoglycan, lipoprotein, phospholipid, protein, lipopolysaccharide and polysaccharide. The cell wall is so closely associated with the cytoplasmic membrane that it is very difficult to remove all of it during the formation of protoplasts. The protoplasts with pieces of cell wall attached are called sphaeroplasts (Birdsell & Cota-Robles, 1967).

Intact gram negative bacteria are less sensitive to many water-soluble antimicrobial agents such as Penicillin than gram positive bacteria although the concentration of agent needed to inhibit the target site is similar in both groups. This is due to the osmotic barrier created by the outer membrane. However, the gram negative cell wall is more susceptible to mechanical damage than the gram positive cell wall which is much thicker

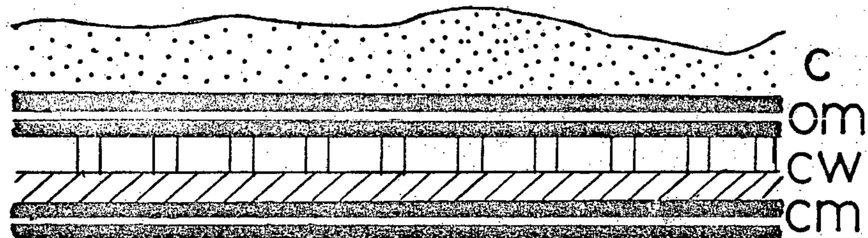
Fig. 2.

Diagramatic Representation of Gram Positive and Gram Negative Cell Envelopes

a Gram positive



b. Gram negative



C = capsule
ta = teichoic acid
pg = peptidoglycan
cm = cytoplasmic membrane
om = outer membrane
cw = cell wall

(Hughes, Wimpenny & Lloyd, 1971).

a) <u>The cytoplasmic membrane</u>

The membranes of both gram positive and gram negative bacteria have many similarities and this discussion, unless specified will apply equally to both.

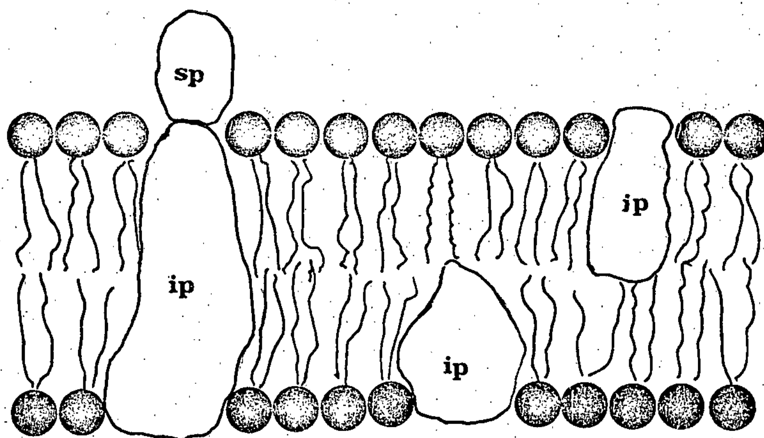
The cytoplasmic membrane forms the osmotic barrier of the cell and is essentially a lipid-protein structure. Much of the membrane lipid exists in bilayer form, which is consistent with Danielli & Davson's (1935) model. However there is evidence to suggest that the proteins are arranged not as a continuous layer on either side of the lipid bilayer, but rather as discrete globules within the lipid matrix, sometimes traversing the lipid bilayer (Singer, 1971) (Fig. 4). The unique properties of lipids are responsible for the semipermeable barrier of the membrane, which behaves as a fluid matrix (Rothfield, 1971). The fluidity of membranes is important to allow diffusion to take place.

Apart from having a permeability barrier function, many complex reactions take place in the membranes of bacteria, as described in Section 1. Enzymes located in the cytoplasmic membrane of bacteria are involved in cell wall synthesis, oxidative phosphorylation, active transport across the membrane and synthesis of fatty acids. It is likely that all "particulate" enzymes are membrane-bound (Rothfield & Romeo, 1971). Many of the membrane-bound enzymes require lipid to be active (Fleischer & Klonwen, 1961) and it is probable that the lipid/protein interaction in the membranes is important in the formation of the active sites of these enzymes (Rothfield & Romeo, 1971).

Although protein and lipid are present in both layers of

Fig. 4.

A Possible Arrangement of Proteins and Phospholipid in Membranes



ip = integral membrane protein

sp = superficial membrane protein

the bilayer it does not follow that their composition is identical. For example in animal cells and in the forespore membrane of Bacillus spp the lipid bilayer has been shown to be asymetric (Ellar, 1975). The lateral movement of lipids within a membrane is very rapid (Poo & Cone, 1974). It has been estimated that a lipid molecule can move from one bacterial cell pole to the other in 5 seconds (Ellar, 1975). However there does not seem to be much interchange of lipids across the bilayer.

How membrane biosynthesis occurs is not clear. Enzymes involved in lipid synthesis tend to be membrane-bound, but it remains to be demonstrated if these are located at specific membrane biosynthetic sites. Membrane proteins could be synthesised on ribosomes bound to the cytoplasmic membranes, although the three proteins coded for by the lac operon are translated from a single mRNA molecule. Two of these proteins, β-galactosidase and the transacetylase are soluble while the third protein, the permease, is tightly bound to the membrane. This would suggest that soluble and membrane-bound proteins are synthesised by the same set of ribosomes.

Parts of the cell which are external to the cytoplasmic membrane, such as the cell wall, and in gram negative bacteria the outer membrane, and periplasmic enzymes, have to be moved across the cytoplasmic membrane at some stage of their biosynthesis, and are assembled in situ. The structural polymers of the cell wall such as peptidoglycan, lipopolysaccharide and teichoic acid, are synthesised on the cytoplasmic membrane, the small precursors being transported across the membrane by lipid carriers. The only known lipid carriers are polyisoprenoid lipids, which function as intermediate carriers of glycosyl groups and are termed glycosyl

carrier lipids (GCL) (Osborn, 1971). How periplasmic enzymes move across the membrane is not known. Schlessinger (1968) showed that APase subunits were secreted across the cytoplasmic membrane by sphaeroplasts of E. coli and proposed that APase subunits dimerise in the periplasmic space. Torriani (1968) showed that there was a pool of APase subunits in the cytoplasm of actively growing E. coli cells, which supports Schlessinger's proposal (1968). If the genes for APase production from E. coli are introduced into Salmonella typhimurium, which does not normally produce the enzyme, then active APase is produced, which is periplasmic in location (Schlessinger & Olsen, 1968). This would suggest that the localisation of APase is associated with the structure of the protein rather than with a specific transport mechanism.

Localisation of membrane-bound enzymes has also been attributed to the structure of the protein. It is proposed that there are hydrophobic areas in the protein which cause the enzyme to migrate to a lipid-rich environment. However in the case of periplasmic enzymes where there is a movement from one hydrophilic area to another across the membrane, there are several questions left unanswered. For example, how and why does this movement take place? Why do APase subunits dimerise in the periplasm but not in the cytoplasm? What changes occur in a protein going from a hydrophilic area to a hydrophobic area and *vice versa*?

b) The cell wall

This is the structural part of the cell, and has been termed the rigid or "r" layer (Braun & Rehn, 1969). It is located between the cytoplasmic membrane and the outer membrane and

corresponds to the electron-dense intermediate layer as seen by electron microscopy.

i) <u>Peptidoglycan</u>

This polymer has a repeating glycan backbone of alternating N'-acetylglucosamine (NAG) and N'-acetylmuramic acid (NAM) residues (Fig. 5). Short peptide chains are attached to the NAM and form crosslinks from one glycan chain to another (Fig. 6) (Ghuysen, 1969; Osborn, 1969). Bacteria differ in the composition of their peptide chains, although in the gram negative bacteria only one type of peptide composition is found (Ghuysen & Shockman, 1973). In <u>E. coli</u> the peptidoglycan forms a single bag-shaped molecule (Weidel & Pelzer, 1965) but in other gram negative bacteria where there is less peptidoglycan, this layer could be discontinuous, with other material filling the spaces.

The peptidoglycan layer when isolated retains the shape of the cell and if it is enzymatically degraded, then the cell is turned into a spherical, osmotically fragile body. Clearly peptidoglycan maintains the cell shape and protects it from osmotic and mechanical damage. It is unlikely that peptidoglycan determines the shape of the cell (Henning & Schwartz, 1973).

Several antibiotics are active against peptidoglycan biosynthesis, e.g. penicillin, cephalosporin, vancomycin and bacitracin. Penicillin is an analogue of D-alanyl alanine (Tipper & Strominger, 1965) and inhibits two enzymes involved in cross-link formation from one peptide chain to another (Fig. 7). Penicillin action causes the formation of weak areas in the cell walls. Cellular lysis does not directly result from penicillin action but is due to autolytic enzymes present in the cell

Fig. 5.

The Glycan Chain of Peptidoglycan

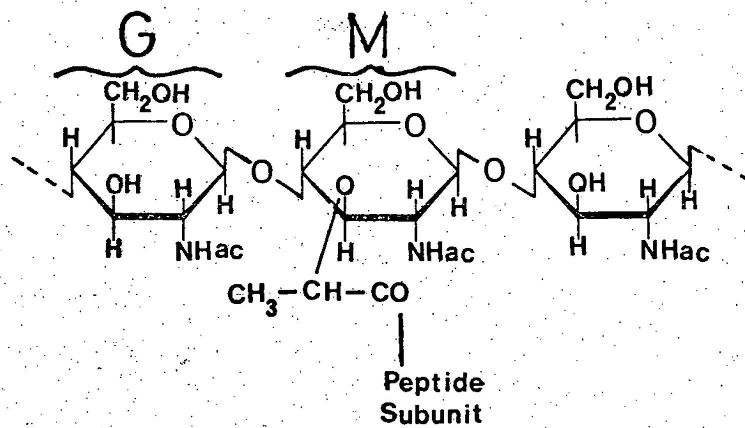


Fig. 6.

<u>*E. coli* Peptidoglycan</u>

---G—M—G---

---G—M—G---   L.Ala-GluOH

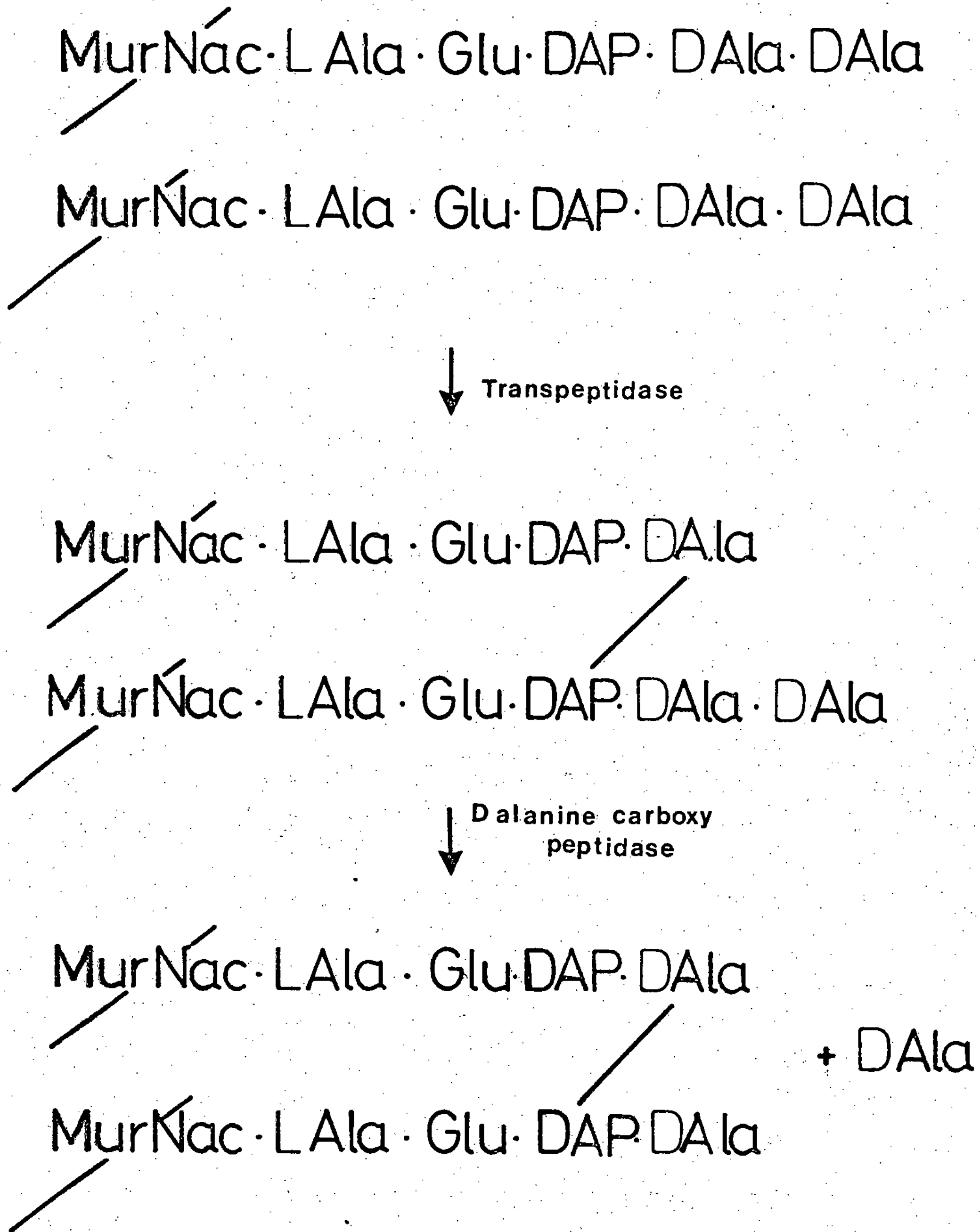
γ

L.Ala-GluOH   DAP-D.Ala---

γ

DAP-D.Ala

Fig. 7. Penicillin Sensitive Enzymes in Peptidoglycan Biosynthesis



envelope which break down the peptidoglycan (Tomasz, Albino & Zanati, 1970). Autolytic enzymes are possibly involved in several surface functions, such as peptidoglycan synthesis, cell division and cell separation, and alteration in cell shape and size (Rogers, 1970; Forsberg & Rogers, 1971; for a review see Tomasz, 1973). Peptidoglycan is also degraded by several enzymes from non-bacterial sources, e.g. egg white lysozyme (N'-acetyl muramidase), which cleaves the glycan backbone (Fig. 5) into disaccharide units (Ghuysen, 1969). Lysozyme is widely used in the formation of sphaeroplasts. Sphaeroplasts can be also formed by growing cells with a sublethal concentration of penicillin in the presence of an osmotic stabiliser but periplasmic enzymes are not released by this treatment, while they are by lysozyme sphaeroplast formation (Heppel, 1971). Periplasmic enzymes are specifically released by an osmotically fragile mutant of *E. coli* when the medium is supplemented with an osmotic stabiliser (Mangiarotti, Apirion & Schlessinger, 1966). It is probable that this mutant is defective in peptidoglycan biosynthesis. Clearly removal of the peptidoglycan has a drastic effect on the periplasm, and it is likely that it has a function in the cell envelope.

Biosynthesis of peptidoglycan takes place on the cytoplasmic membrane but polymerization and cross-linking occur *in situ* in the cell wall. The acceptor molecule in the cell wall is the free end of a peptidoglycan chain. How growth of the cell wall is controlled is not known.

ii) *Cell wall proteins*

Two proteins are associated with peptidoglycan, a lipoprotein of M.W. 7,500 and a large M.W. protein. The lipoprotein

is covalently linked to diamino-pimelic acid of the peptidoglycan, by a lysylarginine dipeptide (Braun & Rehn, 1969). It is also found in the plastic layers of the outer membrane, free lipoprotein exceeds the bound form by a factor of two (Inouye, Hirashima & Lee, 1974). In the cell envelope the lipoprotein is one of the major protein species and is thought to anchor the outer membrane to the cell wall, 90-95% of the bound lipoprotein facing away from the cell, towards the outer membrane (Braun, Bcsch, Hantke & Schaller, 1974). The lipoprotein has an α helical conformation, with hydrophobic amino acids regularly spaced along its length (Braun, et al, 1974), the hydrophobic amino acids being on one side of the helix only. Another protein, a part of erythrocyte porphorin, which passes through the lipid bilayer is also helical (Segrest, Jackson, Marchesi, Guyer & Terry, 1972) which might suggest a relationship between structure and function.

The lipoproteins from Salmonella, Escherichia, Citrobacter, Proteus and Shigella have been isolated and found to be very similar immunologically (Mayer, Schlecht & Braun, 1973). Recently another protein has been demonstrated to be bound to peptidoglycan (Rosenbusch, 1974). It has a M.W. of 36,500 and is possibly identical to the major cell envelope protein. Negatively-stained cell walls of E. coli show a regular arrangement of protein subunits attached to the peptidoglycan (Rosenbusch, 1974). Similar periodic structures have been found by other authors (Weidel, Frank & Martin, 1960; Boy de la Tour, Bolle & Kellenberger, 1965; Bayer & Anderson, 1965). Although the function of this protein is not known Rosenbusch (1974) has suggested that it could have the shape-determining role of the cell wall. Henning & Schwarz (1973) proposed that there would be a self-assembling protein

layer in the cell wall which would be responsible for this function, although this remains to be demonstrated.

c) The outer membrane

The outer membrane consists of protein, phospholipid, lipopolysaccharide and lipoprotein, and appears as a true biological membrane in electron microscope preparations. The outer membrane is unique to the gram negative bacteria, although recently lipopolysaccharide has been detected in the cell envelopes of blue green algae (Weckesser, Katz, Drews, Mayer & Fromme, 1974). The outer membrane acts as a permeability barrier to water-soluble molecules larger than 400 M.W. (Costerton, personal communication). However different mutations affect the permeability of the outer membrane to small M.W. substances in different ways (Normark & Westling, 1971; Tamaki, Sato & Matsuhashi, 1971).

i) Phospholipids, and proteins of the outer membrane

The outer membrane contains fewer proteins than the cytoplasmic membrane. Only one enzyme, phospholipase A, has been located in the outer membrane, while all the enzymes for cell wall biosynthesis and oxidative phosphorylation are located on the cytoplasmic membrane. Of the protein species present in the outer membrane, the two major polypeptides, as shown by polyacrylamide gel electrophoresis, have M.W. of 36-44,000 and 7,500. Depending both on the technique used to isolate the outer membrane and on how the electrophoresis is carried out, the 36-44,000 M.W. protein appears either as a single polypeptide (Schnaitman, 1971; Rosenbusch, 1974) or split into 3 different polypeptides (Inouye & Yee, 1973; Ames, Spudich & Nikaido, 1974) This family of polypeptides which all have a M.W. of around 40,000, form approximately 80% of the outer membrane protein of

E. coli (Schnaitman, 1970; Inouye & Yee, 1973; Leive, 1974). Other proteins may be present in small amounts which are not detected by polyacrylamide gel electrophoresis (Singer, 1974). Phages T_2, T_5, T_6 and λ have all been shown to have protein receptors in the outer membrane (for review see Lindberg, 1973).

The surface area of the outer membrane has been estimated as being divided between protein (60%), phospholipid (20-30%) and lipopolysaccharide (LPS) (11-22%) (Nikaido, 1973). The figures of the LPS are dependent on whether the outer membrane is symmetrical or asymmetrical in the distribution of this molecule between the lipid layers. Phosphatidyl ethanolamine is the major lipid of the outer membrane forming 85-90% of the total outer membrane lipid in E. coli and S. typhimurium. Small amounts of phosphatidyl glycerol and cardiolipin are also found (Osborn, Gander, Parisi & Carson, 1972). In Pseudomonas aeruginosa the composition of the lipids of the cytoplasmic membrane and the outer membrane are similar (Hancock & Meadow, 1967).

ii) Lipopolysaccharide

This molecule is only found in the outer membrane of gram negative bacteria and forms the endotoxin of these organisms. It is a very complex molecule consisting of a lipid moiety to which is attached a short oligosaccharide. To this is attached a long chain polysaccharide made up of repeating sequences (see Fig. 8). The lipid moiety which is termed lipid A, is unique in that the backbone of the lipid is made up of glucosamine, instead of glycerol. It contains D-glucosamine (20%), fatty acids (60%) and phosphorus (Adams & Singh, 1970). The lipid forms part of the endotoxin and is embedded in the hydrophobic region of the outer membrane. The glucosamine is arranged as disaccharide units and

it is thought that LPS molecules are linked in threes by pyrophosphate bridges between the lipid A molecules (Romeo, Girard & Rothfield, 1970) (Fig. 8). All the available hydroxyl groups on the glucosamine are substituted with fatty acids.

Very little is known about the synthesis of lipid A although its fatty acid composition is very different from the glycerophospholipids, being mainly made up of 3-hydroxytetra decanoic acid. Osborn (1974) has isolated a temperature sensitive mutant of E. coli which at the restrictive temperature cannot make lipid A, and dies. It would seem that lipid A is necessary for the cell to function.

The core of LPS is a short oligosaccharide which may or may not be branched. The core LPS of most gram negative bacteria contains ketodeoxyoctonate (KDO), heptose and glucose. The majority of core lipopolysaccharides also contain galactose, although the sequence and bonding of the glucose and galactose residues varies from strain to strain. The LPS core of S. typhimurium has been sequenced (Fig. 9) using mutants defective in LPS synthesis (Stocker & Makela, 1971; Luderitz, Westphal, Staub & Nikaido, 1971). By comparing the phage-adsorption patterns and antibiotic sensitivity of these mutants, it has been shown that core LPS acts as a receptor site for several phages and also forms part of the outer membrane permeability barrier (Schlecht & Westphal, 1970; Sanderson, MacAlister, Costerton & Cheng, 1974).

The structure of LPS cores of E. coli strains has been described and is similar to that of S. typhimurium (Ørskov & Ørskov, 1962; Edstrom & Heath, 1967) but the LPS cores of some strains of E. coli such as E. coli B, do not contain galactose (Boman & Monner, 1975). Several phages which adsorb to E. coli

Fig. 8.

Proposed Arrangement of LPS Molecules in the Cell Envelope (Romeo et al, 1970)

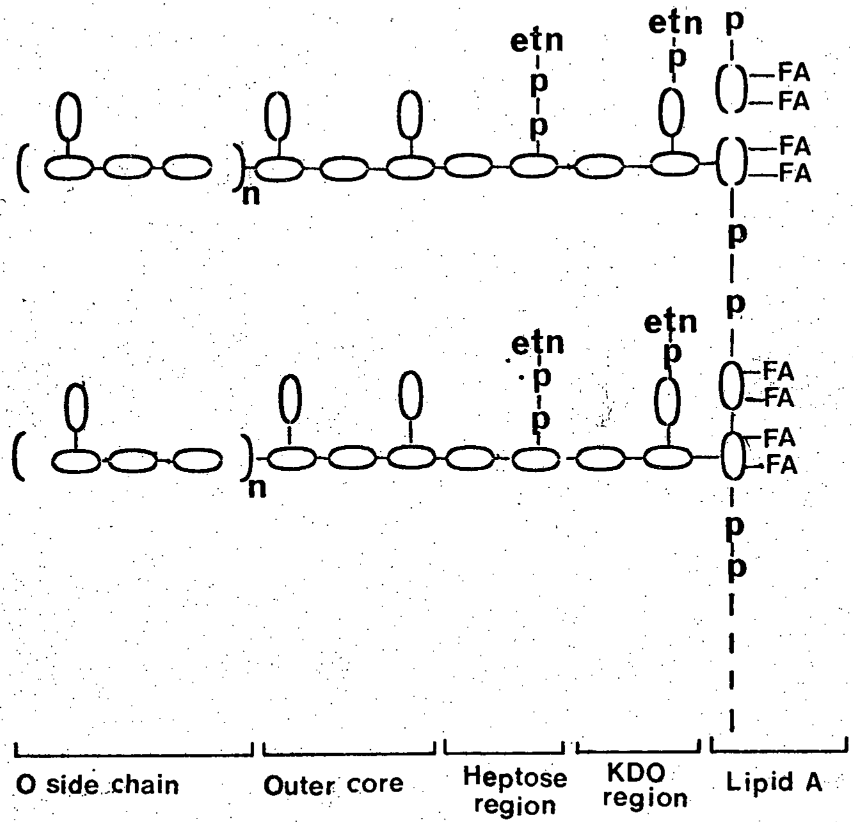
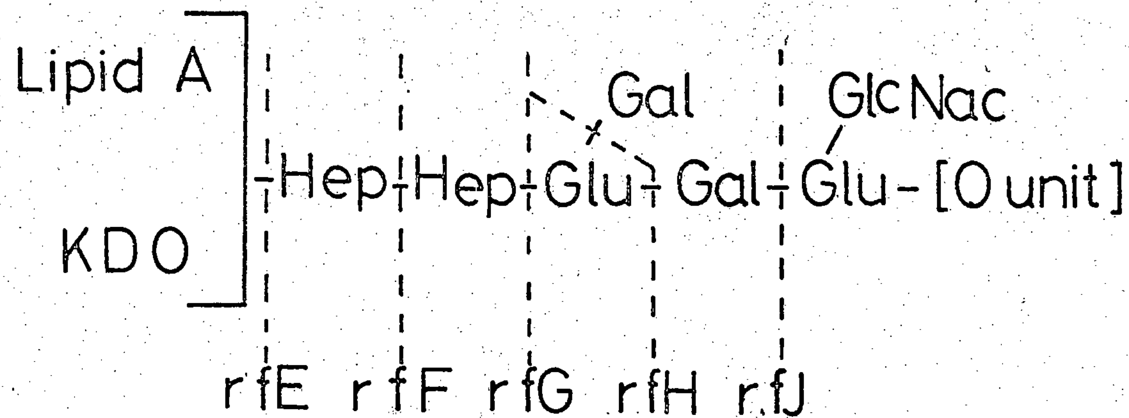


Fig. 9.

The Core Region of *S. typhimurium* Lipopolysaccharide



LPS do not adsorb to S. typhimurium cells, although some phages such as C21 and P1 adsorb to both (Rapin & Kalcar, 1971).

The third part of the LPS molecule is a very long polysaccharide made up of repeating units. The composition of this side chain, which is termed the "O" side chain or "O" antigen, varies from species to species and has been used as a character for classification and identification of bacteria (Kauffman, 1954). Laboratory maintained strains such as E. coli K12 and E. coli B form incomplete "O" side chains, with only one "O" antigen sub-unit attached to the LPS core, but since they appear to grow well it is probable that the side chain has no function under laboratory conditions.

Biosynthesis of LPS occurs in two distinct stages. First, the core and "O" side chains are synthesised independently of each other on the cytoplasmic membrane and secondly are attached in situ in the outer membrane (Cynkin & Osborn, 1968). Synthesis of "O" side chains requires a lipid intermediate carrier while the synthesis of the core molecule does not.

Many of the surface characteristics of gram negative bacteria can be attributed to LPS, such as phage adsorption, antigenic properties and toxicity. However the function of the LPS is not immediately obvious. Clearly different parts of the molecule function in different ways. The "O" antigen is essential for virulence of Salmonella strains. It is possible that the "O" side chain has a protective role, preventing ingestion of the bacteria by macrophage, and also forms part of the endotoxin. The core LPS appears to form part of the permeability barrier of the outer membrane and may also function as a cation-binding structure. Mutants of E. coli and S. typhimurium

which are unable to make complete core oligosaccharides, show alterations in outer membrane protein composition (Ames et al, 1974; Randal, 1975) and it is probable that LPS core stabilises the outer membrane. Little is known of the functions of lipid A although Osborn's work (1974) has suggested that lack of lipid A or KDO is fatal to the cell.

Gram Positive Cell Envelope

The cell envelope of gram positive bacteria appears homogenously electron dense when viewed in thin section by electron microscopy (Salton, 1964; Glauert & Thornley, 1969). Chemical analysis has shown the envelope to contain peptidoglycan, teichoic or teichuronic acid, polysaccharide and protein. Peptidoglycan is the major component of the envelope and can account for up to 90% of its dry weight. Teichoic acid is also present in large amounts, and can form up to 50% of the dry weight of the envelope (for a review see Reavely & Burge, 1972). Unlike the gram negative bacteria, the cell wall of gram positive bacteria can be easily removed from the cytoplasmic membrane by plasmolysis of the cell or degradation of the cell wall with lysozyme. The peptidoglycan in these bacteria forms a multilayered molecule which is highly crosslinked but does not act as a barrier to water soluble molecules. As in gram negative bacteria the peptidoglycan is responsible for maintaining the shape of the cell and also protects the cell from mechanical and osmotic stress. The thickness of the cell wall enables gram positive bacteria to reach much larger dimensions than the thin walled gram negative bacteria.

Teichoic acids are found exclusively in the outer layers of gram positive bacteria. They are phosphorylated polymers of

glycerol or ribitol (for a review see Archibald, 1974). If bacteria are grown under conditions of phosphate limitation then teichuronic acids replace teichoic acids (Ellwood & Tempest, 1972). Teichuronic acids are carboxylated polymers of glycerol or ribitol (Archibald, 1974). Teichoic acids are covalently linked to the outside face of the peptidoglycan layer (Archibald & Baddily, 1965; Hughes, 1970) but are also associated with the outer face of the cytoplasmic membrane (Shockman & Slade, 1964). The main function of this polymer is cation binding (Cutinelli & Galdiero, 1967), and Ellwood & Tempest (1972) have show that cation limited cultures of bacteria produce more teichoic acid. Teichoic acids have also been suggested as possible regulators of autolytic enzymes (Higgins, Pooley & Shockman, 1970).

The majority of the gram positive cell envelope consists of peptidoglycan and teichoic acid. Capsules are often present outside these layers and may be composed of protein, as in the group A Streptococci (McCarty & Morse, 1964) or polysaccharide as in Diplococcus pneumoniae, type III. These outer layers often act as antigenic determinants of the cell, or phage adsorption sites, but molecules deep in the cell wall rarely participate in these functions. Several enzymes have been reported as being associated with the gram positive cell wall (Sargent et al, 1968) and many gram positive bacteria release extracellular enzymes (Pollock, 1964). However there does not appear to be a group of enzymes which is as well defined as the periplasmic enzymes of the gram negative bacteria.

Clearly there are marked differences between the gram positive and gram negative bacterial cell envelopes. The lipid rich nature of the gram negative cell envelope provides a second

selective permeability barrier, the outer membrane. Thus gram negative bacteria have an area in the cell envelope between the cytoplasmic and outer membranes which would be consistent with Mitchell's (1961) hypothesis of the periplasm.

SECTION 3

Localisation of Periplasmic Enzymes

Most of the evidence for the localisation of periplasmic enzymes and binding protein is indirect. Their presence in the cell envelope is inferred from their selective release by treatments which do not release cytoplasmic enzymes, that they have activities that can be measured in whole cells, and they can be shown to be present at the cell surface by cytochemical techniques.

Release of periplasmic enzymes

Malamy & Horecker (1961) were the first to show release of alkaline phosphatase (APase) by turning E. coli cells into sphaeroplasts using lysozyme. Since then other enzymes have been shown to be released during this procedure (Neu & Heppel, 1964).

The effect of lysozyme on the cell is to degrade the glycan backbone of peptidoglycan and thus turn the cell into an osmotically-fragile sphere. Sphaeroplasts are distinguished from protoplasts, because they have some cell wall material still attached to the cell (Birdsell & Cota-Robles, 1967). Treatment of gram negative bacteria with ethylenediaminetetraacetic acid (EDTA) in tris(hydroxymethyl)aminomethane (tris) buffer at an alkaline pH is necessary to alter the permeability of the outer membrane so that lysozyme can reach the cell wall. Sucrose or some other osmotic stabiliser must be added to prevent the sphaeroplasts from lysing.

During sphaeroplast formation the outer membrane becomes disrupted, coils back on itself, and periplasmic enzymes are released. Whether disruption of the outer membrane is solely due to the action of lysozyme, or is enhanced by EDTA/tris is not known. E. coli can also be turned into osmotically fragile spheres by growth in penicillin (Park, 1968) or by lysine

starvation of a lysine auxotroph (see Fig. 10). However these treatments do not release periplasmic enzymes (Heppel, 1971). Both penicillin treatment and lysine starvation interfere with cross-linking of the peptidoglycan molecule, but do not affect its backbone, and do not involve tris/EDTA.

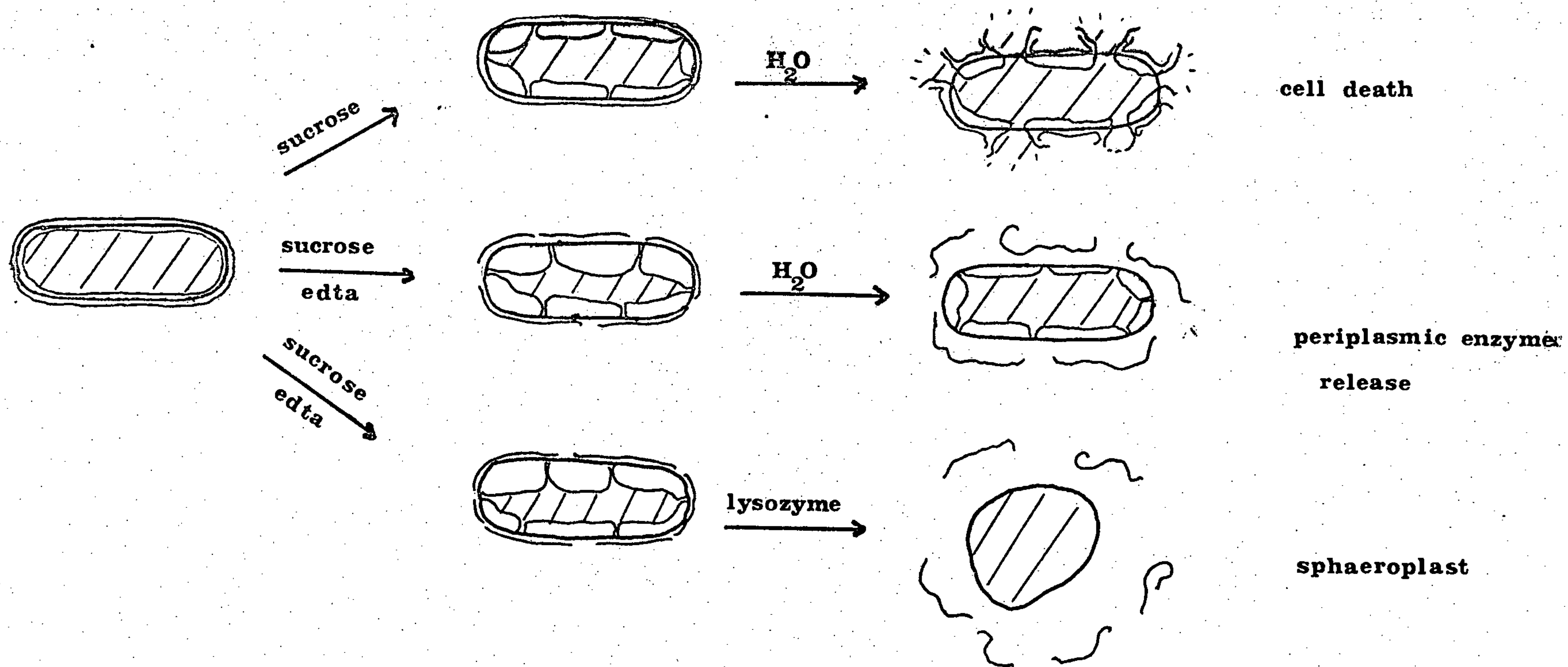
Periplasmic proteins are also specifically released by osmotic shock (Nossal & Heppel, 1966). This involves first treating bacteria with EDTA/tris in 20% sucrose and then quickly transferring them to cold water. Approximately 4% of the cellular protein and all the acid-soluble nucleotide pool are lost in the cold water shock stage. The 4% of the cellular protein contains nearly all the periplasmic enzymes and very little cytoplasmic enzyme (Nossal & Heppel, 1966). If EDTA is not used in the first stage of this treatment, less periplasmic enzyme is released, and viability following osmotic shock is reduced.

When E. coli cells are plasmolysed without EDTA, and then osmotically shocked, finger like extrusions are seen to emerge from the cell wall (Bayer, 1967). Bayer proposed that these extrusions resulted from weak areas in the cell wall. He also showed that when plasmolysed E. coli cells are maintained in sucrose, the protoplasts began to swell after 15 minutes due to the penetration of sucrose into the cytoplasm (Bayer, 1968). If osmotic shock is carried out on bacteria which have been incubated in sucrose for longer than 15 minutes, much less periplasmic enzyme is released (Heppel, 1971).

The role of EDTA in osmotic shock treatment would seem to be to increase the permeability of the outer membrane to sucrose and/or periplasmic enzymes (Anraku & Heppel, 1967). EDTA/tris alters the permeability of the outer membrane to many water

Fig. 10.

Schematic Diagram of Events in Osmotic Shock and Lysozyme Sphaeroplast Formation



soluble agents, such as lysozyme, penicillin and actinomycinD (MacGregor & Elliker, 1958; Repaske, 1954; Leive, 1965a) and it has been shown that EDTA causes the release of up to half the cell LPS, as well as phosphatidyl ethanolamine and some protein (Leive, 1965b).

Both EDTA and tris are chelating agents, and it is thought that they form a complex which chelates more strongly than either on its own (Leive, 1974). It is not clear how EDTA addition protects the cell during osmotic shock.

Rosen & Hackette (1972) showed that the amount of cyclic-phosphodiesterase, acid hexose phosphatase and 5'nucleotidase released by osmotic shock from an unsaturated fatty acid auxotroph decreased if the culture was supplemented with *trans* rather than *cis* unsaturated fatty acids. Cells supplemented with *trans* unsaturated fatty acids also showed increased sensitivity to lysozyme in the absence of EDTA and had an altered cell surface morphology. They proposed that there was an alteration of the characteristics of the outer membrane, and that only a small amount of *trans* unsaturated fatty acid needed to be incorporated into the cell to cause this change. Substituting *trans* for *cis* unsaturated fatty acids in *E. coli* causes an increase in the permeability of the outer membrane, but also affects the release of enzymes by osmotic shock. This would not be expected if the release of periplasmic enzymes during osmotic shock was promoted by increased permeability of the outer membrane. However, it is not clear how substituting with *trans* unsaturated fatty acids would affect the cytoplasmic membrane, and how this would affect the osmotic shock treatment.

When cells of *Spirillum itersonii* are washed with EDTA in

tris buffer, a number of proteins are released, which include APase, ribonuclease 1 (RNase) and cytochrome C, but viability decreases during this treatment (Garrard, 1971). Similar results were reported for Pseudomonas aeruginosa, APase being released by manipulation of the cation concentration and by alteration of the pH of the medium (Cheng, Ingram & Costerton, 1970). The outer membrane of a marine pseudomonad is reported to be destroyed by washing cells with 0.5M sucrose (Thompson & McLeod, 1974). It would seem that the cell envelopes of Pseudomonads and related bacteria, are more dependent on divalent cations than those of members of the enterobacteriaceae. Eagon & Carson (1966) reported that Ps. aeruginosa is made osmotically fragile by treatment with EDTA. It is probable that lysis following EDTA treatment is due to increased activity of autolytic enzymes (Collins, 1964).

Periplasmic enzymes are released from E. coli by treatment with chloramphenicol (Gustaffsson, Nordstrom & Normark, 1973) or polymyxin B (Cerney & Teuber, 1971). Chloramphenicol inhibits protein synthesis. Temperature sensitive mutants of E. coli which are defective in protein synthesis at the restrictive temperature have characteristics associated with an outer membrane defect, such as increased sensitivity to lysozyme, actinomycin D and other drugs (Russel, 1971). It is probable that arresting protein synthesis interferes with outer membrane synthesis (Matzura & Broda, 1968) which could account for the release of periplasmic enzymes. Polymyxin B is a drug which disrupts bacterial membranes (Newton, 1956). On E. coli this drug acts, firstly, by disrupting the LPS containing outer membrane, and then disrupts the cytoplasmic membrane which results in

cellular lysis. Cerney and Teuber (1971) followed protein release when E. coli was treated with polymyxin B. Early in the treatment a group of proteins was released which corresponded to the proteins released by osmotic shock and only after 30 minutes were the cytoplasmic proteins released. They also found that individual periplasmic enzymes were released at different rates, which they interpreted as differential localisation of enzymes in the cell envelope.

Localisation of enzymes by cytochemical techniques

The principle aim of this technique is to locate periplasmic enzymes using specific heavy metal stains and electron microscopy. Two methods have been used, firstly reaction product deposition, which has been used extensively for APase (MacAlister, Costerton, Thompson, Thompson & Ingram, 1972) and secondly, ferritin labelled antibody techniques. Because both techniques involve fixing, embedding and sectioning for electron microscopy it is difficult to interpret the results and to assess the effect that fixing would have on enzyme localisation. Different fixing techniques have been found to yield different results (Wetzel, Spicer, Dvorak & Heppel, 1970).

A study of this nature has shown APase to be preferentially located at the poles of the cell (Wetzel et al, 1970), an observation which was supported by enrichment of periplasmic enzymes in minicells produced by a mutant of E. coli which is altered for cell division (Dvorak, Wetzel & Heppel, 1970). However in a study of several gram negative species, MacAlister et al (1972) found that in all cases APase was evenly distrubuted throughout the cell envelope. Most workers agree that these enzymes are located at the cell surface. However more precise

localisation would seem to require rigorous standardisation of the techniques used.

Measurement of activity in whole cells

The enzymes released by osmotic shock are accessible to substrate when whole cells are used as a source of enzyme. This has been taken as evidence of surface localisation. However the specific activity of whole cells is usually less than that of sonic extracts for these enzymes. This is termed partial crypticity (Brockman & Heppel, 1968). APase shows different crypticity for different substrates, which is thought to be caused by variation in the ease of penetration of the outer membrane by different phosphate esters (Brockman & Heppel, 1968). Mutants of E. coli have been isolated in which periplasmic enzymes are less accessible to substrates (Beacham, Kahana, Levy & Yagil, 1973). It is not clear whether these mutants are altered in permeability of the outer membrane, or if there is an alteration in the binding of the enzyme to the cell envelope. However it has been found that these mutants are altered in their outer membrane proteins (Beacham, personal communication) which would suggest that this defect is a function of the outer membrane.

Effect of cell envelope mutations on periplasmic enzymes

Mutations which affect the cell envelope of gram negative bacteria have been described by many workers, following selection of mutants for a variety of phenotypes, such as phage resistance, increased sensitivity to antibiotics, colicin tolerance, alteration of colonial morphology or cellular morphology, or by conditional lethality (for reviews see Makela & Stocker, 1969).

Mutations which affect the cell surface are usually

pleiotrophic and it is often difficult to relate structure and function when considering cell envelope mutants. Since periplasmic enzymes are located in the cell envelope, it is reasonable to assume that mutants defective in cell envelope biosynthesis might be altered with respect to periplasmic enzymes. Few workers have examined periplasmic enzymes in cell envelope mutants, and where they have the results are often confusing.

Mangiarotti, Apirion & Schlessinger (1968) were the first to show release of periplasmic enzymes in such a mutant. Their mutant was selected for its inability to grow without an osmotic stabiliser, probably because of a defect in cell wall biosynthesis. During normal growth it released 60% of its APase into the medium. This mutant would seem analogous to a sphaeroplast formed from a normal cell with lysozyme.

Other mutants which released periplasmic enzymes are normally defective in their outer membranes. Several classes of _E. coli_ mutants which appeared to release β-lactamase into the medium were isolated as being more resistant to penicillin on plates, but just as sensitive in liquid cultures than the parent (Eriksson-Grenberg, Nordstrom & Englund, 1971). Some of these were LPS deficient, being more sensitive to sodium cholate than the parent and becoming sensitive to phage C21. Another strain which gave the penicillin-resistant phenotype was deficient in sugar transport, and was not altered in its LPS. This gene termed _ctr_ (Wang, Morse & Morse, 1969) is possibly altered in some outer or cytoplasmic membrane function which causes the release of periplasmic β-lactamase.

Mutants of _E. coli_ and _S. typhimurium_ which have no heptose in their LPS core have been found to release 7% of their APase

into the medium (Singh & Reithmeier, 1975; Lindsay, Wheeler, Sanderson & Costerton, 1973). Several LPS mutants have been reported that release RNase 1 into the medium (Leive, 1974; Ennis & Bloomstein, 1974) although the exact nature of the LPS defect was not specified. Mutants of E. coli or S. typhimurium deficient in heptose show increased sensitivity to many antibiotics and to lysozyme (Sanderson, MacAlister, Costerton & Cheng, 1974). This is similar to changes caused by EDTA treatment, and it is probable that there is a change in the permeability of the outer membrane in these mutants.

Lopes, Gottfried & Rothfield (1972) isolated mutants which released periplasmic enzymes into the medium by screening for RNase 1 release. They showed that mutants isolated in this way are pleiotrophic, but did not specify the nature of their cell envelope defects. They concluded that there is both a binding of enzymes to cell envelope structures and a permeability barrier which prevent periplasmic enzymes from being lost from the cell.

Periplasmic enzymes have been shown by many techniques to be located in the cell envelope, but if there is an association with the cell envelope then it must be of a loose nature since the enzymes can be readily released from the cell. The data provided by studies on mutants which release periplasmic enzymes are inconclusive. It is the aim of this project to attempt to elucidate the association of periplasmic enzymes with the cell envelope.

MATERIALS AND METHODS

Bacterial Strains and Phage Stocks

The sources and phenotypes of the bacterial strains used are given in Table 2. All strains were maintained on nutrient agar slopes at $4^{o}C$. The sources of phages are given in Table 3. High titre phage stocks were prepared by the method of Adams (1959), in most cases the phages were grown on E. coli W31106. Phage C21 was grown on E. coli A15.

Media

Nutrient broth (NB) was used for all experiments involving a rich medium; 25 g Oxoid nutrient broth No. 2 per litre of distilled water. For solid media (NA) this was solidified with 1.5% agar (Difco). For experiments requiring minimal media, M9 salts were used (MM) which contained:-

NaH_2PO_4	6 g
K_2HPO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
$MgSO_4$	90 mg
$CaCl_2$	10 mg
H_2O (distilled)	1 litre

This medium was supplemented with a carbon source, usually glucose (0.2%) or glycerol (0.4%), and either amino acids (100 µg/ml) and vitamins (10 µg/ml) as required or casamino acids (Difco technical grade) (0.1% w.v.). Agar (Difco) was added (1.5%) for solid media. Ability to ferment sugars was tested on eosin methylene blue (EMB) indicator plates (Clowes & Hayes, 1968).

Chemicals were routinely purchased from British Drug Houses Chemicals Ltd. (Poole, England). Amino acids and yeast RNA were

Table 2

E. coli Strains and Their Derivation

Strain	Genetic Markers	Parent Strain	Source
E. coli K12 W3110	Prototroph		Dr. J. Jeffries
W31106	Arg^-, Str^r	W3110	This Thesis
M1	Arg^-, Str^r, Pho^c	W31106	This Thesis
ECO	$(Lac - Pro)^{\Delta}$ Str^s, F'ts lac		C.S.H.S.K.
C57B	F^- Ara^- Leu^- Lac^- Ade^- Gal^- Trp^- His^- Arg^- Str^r Xyl^- Mtl^- Met^- B_1^- $T6^r$		C.S.H.S.K.
AB1157	F^- Thr^- Ara^- Leu^- $ProA^-$ Lac^- Gal^- SupE His^- Str^r Xyl^- Mtl^- Arg^- Thi^- $T6^r$		
E. coli B	Prototroph		Dr. J. Dawes
B/4	$T4^r$	B	Dr. J. Dawes
S. typhimurium LT2			
SL1027	Met^- Trp^- Xyl^- Str^r Fla^-		Dr. I. Sutherland
SL1032	RfaG	SL1027	Dr. I. Sutherland
SL878	RfaH (GalE)	SL1027	Dr. I. Sutherland
SL1036		SL1027	Dr. I. Sutherland
SL1543		SL1027	
SL1102	RfaE	SL1027	Dr. G. Schmidt
S. minnesota R595	RfaE		Dr. G. Schmidt

Table 3

Phage Stocks

T1	C.S.H.P.K.
T3	Dr. J. Dawes
T4	Dr. A. Breeze
T6	C.S.H.P.K.
T7	Dr. J. Dawes
C21	Dr. W. Brammar
P1vir	Dr. W. Brammar
P1Kc	Dr. W. Brammar
Ø80	Dr. W. Brammar
λ 1C817	C.S.H.P.K.

C.S.H.P.K. = Cold Spring Harbor Phage Kit

obtained from Sigma Chemical Company (St. Louis, U.S.A.). p-nitrophenyl-phosphate, o-nitrophenyl galactopyranoside bis-p-nitrophenyl phosphate from Kochlight (Colnbrook, Bucks), radiochemicals from the Radiochemical Centre (Amersham), penicillin G and streptomycin sulphate from Glaxo Ltd. (Greenford, England), isopropyl-β-D-thiogalactopyranoside (IPTG) from Calbiochem (Hereford, England) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) from Aldrich Chemical Company (Milwaukee, U.S.A.).

Bacterial Growth and its Measurement

Liquid cultures were grown in either 50 ml amounts in 250 ml Erlenmeyer flasks, or 1 litre amounts in 2 litre Erlenmeyer flasks, in an orbital incubator (Gallenkampf) or reciprocal shaking water bath (Mickle, Surrey, England). Growth was estimated by measuring increase in optical density (600 nm) with a Unicam sp 1800 spectrophotometer (Pye Unicam, Cambridge, England) or by measuring the increase in viable units; a culture of E. coli with an OD_{600} of 0.5 contained approximately 5×10^8 cells/ml, and had a bacterial dry weight of 0.16 mg/ml.

Larger cultures were grown in a 15 litre Porton type vessel fitted to a L.H. Engineering fermentor (L.H.E., Stoke Poges, England).

Mutation of Bacteria

Cultures were treated with NTG to enhance the mutation frequency. Five ml of an exponential culture were washed twice in sterile 50 mM phosphate buffer, (Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 4 g; 1 litre distilled water) pH 7 and resuspended in sterile phosphate buffer containing 100 µg NTG/ml. After incubation for 15 minutes at 37°C the cells were washed twice, resuspended in 50 ml of NB and incubated overnight at 37°C. The

mutated culture was reinoculated into fresh NB and when growing exponentially, sterile glycerol was added to give a final concentration of 15% (v/v). The culture was rapidly frozen and stored at -70°C until needed.

To ensure that possible mutants isolated during the screening procedures were descendants of the parent, and not contaminating bacteria, the parental strain used in this study was made resistant to streptomycin, by spontaneous mutation, and auxotrophic for arginine following NTG mutagenesis. This doubly marked strain, W3110б, was unique to the culture collection of this laboratory.

Release of Enzymes by Osmotic Shock

This was carried out by the method of Heppel (1971) for resting phase cells. Overnight cultures were centrifuged at 8,000 g, washed twice in 0.03M tris buffer pH 7.4 and resuspended in the same buffer containing 20% sucrose and 1 mM EDTA, to give a concentration of 5 x 10^8 cells/ml. This suspension was shaken for 10 minutes at room temperature, centrifuged (8,000 g) and the pellet quickly resuspended in an equal volume of cold distilled water. This was stirred on ice for 2 minutes, 5 ml was removed for sonication and the remainder centrifuged at 8,000 g for 10 minutes. Periplasmic enzyme release was determined as the percentage of the total enzyme (as measured in sonicated cells) found in the distilled water supernatant.

Release of Enzymes by Sphaeroplast Formation

Overnight cultures were harvested and treated as in the first stage of osmotic shock. Lysozyme 1 mg/ml was added to the suspension of bacteria in sucrose and this was incubated at room temperature for 20 minutes. Sphaeroplast formation was checked

by phase contrast microscopy. Sphaeroplasts were removed by centrifugation (8,000 g) for 10 minutes and the supernatant assayed for enzyme activities.

Measurement of Enzyme Release During Growth

i) Release by overnight cultures

Cultures were grown for 18 hours in NB plus 1×10^{-4} M IPTG to induce β-galactosidase (β-GSDase) production. Five ml of culture was centrifuged (8,000 g) for 10 minutes and 5 ml was sonicated. The supernatants from both were assayed for APase and β-GSDase.

$$\text{Enzyme release (\%)} = \frac{\text{Enzyme Activity in spent media}}{\text{Enzyme Activity in culture sonicate}} \times 100$$

ii) Release by exponential cultures

Cultures were grown in NB supplemented with IPTG at 37°C. Three ml of culture was removed. One ml was centrifuged for 2 minutes in a microcentrifuge (Quickfit, England) and the remainder sonicated. Enzyme assays were performed on the culture supernatant and on the culture sonicate.

Sonication of Cells

From 2 to 25 ml of cells were disrupted by sonication, in an ultrasonic disintegrator (MSE, London, England) at maximum amplitude for a total of 2 minutes. Throughout this process the sample was cooled in an ethanol ice bath.

Enzyme Assays

i) Alkaline phosphatase

This enzyme was assayed by the spectrophotometric estimation at 405 nm of p-nitrophenol formation from nitrophenylphosphate (pNPP), in the following incubation mixture:- 1 ml pNPP, 0.7 mg/ml; 0.1 ml enzyme sample and 1.9 ml 1.0 M tris buffer pH

8.0. One unit of enzyme activity causes the release of 1 μmole of pNPP per minute at 25°C. This assay was modified by adding 1 mM Zinc chloride to the tris buffer when the enzyme sample had been exposed to, or contained EDTA.

ii) β-Galactosidase

This enzyme was assayed in toluenised cells or in cell sonicates. The amount of activity was the same in samples treated by both methods. Its activity was measured by the estimation of nitrophenol production spectrophotometrically at 410 nm from nitrophenyl galactopyranoside (oNPG) in the following incubation mixture:- 0.2 ml oNPG, 4 mg/ml; 0.1 ml enzyme sample; 1.7 ml 0.05 M phosphate buffer pH 7.0 containing 0.01 M potassium chloride, 0.001 M magnesium chloride and 0.05 M mercapto ethanol. The reaction was stopped and the assay made alkaline by adding 0.5 ml of 1 M Na_2Co_3. Enzyme units were calculated as follows:- One unit is expressed as the amount of enzyme that will produce 1 μg o-Nitrophenol from oNPG in 1 minute at 25°C.

iii) Asparaginase II

This enzyme was assayed by the phenol-hypochlorite estimation of ammonia produced from L. asparagine (Muftic, 1964). The following incubation mixture was used:- 0.7 ml 50 mM acetate buffer, pH 5.5; 0.2 ml asparagine 4 mM and 0.1 ml washed cells. After incubation at 37°C for 30 minutes the reaction was stopped by adding 0.5 ml phenol (20% in ethanol) and 1.0 ml hypochlorite solution. (25 g of calcium hypochlorite in 300 mls of hot water, add 135 mls of 20% potassium carbonate solution. Heat and dilute to 500 mls and then filter while hot.)

This was allowed to stand for 10 minutes and the extinction at 655 nm was measured against a control.

One unit is expressed as the amount of activity that produces 1 μmole of ammonia per minute from asparagine at 37°C.

iv) Ribonuclease I

Strains were streaked on NA plates, incubated overnight at 37°C and ribonuclease 1 (RNase 1) tested for by the method of Gestland (1966). Plates were exposed to chloroform for 15 minutes and completely covered with 3 ml of molten sloppy water agar containing 1.5% yeast RNA. When set, the plate was incubated at 42°C for 2 hours and the surface flooded with 1 M hydrochloric acid, which precipitates the RNA except where it has been degraded by RNase 1.

v) Cyclic phosphodiesterase

The activity of this enzyme was estimated by measuring the production of nitrophenyl from bis(p nitrophenyl) phosphate, spectrophotometrically at 405 nm in the following incubation mixture:- 0.1 ml enzyme sample; 1.0 ml 0.15 M acetate buffer pH 6.0 containing 1.4 mg $MgCl_2$/ml and 0.4 mg $CoCl_2$/ml; 0.5 ml bis(pNP)P 6 mg/ml and 1.4 ml distilled water. The reaction was stopped and the assay made alkaline by adding 2 ml 0.3 M NaOH. Units were calculated as for alkaline phosphatase.

vi) Acid phosphatase

The enzyme activity was estimated by measuring p-nitrophenyl formation from pNPP at room temperature and pH 5.0 in the following mixture:- 0.1 ml enzyme sample; 0.2 ml of 5 mM pNPP; 2.7 ml 0.2 M acetate buffer pH 5.0. The reaction was stopped and the assay made alkaline by the addition of 2 ml of 0.3 M NaOH. Units were calculated as for alkaline phosphatase.

Phage Sensitivity Tests

These were performed by placing small drops of phage

suspensions (about 10^9 p.f.u./ml) onto well dried lawns of the bacterium on NA plates. These were incubated overnight at 37°C and the plates read. A clearing of the lawn indicated sensitivity, partial clearing of the lawn or +/-, a negative result was shown by absence of lysis.

Adsorption of Page T_4 to E. coli

All adsorption experiments were carried out in 10 mM tris-HCl buffer pH 7.8 containing 0.08% NaCl, at 30°C, with a bacterial concentration of 5×10^8 cells/ml. At time zero T_4 phage was added to give a final concentration of 2×10^6 p.f.u./ml. At 3 minute intervals quantities of this mixture were removed and centrifuged to sediment the bacteria and adsorbed phage. The phages remaining in the supernate were assayed by the top agar method, using E. coli W3110б as the indicator strain.

Antibiotic Sensitivity Tests

Strains wereeither streaked onto NA plates containing varying amounts of antibiotic, and the amount of growth relative to the parent strain recorded after incubation for 18 hours, or 5 ml amounts of NB containing the required concentration of antibiotic were inoculated with 0.1 ml of an exponential culture at a bacterial concentration of 5×10^8 cells/ml and the amount of growth after 18 hours incubation with shaking estimated by the turbidity of the culture.

Genetic Mapping by Conjugation

Mutant strains were made into Hfr strains by introducing an F'tslac from ECO into a lac⁻ derivative of the mutant. At 42°C colonies which were lactose positive on EMB lactose plates, had the F'lac integrated into the chromosome. The Hfr containing the mutant lesion were mated with two well marked F⁻ strains.

Overnight cultures of both Hfr and a T6 resistant F^- strain were diluted 1 in 10 in fresh NB and incubated at 37^oC for 1 hour. Five ml of each were mixed in a prewarmed 250 ml Erlenmeyer flask, and incubated at 37^oC with gentle shaking on a reciprocal shaker (at 40 r.p.m.) for 3 hours. The exconjugants were then diluted 1 in 5 into NB containing T6, 1×10^9 p.f.u./ml, and incubated for a further hour at 37^oC. The culture was diluted and plated on M9 salts agar containing all the growth requirements of the F^- recipient and plates containing progressively fewer of these requirements. A gradient of transmission was plotted of the number of recombinants for each marker against the distance of that marker in minutes of chromosome time from the origin of chromosome transfer, which in this case was at 9 minutes, lactose being the last marker introduced, and _pro_A one of the earliest. Recombinants for each selected marker were tested for the mutant phenotype and the position of the mutation on the chromosome estimated from the gradient of transmission.

Incorporation of Radiolabelled Substrates into Bacteria

DNA, RNA and protein biosynthesis were studied by following the incorporation of [6-^{3}H] thymidine (^{3}HTdr), [5-^{3}H] uridine (^{3}HUr) or L[u-^{14}C] arginine, respectively, into trichloroacetic acid (TCA) insoluble material. All experiments were carried out with aerobically growing NB cultures, with addition of 10 μCi/ml of ^{3}HTdr and ^{3}HUr and 5 μCi/ml of ^{14}CArg. To ensure that ^{3}HTdr was only incorporated into DNA, 100 μg 2-deoxyadenosine/ml was added to these cultures.

To measure the radioactivity of the TCA insoluble material 10 μl of culture was placed onto a Whatman No 1 filter disc, diameter 2.1 cm. When the liquid was absorbed by the paper the

disc was immersed in cold 5% TCA. After all the samples had been taken, the discs were left in 5% TCA on ice for 10 minutes and washed 3 times with fresh cold TCA. Each time the discs were left in TCA on ice for 10 minutes. The discs were washed twice in absolute alcohol to remove the TCA, and spread on a piece of blotting paper and dried at 45°C. Each dry disc was placed in a scintillation vial, and 5 mls of toluene based liquid scintillant NE 233 (Nuclear Enterprises, Edinburgh, Scotland) was added to each vial. The amount of radioactivity on each disc was measured in a Packard tricarb liquid scintillation spectrometer model 3330 (Packard Instruments Ltd., Reading).

Polyacrylamide Gel Electrophoresis (P.A.G.E.)

Gels (7.5%) were prepared by the method of Weber & Osborn (1969). Acrylamide stock solution 13.5 ml (containing 22.2 g acrylamide recrystallised from chloroform, and 0.6 g bis acrylamide in 100 mls of water) was mixed with 15 ml of double strength gel buffer pH 7.0 (7.8 g $NaH_2Po_4.2H_2O$ + 51.0 g $Na_2HPo_4.12H_2O$ + 2 g sodium dodecyl sulphate (SDS) in a litre of water). This was de-aerated and 1.5 ml of a freshly prepared ammonium persulphate solution (15 mg/ml) and 0.035 ml of N,N,N',N' tetramethylethylene diamine (TEMED) were gently added, and the mixture gently swirled. 1.7 ml of this solution was rapidly transferred to each stoppered gel tube and a small amount of water layered on top of each to ensure that the gel top would be flat when set.

Samples to be run on gels were solubilised first by boiling for 10 minutes in gel buffer, and then to 0.8 ml of the sample 0.1 ml of 80% glycerol and 0.1 ml of 0.05% Bromothymol blue were added. Approximately 100 μg of protein was loaded into each gel, although rarely more than 50 μl of liquid was applied. Gels

were run in gel buffer with a constant current of 8 mA on a Shandon voKam electrophoresis unit (Shandon, London, England) until the bromothymol blue tracker dye had run three-quarters of the length of the gel. The length of the gel and the distance that the dye had moved were measured.

Gels were stained and destained by the 5-stage procedure of D. F. H. Wallah (Max-Planck-Institut für Immunbiologie, Freiburg, Germany):-

i) 1.25 g coomassie blue; 650 ml isopropanol; 250 ml acetic acid and 1,650 ml distilled water - 18 hours.

ii) 125 mg coomassie blue; 250 ml isopropanol; 250 ml acetic acid; 2,000 ml distilled water - 18 hours.

iii) 63 mg coomassie blue; 250 ml acetic acid; 2,250 ml distilled water - 18 hours.

iv) 800 ml methanol; 200 ml acetic acid and 1,000 ml distilled water - 6 hours.

v) 10% acetic acid - 12 hours.

The gels were stored in 10% acetic acid. The mobility of the various proteins were calculated as -

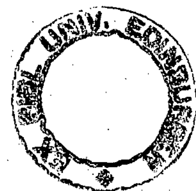
$$\text{mobility} = \frac{\text{Distance of protein migration}}{\text{Gel length after destaining}} \times \frac{\text{Gel length at the start}}{\text{Distance of dye migration}}$$

Gels were read on a Joyce Loebel UV gel scanner (Gateshead, England).

Preparation of samples for P.A.G.E.

1) Periplasmic proteins

Periplasmic proteins were obtained from both the spent medium and the osmotic shock fluid of 100 ml of M9-salts-grown culture. Both were dialysed overnight against running tap water and then freeze dried. The resulting solids were resuspended in



5 ml of gel buffer.

2) Outer membrane proteins

Outer membrane was isolated by a modification of the method of Schnaitman (1970). A litre of NB was inoculated with 50 ml of an overnight culture and incubated for 2½ hours at 37°C. The culture was harvested by centrifugation at 8,000 g for 10 minutes at 4°C and the cells resuspended in 50 ml of 25% sucrose in 10mM tris buffer pH 7.4 containing 1 mg lysozyme/ml. The mixture was kept on ice for 2 minutes and then diluted with gentle swirling over a period of 8 minutes with 100 ml of 1 mM EDTA in 10 mM tris buffer pH 7.4. Sphaeroplast formation was checked by phase-contrast microscopy. The sphaeroplasts were disrupted by sonication, and the unbroken cells pelleted by centrifugation at 5,000 g for 10 minutes. The supernate was centrifuged at 38,000 r.p.m. for 1 hour in a MSE Superspeed 50 centrifuge at 4°C. The resulting pellet was drained of liquid and resuspended in a total of 15 ml 10 mM N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) buffer pH 7.4 containing 10 mM $MgSO_4$ and 2% triton X100. The mixture was incubated at room temperature for 20 minutes and centrifuged again at 38,000 r.p.m. for 1 hour. The pellet was resuspended in 3 ml of gel buffer.

Isolation of Lipopolysaccharide by the Method of Westphal & Jann (1965)

Fifteen litres of M9-salts-grown bacteria were harvested by centrifugation at 8,000 g for 10 minutes at 4°C, washed with fresh medium and freeze dried. One gram of freeze dried cells were dispersed in 10 ml of distilled water, heated to 70°C, and mixed with an equal volume of 90% phenol also at 70°C. The mixture was stirred for 10 minutes at this temperature, cooled in

ice, and centrifuged, using glass centrifuge tubes, to separate the aqueous and phenolic phases. The aqueous phase was collected, dialysed overnight against tap water, and centrifuged at 35,000 r.p.m. for 3½ hours to sediment the LPS. This was resuspended in distilled water and freeze dried.

Hydrolysis and Chromatography of LPS

Five mg of LPS was hydrolysed for 3 hours in 2 ml of 2 M hydrochloric acid in a sealed tube at 100°C, and the HCl neutralised by adding an excess of Dowex resin, and deionised by washing through a short Dowex/amberlite resin column and taken to dryness in a rotary evaporater. The hydrolysed LPS was resuspended in 0.2 ml distilled water, 50 µl spotted onto Whatman No. 1 chromatography paper and run with standards for 24 hours in butanol: pyridine:water (6:4:3). The chromatogram was developed by the alkaline-silver nitrate reagent of Trevelyan, Procter and Harrison (1950).

Quantitative Analytical Procedures

i) Protein was determined either by the method of Lowry, Rosenburgh, Farr and Randall (1951) using bovine serum albumin as a standard, or by the ratio of extinctions at 260 nm and 280 nm by the following equation -

Protein concentration (mg/ml) = E_{280nm} x Factor x $1/d$

d = length of light path in cm. The factor is obtained from Data for Biochemical Research, Dawson et al (1969).

RESULTS

SECTION 1

Affect of Physical Conditions on Periplasmic Enzyme Release

APase is the best known and characterised of the periplasmic enzymes. It is also the most amenable to study, having a simple colorimetric assay.

The APase of E. coli is repressed by high concentrations of phosphate and induced when the concentration of inorganic phosphate falls to a low level (Echols, Garen, Garen & Torriani, 1961). Phosphate is commonly used to stabilise the pH of culture media. However low phosphate medium for the induction of APase must be buffered by some other substance. Tris (Hydroxymethyl) aminomethane (Tris) has often been used for this purpose but is a strong chelating agent and is necessary for the removal of LPS by EDTA. The effect that Tris has on the cell envelope is not clear, but for a study of cell envelope enzyme relationships it was decided not to use Tris to buffer the growth medium. Few other substances are suitable for buffering growth media and these are prohibitively expensive. To overcome this problem a strain which was constituitive for APase was constructed.

Isolation of mutants of E. coli W31106 constituitive for alkaline phosphatase

Both NTG mutated and non-mutated cells of E. coli W31106 were plated on Tris-buffered minimal agar containing 0.01M glycerophosphate as the sole carbon source, and a high concentration of inorganic phosphate. APase is necessary for growth when glycerophosphate is the sole carbon source. Clones that can grow on glycerophosphate in the presence of high concentrations of inorganic phosphate must be derepressed for APase production (Torriani & Rothman, 1961). Colonies growing through the residual lawn were picked off and purified by streaking for single colonies on high phosphate, glycerophosphate minimal medium. In

this way four strains constituitive for APase were isolated. Their APase activities when growing in high and low phosphate media are given in Table 4. The spontaneous mutant M1 was used for further experiments, as it is less likely to be a double mutant than those derived from NTG treated populations.

Release of enzymes by osmotic shock and lysozyme sphaeroplast formation

E. coli M1 did not release APase or β-galactosidase (βGSDase) into the growth medium, or during the first stage of osmotic shock treatment. However large amounts of APase were released during the second stage of osmotic shock, and during lysozyme sphaeroplast formation (Table 5). These procedures cause the release of 4% of the total cellular protein, but no cytoplasmic β-GSDase (Heppel, 1971).

Polyacrylamide gel electrophoresis of the proteins released from M1 by osmotic shock demonstrated that only 10-12 protein species were released (Fig. 11). Three protein bands, 5, 6 and 7, form the majority of the protein released by osmotic shock. These correspond to proteins of M.W. 60,000, 52,000 and 40,000 respectively (Table 6). APase is a dimer of M.W. 80,000 and could give rise to band 7. This band was absent in gels of proteins released by osmotic shock from E. coli W31106 grown under conditions when it would not produce APase. On a M.W. basis bands 5 and 6 could correspond to cyclicphosphodiesterase and 5' Nucleotidase and band 11 to Ribonuclease 1 (RNase 1).

Osmotic shock was carried out in two stages. During the first stage the cells were treated with Tris/EDTA at pH 7.4 in the presence of 20% sucrose. The Tris/EDTA treatment causes removal of LPS and other outer membrane material (see introduction:

Table 4

Specific Activities of APase in APase Constituitive Mutants of *E. coli*

Strain	Mutagen	Sp Activity of APase in Low Phosphate medium[(a)] units/mg protein	Sp Activity of APase in High Phosphate medium[(b)] units/mg protein
W31106	WT	0.715	0.00112
M1	Spont	0.587	0.392
M2	NTG	0.54	0.455
M3	NTG	0.48	0.37
M4	NTG	1.09	0.274

(a) Low phosphate medium was that of Neu & Heppel, 1964.

(b) The high phosphate medium was M9 salts.

Table 5

Release of APase and βGDSase by Various Treatments, from E. coli M1

Sample	APase[d]	βGSDase[d]
Total cells (sonicate)	0.58	2.0
Spent medium	0.005 (0.8)[a]	0.002 (0.1)
Osmotic shock (stage I)[b]	0.006 (0.1)	-
Osmotic shock (stage II)[c]	0.383 (66.0)	0.008 (0.4)
Lysozyme sphaeroplast formation	0.44 (76.0)	0.0085 (0.4)

(a) Figures in brackets refer to % of total enzyme.

(b) Stage I is the tris/EDTA sucrose plasmolysis stage.

(c) Stage II is the cold water shock stage.

(d) Units/ml of culture.

Fig. 11.

Scan of Proteins Released by E. coli M1 (A) Following Osmotic Shock; (B) Into the Medium. Separated by P.A.G.E. and Stained with Coomasie Blue

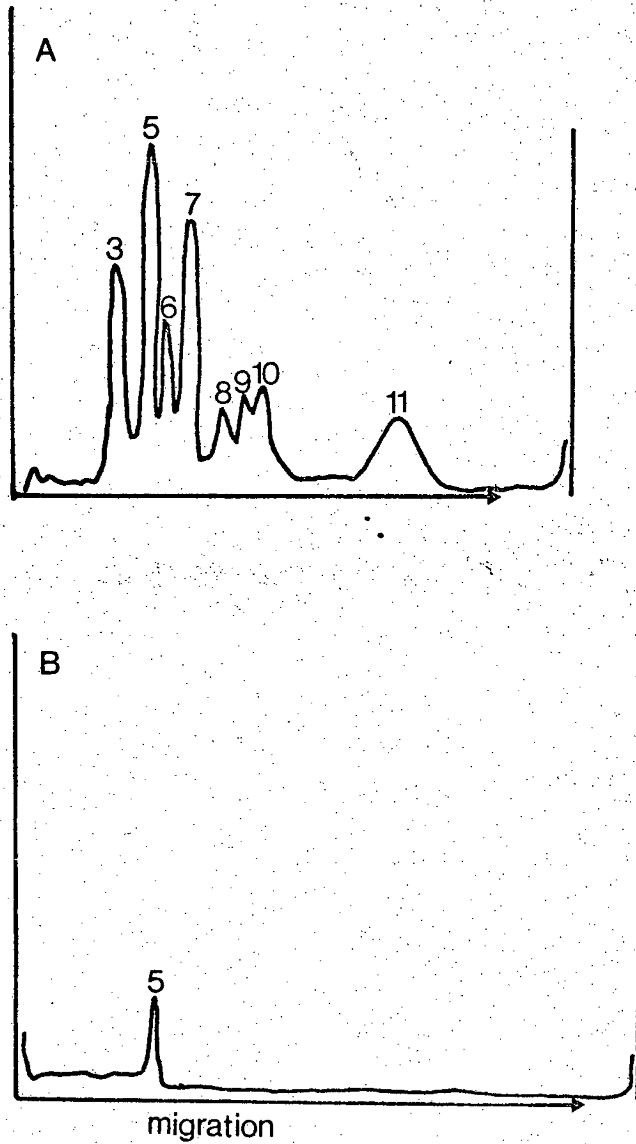


Table 6

M.W. of Proteins Released by Osmotic Shock

Band No.	Mobility	Estimated M.W. x1000	Postulated Protein
3	0.215	78	
4	0.251	69	
5	0.305	60	Cyclic Phosphodiesterase
6	0.359	52	5'Nucelotidase
7	0.412	44)	APase
8	0.439	41)	
9	0.560	30	
10	0.610	26	
11	0.914	11	RNase 1

section 2) while 20% sucrose plasmolyses the cells. In stage II the plasmolysed cells were subjected to a sudden reduction in the osmotic pressure of the medium, resulting in swelling of the cells and rupture of the outer membrane (Bayer, 1968).

When EDTA was omitted from stage I of osmotic shock, only 15% of the APase was released in stage II, compared to 60% when 1 mM EDTA was present in stage I. The effect of increasing the EDTA concentration in stage I was to decrease the amount of APase released in stage II while increasing its release in stage I (Fig. 12). However 10mM EDTA only releases 1% of the APase during stage I.

Release of enzymes from M1 in HEPES buffer

When exponentially growing bacteria were incubated in 10mM HEPES buffer pH 7.4 at a concentration of 4×10^8 cells/ml both APase and βGSDase were released at the same rate (Fig. 13). This indicates that the bacteria were lysing and that there was no specific release of periplasmic enzymes. However when 5mM EDTA was added to the HEPES there was an immediate release of APase into the medium. No further release occurred even after prolonged incubation (Fig. 14). Nearly 4% APase was released from actively growing cells treated with EDTA compared with 0.1% released from resting phase cells, as shown by the tris/EDTA treatment during osmotic shock (Fig. 12, a and b). This may reflect differences in the stability of the cell envelope in resting and exponential phase cells. Clearly EDTA treatment does not cause substantial release of APase, although there was a detectable increase in enzyme release due to treatment with this agent.

Affect of cation conc and pH on APase release

Fig. 12.

Release of APase From M1 By Osmotic Shock: Effect of Increasing EDTA Concentrations in Stage I

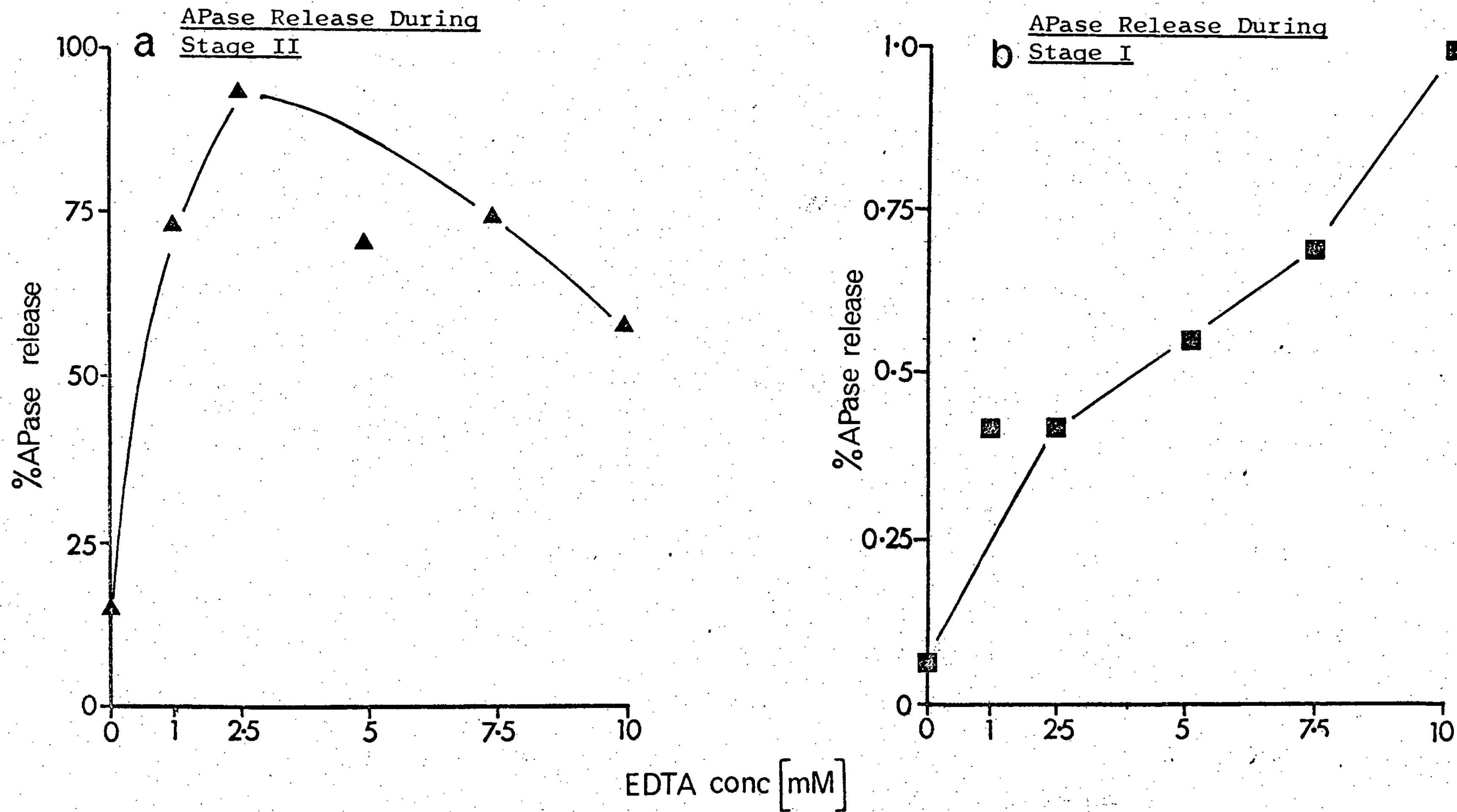
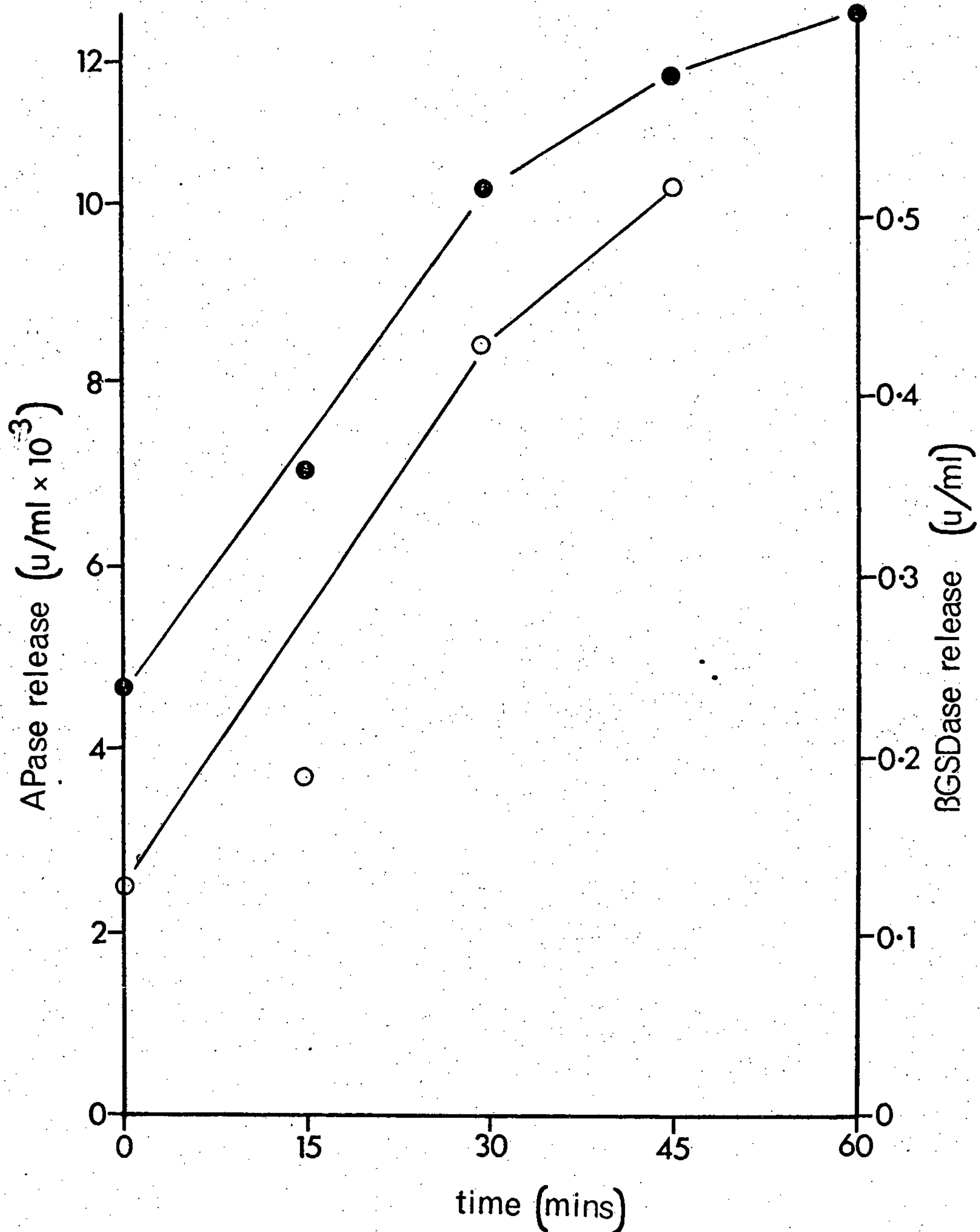


Fig. 13.

Release of APase and βGalactosidase From M1 Incubated in Hepes pH 7.4



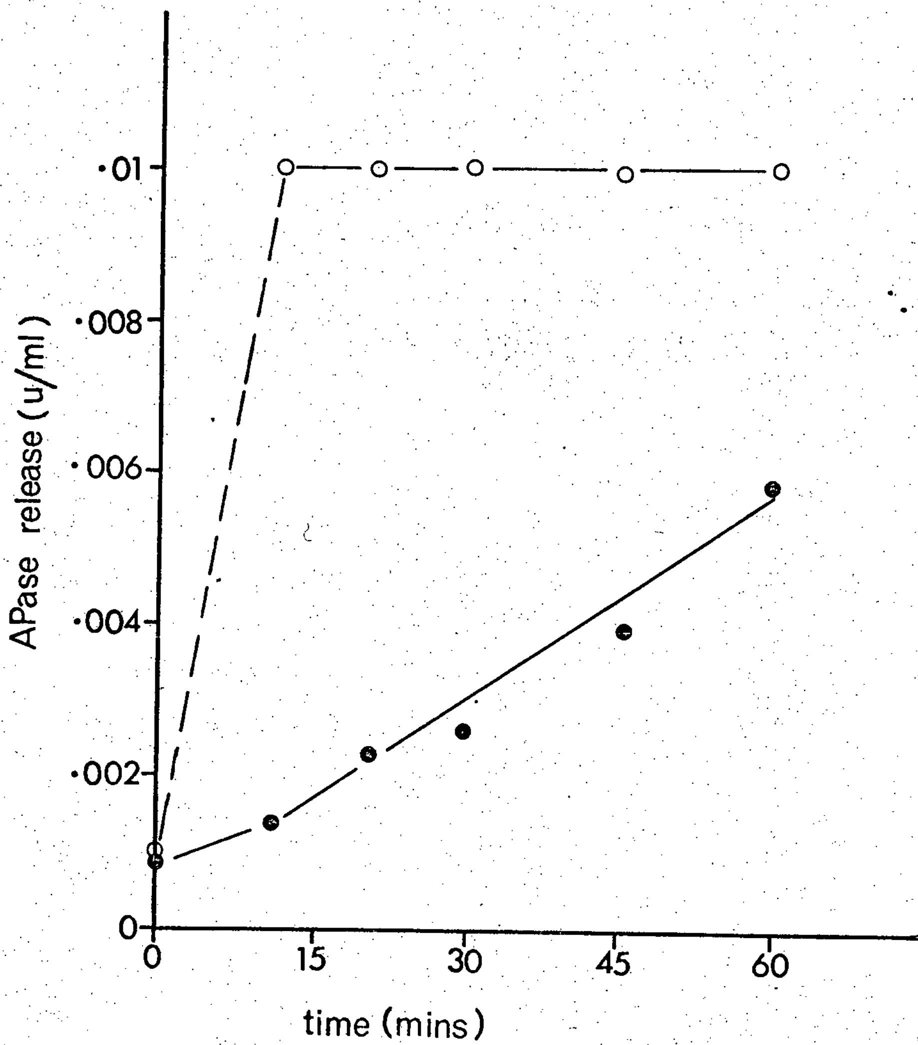
● APase release;

○ βGSDase release.

Cells grown in NB containing IPTG, 37°C, to 5 x 10^8 bacteria/ml, harvested, washed and resuspended in an equal volume of 10 mM Hepes buffer pH 7.4, and then incubated at 37°C.

Fig. 14.

Release of APase From Washed Cells of M1
Effect of EDTA



○ M1 cells incubated in HEPES + 5mM EDTA;

● M1 cells incubated in HEPES alone.

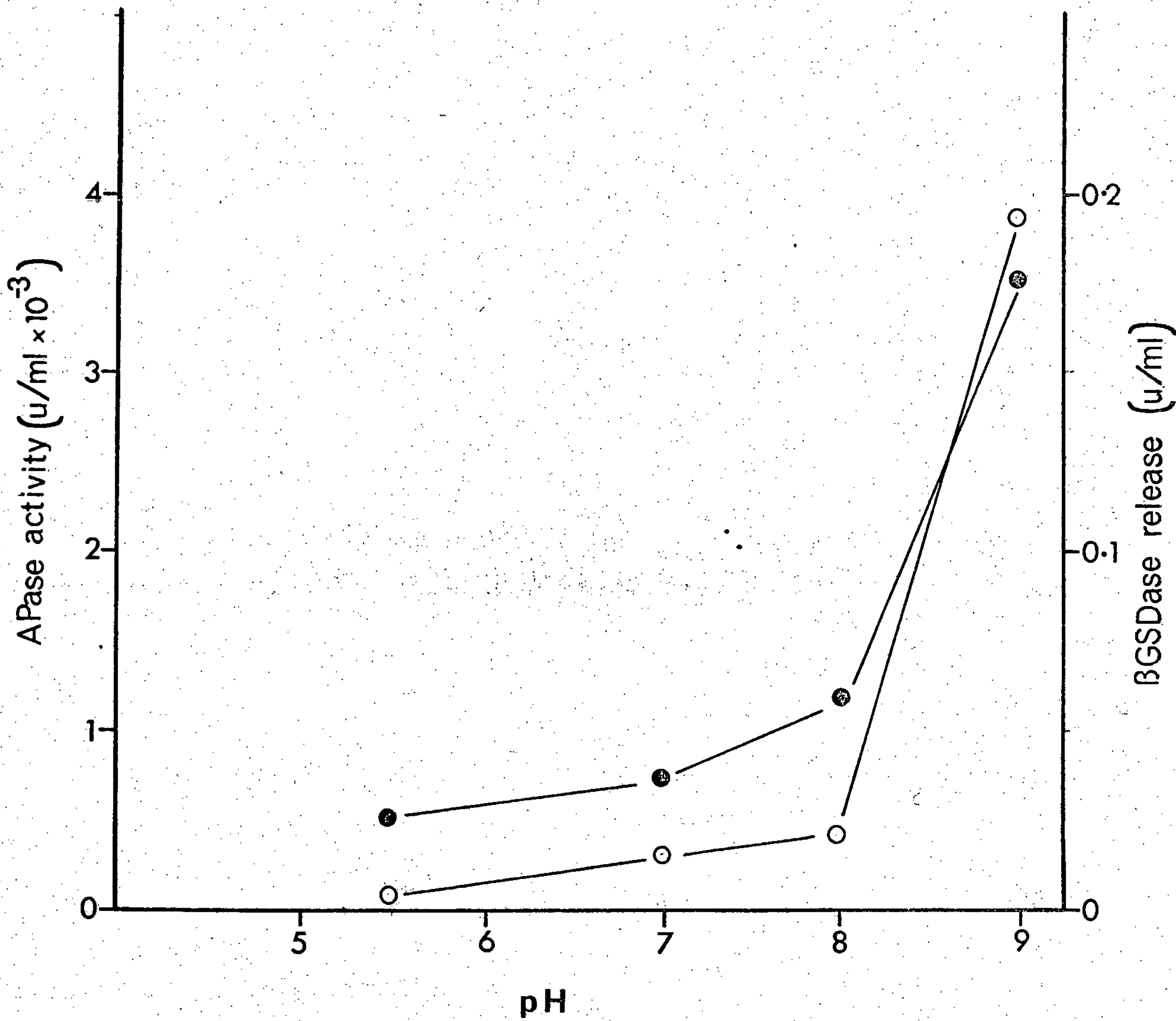
The APase of Pseudomonas aeruginosa can be released by washing cells with elevated Mg^{2+} concentrations or by manipulating the pH (Cheng et al, 1970). If the association between APase and the E. coli cell envelope is purely of an ionic nature, alteration of cation concentration and the pH of the medium should cause enzyme release.

An exponentially growing culture of M1 was washed in 10mM HEPES buffer pH 7.4 and resuspended in various buffers at a concentration of 4×10^8 cells/ml. The pH of the buffers ranged from pH 5.5 to 9.0. There was no peak of APase release (Fig. 15). Throughout the experiment release of APase and β-GSDase was identical, indicating that enzyme release was due to cell lysis. Similarly washing the cells with HEPES buffer containing various concentrations of NaCl, did not cause release of the APase (Table 7).

Clearly in E. coli the APase is not released by manipulating the physical conditions of the medium and it is unlikely that the association of the enzyme with the cell envelope is by purely ionic attraction. However the association must be of a loose nature, since the periplasmic enzymes can be released by osmotic shock and lysozyme sphaeroplast formation. Removal of some of the LPS from the cell by EDTA treatment has been shown to cause an increase in permeability of the cells to various water soluble molecules (Leive et al, 1968) including lysozyme, which is a protein of M.W. 14,000. EDTA treatment did not cause the release of APase which has a M.W. of 80,000 but RNase 1 of M.W. 11,500 was released when E. coli M1 was grown on plates containing EDTA. The release of periplasmic enzymes in EDTA-damaged cells may be related to the size and shape of the molecules which would

Fig. 15.

Release of APase and βGSDase From M1
Effect of pH Manipulation



●APase release;

○βGSDase release.

Cells prepared as in legend to Fig. 13, and resuspended in an equal volume of buffer - acetate/acetic acid pH 5.5, Hepes/HaOH Tris/HCl pH 8.0.

Table 7

Release of APase from M1 Cells Washed with Various Concs of NaCl

Salt Conc (M)	APase Release Units/ml	% Release of Total Enzyme
0	0.0065	4.2
0.086	0.005	3.5
0.172	0.0057	3.7
0.345	0.0076	5.0

Cells were grown in NB to 4 x 10^8 cells/ml, harvested, washed in 10mM Hepes/HCl buffer pH 7.4, and then resuspended in this buffer and incubated at room temperature for 15 minutes. Enzyme release was determined as in the materials and methods.

indicate that EDTA treatment creates weak areas or small holes but not generalised breakdown of the outer membrane. However the periplasmic enzymes may be associated with different structures within the cell envelope which are affected by EDTA to different extents.

SECTION 2

Mutations Affecting the Cell Envelope

Mutations which affect the gram negative bacterial cell envelope are often pleiotropic. It is reasonable to assume that altering the cell envelope may affect its association with the periplasmic enzymes. Cell envelope mutants may be isolated by screening for such phenotypes as antibiotic sensitivity, phage resistance and altered morphology. Several of these mutants were obtained and examined for alteration of their cell envelope/ periplasmic enzyme associations.

Mutations Affecting LPS

i) Phosphoglucomutase mutants

Phosphoglucomutase (Pgm) catalyses the interconversion of glucose.6.(P) to glucose.1.(P) (Fig. 16). Glucose.1.(P) is a precursor of uridine diphosphoglucose (UDPGlu) and uridine diphosphogalactose (UDPGal) (Adhya & Schwartz, 1971). Mutants deficient in Pgm, when growing on glucose or amino acid medium, cannot make UDPGlu or UDPGal, but can if grown on galactose. However when growing on galactose or maltose glucose.1.(P) accumulates but is turned into maltodextrin and amylose which are stainable with iodine to give the "blu" phenotype of Adhya and Schwartz (1971).

Mutants which stained dark blue with iodine when grown on maltose were isolated following NTG mutagenesis of M1. Survivors were plated on EMB base agar containing 0.4% maltose. These were replicated onto fresh plates. After incubation the plates were flooded with a 0.1% solution of iodine in 1.0% potassium iodide. Colonies staining dark blue were easily identified and picked off the master plate.

Three classes of "blu" mutant are known; (1) maltodextrin-phosphorylase-less mutants, (2) 6-phosphogluconolactonase-less

Fig. 16.

Interconversions of Glucose and Galactose to Form Cell Wall Polysaccharide Precursors

polysaccharide biosynthesis

UDPGlu ⟷ UDPGal

2

Glu·1·P

UDPGlu

Malto-dextrins

1

Gal·1·P

Glu·6·P

ATP

Maltose

PEP

Galactose

Glucose

Lactose

1 = phosphoglucomutase

2 = UDP glucose pyrophosphorylase

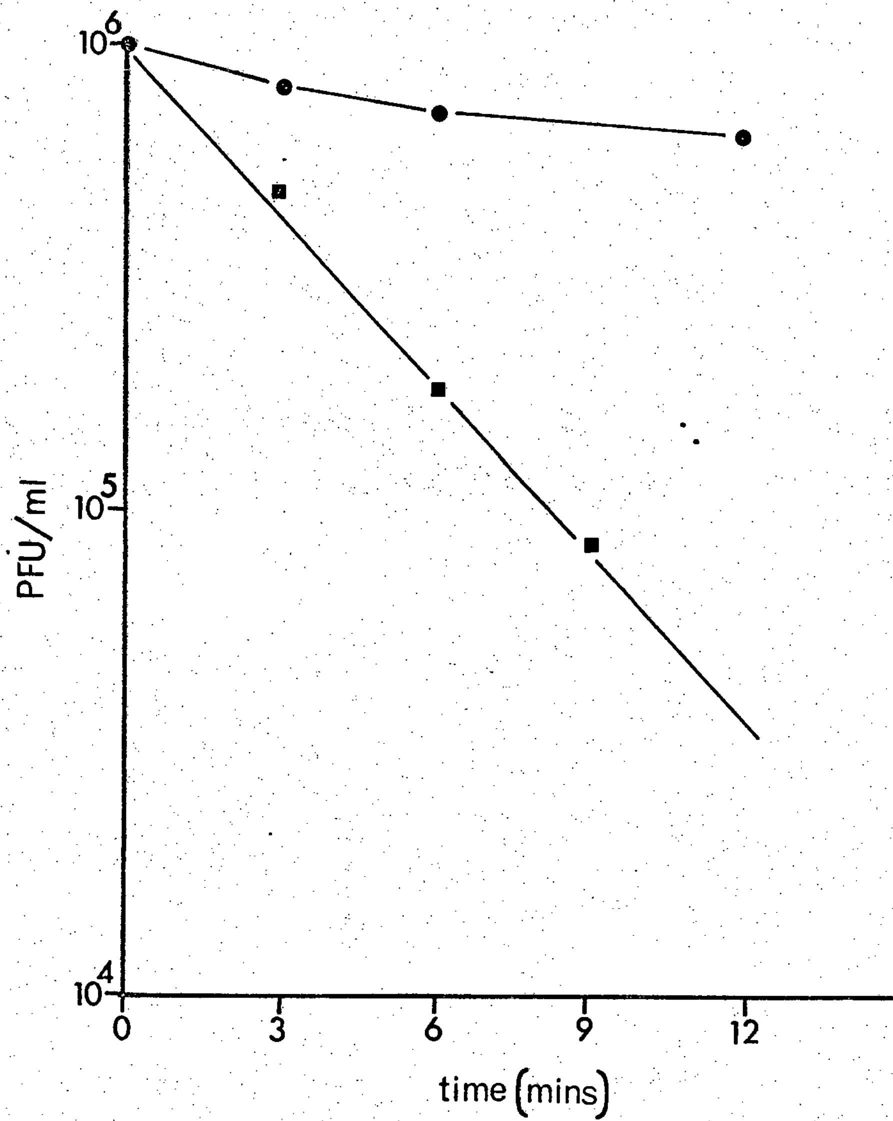
mutants, which accumulate gluconolactone, a strong inhibitor of maltodextrin phosphorylase, and (3) phosphoglucomutase-less mutants. Pgm mutants give the "blu" phenotype on both maltose and galactose and can therefore be readily differentiated from the other two classes of "blu" mutant.

Phage typing of the Pgm mutant, designated A15, showed that it grew the phages T1, T4, T6, T7, λ , P1 and Ø80. However, unlike M1, it was sensitive to phage C21 when grown on glucose medium but was resistant when grown on galactose medium. Phage C21 binds to cells which have LPS deficient in galactose (Shedolovsky & Brenner, 1963). If the mutant, when growing on glucose or amino acid, is unable to make UDPGlu glucose or galactose would not be expected to be incorporated into cell envelope polymers such as LPS. The T4 adsorption site is absent in cells deficient in UDPGlu pyrophosphorylase (Hattman & Fukasawa, 1963) and high glucose concentrations inhibit the adsorption of T4 to *E. coli* B (Dawes, 1975). It would seem that glucose is part of the adsorption site of T4 in *E. coli* LPS. Clearly A15 must have some complete T4 adsorption sites or it would not grow the phage and therefore must incorporate some glucose into the LPS. If adsorption of T4 to A15 is compared with that to M1 (Fig. 17) only 25% was adsorbed by A15 and over 95% by the parent M1. Thus there is a reduction in the number of T4 adsorption sites. Either this mutant is not completely blocked in phosphoglucomutase activity or there is some other way of producing glucose.1.(P) in the bacterial cell. A15 did not leak RNase 1 or APase into the growth medium nor was it more sensitive to DOC or GV than M1 (Table 8 and 9).

The postulated function of EDTA in osmotic shock treatment

Fig. 17.

Adsorption of T4 to *pgm*$^{+}$ and *pgm*$^{-}$ Strains



● A15;

■ M1.

Table 8

Release of APase and βGSDase During Growing of M1 and A15

Strain	APase Units/ml	βGSDase Units/ml
M1	0.006 (1.0)	0.00012 (0.3)
A15	0.003 (0.87)	0.00007 (0.2)

Figures in brackets % of total enzyme released.

Table 9

Sensitivity to Phage, DOC and GV, and RNase 1 Release by M1 and A15

Strain	T4	T7	C21	DOC 30 mg/ml	GV 5 µg/ml	RNase 1 release
M1 NB	+	+	-	r	r	-
M1 NBG[a]	+	+	-	r	r	-
A15 NB	+	+	+	r	r	-
A15 NBG	+	+	-	r	r	-

(a) NBG = Nutrient Broth + galactose (0.2%).

is to make the cell envelope more permeable (Heppel, 1971). EDTA causes an alteration in permeability by removing a large amount of LPS and some outer membrane material (Leive, 1965). The release of enzymes during osmotic shock treatment with and without EDTA, was examined using E. coli A15, which has an LPS defect suppressible by growing the cells in the presence of galactose, to see if a defective LPS would have the same effect as treating the cell with EDTA. The results are summarised in Table 10. Very little APase was released by osmotic shock of M1 without EDTA, irrespective of the growth medium. With EDTA approximately 70% of the enzyme was released. A15 released 60% of the APase by osmotic shock without EDTA when grown on non-galactose media, but only 1.5% when grown in the presence of galactose. Galactose grown A15 cells released 80% APase by osmotic shock in the presence of EDTA. Clearly the defective LPS allowed the release of APase by osmotic shock whether EDTA was present or not. The effect of EDTA appears to be to alter the outer membrane and this alteration is required for successful release of periplasmic enzymes by osmotic shock. The Pgm mutation however, does not cause a change in the association of periplasmic enzymes with the cell envelope.

ii) Penicillin super sensitive mutants of E. coli

Mutants of E. coli M1 which were more sensitive to penicillin were isolated following NTG mutagenesis, and screening the survivors for the inability to grow on 30 μg penicillin/ml. In this way Ps2 and Ps4 which had identical phenotypes were isolated. They were found to be more sensitive to novobiocin and penicillin than M1, and T4 resistant, but they were not tested for T7 resistance. These strains released RNase 1 as shown by the plate

Table 10

Effect of EDTA on Release of APase by Osmotic Shock by M1 and A15 Grown With and Without Galactose

	APase Release by Osmotic Shock Units/ml	
Strain	+EDTA	-EDTA
M1 NB	0.252 (72)[b]	0.006 (1.7)
M1 NBG[a]	0.246 (71)	0.014 (4.0)
A15 NB	0.11 (77)	0.083 (60)
A15 NBG	0.12 (80)	0.0022 (1.5)

Osmotic shock carried out as in materials and methods.

(a) NBG = Nutrient Broth + 0.2% galactose.

(b) Figures in brackets % of total enzyme.

assay, but did not release APase (Table 11). The mutants had a slower growth rate in NB than the parent. As these mutants were T4 resistant there is likely to be an LPS alteration.

Similar mutants have been described which have altered LPS and are T4 resistant (Tamaki et al, 1971). These mutants were reported to leak periplasmic APase (Singh & Reithmeier, 1975), which is in contrast to the results obtained in this study. Singh & Reithmeier (1975) obtained their novobiocin-sensitive strains from Tamaki et al (1971) and APase production was induced in tris-buffered minimal media. Under these conditions they found that 4-8% of the APase activity was released into the culture medium. This amount of enzyme release might have been caused by the action of tris on these deep rough LPS mutants.

iii) E. coli M1 mutants resistant to phages T4 and T7

Spontaneous mutants resistant to phages T4 and T7 were isolated as colonies that grew through a top agar plate containing enough phage to give confluent lysis of the bacteria. Several mutants were isolated and these fell into 3 phenotypic classes (Table 12). Class 1, isolated for T4 resistance, were sensitive to GV but not to DOC, sensitive to T7 and C21 and also released RNase 1 into the growth medium. Clearly this group has an LPS defective in galactose and also in the T4 receptor site, but not the T7 receptor site. Class 2, isolated as resistant to T4 or T7 were mucoid, resistant to C21, but did not show any of the other phenotypes normally associated with LPS deficient mutants. They did not release RNase 1 into the medium. It is probable that the receptor sites for the phages are blocked by extracellular material known to occur in other mutants (Radke & Siegal, 1971). Class 3, isolated as resistant to Phage T7 were resistant to T4,

Table 11

Characterisation of Penicillin Sensitive Mutants of *E. coli*

	Sensitivities to						
Strain	T4	T7	T1	T6	Novo-bio-cin 30 mg/ml	Peni-cil-lin 30 mg/ml	Dbl-ing Time NB $37^{o}C$	RNase 1 re-lease
W31106	+	+	+	+	r	r	27	-
Ps2	-	+	+	+	s	s	55	+
Ps4	-	+	+	+	s	s	50	+

Table 12

Characterisation of T4 and T7 Resistant Mutants of *E. coli*

Class	DOC 30 mg/ml	GV 5 µg/ml	T4	T7	C21	RNase 1 re-lease
WT	r	r	+	+	-	-
1	r	s	-	+	+	++
2	r	s	-	-	-	-
3	s	s	-	-	-	+++

T7 and C21. They were also very sensitive to DOC and GV and released large amounts of RNase 1. This group of mutants show alterations consistent with a mutation affecting the innermost part of the core LPS and are likely to be defective in the adsorption sites of phages T4, T7 and C21.

None of these classes of mutants released APase at significantly higher levels than can be explained by cellular lysis, as shown by βGSDase release (Table 13). Extensive lysis occurred in some of these strains, notably those from Class 3. Why this should occur is not clear.

Release of RNase 1 by mutants from Classes 1 and 3 was tested on plates. This release was distinguished from release due to cellular lysis by incorporation of 20% sucrose into the agar to stabilise the bacteria. APase however was not released. Clearly in both Classes 1 and 3 there is an alteration in the permeability barrier of the outer membrane. This was shown by increased sensitivity to GV which is a monitor of permeability of the outer membrane (Gustafsson, Nordstrom & Normark, 1973) and also by the susceptibility of Class 3 to DOC. The difference in patterns of release of RNase 1 and APase in these mutants could be due either to a difference in localisation within the cell envelope or to a difference in molecular size. It is not clear what part the LPS plays in maintaining periplasmic enzymes within the cell envelope. To study this in more detail known LPS mutants were obtained.

iv) LPS deficient mutants of Salmonella typhimurium

The structure of the LPS of S. typhimurium has been determined by the isolation and characterisation of defective mutants (for reviews see Stocker & Makela, 1971; Lüderitz, Westphal, Staub & Nikaido, 1971). Mutants for most steps in the biosynthesis of the

Table 13

Release of Enzymes by T4 and T7 Resistant Mutants of *E. coli*

Class	RNase 1	APase	βGSDase
WT	-	0.8%	0.1%
1	++	6.0%	6.8%
2	-	2.4%	0.3%
3	+++	11%	8.5%

LPS core are available. The structure of the core of S. typhimurium and its core-deficient mutants is shown in Fig. 9. A range of these mutants was obtained and tested for various characters such as increase in sensitivity to novobiocin, DOC and GV, and also for release of RNase 1 and cyclicphosphodiesterase (CPDase). APase is not produced by S. typhimurium.

As the LPS lesion affects more of the core region so the strain becomes more sensitive to GV. rfaG, which has no glucose or galactose in its LPS, is more sensitive to GV than the WT, while rfaH, which has one residue of glucose attached to the core is not. Similarly it is only the heptose-less strain rfaE which becomes sensitive to novobiocin and DOC (Table 14). The release of RNase 1 and CPDase increased as the mutation affected more of the core and reached a maximum in the rfaE strain (Table 15). It is interesting that even the rfaK strain has some effect on release of RNase 1 and CPDase, although this is slight. A control for cellular lysis was not included in this experiment but the total growth of these strains was similar to that of the wild type and it is unlikely that 74% of the culture had lysed in the case of the rfaE strain. More acid phosphatase (AcPase) was released from the rfaE strain than from the WT, but the level of enzyme released by the WT was very high. The reason for this is not clear. CPDase has a M.W. of approximately 60,000 and a large amount of this enzyme is released by the rfaE strain. Clearly there is not much of a permeability barrier presented by the outer membrane of the rfaE strain to this enzyme.

Lindsay et al (1973) reported that APase was released from a S. typhimurium rfaE strain which contained an F' carrying the APase genes from E. coli. Their results were similar to those of

Table 14

Sensitivity of *S. typhimurium* LPS Mutants to Deoxycholate, Gentian Violet and Novobiocin

Strain	Mutation	DOC 30 mg/ml	GV 5 μg/ml	NOV 30 μg/ml
SL1027	Wild type	R	R	R
SL1032	rfa G	R	S	R
SL878	rfa H	R	R	R
SL1036	rfa K	R	R	R
SL1102	rfa E	S	S	S

Table 15

Enzyme Release by *S. typhimurium* LPS Mutants

Strain	Mutation	RNase 1	CPD	Acid Phos-phatase
SL1027	Wild type	-	10%	20%
SL1032	rfa G	++	23%	nd
SL878	rfa H	++	22%	nd
SL1036	rfa K	+	16%	nd
SL1102	rfa E	++++	74%	50%

nd = not done

Singh & Reithmeier (1975) for heptose-less mutants of E. coli, although Lindsay et al found that release of APase followed shedding of LPS into the medium. Only 7% of the APase was released. The protein composition of the outer membrane is altered in deep rough strains of S. typhimurium and E. coli (Ames et al, 1974) with decreased amounts of the major proteins. Randall (1975) showed that one of the minor proteins of the outer membrane, which forms the adsorption site of phage λ, is present in decreased amounts in E. coli with defective LPS. The LPS seems to have a stabilising function in the outer membrane, probably of an ionic nature. It is not clear whether the effects of LPS removal on the periplasmic enzyme is due to the absence of LPS itself, or through changes in other constituents of the outer membrane caused by its absence. It is possible that CPDase and RNase 1 are either more closely associated with LPS than APase, or that these enzymes are available for release through the outer membrane defect caused by LPS removal, but APase is not.

Release of Enzymes by Other Cell Envelope Mutants

Several other mutants of E. coli K12 and E. coli B were obtained and tested for permeability barrier defects and release of RNase 1 (Table 16). The T4 resistant mutant of E. coli B (B/4), was sensitive to GV and released RNase 1 into the medium, while the T4 resistant mutant of E. coli K12, (C32), did not and was not sensitive to GV. This strain plated some mutants of T4 which have altered base plates, and it is likely that part of the T4 adsorption site is present in this strain. Two strains resistant to T6 were obtained. These did not release RNase 1, nor were they sensitive to GV or DOC. The Tonγ strain, PA340, behaved similarly. The adsorption sites for T6 and T1 are known not to

Table 16

RNase 1 Release, Phage Sensitivity and Sensitivity to Deoxycholate and Gentian Violet of Various *E. coli* Cell Envelope Mutants

E. coli Strain	Envelope Mutation	RNase 1 release	T1	T3	T4	T6	T7	C21	DOC 30 mg/ml	GV 5 µg/ml
M1	Wild type	-	s	s	s	s	s	r	r	r
B	Wild type	-	s	s	s	s	s	r	r	r
B/4	UDPGlucose pyrophosphorylase less	+	s	s	r	s	s	r	r	r/s
C32	T4 resistant mutant of *gal E* strain	-	s	s	r	s	s	nd	r	r
CSH57B	*tsx*	-	s	s	s	r	s	nd	r	r
AB1156	*tsx*	-	s	s	s	r	s	nd	r	r
PA340	*tonA*	-	r	s	s	s	s	nd	r	r

be present on the LPS and are thought to be protein.

SECTION 3

Mutations Causing Periplasmic Enzyme Release

Isolation and Characterisation of Mutants Which Release RNase 1

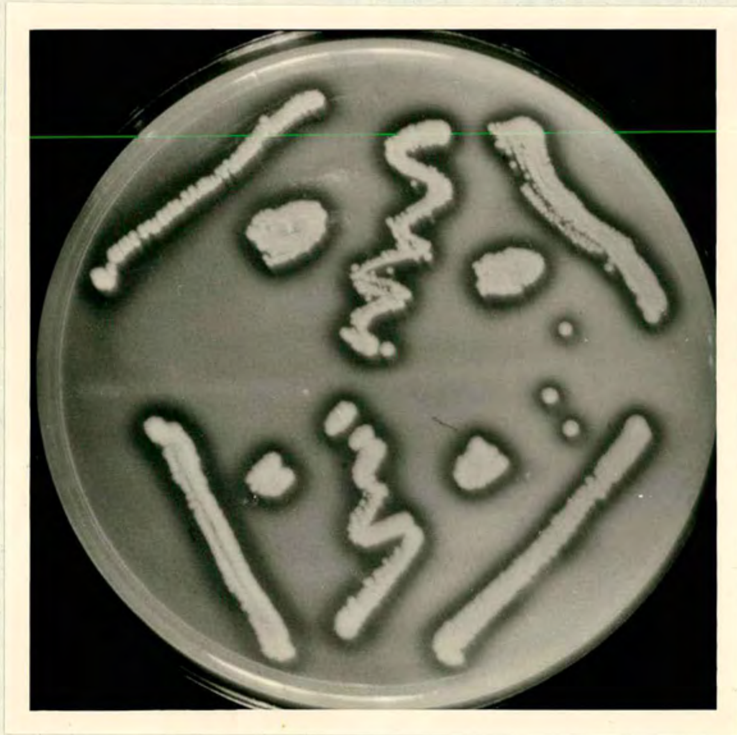
Mutants of E. coli M1 which release periplasmic RNase 1, were isolated following NTG mutagenesis and screening for RNase 1 release on plates by the method of Lopes et al (1971). Colonies that release RNase 1 were easily detected (Fig. 18) and differentiated from non-releasing colonies. Mutants that release RNase 1 were isolated at a frequency of 3×10^{-3}, from a mutagen treatment that yielded 1.4×10^{-1} auxotrophs as determined by colonies unable to grow on minimal medium. Fifteen mutants which release RNase 1 were isolated in this way.

The mutants were tested for various parameters which might be expected to be altered, e.g. release of other periplasmic enzymes, release of cytoplasmic enzymes, sensitivity to antibacterial agents, phage sensitivity and colonial morphology. The results are shown in Tables 17 and 18 and Fig. 19.

Strains B15 and B20 were very similar, releasing large amounts of RNase 1, very little APase, and being more sensitive to DOC and GV than the wild type. Increased sensitivity to GV and DOC were found to be useful parameters to distinguish different mutations resulting in release of RNase 1. GV sensitivity is a good indicator of outer membrane permeability defects (Gustafsson et al, 1973) and it is probable that strains B6, B15 and B20 have an altered outer membrane permeability barrier. It is not clear how DOC acts on the cell, although it is probable that being a lipid solvent it exerts its effect on the cytoplasmic membrane. Two types of mutation might be expected to result in DOC sensitivity; an alteration in the permeability of the outer membrane to DOC, and mutations which would render the outer membrane susceptible to DOC dissolution. Several strains

Fig. 18.

Detection of Ribonuclease I Release By The Plate Assay



B19 was streaked on NA and the plate assay carried out as described in the materials and methods.

Table 17

Release of Enzymes by RNase 1 Releasing Mutants of E. coli

Strain	APase units/ml	RNase	ASNase units/ml	βGDSase units/ml
M1	0.005 (1%)[a]	-	0.11	0.0036 (0.35%)
B19	0.26 (50%)	+++	0.026	0.0044 (0.4%)
B15	0.007 (1.6%)	+++	0.082	0.005 (0.45%)
B18	0.041 (7.2%)	+	0.068	0.0184 (1.6%)
B3	0.014 (3%)	+	0.117	0.004 (0.39%)
C9	0.0078 (3.7%)	+	nd[b]	0.005 (0.5%)
C14	0.0117 (2.2%)	+	nd	0.01 (0.80%)
B16	0.048 (10%)	+++	nd	nd
B20	0.0034 (1%)	+++	nd	0.005 (0.45%)
B17	0.0162 (3%)	++	nd	nd
B6	0.0046 (1%)	++	nd	nd

(a) Figures in brackets are the % of total enzyme released.

(b) nd = not done.

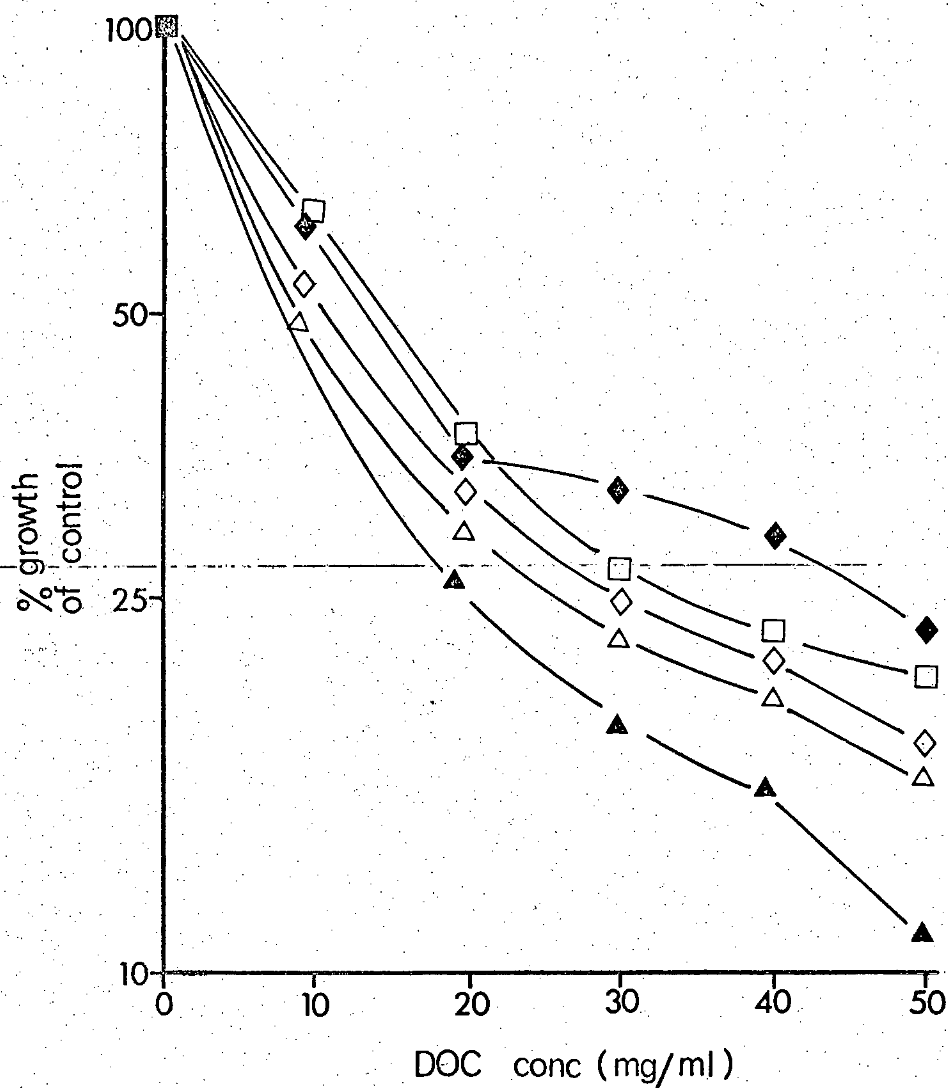
Table 18

Colonial Morphology and Sensitivity of RNase 1 Releasing Mutants to Various Antibacterial Agents

			Sensitivity to			
Strain	Colonial morphology	RNase 1 release	Pen µg/ml	Novo µg/ml	GV µg/ml	DOC mg/ml
M1	R	-	50	>30	7.0	>50
B3	R	+	20	10	7.0	>50
B6	R	++	50	>30	1.0	10
B15	Muc	++++	50	>30	1.0	10
B16	R	++	50	>30	5.0	20
B17	R	+	50	>30	7.0	30
B18	R	+	50	>30	7.0	>50
B19	R	++++	50	>30	7.0	30
B20	Muc	++++	50	>30	1.0	10
C9	R	+	50	>30	5.0	30
C14	R	+	30	>30	5.0	>50

Fig. 19.

Resistance of RNase 1 Releasing Mutants of *E. coli* to DOC



Cultures were all grown at 37°C in NB containing the indicated concentration of DOC.

(□ C14;
◆ M1;
◇ C32;
△ B15;
▲ B19.)

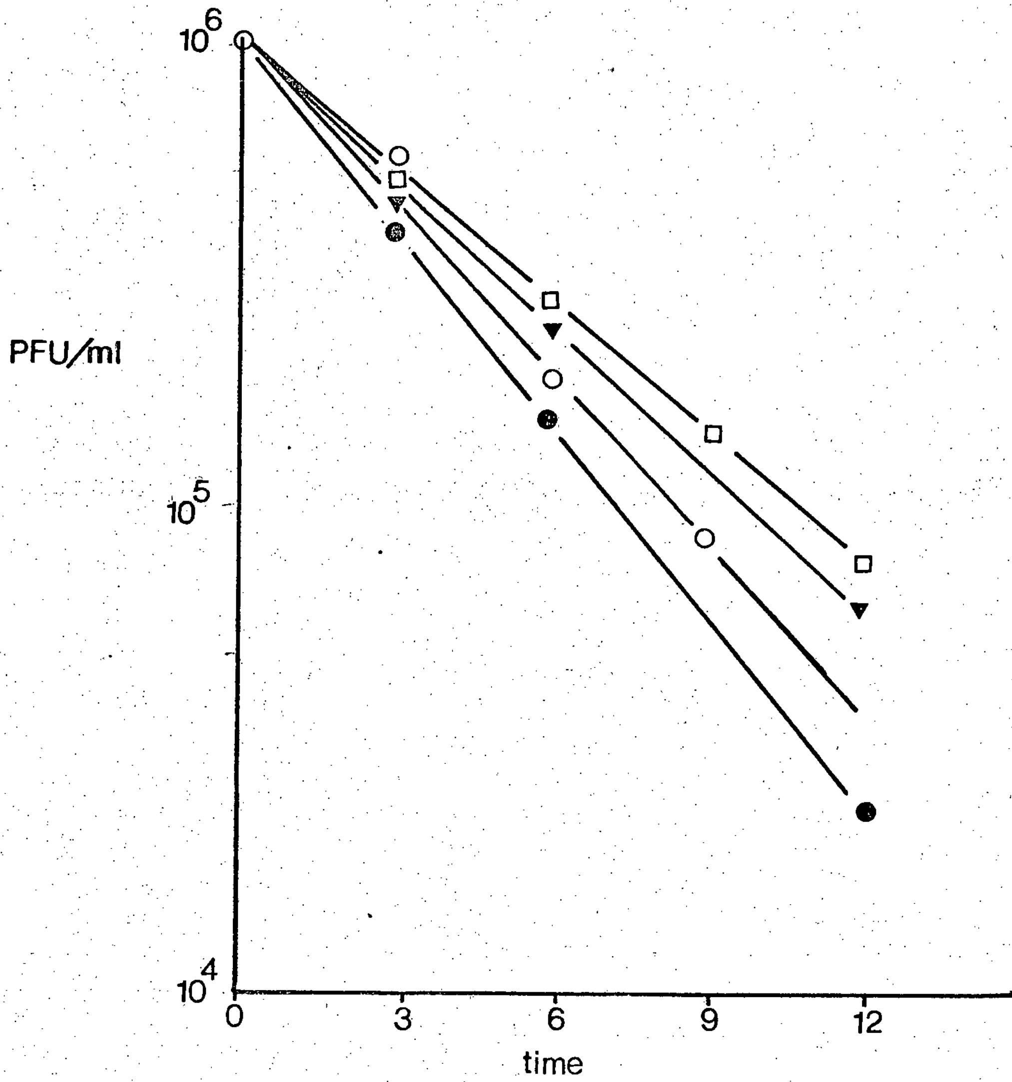
such as B16, B17, B19 and C9 showed increased sensitivity to DOC but not to GV, although all strains sensitive to GV were sensitive to DOC. It is difficult to compare the permeability across the outer membrane of two molecules which are so different in size and charge as GV and DOC, although mutants which were very sensitive to DOC did seem to be sensitive to GV.

Novobiocin and penicillin were not very useful in classifying these mutants, only one strain, B3, being sensitive to either. None of the mutants isolated for RNase 1 release were altered in their sensitivity to any of the common E. coli phages. This is surprising, since several mutants isolated for T4 or T7 resistance were found to release RNase 1 into the medium (see Results - Section 2). If any of the RNase 1 releasing mutants were deficient in the inner core of their LPS, it would be expected that they would be resistant to T4 or T7. It is however possible that a mutation affecting the LPS core which is leaky or which causes a decrease in the overall amount of LPS in the cell could cause the RNase 1 releasing phenotype but retain phage sensitivity. To check this possibility, the ability of several of these mutants to adsorb phage T4 was measure (Fig. 20). B15, B18 and B19 all adsorbed T4 as efficiently as the parent M1, while in A15, a pgm$^-$ mutant, there is markedly reduced adsorption of T4.

Release of periplasmic enzymes was found to vary from mutant to mutant. B15 and B20 were found to release large amounts of RNase 1 by the top agar method although only 1% of their APase was released. B19 however released large amounts of RNase 1 and 50% of the APase activity during normal growth. This mutant also had much reduced amounts of cell bound Asparaginase II. None of

Fig. 20.

Adsorption of T4 to Various *E. coli* Mutants that Release Periplasmic Enzymes



(○ M1;
▼ B19;
● B15;
□ B18.)

the mutants released significant amounts of the cytoplasmic enzyme β-galactosidase.

Assessment of Enzyme Release

a) Standardisation of experiments

The amount of periplasmic enzyme released by a bacterial culture will be dependent on the cultural conditions, the phase of growth that the culture has reached and the assay system used to detect enzyme activity in the culture media. Conditions in these experiments were standardised as far as possible. All cultures were grown for 18 hours in NB at 37°C. It was found that the temperature of growth had more effect on APase release in some strains than in others (Table 19) but in any one strain the amount of APase released by an 18-hour culture grown in NB at 37°C was constant.

Various methods of obtaining APase preparations to measure the release of this enzyme were tried. It was found that sonication of the bacteria in spent medium was the most convenient method and was used to obtain a total enzyme sample. Sonication for the length of time needed to disrupt the bacteria did not affect the activity of the enzyme that had been released into the culture fluid. All APase assays were carried out using 0.1 ml of enzyme sample to dilute out any inorganic phosphate which would be present in the spent medium, and which might interfere with the assay.

b) Methods of assessing release of Asparaginase II and RNase 1

Release of asparaginase II was inferred from its disappearance from whole cells. The assay system used depends on the release of ammonia from asparaginase, and as the enzyme is only produced by bacteria growing in ammonia rich culture media

Table 19

APase Release by RNase 1 Releasing Mutants Grown at Various Temperatures

Strain and Growth Temperature		APase release
M1	30	0.8%
	37	0.7%
	40	0.8%
B18	30	0.83%
	37	8.0%
	40	14.0%
B19	30	1.0%
	37	47.0%
	40	50.0%
B3	30	0.8%
	37	3.0%
	40	4.0%

(Jeffries, 1974), detection of this enzyme in the culture medium is not practicable.

Problems were encountered in devising a reproducible assay system for RNase 1 in spent culture media. Using the method of Neu & Heppel (1964) very high background activity was present in the control assay possibly due to EDTA action, the presence of which is necessary to breakdown the endogenous RNA. Addition of excess Mg^{2+} ions interfered with the enzyme's activity. Reproducible results were obtained by the plate assay method of Gesteland (1965) modified by Lopes <u>et al</u> (1971).

<u>Characterisation of the B19 mutation</u>

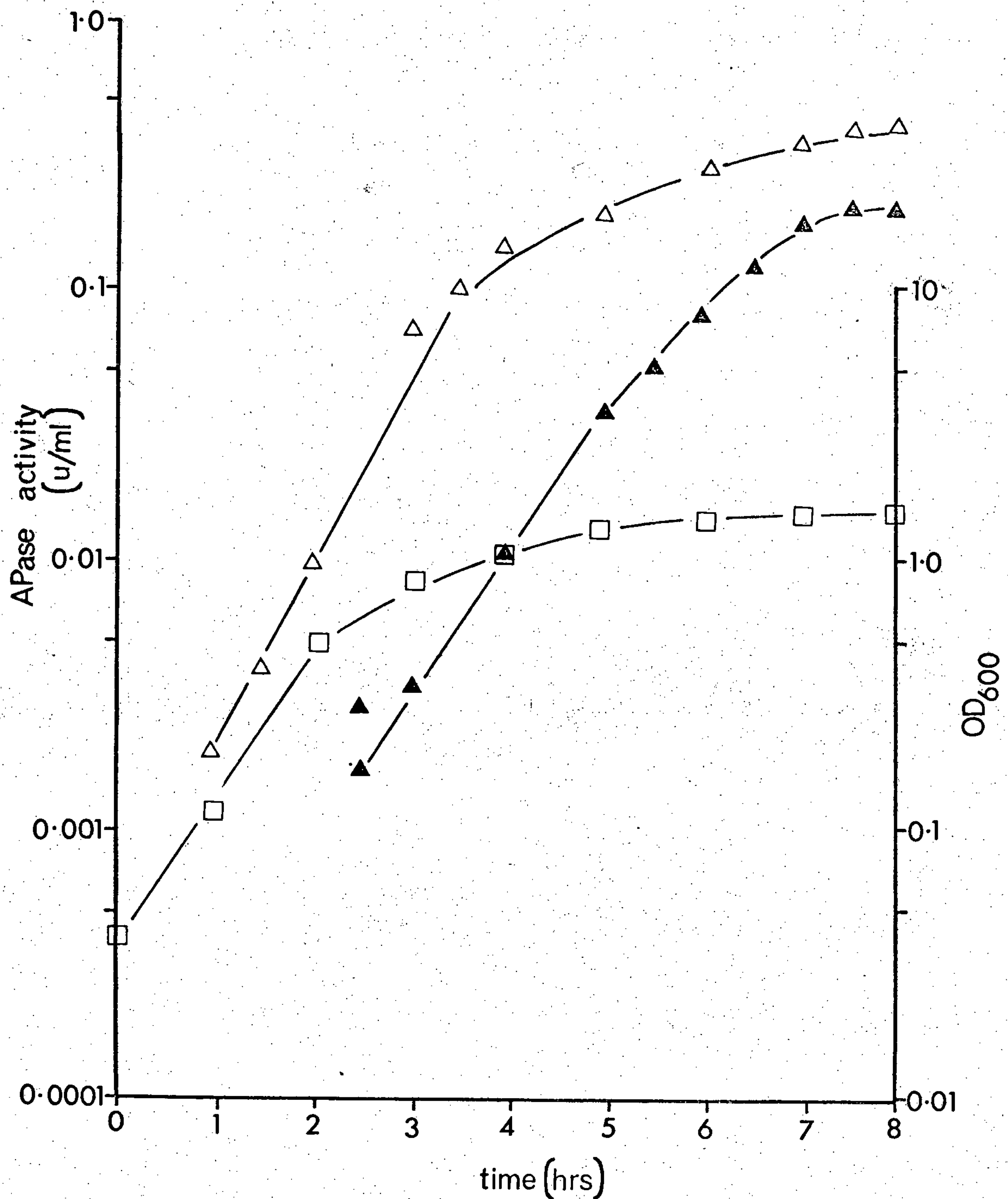
B19 releases large amounts of APase into the culture medium during normal growth but very little of the cytoplasmic enzyme β-galactosidase. This would suggest that the release of APase is not caused by cellular lysis in this mutant. B19 is more sensitive than the parent M1 to DOC but is the same in its sensitivity to phages, GV and antibiotics. This mutant also released large amounts of RNase 1 and asparaginase II but seems to have only minor outer membrane permeability defects.

(1) <u>Factors affecting APase release in B19</u>

When APase production and release were followed throughout the growth cycle in NB (Fig. 21), as growth slowed down so did the production of APase but after a delay of 30 minutes. However the release of APase carried on until 50% of the enzyme had been released when it slowed down and from then on followed enzyme production, i.e. 50% of the APase produced was always released. The enzyme that remains associated with the bacteria can be released by sonication, and also by osmotic shock (Table 20), indicating that it was still located in the periplasm. If the

Fig. 21.

Release of APase from B19



B19 was grown at 37°C in NB. Under identical experimental conditions, no APase release was detected from M1.

(△ total APase activity;

▲ APase activity in the medium;

□ optical density of the culture.)

Table 20

Release of APase into the Medium and During Osmotic Shock of B19

Sample	APase activity units/ml
Total cells	0.243 (100)[a]
Spent medium	0.146 (60)
Osmotic shock stage II	0.1165 (48)

(a) Figures in brackets are % of total enzyme.

release of enzyme is followed during growth in MM, but protein release is also monitored (Fig. 22), protein release follows growth, but production and release of APase are faster than either. Thus APase release is limited by the rate of production of the enzyme, and not all of the proteins that are released are released at the same rate.

a) Effect of chloramphenicol on release of APase from B19

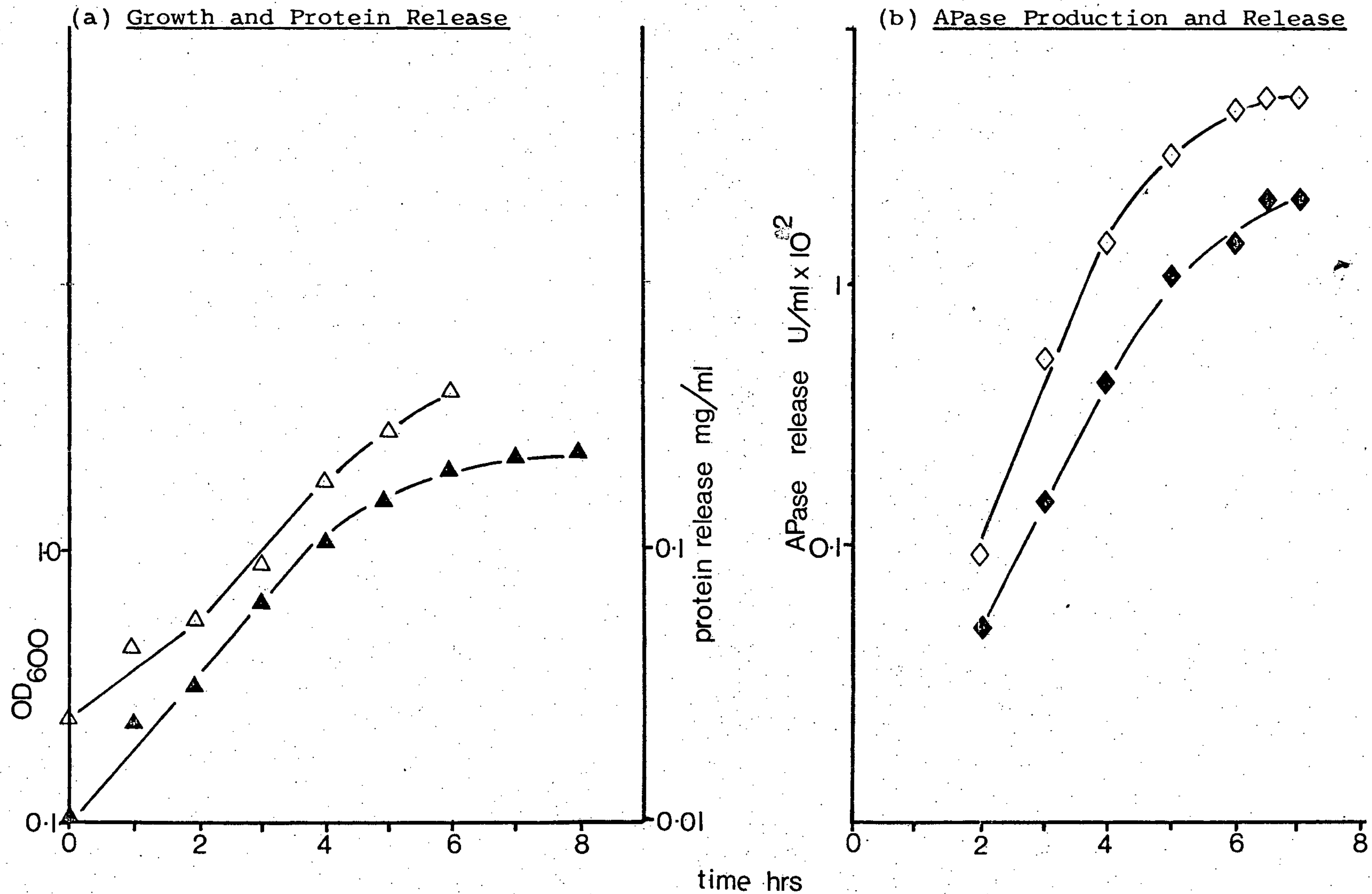
Chloramphenicol (CAP) 10 μg/ml was added to an exponentially growing culture of B19 and APase production and release followed (Fig. 23). Within 15-30 minutes growth and APase production had ceased. APase release however, continued until 50% of the enzyme had been released. No APase release was detected in cultures of the parental strain treated in this way. Clearly APase release does not depend on continued production of the enzyme, which might suggest that APase diffuses through the outer membrane and is not forced out of the periplasm. Half of the APase is released by this mutant during growth. The remaining APase could be located in a different part of the periplasm, or the mutation might only partially reduce a component in the cell envelope which is responsible for maintaining the enzyme in the periplasm.

b) Effect of temperature on APase release and other phenotypes

B19 releases very little APase at 30°C, but large amounts at 37°C or 40°C (see Table 19). This cannot be correlated with differences in the growth rate at these temperatures (Fig. 24). The amount of APase released by cultures of B19 increases with the growth temperature above 30°C reaching a maximum at 37°C to 40°C (Fig. 25). The parent M1 did not release APase over this temperature range. It is possible that B19 has a temperature

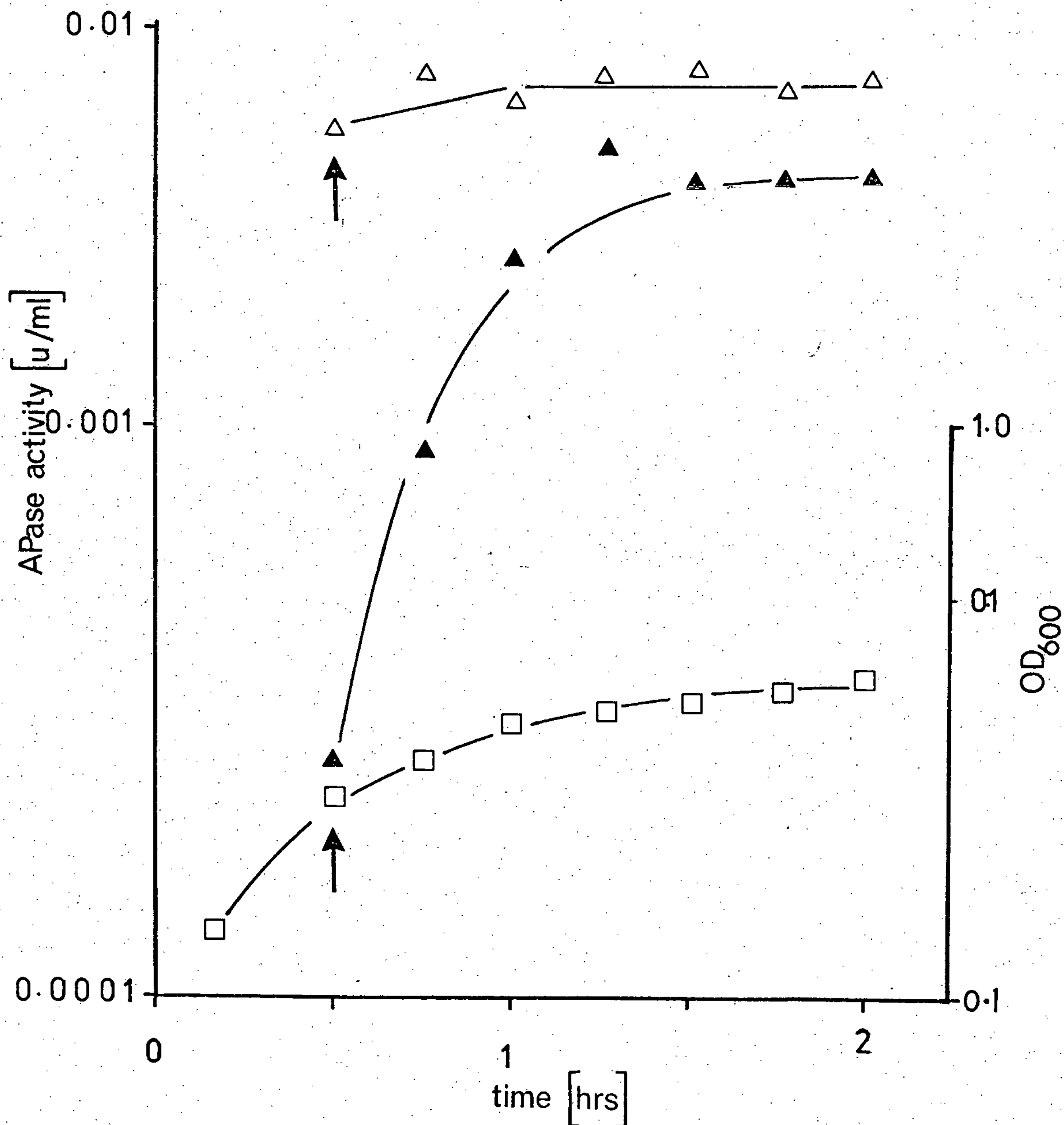
Fig. 22.

Release of APase and Protein From B19



B19 was grown in M9 medium supplemented with 0.15% casamino acids and 100 μg/ml arginine, at 37°C. Protein concentration was determined by the method of Lowry *et al* (19).
(▲ optical density; △ protein concentration in the medium; ◇ total APase activity; ◆ APase activity in the medium.

Fig. 23.



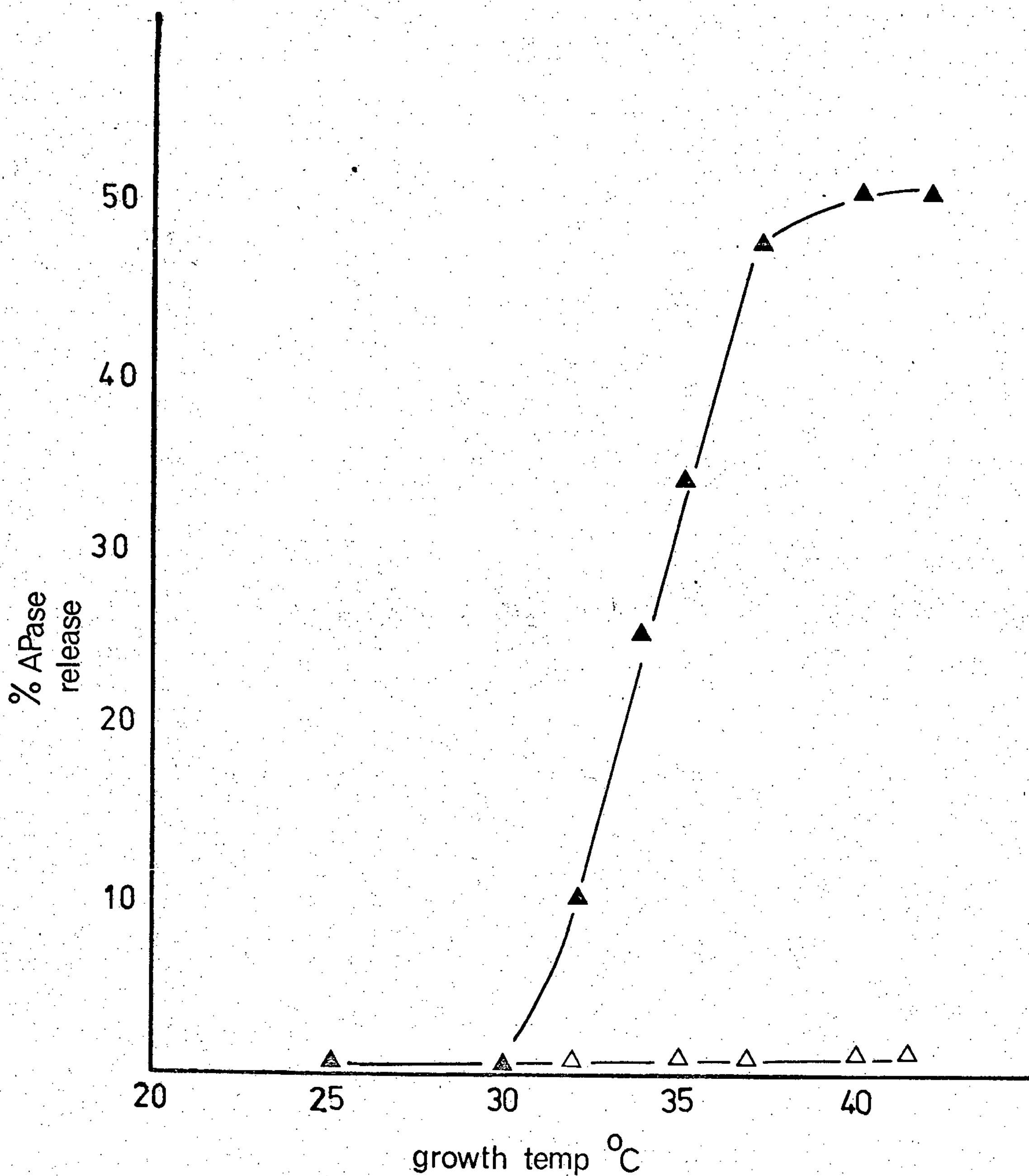
B19 was grown in NB at 37°C to an approximate cell concentration of 2 x 10^8 cells/ml, chloramphemicol was added to give a final concentration of 10 μg/ml (arrowed on the graph).

(□ optical density of the culture;

△ total APase activity;

▲ APase activity in the medium.)

Fig. 24.



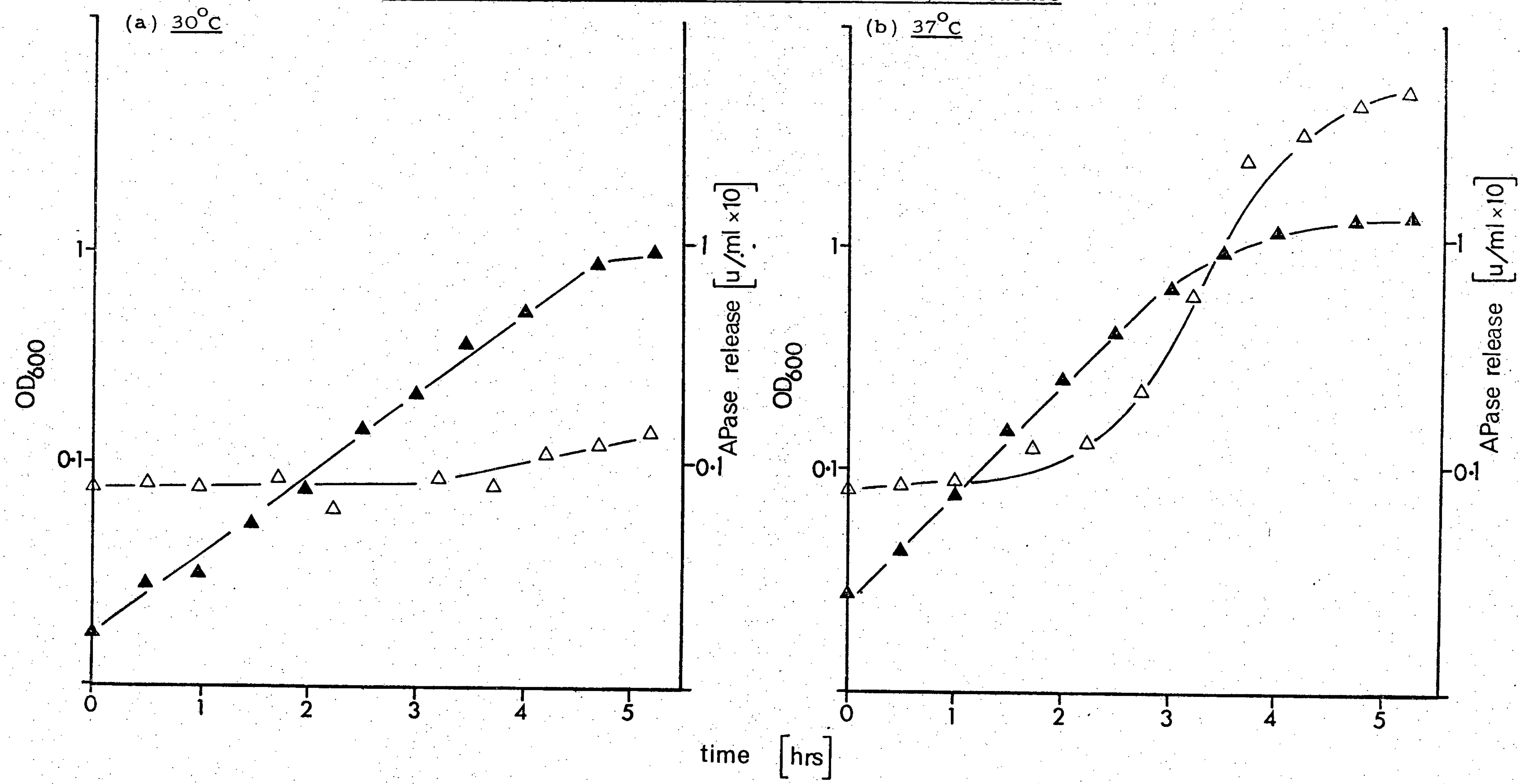
Cultures grown in NB at the temperatures indicated. Enzyme release was measured as described in the materials and methods.

(▲ % APase released by B19;

△ % APase released by M1.)

Fig. 25.

Release of APase by B19
Effect of Temperature on Growth Rate and Enzyme Release



Open symbols - enzyme release, closed symbols - optical density of the culture.

sensitive mutation which is responsible for the APase release. To verify this, the sensitivity of B19 and M1 to DOC at various temperatures was tested (Table 21). B19 was sensitive to DOC at temperatures below 30°C. Either B19 is a double mutant or the APase-releasing phenotype is not caused by a temperature sensitive protein, but by a temperature dependent change in the cell envelope. B19 released less RNase 1 at 30°C than at 37°C (Table 22). However the fact that RNase 1 is released at 30°C indicates that there is a cell envelope defect in the bacterium grown at this temperature.

c) Effect of cation concentration on APase release

When divalent cations, such as Mg^{2+} or Ca^{2+} were added to the growth medium the percentage of the APase released decreased (Fig. 26) but the amount of enzyme produced also decreased. Magnesium did not inhibit the enzyme at the concentrations used. It is likely that divalent cations stabilise membranes and thus not only affect the release of APase across the outer membrane, but also affect the amount of enzyme passed across the cytoplasmic membrane into the periplasm. Monovalent cations, e.g. Na^{+}, at the same concentrations did not affect enzyme release.

Polyacrylamide gel electrophetic study of proteins released by B19

Most of the proteins that released from M1 by osmotic shock were released by B19 into the culture medium during growth (Fig. 27). Thus the proteins released by B19 were periplasmic. Protein bands 5, 6 and 7 of M.W. 60, 52 and 40,000 respectively were the most prominent. As stated earlier, these bands could be equivalent to CPDase, 5' nucleotidase and APase. In the gel scan of proteins from B19 medium a large disperse band which ran between

Table 21

Effect of Growth Temperature on Sensitivity of M1 and B19 to Deoxycholate

Strain	NB					DOC 30 mg/ml				
	15	25	30	37	42	15	25	30	37	42
M1	±	+	+	+	+	±	+	+	+	+
B19	±	+	+	+	+	-	-	-	-	-

Plates were incubated until the controls had grown, and then scored for DOC sensitivity.

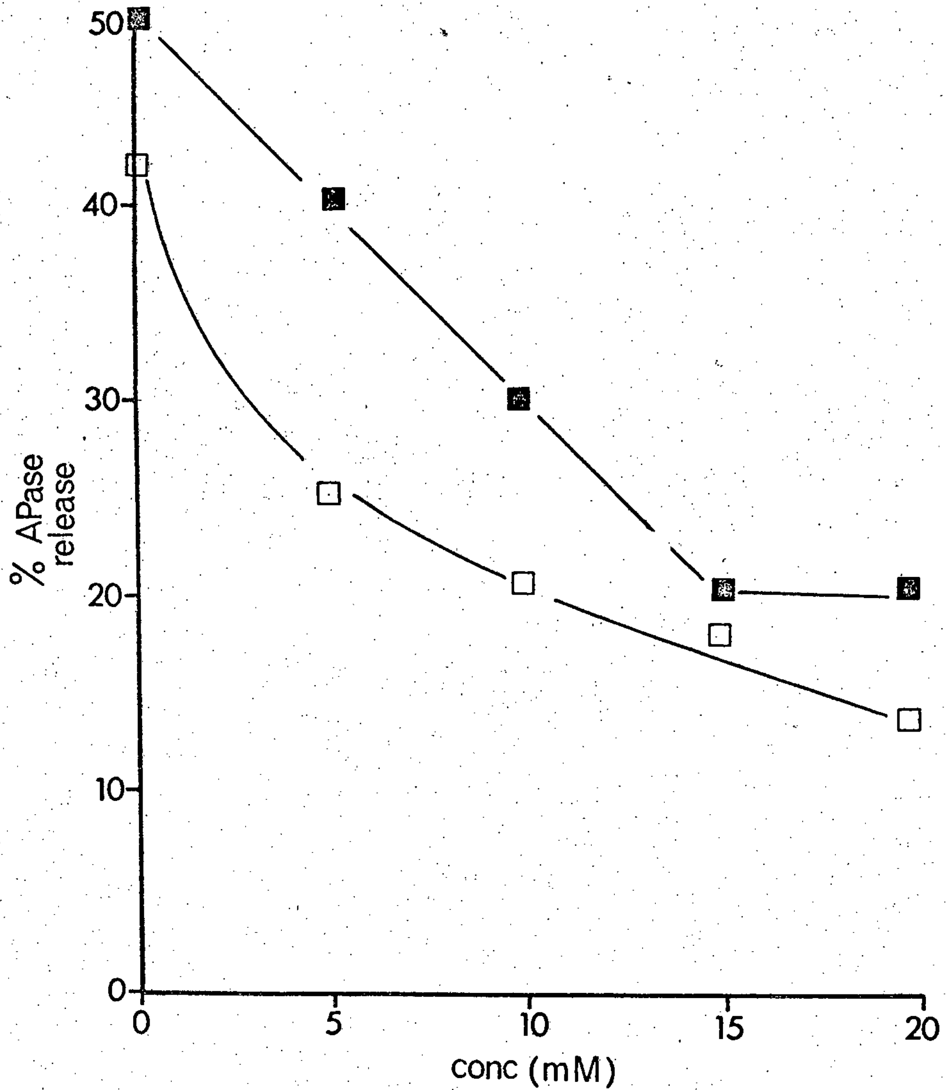
Table 22

Release of RNase by B19 at Different Growth Temperatures

Strain	$30^{o}C$	$37^{o}C$	$42^{o}C$
M1	-	-	-
B19	+	+++	+++

Fig. 26.

Release of APase From B19
Effect of Divalent Cations



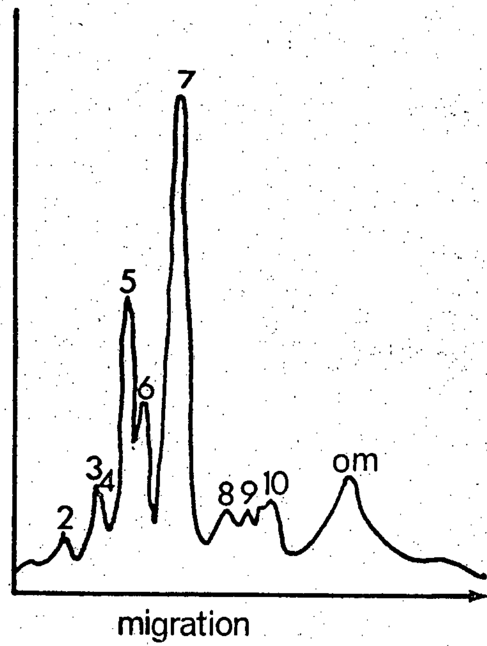
Cultures were grown in NB at 37°C, with the indicated concentration of cation.

(▩ % APase release at various calcium concentrations;

□ % APase release at various magnesium concentrations.)

Fig. 27.

Densitometer Trace of Proteins Released by B19 into the Medium, Separated by P.A.G.E. and Stained with Coomassie Blue



protein bands 10 and 11 can be seen. This band did not stain with coomassie blue, so is not protein. This material was not present in gels of the osmotic shock fluid of M1, or of the spent medium from M1 cultures. It is probable that this band contains lipid and/or carbohydrate and represents some part of the outer membrane which is also released into the medium. There was much less of this non-protein band in gels of the spent medium of B19 cultures grown at 30°C, when there were fewer protein bands present (Fig. 28). Similar white disperse bands were seen in gels of outer membrane preparations.

Effect of the mutation on the cell envelope

a) Lipopolysaccharide

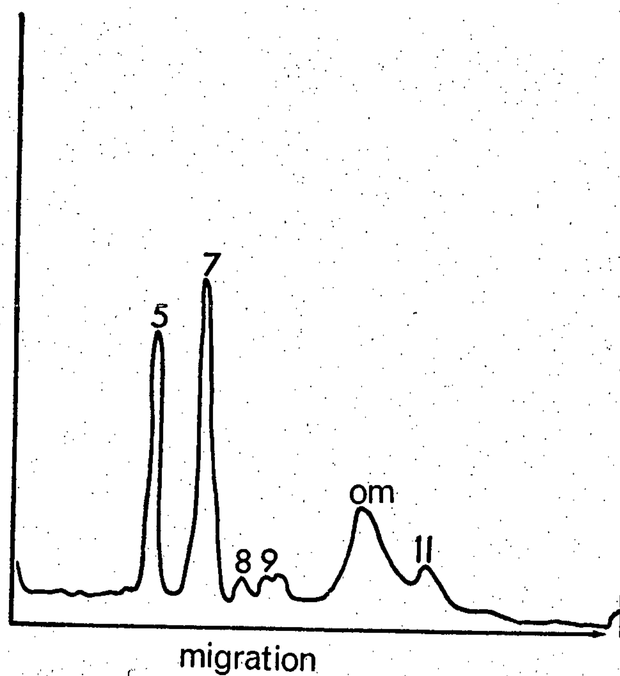
It is probable that B19 has an altered cell envelope which would account for the observed phenotypes. LPS was isolated from both B19 and M1 by hot phenol/water extraction. The isolated LPS samples were hydrolysed and chromatograms prepared from them (Fig. 29). The LPS from both B19 and M1 contained similar sugars namely, glucose, galactose and a trace of rhamnose. Glucosamine was not present, but it is unlikely that glucosamine-sugar linkages would be hydrolysed by this treatment. M1 and B19 had very similar LPS compositions. Although it is possible that there is a defect in the LPS which would only be detected by more detailed analysis, it seems unlikely that a mutation affecting the LPS, which does not significantly decrease the number of T4 binding sites or the amounts of glucose, galactose and rhamnose in the core LPS, would result in the B19 phenotype.

b) Outer membrane proteins

Outer membranes were prepared by isolation of triton X100-insoluble cell envelope material from lysozyme sphaeroplasts

Fig. 28.

Densitometer Trace of Proteins Released by B19 Grown at (a) 37°C and (b) 30°C, Separated by P.A.G.E. and Stained with Coomassie Blue



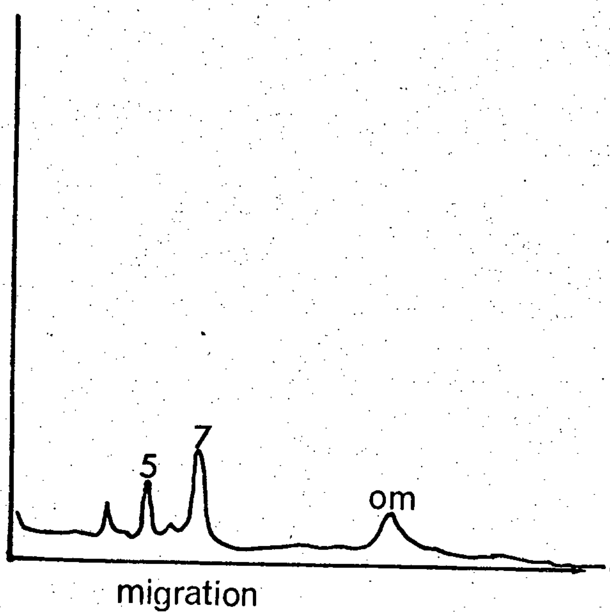
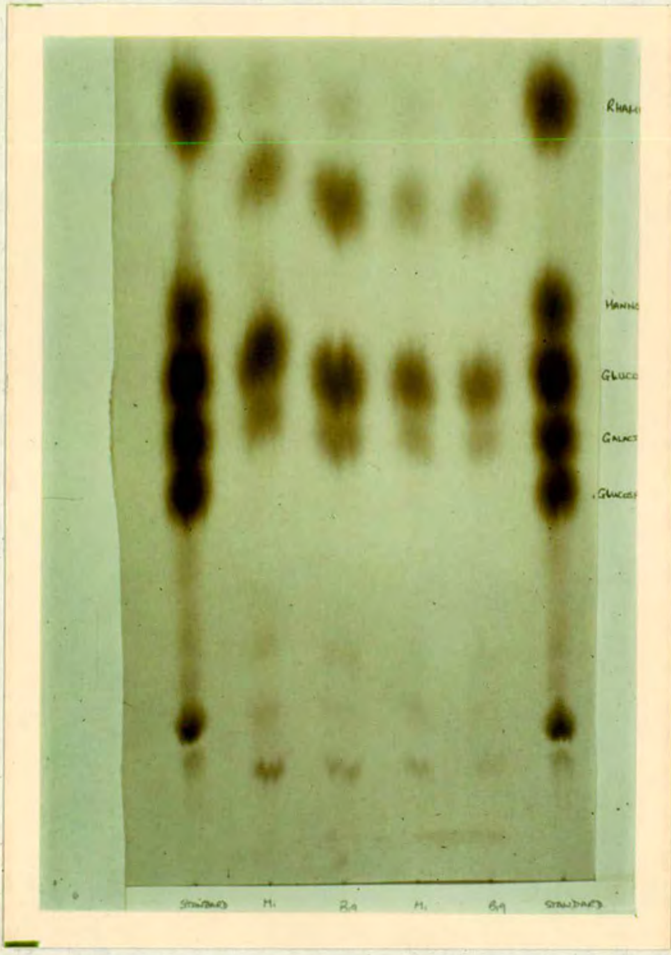


Fig. 29.

Chromatogram of Hydrolysed LPS Isolated from M1 and B19, with Sugar Standards



(Schnaitman, 1970). The protein patterns from B19 and M1 outer membranes showed several differences (Fig. 30). Bands 10, 11 and 13 were greatly reduced in B19 gels, but bands 9 and 12 were much larger. Band 13 has a M.W. of approximately 8,000 and would be equivalent to the small molecular weight lipoprotein of the outer membrane. Band 12, however, which has a M.W. of approximately 10,000 is almost absent in M1 gels, but is present in large amounts in the B19 outer membrane becoming the major protein of this membrane. The other major outer membrane protein, band 7, with a M.W. of approximately 40,000 was unaffected by the mutation.

Whether the alteration of one protein in the outer membrane of this mutant is responsible for all the phenotypes described, or whether there is more than one mutation affecting the outer membrane is not clear. If one major protein was substantially reduced it could affect the regulation of the other outer membrane proteins, so that they are overproduced to compensate for loss of the original protein.

The increase in bands 9 and 12 might be due to overproduction or due to alteration in the migration of other protein bands such as 10, or 11 and 13.

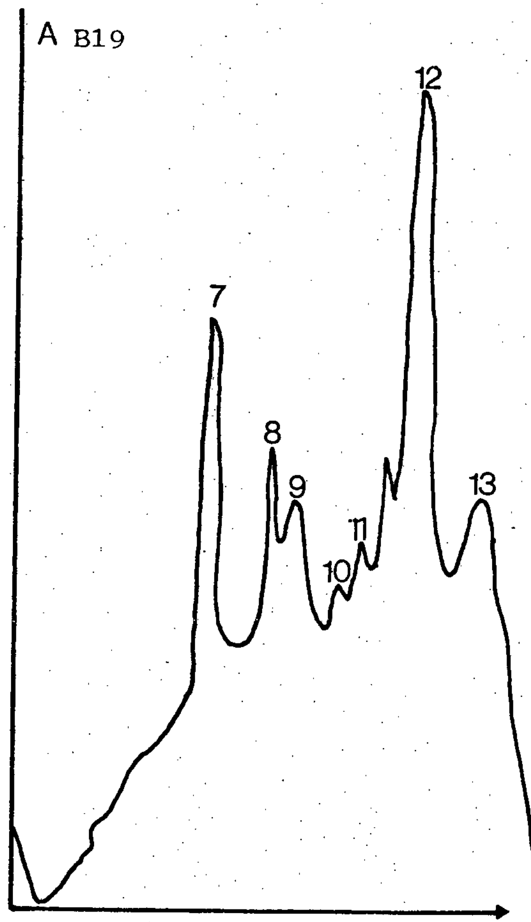
<u>Genetic Analysis of the B19 Mutation</u>

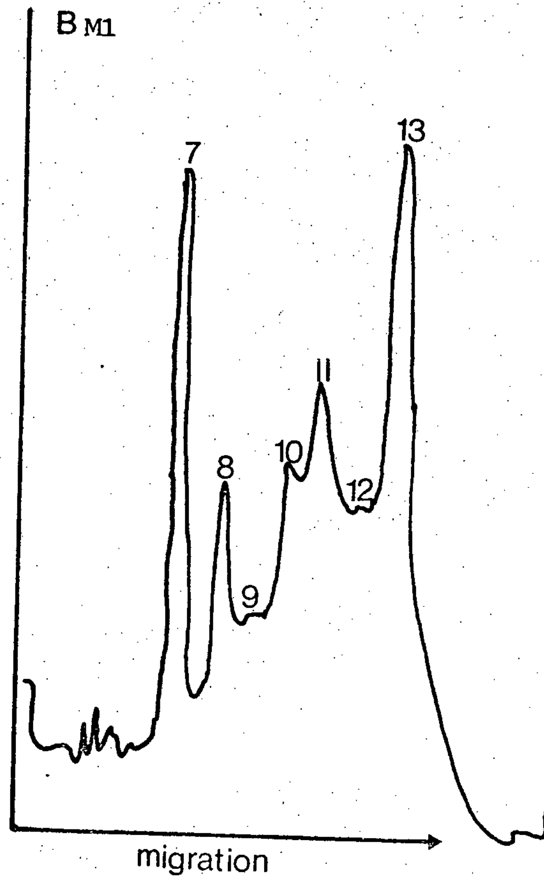
a) <u>Reversion to parental phenotype</u>

Mutations affecting the cell envelope of gram negative bacteria are often pleiotropic (Bernstein, Rolfe & Onodera, 1972). It is necessary to distinguish between pleiotropism and the possible effects of two or more mutations. All the RNase 1 leaky mutants were isolated followed NTG mutagenesis, which can easily produce double mutations (Guerola, Ingraham & Cerda-Olmedo, 1971). To check for possible double mutations, B19 was reverted to non-

Fig. 30.

Densitometer Trace of the Triton x100 Insoluble Cell Envelope Proteins Separated by P.A.G.E. and Stained with Coomassie Blue





release of RNase 1 or to DOC resistance, and the reversion of the other phenotypes was tested.

Eighty percent of the revertants of B19 isolated for DOC resistance did not release RNase 1 (Table 23). Similarly 75% of mutants isolated for the non-release of RNase 1 were DOC resistant. It is probable that B19 carries a single mutation, although as NTG was used to revert the mutation, it is possible but unlikely that RNase 1 release and DOC sensitivity are due to two mutations in very closely linked genes. The strains which had reverted for only one of the phenotypes, showed partial recovery of the other. These could be the result of a mutation in another part of the same gene or in a further gene, which affects the cell envelope.

One of the revertants, R5, was studied in detail. It was as resistant to DOC as M1 and did not release RNase 1. This revertant also released much less APase than the B19 parent (Table 24). Polyacrylamide gel electrophoresis of the proteins released from this revertant into the medium (Fig. 31) show that fewer proteins, and very little outer membrane material, were released. Clearly although this mutant released some periplasmic enzymes, the reversion affects the release of APase and RNase 1 and also DOC sensitivity, suggesting that all three are the result of a single mutation.

b) Mapping of the B19 mutation on the *E. coli* chromosome

To identify the position of the mutant gene in B19 on the *E. coli* chromosome map, an Hfr was constructed from B19. To do this an *F'ts lac* was introduced into a *lac*$^-$ derivative of B19. Colonies that fermented lactose were picked off lactose-EMB plates incubated at 42°C. At this temperature the *F'ts lac* is

Table 23

Reversion of B19 to RNase 1 Non-Releasing and to DOC^r

Selected Phenotype	No. of Revertants Tested	No. of Revertants DOC^r	No. of Revertants RNase 1 non-Releasing
RNase 1 non-release	15	11 (75%)	-
DOC^r	18	-	14 (80%)

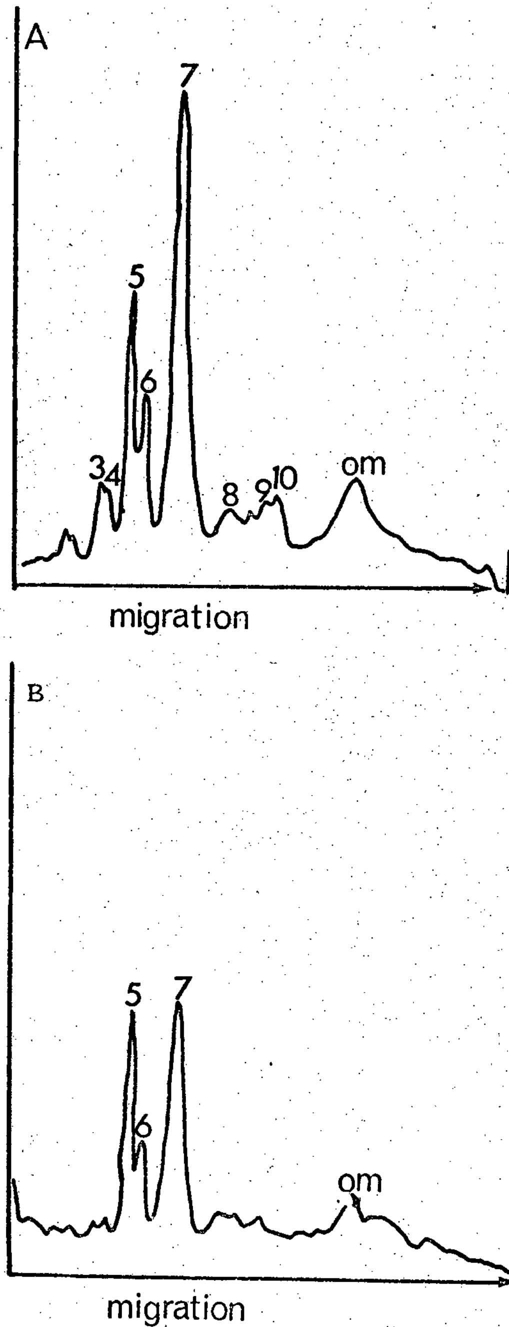
Table 24

Deoxycholate Sensitivity and Periplasmic Enzyme Release of R5

	RNase 1 Release	APase Release units/ml	DOC 30 mg/ml
M1	-	0.0065 (1.1%)	++
B19	+++	0.16 (50%)	-
R5	-	0.034 (10%)	++

Fig. 31.

Densitometer Trace of Proteins Released by (a) B19 and (b) R5, Grown at 37°C: Separated by P.A.G.E. and Stained with Coomassie Blue



inactivated unless integrated into the chromosome.

The resultant Hfr, B19/14 was crossed with C57B to determine the direction of transfer of the chromosome . The F'ts lac had integrated at lactose and transferred the leu marker early and the his marker late. Phage T6 was used to counter-select against the Hfr, tsx being one of the last markers to be transferred.

In the preliminary cross of B19/14 against C57B, the mutation leading to release of RNase 1 and DOC sensitivity was introduced early in the cross (Table 25). For more accurate positioning of the mutation, AB1157 was used instead of C57B. AB1157 has several markers in the part of the genome that is transferred first by B19/14 (Table 2). For the cross AB1157 against B19/14, a gradient of transmission was drawn of the number of recombinants per selected marker, against the distance of that marker (in minutes) from the origin of transfer (Fig. 33). For this Hfr the origin of transfer was taken as 9 minutes. The results of this cross are shown in Table 25. The position of the mutation on the chromosome can be estimated from the gradient of transmission if the number of recombinants inheriting the mutant phenotypes are known (de Haan et al, 1969). The B19 mutation termed omd (outer membrane defect) mapped closely to proA at approximately 6 minutes on the E. coli K12 chromosome map (Fig. 34). Recombinants selected for RNase 1 release or DOC sensitivity also were recombinant for the other B19 phenotypes.

Characterisation of the B15 mutation

B15 and B20 were very similar in their properties. B15 released large amounts of RNase 1 but very little APase, and was sensitive to GV and DOC. It was mucoid when grown on NA plates

Table 25

Results of Crosses Between B19/14 and C57B and B19/14 and AB1157

Selected Markers	No. of Recombinants	% Recombinants Releasing RNase 1
B19/14 x C57B		
Leu, Tsx^r	1.5×10^5	94
Leu, Ileu, Tsx^r	1.3×10^4	100
Leu, Ileu, Arg, Tsx^r	6.0×10^3	100
Leu, Ileu, Arg, His, Tsx^r	2.0×10^2	100
B19/14 x AB1157		
Pro, Tsx^r	2.1×10^6	82
Pro, Leu, Tsx^r	1.2×10^5	100
Pro, Leu, Thr, Tsx^r	5.0×10^4	100
Pro, Leu, Thr, Arg, Tsx^r	-	-

Fig. 33.

Gradient of Transmission of Selected Markers in a Mating Between B19/14 and AB1157

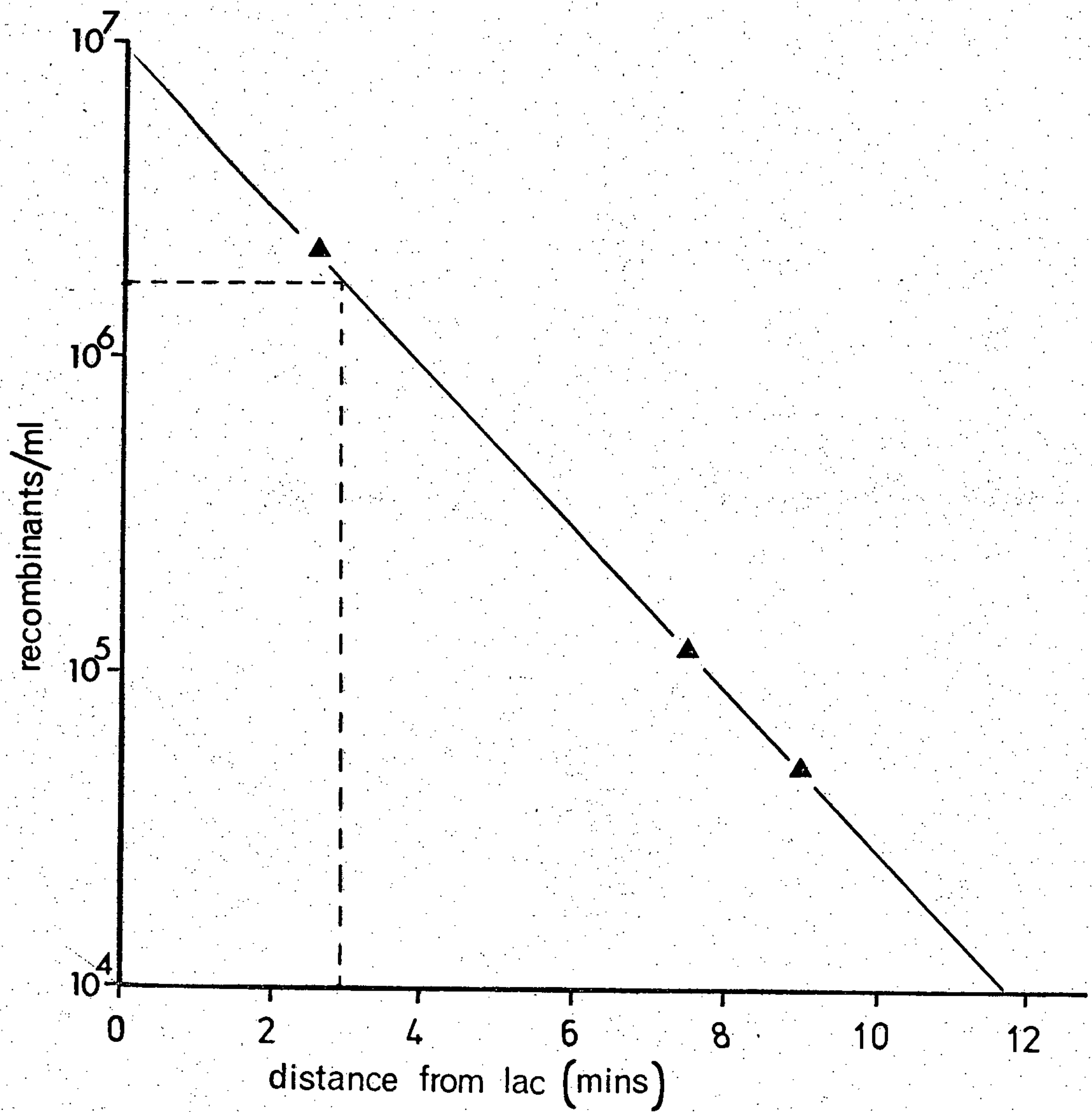
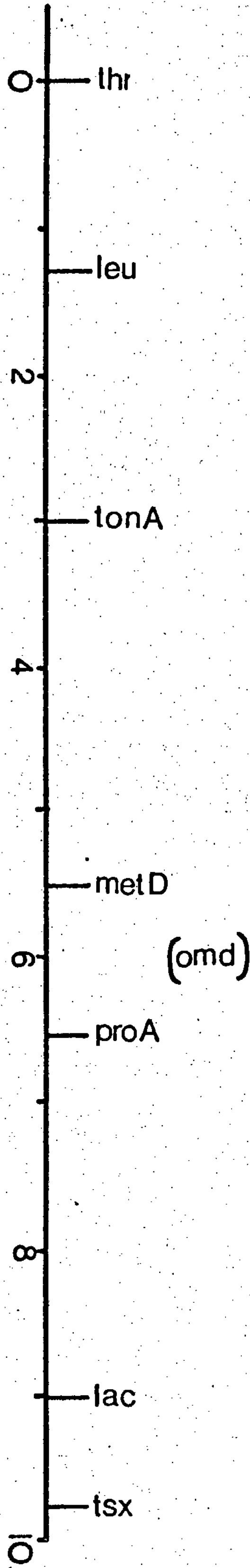


Fig. 34.

E. coli Chromosome Map



(Table 18). Although isolated for release of RNase 1 at 37°C, it was temperature sensitive for growth. As stated before this mutant was not sensitive to penicillin or novobiocin and did not differ from the parent strain M1 in phage sensitivity.

It would seem that there is an alteration which causes increased permeability of the outer membrane to GV and DOC. However as these characters were tested at 37°C and the mutant is unable to grow at 42°C, it is possible that there is a temperature sensitive mutation, which is only partly expressed at 37°C, and which causes the observed phenotypes. At the higher temperature the mutation would be fully expressed, causing cell death. Temperature sensitive mutants of E. coli have been isolated which are defective in protein synthesis but are able to grow when the osmotic strength of the medium is increased. These mutants show defects associated with an alteration of the outer membrane (Russell, 1971). It is possible that a similar mutation is present in B15.

Effect of temperature on B15

a) The effect of temperature on growth

B15 grew well at 30°C and 37°C, but not at 42°C. If the medium was supplemented with 12.5% sucrose then B15 could grow at 42°C. Clearly the temperature sensitive phenotype is osmotically remedial (Table 26). B15 was mucoid when grown on NA. However when grown at 30°C it had the same colonial morphology as M1.

When cultures of B15 growing at 30°C were raised to 42°C the OD of the culture rose rapidly at first but soon began to slow down, and eventually fell (Fig. 35). The viable count of the culture however began to fall soon after the temperature

Table 26

Effect of Temperature on Growth of B15 With and Without 12.5% Sucrose

Strain	NA			NA + 12.5% Sucrose		
	30°C	37°C	42°C	30°C	37°C	42°C
M1	+	+	+	+	+	+
B15	+	+	-	+	+	+

Fig. 35.

Affect of a Temperature Shift on Growth, Viability and Release of βGSDase, of B15

Cultures were grown at 30°C in NB supplemented with IPTG and changed to 42°C at the time indicated by the arrow.

(a) ▲ total βGSDase activity; βGSDase activity in the medium.

(b) ◉ viable count; ○ optical density of the culture.

change (Fig. 35).

The effect of the change was delayed at higher cell concentrations (Fig. 36), implying that high cell concentrations might have a stabilising effect on the culture. When changed to the high temperature, cultures did not just stop growing, but died. It is not clear how death was caused, whether by cessation of protein synthesis, or through loss of some other function.

b) <u>Effect of temperature on RNA, DNA and protein synthesis</u>

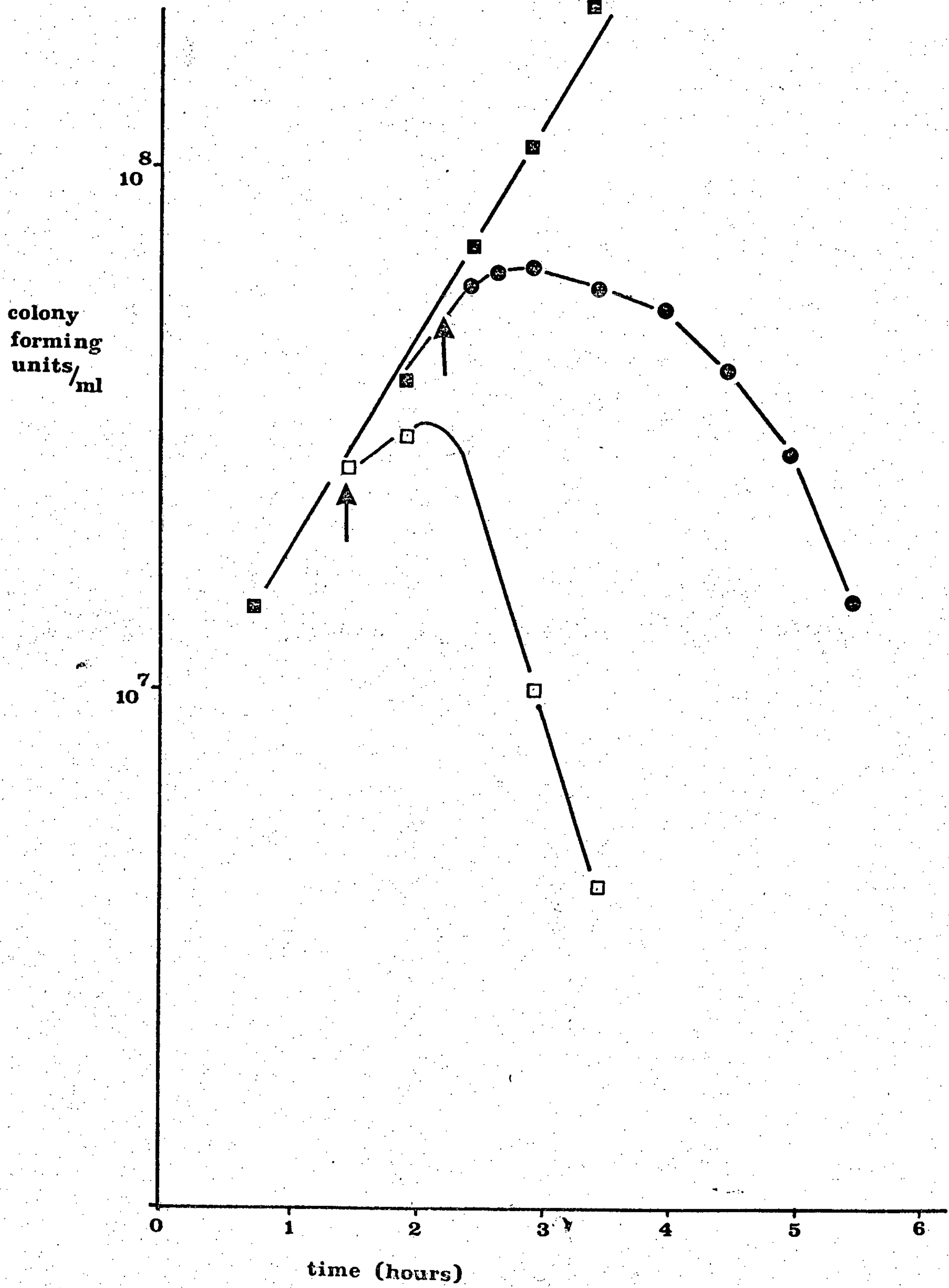
Synthesis of these macromolecules by a culture of B15 was not affected during the first 90 minutes after a shift from 30°C to 42°C, as shown by continued incorporation of labelled uridine, thymidine and arginine (Fig. 37). The culture began to lose viability 30 minutes after the temperature shift (Fig. 35). It is unlikely that this mutation affects RNA, DNA or protein synthesis.

c) <u>Effect of temperature on enzyme release and sensitivity to DOC</u>

When growing at 37°C or at 42°C in the presence of 12.5% sucrose, B15 did not release more APase into the medium than was released by the parent, but did release large amounts of RNase 1 (Table 27). No RNase 1 was released when the growth temperature was reduced to 30°C (Table 27). B15 was not sensitive to DOC at 30°C, but was at 37°C and 42°C. There would seem to be a temperature sensitive mutation in B15, so that at 30°C it has the parent phenotype, but at 42°C has the full mutant phenotype which results in lethality. At 37°C it has an intermediate phenotype; it becomes sensitive to DOC, mucoid, releases RNase 1 but remains viable. RNA, DNA and protein synthesis are normal at 42°C, so it is probable that the mutation directly affects

Fig. 36.

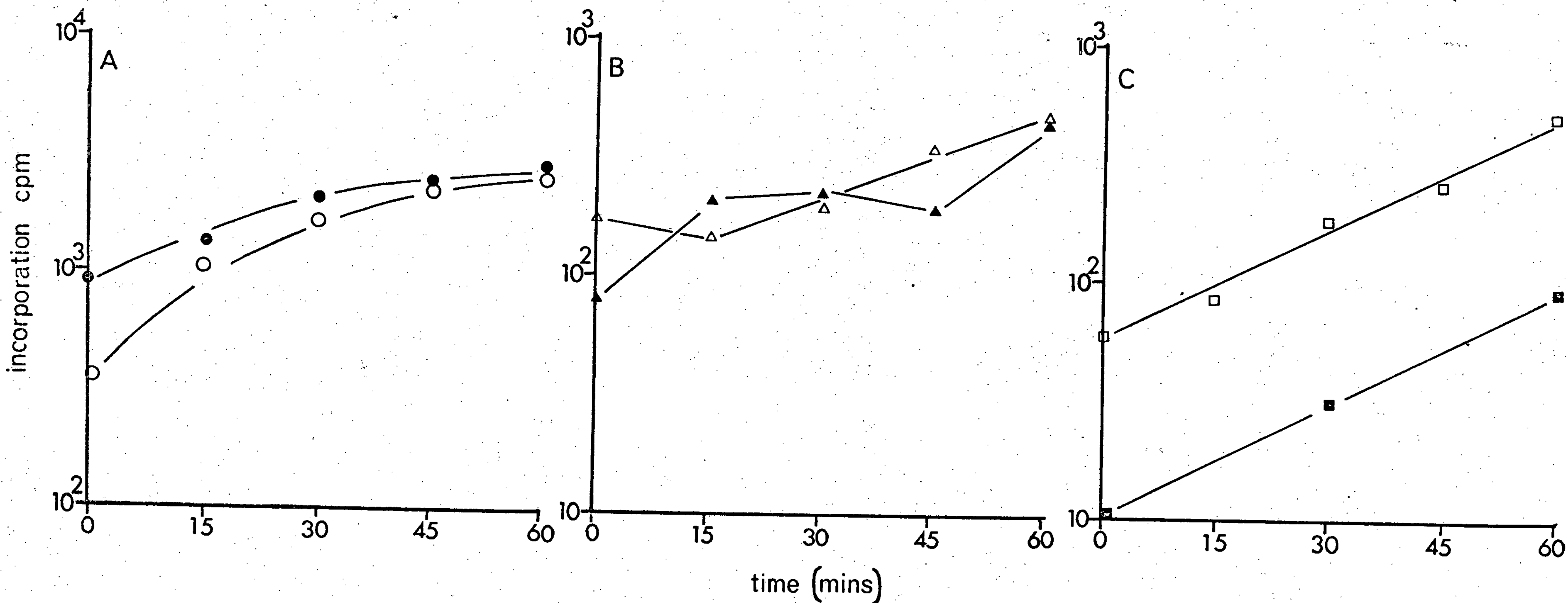
Affect of a temperature shift on B15 at different cell concentrations



Cultures grown in NB at 30°C and shifted to 42°C at times indicated by arrows.

Fig. 37.

Incorporation of Radioactive Precursors by B15 at 42°C into Acid Insoluble Material: (a) ^{14}C Arginine, (b) ^{3}H Uridine, (c) ^{3}H Thymidine



Cells grown in NB at 30°C to a cell density of 2 x 10^8 then shifted to 42°C, labelling was performed as described in the materials and methods. Open symbols - M1, closed symbols - B15.

Table 27

RNase 1 Release by B15 at Different Growth Temperatures

Strain	Growth				RNase Release			
	30°C	37°C	42°C	45°C	30°C	37°C	42°C	45°C
M1	++	++	++	-	-	-	-[a]	-
B15	++	++	-	-	-	++	++[a]	-

(a) Determined when growing in the presence of 12.5% sucrose.

some cell envelope structure.

The fate of β-galactosidase was followed in a growing culture of B15 during a temperature shift from 30°C to 42°C. The enzyme was released into the medium soon after the temperature shift, at the same time as the culture began to decrease in viability (Fig. 35). Clearly cell death and lysis occur at the same time. Whether death is the result of lysis or vice versa is not apparent from this experiment. However as both β-galactosidase release and cell death can be prevented by osmotically stabilising the medium it would seem that cell death is caused by lysis. Several mutants of *E. coli* defective in protein synthesis and osmotically remedial have been described (Russell, 1972). The conclusion that this author reaches is that the temperature sensitive proteins produced by these mutants may be stabilised by increasing the osmotic strength of the medium. However it would appear that increasing the osmotic strength prevents the cells from lysing in B15. Temperature sensitive lytic mutants of *E. coli* all having peptidoglycan defects, were described by Lugtenberg *et al* (1971). It is probable that B15 also has a defect in this polymer. The mucoid nature of both this mutant and B20 when growing on NA at 37°C is due to overproduction of colanic acid. Synthesis of colanic acid, LPS "O" antigen and peptidoglycan all involve an isoprenoid carrier lipid, the amount of which limits polymer biosynthesis (Archibald, 1974). If the synthesis of one of these polymers is stopped, there would be more carrier lipid available for synthesis of the other two polymers (Sutherland, 1975). *E. coli* K12 does not produce much, if any, "O" antigen, so if peptidoglycan synthesis is defective, colanic acid would be overproduced. A peptidoglycan

defect could alter the outer membrane allowing periplasmic enzyme release and causing the outer membrane to become permeable to GV and DOC.

Release of periplasmic enzymes from B15

Polyacrylamide gel electrophoresis of the proteins released by osmotic shock, and those released into the medium by B15, shows that only protein bands 5, 8 and 11 are present in the medium. These correspond to 3' nucleotidase, an unknown protein of approximate M.W. 40,000, and RNase 1 respectively (Fig. 38). All the proteins released by osmotic shock from the parent are released by osmotic shock from B15.

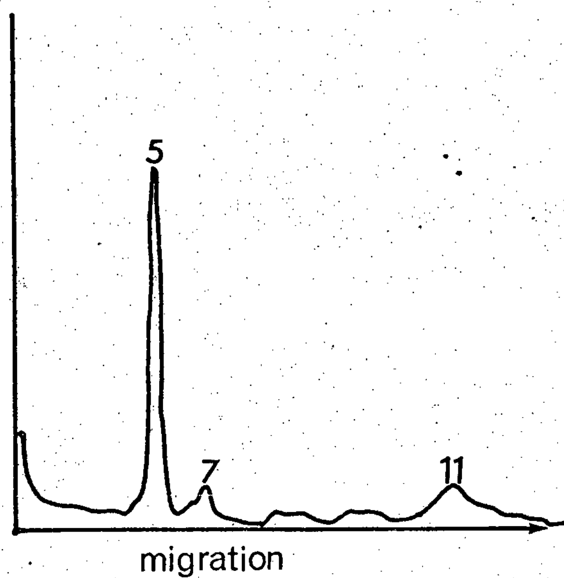
It is difficult to explain the release of some but not all periplasmic proteins by this mutant solely on a size basis. Several proteins which are smaller than 3' nucleotidase (approximately 60,000 M.W.) are not released into the growth medium. It is possible that there is differential localisation of these enzymes in the periplasm.

Other mutants that release RNase 1 (see Table 17)

B18 and B16 released approximately 10% of their APase into the medium during growth. B18 did not release very much RNase 1 and was not sensitive to GV or DOC. B16 however released larger amounts of RNase 1 and was sensitive to DOC. B3 had a permeability defect shown by sensitivity to novobiocin and penicillin, but was not sensitive to DOC or GV. Clearly increased permeability to GV and to penicillin can occur by mutations affecting different parts of the cell envelope. This mutant did not release much APase or RNase 1, neither was there a decrease in the cell-bound asparaginase II. The mutation has little effect on the release of periplasmic enzymes. Similarly B6 which was not sensitive to

Fig. 38.

Densitometer Trace of Proteins Released During Growth of B15, Separated by P.A.G.E. and Stained with Coomassie Blue



penicillin or novobiocin was extremely sensitive to DOC and GV, but did not release any APase, and very little RNase 1.

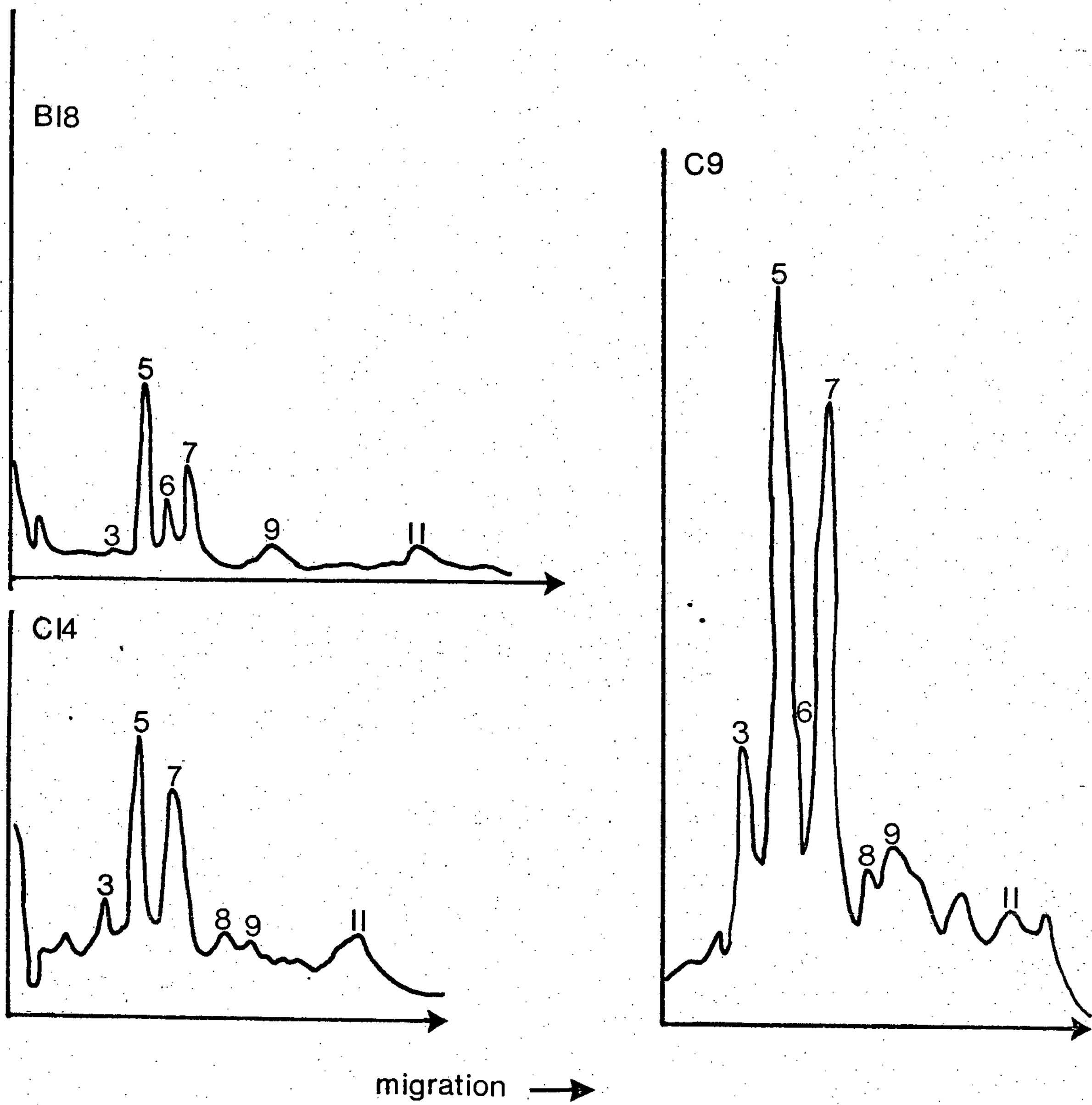
The mutant C9, which released very little APase or RNase 1 when growing in NB released large amounts of periplasmic enzymes when growing in MM (Fig. 39). In this medium it also released non-protein material similar to that released by B19 and detected on gels. There could be a nutritional requirement which is only partially satisfied by NB, or an alteration in the outer membrane caused by the physical conditions of M9 media. Further characterisation of this phenomenon and of the possible requirement may prove useful in elucidating the association of periplasmic enzymes with the cell envelope.

Although the effect of the mutations on the cell envelopes of these strains is not known there does not seem to be an LPS core defect in any of them.

These mutants, with the exception of C9, do not release large quantities of periplasmic enzyme and were not studied further.

Fig. 39.

Scan of Proteins Released into Medium by Various Ribonuclease 1 Releasing Mutants of E. coli Separated by P.A.G.E. and Stained with Coomasie Blue



DISCUSSION

a) Localisation of Periplasmic Proteins

The two membranes of the gram negative cell envelope provide a compartment in the cell which is separate from both the cytoplasm and the medium. Both the periplasmic enzymes and the periplasmic binding proteins are located in this area.

If the periplasmic binding proteins are involved in transport across the cytoplasmic membrane, it is reasonable to assume that they must be associated with, or become associated with, this membrane. However, such an association has not been demonstrated. Membrane vesicles made by the method of Kaback (1971) might be expected to contain periplasmic binding proteins if they are associated with the cytoplasmic membrane. However this is not the case. Periplasmic enzymes are not thought to be directly involved in transport, but with modification of substrates so that they can be transported into the cell. Evidence suggests that under normal physiological conditions, periplasmic enzymes do not protrude into the medium. Thus substrates must become available to these enzymes in the periplasm. Clearly the outer membrane is not as stringent a permeability barrier as the cytoplasmic membrane. This is substantiated by the crypticity of periplasmic enzyme activity when measured in whole cells.

There is little evidence to suggest association of periplasmic enzymes with particular cell envelope structures. However if these enzymes are free in the periplasm, they could be released by altering the permeability of the outer membrane. EDTA/tris treatment is essential for release of periplasmic enzymes by osmotic shock and also for the formation of sphaeroplasts by lysozyme. This treatment causes the release of outer membrane structures, mainly LPS, with a resulting increase in permeability

of this membrane. However treatment of E. coli with EDTA/tris causes little release of periplasmic enzymes, even though the outer membrane becomes more permeable to lysozyme.

b) Mutants isolated for RNase 1 release

The characteristics of several mutants of E. coli which release periplasmic enzymes and which show alterations in sensitivity to GV and DOC are reported in this thesis. One of these, B19, releases up to 50% of its APase during growth. This mutant is altered in its outer membrane proteins but does not show an increase in permeability to GV, novobiocin or penicillin. How 50% of the APase and most of the other periplasmic proteins are released into the medium without a permeability defect in the outer membrane is not clear. It is possible that the periplasmic enzymes are associated with the outer membrane proteins, and that alteration of these proteins in the mutant causes the release of the periplasmic enzymes. The release of enzymes by B19 is temperature dependent, with a threshold at 30°C. This is probably due to an alteration in the physical properties of the outer membrane, such as a change of state of its lipids. It would be interesting to examine the affect of growth at 30°C on the outer membrane proteins of this mutant since this would establish a cause and effect relationship between the proteins and lipids of the outer membrane. Material shed into the medium by B19 could be derived from the outer membrane. However no protein was found to be associated with it and no outer membrane proteins were present in the medium. Less of this outer membranous material was released at 30°C. Clearly periplasmic enzymes and outer membrane material are released simultaneously by raising the growth temperature.

The effect of DOC on this mutant was surprising. Colonies grew overnight on plates containing DOC, but dissolved during the following day. The relatively high concentration of DOC needed to produce this effect suggests that the outer membrane has not become more permeable to DOC but since the colonies dissolve, DOC must pass through the outer membrane. The mutation affects the outer membrane proteins which might cause this structure to become more lipid rich and thus more susceptible to DOC. However B19 is sensitive to DOC at all temperatures tested, which suggests that the enzyme release does not solely result from protein alterations in the outer membrane, but also from a temperature-induced change which causes shedding of some outer membrane material.

APase was reported to enter into a complex and to associate with LPS in the cell (Lindsay et al, 1973; Ingram et al, 1973). The results obtained with B19, which has normal LPS, are in disagreement with these findings, and suggest that at least 50% of APase is not LPS associated in E. coli. Lindsay et al (1973) described simultaneous release of APase and KDO from an LPS-core deficient mutant of S. typhimurium. They did not examine the nature of the LPS shed into the medium, nor did they suggest why KDO should be shed from a mutant which is deficient in LPS-core synthesis. However only 7% of the total APase was released into the medium by overnight cultures of this strain. Singh and Reithmeier (1975) studied a similar LPS mutant in E. coli, with similar results. However they interpreted the data in a different way, and suggested that APase release could be mediated by alterations in the amounts of major polypeptides of the outer membrane which occur in LPS-core deficient mutants of E. coli

and S. typhimurium (Ames et al, 1974; Koplow & Goldfine, 1974).

CPDase and RNase 1 are released in large amounts by LPS-core deficient strains of S. typhimurium. It would seem that these enzymes and APase are affected in different ways by LPS mutations, and may either reflect a difference in their associations with the cell envelope, or differences in permeability across an outer membrane with part of the LPS molecule removed. CPDase was found in the spent culture fluid of E. coli M1, and higher levels were found in the medium following growth of most of the E. coli mutants isolated for release of RNase 1. Similarly CPDase is totally released by polymyxin B treatment of exponentially growing E. coli cultures, but only 50% of the RNase 1, APase and acid-phosphatase are released (Cerney & Teuber, 1971).

In B15 which releases large amounts of RNase 1 but no APase, there is a presumed peptidoglycan defect. Altering this molecule could have an affect on the outer membrane, which is anchored to the cell wall. This results in an increase in sensitivity to GV and DOC, due to altered permeability of the outer membrane. In most mutants which have increased sensitivity to GV and DOC, RNase 1 is released, which suggests that some fraction of the RNase 1 is either free, and can diffuse through the defective outer membrane, or is located superficially in the cell. Similarly CPDase is readily released by treatments or mutations which alter the outer membrane. Cerney and Teuber's work (1971) with polymyxin B, would indicate that only half the RNase 1 and APase but all the CPDase are associated with, or released by, removal of the outer membrane.

Clearly a defect in the permeability barrier of the outer membrane is not sufficient for APase release from the periplasm.

As APase can be released when there is no permeability barrier defect even to DOC or GV, there must be some kind of association between this enzyme and the cell envelope. It is difficult to imagine why release of large amounts of periplasmic proteins can occur from a mutant, such as B19, with relatively few other associated changes. It is possible that the lamellae of the outer membrane lipid bilayer are different. Most evidence suggests that LPS faces away from the cell, and it is difficult to imagine why LPS should be present on the inside of the outer membrane. Relatively little is known about the distribution of other outer membrane components, but if periplasmic enzymes can pass out through the outer membrane with no increase in ease of passage of molecules into the cell, it could be due to an alteration of the inner of the two lamellae of the outer membrane. However this phenomenon could also be explained by localisation of periplasmic enzymes at discrete sites of the cell envelope. Such sites are known to occur in the envelope where LPS addition to the outer membranes takes place (Bayer, 1969). However there is little evidence for discrete localisation of periplasmic enzymes. Ferritin-antibody labelling and reaction product deposition suggest that APase is dispersed throughout the periplasm (Costerton et al, 1972). Experiments with E. coli mutants having outer membranes which are more permeable to antibiotics have shown that substantial release of periplasmic enzymes is not found except in deep rough LPS-core mutants. These mutants have decreased amounts of outer membrane proteins (Koplow & Goldfine, 1974; Randall, 1975).

One strain of E. coli which contains the envA mutation, is extremely sensitive to DOC and GV, but does not release

periplasmic β-lactamase. This strain has decreased amounts of phosphatidylglycerol in its outer membrane (Wolf-Watz, Normark & Bloom, 1973) but it was not examined for the release of other periplasmic enzymes. While alteration of the lipid composition does not appear to have much effect on the periplasmic enzymes, it does have a great effect on the outer membrane. Similarly when supplementing _trans_ for _cis_ unsaturated fatty acids in a fatty acid auxotroph of _E. coli_ a change in permeability was noted. Although periplasmic enzyme release was not monitored, the amount of activity of periplasmic enzymes did not appear to be significantly decreased (Rosen & Hackette, 1972). So it would seem that the outer membrane proteins play the largest role in maintenance of enzymes in the periplasmic space.

In the mutant B19 there are multiple changes in the outer membrane proteins, although the largest change is in the small molecular weight proteins, probably the lipoprotein. It would be interesting to examine these proteins further, and to characterise which ones have decreased and which have increased. The changes that were observed in the B19 outer membrane proteins suggest that the alteration of one protein will upset the equilibrium of the membrane and lead to overproduction of other proteins to compensate. The regulation of membrane proteins and membrane synthesis remains an intriguing area for conjecture, but if lessening of one protein causes the increase of others, the membrane proteins must be under some kind of feed back control mechanism. Alterations in outer membrane proteins have been described by other workers (Randall, 1975; Beacham, person. comm.) but little function has been ascribed to these proteins. From B19 it would appear that the lipoprotein and other, more

minor, proteins are not involved in the permeability barrier of the cell, but alteration of these proteins does cause susceptibility of the cell to DOC. However few other phenotypic characters have been attributed to this mutation. Only one enzyme is known to be located in the outer membrane, phospholipase A (Machtiger & Fox, 1973). This enzyme may be involved in alterations to the cell envelope during division or in changing the lipids of the outer membrane. It is also necessary for complete lysis of E. coli cells following T4 infection (Hardaway, Maten & Buller, 1975) and so this enzyme may have a role analogous to the autolytic enzymes that affect peptidoglycan.

c) Osmotic shock

When osmotic shock is carried out on cells pretreated with EDTA, it causes the release of periplasmic enzymes but does not kill the cells. EDTA treatment could be substituted by an LPS mutation. Thus its affect in the osmotic shock process is presumably to increase the permeability of the outer membrane. However substituting trans for cis unsaturated fatty acids in E. coli, while causing the outer membrane to become permeable to actinomycin D and lysozyme, causes a decrease in the amount of periplasmic enzymes released by osmotic shock (Rosen & Hackette, 1972). Cell death during osmotic shock treatment would result from rupture of the cytoplasmic membrane. Periplasmic enzyme release however is brought about by disruption of the outer membrane. During plasmolysis water is lost from the protoplast which shrinks. If plasmolysed cells are maintained in sucrose, which penetrates the protoplast slowly, the protoplast will regain its normal size due to sucrose uptake. If this is now transferred to a dilute medium, water will be taken up much

faster than sucrose can pass out of the cell, and so the protoplast will expand rapidly. Sucrose would pass through an EDTA treated outer membrane faster than through a normal outer membrane but the cytoplasmic membrane would remain unchanged by EDTA treatment. This would allow sucrose to enter the periplasm faster, thus the cell would become plasmolysed more quickly. However the speed of sucrose entry into the protoplast would not be increased. Osmotic shock if carried out now would mainly act against the outer membrane, and cause release of periplasmic enzymes.

Increasing the EDTA concentration in osmotic shock treatment causes less periplasmic enzyme release, which might reflect action against the cytoplasmic membrane. Similarly substituting trans for cis unsaturated fatty acids would affect the cytoplasmic membrane as well as the outer membrane, thus reducing the amount of periplasmic enzyme release by osmotic shock.

Biological importance of the periplasm

It is interesting that strains of *E. coli* can lose so much of their periplasmic enzymes yet appear to remain fairly normal, which raises the question of the biological significance of the periplasm. Clearly it is more efficient for the bacterium to have its "extracellular" enzymes associated with the cell, where they can be maintained under conditions necessary for optimal activity, and the products of their actions used exclusively by the bacterium. It is to be expected that this facility would be most useful when the bacterium is growing in an aqueous environment which is low in nutrients and metal ions. This may explain why in many such environments the bacterial flora is represented entirely by gram negative species (Costerton, personal

communication). Several enzymes which are potentially harmful to the cell, such as nucleases and nucleotidases, are found in the periplasm and have cytoplasmic inhibitors. These enzymes may be necessary for protecting the cell against alien genetic material, or may even have a role in endogenous metabolism. The periplasm provides a suitable compartment for localisation of these enzymes. Similarly β-lactamase would need to exert its effect on penicillin before the antibiotic disrupts peptidoglycan cross-link formation, and an envelope localisation would appear to be essential.

Periplasmic enzymes are not substantially released simply by creating a permeability defect in the outer membrane. Therefore there is probably an association between the periplasmic proteins and the cell envelope. This association must be of a loose nature and probably takes the form of hydrogen bonding. The most likely outer membrane/cell envelope structures to take part in this association would be the outer membrane proteins.

The release of periplasmic proteins by osmotic shock and lysozyme sphaeroplast formation involves disruption of the outer membrane. It is most likely that the spatial arrangement of outer membrane/cell envelope structures is important in maintaining the periplasmic enzyme association.

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