TITLE

Sensitivity to Antibacterial Agents Associated with Envelope Changes in Escherichia coli.

Submitted for PhD. examination by

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ABSTRACT

Gram-negative bacteria are resistant to many hydrophobic antibacterial agents due mainly to the fact that their outer membrane acts as a very efficient permeability barrier against the entry of such compounds. Sensitivity to hydrophobic, cationic and 4-quinolone antibacterial agents was investigated using strains altered in outer membrane permeability due to the effects of cationic permeabilisers, Col V plasmids and mutations affecting outer membrane proteins .

The cationic agents N-(2-pyrimidinyl)piperazine, 4,5,6-triamino pyrimidine, 1,4-diaminopiperazine, methylglyoxyl bis(guanyl-hydrazone), spermidine, agmatine sulfate, 1,3-diaminoacetone, 3,3'-diaminobenzidine and 1,4-diaminobutanone were found to considerably enhance the growth inhibitory effects of novobiocin. In constrast, the agents 1,3-diaminoguanidine, triethylenetetramine, tetraethylene pentamine, N,N,N',N'-tetramethyl-p-phenylenediamine and formamidine disulfide had no effect on novobiocin activity. Diaminoacetone also enhanced the activity of erythromycin, rifampicin, nalidixic acid, bacitracin, serum, polymyxin B, hydrogen peroxide and hexanoic acid (at pH 4), but failed to have any significant effect on the inhibitory activities of rifamycin, fusidic acid, vancomycin, oxacillin, nafcillin, nitrofurantoin and deoxycholate. The growth inhibitory activity of EDTA and hexanoic acid were partially reversed by diaminoacetone. Magnesium ions were found to reverse novobiocin acitivity and also to compete with the enhancers of novobiocin activity. Inhibitory effects of the DNA gyrase inhibitors norfloxacin and ofloxacin were unaffected by the cationic agents diaminoacetone and methylglyoxyl bis(guanyl-hydrazone), but that of flumequin was significantly increased by both the cationic agents. Norfloxacin and ofloxacin are hydrophilic and amphoteric compounds respectively and hence use the porin pathway for entry into the Gram-negative cell. Flumequin, on the other hand is the most hydrophobic of these three DNA gyrase inhibitors tested, and may enter the cell via one of the other two pathways. It

seems that the cationic agents act as permeabilisers of hydrophobic agents and therefore act at the surface of the cell ie. at the outer membrane ; the possible mechanism of this effect is discussed.

The effects of the Col V plasmids ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30, ColV-8 and ColV-41 were investigated on sensitivity of their *E.coli* K12 host strain to various antibacterial agents. Plasmids ColV,Ia-K94, its derivative ColV,Ia-K94 Tn10 and ColV-41 were found to considerably increase the sensitivity of their host strain to erythromycin, rifampicin, gentamicin, novobiocin, but only to flumequin (and some to ofloxacin) out of the five 4-quinolones tested. Plasmid ColV-8 sensitised its host to most of the quinolones tested whereas plasmid ColV-41 conferred no effect on the sensitivity to these agents. With ColV,Ia-K94, colicin V and its immunity component, transfer components and an unidentified factor were found to contribute towards the observed increased antibiotic sensitising effect. I propose that the increased antibacterial agent sensitivity effect associated with the presence of most of the Col V plasmids is due to a change in the proportion of the outer membrane components and possibly also due to changes in LPS structure, but not due to any specific uptake system encoded by these plasmids; ColV-8 may however provide an additional route of entry for the quinolones.

Novobiocin was found to be very active at low pH and this change in activity may be related to an observed change in its conformation at pH 5. On the other hand, novobiocin may be using a different pathway of entry into the cell at pH 5. This latter concept was investigated using strains containing mutations in the various major outer membrane proteins eg. porin proteins and those involved in the iron-assimilation system.

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Preface

This thesis is submitted for PhD. examination under the regulations of University of London.

Some of the results presented here have also appeared in the following publication :

Modha, J. L., K. J. Barrett-Bee & R. J. Rowbury. 1989 Enhancement by cationic compounds of the growth inhibitory effect of Novobiocin in *E.coli.* *Letters in Applied Microbiology* **8** : 119-122.

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1. INTRODUCTION

1.1. Escherichia coli and its virulence :

Escherichia coli, a Gram-negative rod-shaped bacterium which is found commonly in the intestinal flora of humans and animals, has been identified as the causative agent in a variety of diarrhoeal illnesses eg. infantile diarrhoea, cholera-like disease in adults (Sack *et al*, 1971), travellers diarrhoea and colibacillosis in young domestic animals (Smith, 1976). Many strains of *E.coli* have also been isolated from extraintestinal infections in man eg. from meningitis, septicaemia and urinary tract infections.

The intestinal infections are mainly caused due to the production of enterotoxins or colonization antigens eg. K88 or CFA's by the infecting organism eg. enterotoxigenic *E.coli* (ETEC; Sussman, 1985). The extraintestinal infections are usually caused by those organisms which have a specific invasive property ie. enteroinvasive E.coli (EIEC) that enables them to adhere to the intestinal lining, invade it and then resist the host defence mechanisms eg. bacteriocidal activity of serum, iron-starvation, avoiding or surviving phagocytosis and still be viable enough to cause an infection such as meningitis.

Many infections caused by Gram-negative bacteria are difficult to treat as many of these organisms are rather resistant to the common antibacterial agents used to treat Gram-positive bacteria such as macrolide antibiotics like erythromycin, rifamycins like rifampicin and other agents such as lincomycin, clindamycin and fusidic acid (Nikaido & Nakae, 1979). The main reason for this resistance is not genetic but due only to the fact that there is a very effective barrier present on the cell surface (Leive, 1974) of such bacteria that prevents or greatly reduces the antibiotics from entering the cell (Nikaido, 1989) and reaching their site of action.

1.2. The Gram-negative Cell Envelope :

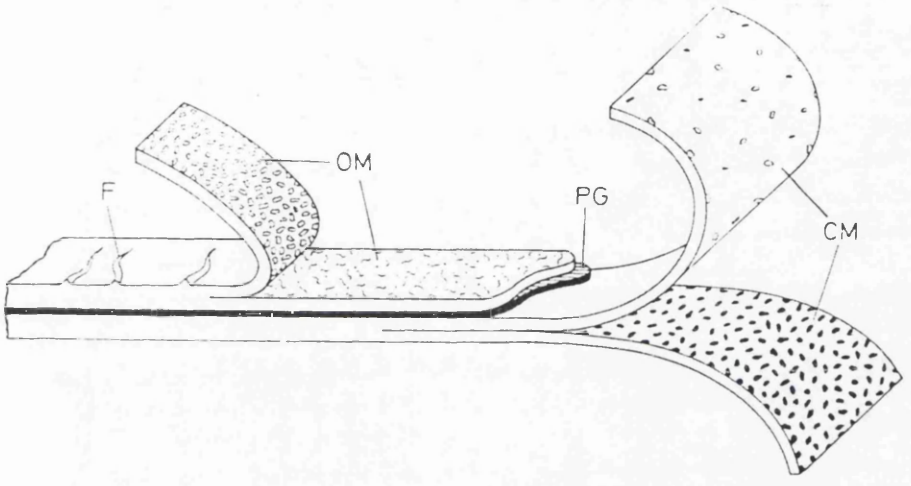
This cell-envelope is a multilayered structure (Fig.1) consisting of the cytoplasmic membrane, the periplasmic region containing a gel with peptidoglycan and the outermost and unique outer membrane (Glauert & Thornley, 1969).

1.2.1. The Cytoplasmic Membrane :

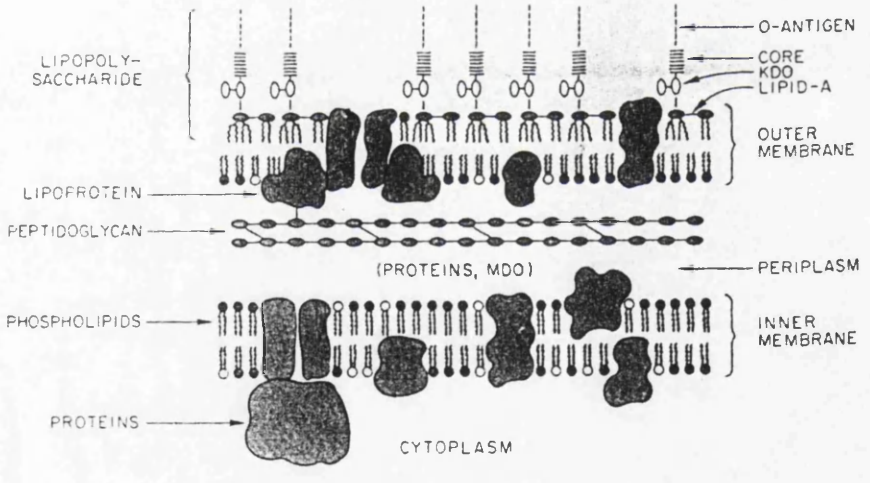
This is a phospholipid bilayer membrane like that found around other cells. The bilayer also contains proteins that span either one layer only (intrinsic proteins) or both bilayers (extrinsic proteins ; Lehninger, 1982). Most of these proteins are either structural or involved in transport functions eg. uptake of nutrients and export of toxic metabolic byproducts, or in energy generation by proton-translocation along an electrochemical gradient containing cytochromes, or are enzymes involved in the synthesis of peptidoglycan, lipids, LPS, etc. Transport across the cytoplasmic membrane is by diffusion, facilitated for certain compounds, or by energy consuming active transport. This membrane is a very strong barrier against the entry of hydrophilic or charged molecules into the cell if a specific transport system for them is absent. However, hydrophobic molecules can enter the lipid bilayer by displacement of the phospholipids (Nikaido, 1976) and hence readily cross the membrane. In bacteria, the main functions of the cytoplasmic membrane are firstly, energy generation and it is this process that is the target of many toxins eg. colicins V and I, and secondly biosynthesis of envelope components.

Fig. 1 :

(a) Organisation of the Gram-negative Cell Envelope

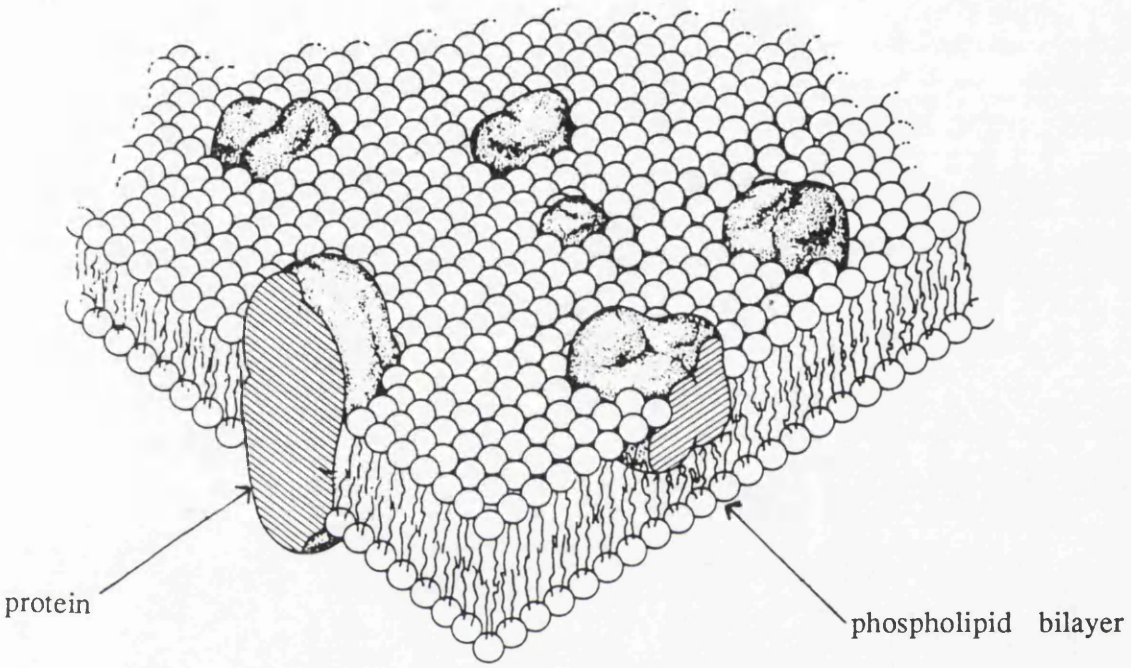


(b) Detailed Structure of the Envelope Components



Adopted from Nanninga (1985) & Raetz (1987). Abbreviations : F, flagella; OM, outer membrane; CM, cytoplasmic membrane; PG, peptidoglycan; KDO, 2-keto-3-deoxyoctonate; MDO, membrane derived oligosachharides.

Fig. 2 : Structure of the Cytoplasmic Membrane



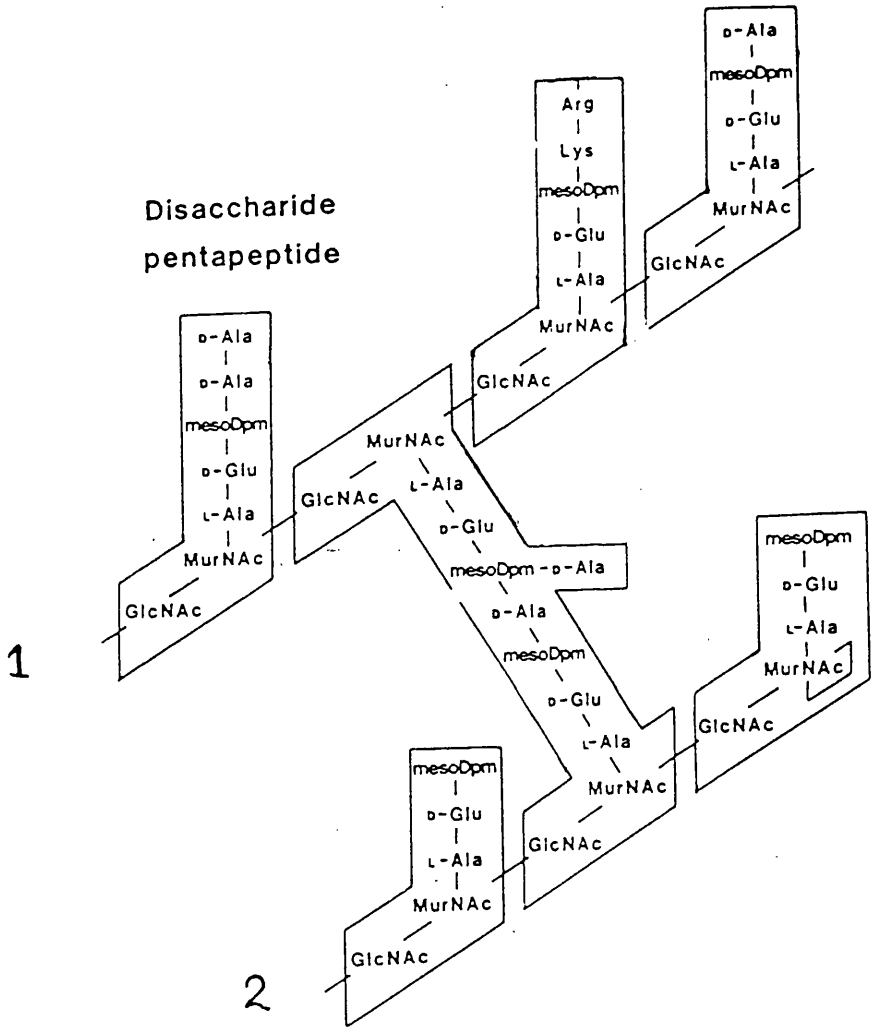
Adopted from Gale *et al* (1982). The lipid-globular protein mosiac model with a lipid matrix.

1.2.2. The Periplasmic Gel and Peptidoglycan :

The compartment separating the cytoplasmic and the outer membrane in Gram-negative organisms has been thought until recently to be subdivided distinctly into the peptidoglycan or murein layer and the periplasmic space. In this model, the periplasm is an aqueous area containing proteins many of which are enzymes eg. firstly, scavenging enzymes such as asparaginase, alkaline phosphatase, acid hexose phosphatase & nucleosidases; enzymes catabolising deoxyribonucleosides such as deoxyribomutase, deoxyriboaldolase, purine deoxyribonucleoside phosphorylase and deoxythymidine phosphorylase ; nucleases such as ribonuclease I and deoxyribonuclease I ; secondly, detoxifying enzymes such as beta-lactamase, aminoglycoside-3'-phosphotransferase, -2'-acetylase, 2'-adenylase and alkyl sulphohydrolase ; and thirdly, binding components involved in transport and chemotaxis. The detoxifying enzymes are very important in conferring resistance on the organism to various antimicrobial agents (Beacham, 1979 ; Hammond *et al*, 1984).

The peptidoglycan is a rigid polymer with a disaccharide backbone containing alternating N-acetyl-muramic acid and N-acetyl-glucoseamine residues with a tetrapeptide covalently linked to each N-acetyl-muramic acid. Peptidoglycan is the main structural component of the bacterial cell envelope and it provides shape to the cell by extending over the whole cell surface (Weider & Pelzer, 1964) and forming a sacculus as well as preventing cell death under conditions of high osmotic pressure. Peptidoglycan is cross-linked within its own polymers via its tetrapeptide branches (Fig.3). The number of tetrapeptide chains that participate in cross-linking varies from organism to organism eg. in *E.coli*, there is only 20% cross-linking with the majority of the tetrapeptides remaining unattached (Hammond *et al*, 1984). Peptidoglycan is also linked to the outer membrane by lipoprotein (Braun, 1975).

Fig. 3 : Composition of the murein or peptidoglycan



Adopted from Nanninga,N. (1985). Chains 1 and 2 are crosslinked via a pentapeptide. Abbreviations : D-Ala, D-Alanine; mesoDpm, meso-Diaminopimelic acid; D-Glu, D-Glucose; Arg, Argnine; Lys, Lysine; GlcNAc, N-Acetylglucoseamine; MurNAc, N-Acetylmuramic acid.

A new model for the organization of the periplasmic components and the peptidoglycan has recently been proposed by Hobot *et al* (1984). Instead of there being a distinct boundary between the peptidoglycan layer and the periplasm, as has been observed in earlier electron micrographs of the cell envelope, the new proposal is that peptidoglycan covers the whole area present between the two membranes and that the periplasmic components exist in a semi-aqueous gel-like texture in gaps between the peptidoglycan chains. In addition, it is proposed that the peptidoglycan is more cross-linked in the area nearer to the outer membrane edge but considerably less so or not at all nearer the cytoplasmic membrane edge. A potential advantage proposed with this arrangement is that in such a gel, the high viscosity of the area prevents convection and only allows diffusion of molecules across it, making possible the existence of linear charge and concentration gradients between the two membranes.

This latter proposal may well be nearer to the true organisation of the envelope components between the cytoplasmic and outer membranes as compared to the previous model. But for this to be accepted, the distinct peptidoglycan layer seen in electron micrographs and implying the presence of two separate compartments between the two membranes must be seen as an artefact of sample preparation as the new proposals are based on results obtained using different sample preparation techniques. Another advantage from the new model is that the transport of large molecules such as porin proteins, iron complexes with siderophores, LPS, etc. between the two membranes need not be restricted to adhesion sites between the two membranes ie. Bayers junctions, and can freely occur all over the cell surface area. This seems to be a more realistic idea considering the swift-adaptation requirements in cell growth rates and metabolism that occur under frequently changing environmental conditions.

1.2.3. The Outer Membrane :

The outermost layer of the envelope of Gram-negative bacteria is a topic of much interest as it is this that plays a major role in differentiating these bacteria from Gram-positives. Also, it is this membrane that protects the cell from the detergent action of bile salts and degradation by digestive enzymes (Nikaido & Nakae, 1979), may allow it to evade phagocytosis, confers some complement resistance and the capacity to avoid a specific immune attack by altering the surface antigen constitution (Makela *et al*, 1980), as well as conferring the above mentioned resistance to hydrophobic antibiotics. Hence, in any rational attempt to design antimicrobial agents effective against Gram-negative organisms or potentiate the effect of those which are already available but are ineffective, the outer membrane appears to be an attractive target. This structure is unique to the Gram-negative bacteria and crucial for their survival in their natural habitat.

The components of the outer membrane are proteins, lipopolysaccharide (LPS) and phospholipids (DiRienzo *et al*, 1978). The distribution of these components is very interesting and seems to be highly evolved and sophisticated. Thus this membrane is also a bilayered structure like the cytoplasmic membrane but the constituents of the two bilayers are different. The inner leaflet contains virtually all the phospholipids whereas the outer leaflet contains LPS (Nikaido & Nakae, 1979 ; Lugtenberg & van Alphen, 1983) and very little if any phospholipid. The proteins are distributed throughout the membrane bilayer and may protrude out of both sides of the membrane depending on their structure and function. The outer membrane has almost no enzymatic activity except for the presence of a phospholipase (Osborn *et al*, 1972) and a protease.

1.2.3.a Outer Membrane Proteins :

Nearly half of the outer membrane mass can be attributed to proteins. Some proteins are present in huge amounts eg, > 100,000 copies per cell and these are called the

Table 1 : Major Outer Membrane proteins

Protein	ca. Mol. wt.	Genes (map position)
OmpF	37,000	opmF(21)
OmpC	36,500	ompC(47)
LamB	50,000	lamB(91)
Protein 2	36,000	phage(PA-2)
NmpAB	37,000	nmpA(83) or nmpB(9)
NmpC	37,000	nmpC(12)
MRB	29,000	R-factor
OmpA	35,000	ompA(21)
Lipoprotein	7,500	lpo(36.5)
Protein 3b	40,000	?

(Adopted from Hall & Silhavy, 1981)

"major" proteins. The actual number of the major protein species present in the cell envelope at any one time is variable and is influenced by the growth medium & temperature, by lysogeny, mutation or strain background. At least ten major outer membrane proteins have so far been identified (Hall & Silhavy, 1981). These include among others the OmpF, OmpC, OmpA, LamB protein, and the murein lipoprotein (Table 1). There are many other species of proteins present in the outer membrane which are present in smaller quantities, and these are termed the " minor " proteins (Table 2). Some of these may be induced to higher levels under certain environmental conditions.

(i) **The Murein Lipoprotein :**

This is a small (7.2 kDa) major outer membrane protein (7×10^5 copies) that occurs partially (one third in *E.coli* K12) in a bound form and the rest as free protein in the outer membrane (Inouye *et al*, 1972). The bound form is covalently linked to the underlying peptidoglycan, and from sequence analysis, its polypeptide chain appears to exist mostly in an alpha-helical form (Braun *et al*, 1976) and there may be some cross-linking between the chains to give oligomers (Reithmeier & Bragg, 1977). There is no clear evidence for its exposure at the outer cell-surface and considering its strongly hydrophilic amino acid composition, it seems probable that only its lipid portion penetrates into the outer membrane, the rest being on the inner surface of the outer membrane. Mutational analysis shows that this protein is not essential for growth, viability (Hirota *et al*, 1977) or normal diffusion processes (Nikaido *et al*, 1977). However, *lpo* mutants seem to have unstable cell walls as outer membrane vesicles and periplasmic enzymes are constantly released (Hirota *et al*, 1977). These observations suggest a structural role for this murein lipoprotein in maintaining a bond between the outer membrane and the peptidoglycan.

(ii) **OmpA Protein** :

This is a larger (ca. 35 kDa), heatmodifiable major outer membrane protein which is similar to the porins in size and in their rich beta-sheet structure (Nakamura & Mizushima, 1976) but is functionally different from them. Studies of *ompA* mutants have shown reduced rates for amino acid and peptide uptake (Manning *et al*, 1977), resistance to phages TuII* and K3, and to colicin L, unstable outer membranes and defective conjugation (Manning & Achtman, 1979). Mutants altered in OmpA protein are also unable to grow at 44°C in minimal salts medium and have low inhibitor sensitivities (Deeney *et al*,1986; Manoil & Rosenbusch, 1982; Reakes *et al*,1988). The OmpA protein can be crosslinked to the peptidoglycan (Endermann *et al*, 1978) and appears to come to the outer surface as it can be labelled by a nonpenetrating reagent acting at the surface of intact cells. Hence, OmpAp seems to span the thickness of the outer membrane. An interesting property of the OmpA structure is that it contains a region similar to the hinge region of immunoglobulins which, when treated with a protease splits the protein into two large domains. The N-terminal domain is inserted into the outer membrane and spans it whereas the C-terminal domain is presumably associated with the peptidoglycan layer or is free in the periplasm (Chen *et al*, 1980).

(iii) **The Porins** :

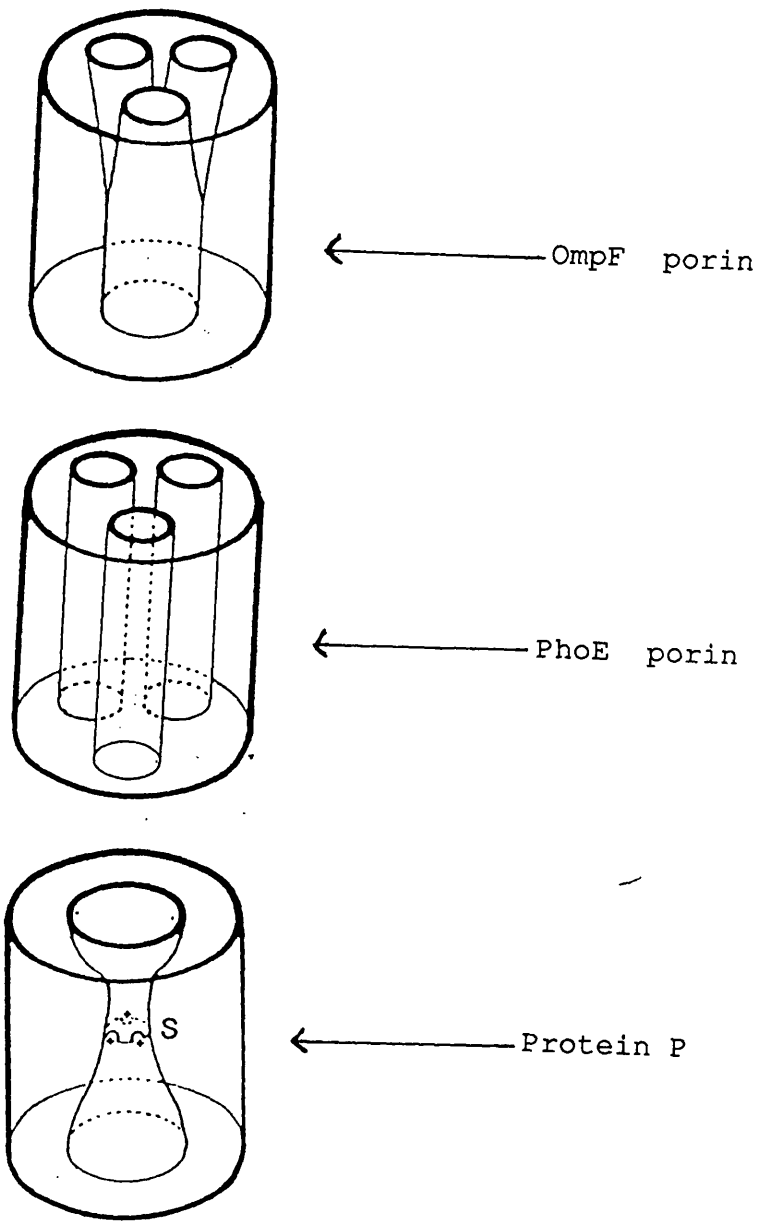
These proteins are large (Hancock, 1987) and exist as oligomers, usually trimers, and are often strongly but non-covalently associated with the underlying peptidoglycan and the LPS. Their prime function of forming non-specific channels in the outer membrane, with an upper size exclusion limit of ca.600 Da (Nakae & Nikaido, 1975; Decad & Nikaido, 1976) has earned them the name "porin". Of the four well-studied porins in *E.coli*, the genes for OmpFp, OmpCp and PhoEp show striking DNA sequence homology (Mizuno *et al*, 1983) and this suggests a common evolutionary origin, whereas LamB is less similar but contains several regions of local homology. Circular dichroism has shown that porins are unusually rich in beta-sheet

structure but have undetectable alphahelical segments (Rosenbusch, 1974; Nakamura & Mizushima, 1975), and infra-red analysis has showed that the beta-sheet structures of porins are orientated so that the backbones are roughly perpendicular to the surface of the membrane (Garavito *et al*, 1982). *E.coli* porins OmpFp and OmpCp are receptors for phages TuIa and Me1 respectively, but addition of LPS is required for them to successfully inactivate the phages upon attachment (Nikaido & Vaara,1985). Three types of structures have been proposed for the porin channels taking the pore sizes and various solute uptake data into consideration (Fig.4 ; Hancock, 1987). OmpFp and OmpCp porins of *E.coli* have channel diameters of 1.2nm and 1.1nm respectively (Nikaido & Rosenberg, 1983)

(iv) **Minor / Inducible Outer Membrane Proteins :**

These proteins are usually present in the outer membrane but only in very small quantities when compared to for example the porins. Some of these eg. the Tsx protein, are constitutively synthesised whereas others like the PhoE, LamB and BtuB proteins, and those involved in the iron assimilation systems are induced under appropriate nutritional conditions. Many of the minor proteins facilitate specific diffusion processes, usually of essential solutes that do not use the general porins efficiently (Table. 2). Protein Tsx is a receptor for colicin K and phage T6 and also has a role in transport (as a porin, not as a carrier) of nucleosides (Hankte, 1976). The PhoE protein is induced to the level of a major protein under conditions of phosphate starvation. It is related immunologically to OmpFp and OmpCp and seems to be produced at their expense under phosphate limited conditions. The PhoE protein exhibits a preference for anionic solutes, particularly pyrophosphates, and also has a specific phosphate binding site (Nikaido & Rosenberg, 1983). The LamB protein is the receptor for phage lambda and is also involved in maltose uptake; it is encoded by the *lam*B gene which is a part of the maltose operon (Hazelbauer, 1975; Szmelcman & Hofnung, 1975). Synthesis of the LamB protein is controlled by the maltose regulatory gene *mal*T (product molecular weight ca.50kDa) and is present only

Fig. 4 : Structures of porin channels



Adapted from Hancock (1987).

in the outer membrane. The LamB protein forms a pore in the outer membrane and is utilised by a variety of solutes for entry but maltose is given top priority (Luckey & Nikaido, 1980(a) & (b)). However, the main role of LamB protein is in the uptake of maltodextrins into the cell; these are too large to enter through the regular porins (Wandersman *et al*, 1979). The BtuB protein is present for the uptake of vitamin B_{12} into the cell, however it requires divalent cations and LPS for maximum activity. BtuBp is also a receptor for phage BF23 and E-group colicins (Reynolds *et al*, 1980).

Under conditions of iron stress, *E.coli* produces at least five outer membrane proteins eg. Cir, TonA, FecA, FepA and an 83kDa protein. FepA is the receptor for colicin B and is thought to be the binding site for enterochelin-Fe(III) complex. TonA protein (also called fhuA) is the receptor for phages T1, T5, ϕ80 and colicin M and is involved in ferrichrome uptake, independently of the enterobactin system (Luckey *et al*, 1975; Wayne & Neilands, 1975). FecA and FecB are proteins involved in ferric citrate uptake, with only FecA being present in the outer membrane.

Protein Cir is a receptor for colicin I but its exact role in iron uptake is not known. The TonB protein influences the processing of ligands bound to the Cir, TonA, FecA, FepA and BtuB proteins and mutation at this genetic locus abolishes high affinity transport by any of the iron-specific membrane proteins and additionally prevents the lethal action of colicins B, D, I, M and V, or infection by phages T1 and ϕ80 (Reynolds *et al*, 1980). The action of the enterobactin-Fe(III) transport system appears to be directly controlled by the energy state of the cytoplasmic membrane and the TonB protein serves as a device to couple the outer and cytoplasmic membrane in some way. Similarly, the energy-dependent uptake of vitamin B_{12} by *E.coli* also requires a functional TonB protein (Neilands, 1982; Hammond *et al*, 1984).

Table 2 : Minor / Inducible Outer Membrane Proteins

Protein	Molecular weight	Conditions for induction
Tsx	26 kDa	(constitutive)
PhoE	40 kDa	Phosphate limitation
LamB	50 kDa	Presence of maltose
BtuB	60 kDa	Vitamin B12 limitation
Cir	74 kDa	Iron limitation
TonA	78 kDa	
FecA	80.5 kDa	
FepA	81 kDa	
83k protein	83 kDa	

Adopted and modified from Hammond *et al*, 1984.

1.2.3. Lipopolysaccharide :

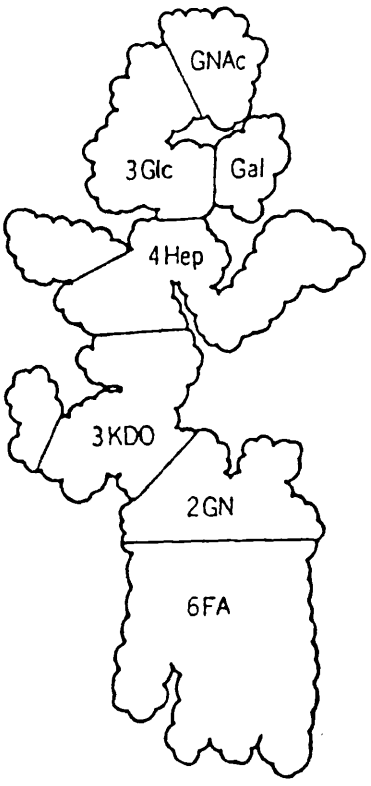
Lipopolysaccharide (LPS), also commonly known as the endotoxin, is an outer membrane macromolecular complex of polysaccharide and lipid. LPS from Enterobacteriaceae has been generally found to be made of three well-defined regions : the O-specific antigen, core-oligosaccharide and the lipid A.

The O-specific antigen, also called the O-antigen or the somatic antigen is a long chain polysaccharide consisting of repeating units with one to seven monosaccharides (Orskov *et al*, 1977 ; Jann & Jann, 1984). The oligosaccharide core is composed of approximately ten monosaccharides which include 3-keto-2-deoxyoctonate (KDO), heptose, glucose, galactose and N'-acetylglucoseamine. These sugars are added on sequentially to the growing LPS precursors by unique glycosyl transferases.

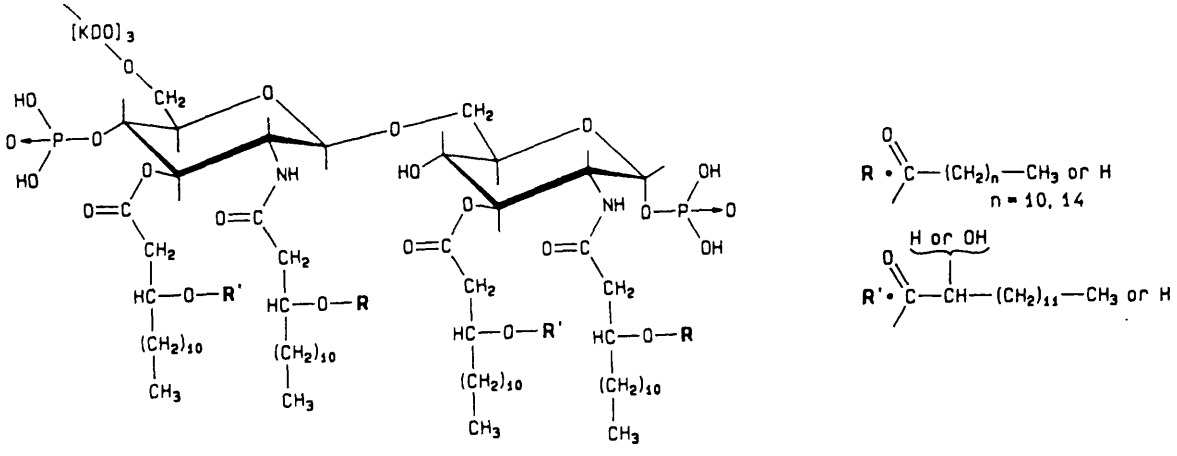
KDO is a unique sugar present only in the LPS of Gram-negative bacteria (Droge *et al*, 1970). The structure of KDO was first discovered by Heath & Ghalamber (1963) in *E.coli* LPS. There has been some controversy as to how many KDO residues are present in *E.coli* K12 LPS. Recent evidence suggests that there are two KDO residues linking the lipid A and the core polysachharide instead of three residues as was formerly believed (Qureshi *et al*,1988). There is some evidence that the KDO region is important for the translocation and integration of LPS into the outer membrane (Rick & Osborn,1977). In addition, the KDO region could have a secondary role in the formation of the LPS-dependant barrier function of the outer membrane as it is these residues that may form an umbrella to block the entry of any compounds to the hydrophobic regions of the outer membrane such as the fatty acids of the Lipid A component or the phospholipids in the inner leaflet of the outer membrane. Phosphate-mediated ionic bridges linking LPS-LPS or LPS-protein complexes in the outer membrane may well be the major force in establishing the permeability barrier. Being in the middle of the LPS molecule, the KDO residues can also affect the orientation of the other components of LPS on

Fig. 5 : Structure of E.coli K12 LPS

(a) Whole LPS molecule :

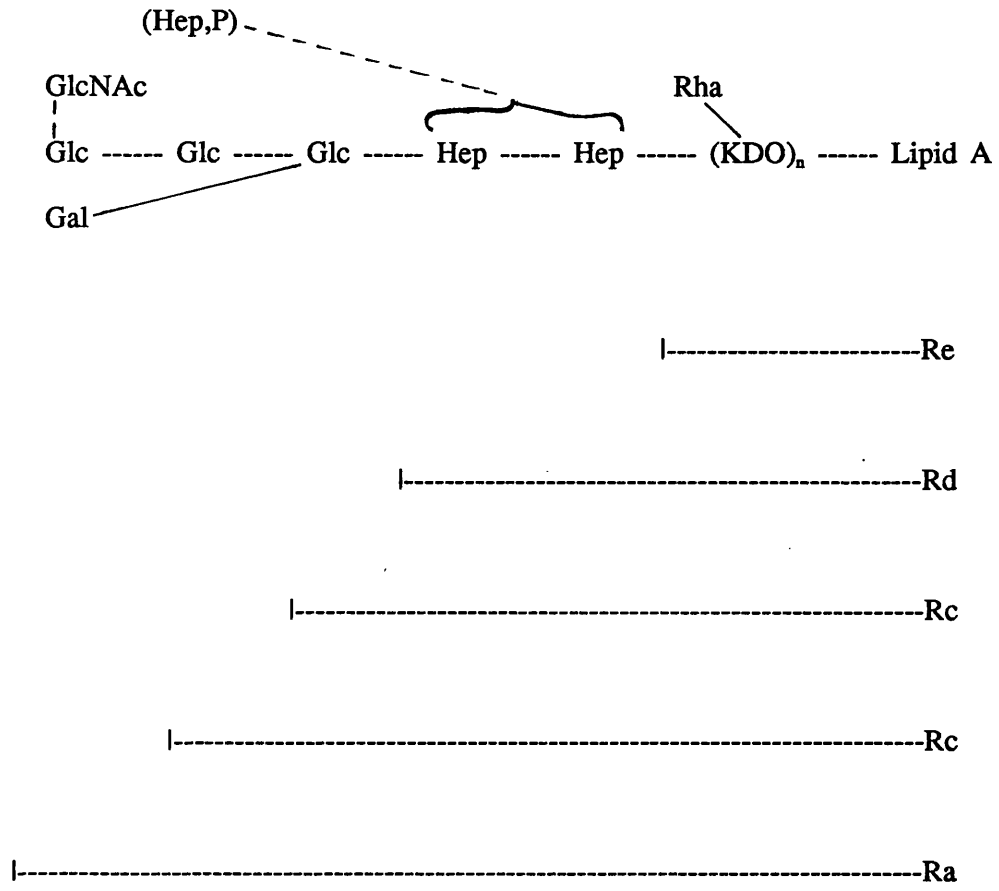


(b) Structure of Lipid A :



Adopted from Mizushima (1985). Abbreviations : GNAc, N-acetylglucoseamine; Glc, glucose; Gal, galactose; Hep, heptose; KDO, 2-keto-3-deoxyoctonate; GN, glucoseamine; FA, fatty acids.

Fig. 6 : Mutations (Ra-Re) of E.coli K12 core LPS



Adopted from Nikaido & Vaara,1985. KDO is 3-deoxy-D-manno-octulosonic acid and the value of n is believed to be either 2 or 3. Broken lines indicate incomplete subtitution. Other abbreviations : Hep, L-Glycero-D-manno-heptose; Glc, D-Glucose; GlcNAc, N-Acetyl-D-Glucoseamine; Gal, D-Galactose; P, Phosphate.

either side and consequently promote local hydrogen-bonding thus resulting in a more rigid and impermeable membrane (Schindler & Osborn, 1979).

The Lipid A is a backbone consisting of diglucosamine in B1-6 linkage with ester, amide and diester-linked fatty acids. Free Lipid A has most of the endotoxic and biological characteristics of the intact LPS. LPS is anchored in place in the outer leaflet of the outer membrane by the Lipid A moeity. The complete structure of free Lipid A derived from LPS of *Salmonella* strains has been established and the covalent structure of Lipid A from both *E.coli* and *Salmonella* strains has been found to be identical (Imoto *et al*, 1985).

Fig. 5 shows the structure of *E.coli* K12 LPS. *E.coli* K12 LPS does not possess any O-specific antigen polysaccharide, but only the core oligosaccharide and the lipid A moiety (Reitschel *et al*,1984). Lack of O-antigen side chain confers two phenotypes on the K12 strains : (i) the surfaces of the bacterial colonies are rough and irregular and, (ii) the bacteria agglutinate in saline. These two characters of LPS lacking the O-specific antigen has earned it the name rough LPS (Hitchcock *et al*, 1986).

The two major outer membrane lipid components ie. LPS (outer leaflet) and the phospholipid (inner leaflet) differ in at least three ways. Phospholipids only have two fatty acids connected to the backbone structure, whereas LPS has six or seven fatty acid chains linked to the diglucosamine backbone. Secondly, unlike the phospholipids, all the fatty acids in the LPS are saturated, and some are 3-hydroxy fatty acids, plus some fatty acid residues are linked to the 3-hydroxy group of another fatty acid producing the 3-acyl-oxy-acyl structure. Thirdly, many negatively charged groups exist on the backbone of LPS itself as on proximal sugar residues such as KDO (Nikaido & Vaara, 1985).

LPS is found to bind divalent cations quite strongly. In addition to reduction of charge repulsion between the highly anionic LPS molecules, divalent cations eg. magnesium ions, are thought to bridge adjacent LPS molecules and to link LPS with outer

membrane proteins. Chelating agents such as EDTA effect the release of up to 50 % LPS from whole cells and cause an increase in permeability to certain hydrophobic compounds that cannot normally enter the cell (Leive *et al*, 1968). In *E.coli* K12 strains the outer membrane was found to be enriched in both calcium and magnesium ions relative to the cytoplasmic membrane. Both membranes were found to contain signicant levels of iron, aluminium and zinc and the multivalent cation content of the LPS resembled that of the outer membrane. LPS has been shown to associate with many cations other than magnesium and calcium eg. sodium, certain polyamines like spermine and spermidine (Coughlin *et al*, 1983) as well as a paramagnetic cation of the element Europium with molecular weight 63 (Ferris & Beveridge, 1984) depending on the availability of the cation in the growth medium. The association of these cations is thought to be with the phosphoryl and some carboxyl groups present on the LPS.

To determine the role of LPS side chains in outer membrane rigidity, Rottem & Leive (1977) (using fatty acid probes) found that outer membrane preparations containing long chain polysaccharide LPS were much less fluid than were preparations from LPS mutants containing short chain polysaccharides. Also removing approximately 50 % LPS by EDTA treatment from long chain polysaccharide LPS resulted in greatly increased fluidity of the outer membrane. These effects were independent of whether phospholipid was also lost on EDTA treatment or not. When compared, the cytoplasmic lipid was more fluid than the lipid in the outer membrane. These results indicate that LPS and especially the polysaccharide portion, is responsible for the restricted mobility of the lipid hydrocarbon chains in the outer membrane. The involvement of the sugars in the barrier function of the outer membrane is further illustrated by the deep rough mutants of *E.coli*. Fig.6 shows the series of *Ra-Re* LPS core mutants. The deep rough mutants have increased permeability to hydrophobic agents (Gustafsson *et al*, 1973 ; Roantree *et al*, 1977 ; Rottem *et al*, 1979). These mutants are thought to be hypersusceptible either because there are fewer LPS-LPS interactions or because they have increased levels of

phospholipid in the outer leaflet of the outer membrane (Nikaido & Vaara, 1985).

Alterations in the amino- or phosphate groups either in the fatty acid of lipid A (Conrad & Galanos, 1989), KDO residues (Schindler & Osborn, 1979) or the other sugars in the core region will have a profound effect on the integrity of the LPS layer and consequently on outer membrane permeability (Roque *et al*, 1988). Some such changes in LPS composition may be induced by the presence of plasmids and during this work an attempt was made to investigate the effect of ColV plasmids on LPS.

1.2.3.c Phospholipids :

This component of the outer membrane is located almost exclusively in the inner leaflet and the constituent phospholipids found here are the same as the ones found in the cytoplasmic membrane. In *E.coli* K12, the phospholipids are phosphatidylethanolamine (91%), phosphatidylglycerol (3%) and diphosphatidylglycerol (6%) of which 55% of the total lipid is unsaturated (Osborn *et al*,1972). However, the phospholipid composition particularly the fatty acid moiety, is affected by various factors including growth phase, rate and temperature (Cronan, 1968), by low pH and low oxygen tension (Cronan & Vagelos, 1972) and by high concentrations of sodium chloride, other salts or sucrose (McGarrity & Armstrong, 1975; 1981) if these are present in the growth medium (Ratledge & Wilkinson, 1988). The structures of the phospholipids are given in Fig. 7.

Fig. 7 : Structure of Phospholipids :

(a) <u>The polar head group</u> :

CH_2OCOR^1

R^2COOCH O

CH_2--P--OX

OH

(b) <u>The groups at X in (a)</u> :

Phosphatidylethanolamine --$CH_2CH(OH)CH_2PO_3H_2$

Phophatidylglycerol --$CH_2CH(OH)CH_2PO_3H$

Diphosphatidylglycerol --$CH_2CH_2NH_2$

Adopted from Ratledge & Wilkinson (1988). Groups R^1 and R^2 can be from C_{12} to C_{19} in length.

1.2.4. Interaction between Cytoplasmic and Outer Membrane

In spite of the presence of the peptidoglycan layer between the cytoplasmic and the outer membranes, zones of adhesion have been observed in electron micrographs of plasmolysed cells. These areas are called Bayer's junctions. As many as 200-400 of such sites are reported to be present per cell and these zones seem to play an important role in the transport of molecules from inside the cell to outside and vice versa. Bayer *et al* (1982) observed that bacteriophages MS2, T4 and T2 adsorbed at such sites contained no nucleic acid and concluded from this that these junctions were used by the phages to inject their DNA. On the other hand such sites may be formed as a result of phage attachment. The adhesion zones have also been proposed as sites for exporting LPS (Muhlradt *et al*, 1973) and porin proteins (Smit & Nikaido, 1978) from the cytoplasm to the outer membrane. From the transport of porins suggested above, it would seem that either a continuous series of adhesion zones would need to be formed so that the proteins would be placed at intervals in the outer membrane or that the exported proteins could diffuse to their insertion sites. The porins are found all over the cell surface and a simple lateral diffusion of porins through the fairly rigid structure of the outer membrane is difficult to visualise. Membrane vesicles carrying markers of the adhesion zones have been isolated (Bayer *et al*, 1982) and hence these adhesion zones may well be transient structures which form as a result of translocation of outer membrane components like LPS or porins through the cell envelope. This still leaves the peptidoglycan as a barrier, however, there are large pores in the peptidoglycan and it may be that it is only at such areas that the translocation of secretary components can take place.

1.2.5. Transport across the Outer Membrane :

Three pathways for transport across the outer membrane have been postulated. These are termed the hydrophilic, the hydrophobic and the self-promoted pathway.

(a) **The Hydrophilic Pathway :**

The existence of the hydrophilic pathway is now well established and the porins, the outer membrane channel-forming proteins, are the key components of this pathway (Benz *et al*, 1982; Nikaido & Nakae, 1979). A variety of factors influence the rate of entry of a hydrophilic compound through these channels.

The channel area of the individual porin pores and hence the size of the particles allowed through varies from organism to organism. *E.coli* allows the passage of trisaccharides (Nikaido & Nakae, 1979) and tetrapeptides (Payne & Gilvarg, 1968). Also, the rate of uptake by the various porin proteins varies depending upon the size of the porin channels and the selectivity with respect to the relative size, charge and hydrophobicity of the entering compound. OmpF, OmpC and PhoE porins of *E.coli* have channel diameters of 1.13nm as determined by liposome dilution studies and the sugar raffinose is the largest molecule capable of penetrating through these channels (Nakae, 1976).

The structures of the porin channels can also differ although they are all now believed to be formed from trimers of a single porin subunit (see Fig.4 ; Hancock, 1984). Most porins demonstrate ion and charge selectivity for different substrates but there does not seem to be much differetiation with respect to the chemical nature of the entering compounds (Nikaido & Rosenberg, 1983 ; Nikaido & Vaara, 1985).

There are charged amino acids present in the interior of the channels (Hancock *et al*, 1986 ; Nakae, 1986 ; Schlaeppi *et al*, 1985) and it is the number and position of these charges relative to the most constricted part of the channel that determines the ion selectivity of the channel (Hancock *et al*, 1986). With ion selectivity there is further differentiation as to whether the channel favours cations or anions. This differentiation is illustrated quite well by the specificity for the entry of hydrophilic antibiotics that is observed. OmpF channels favour the passage of zwitterionic over anionic beta-lactams, but this selectivity is found to be reversed for the PhoE channels (Nikaido *et al*, 1983). However, for small ions eg. K^+ and Cl^-, these selectivity differences are present but to a much

lesser extent although they do exist (Benz *et al*, 1985). The presence of the charged groups in the interior of the channels prevent hydrophobic compounds from entering (Nikaido & Rosenberg, 1983) even though they may be very small in size (Nikaido, 1976).

Lastly, the rate of entry of a compound depends on the number of avialable channels on the cell surface. The availability of channels depends on the expression of the porin proteins due either to genetic reasons eg. *E.coli* K12 encodes both OmpF and OmpC porins whereas *E.coli* B encodes only the OmpC porin ; or their regulation eg. in *E.coli* the expression of OmpF and OmpC porins is regulated by the osmolarity of the growth medium with the level of expression of one porin being higher than that of the other under specific environmental conditions. Porin channels are also voltage regulated, and the number of open channels as a proportion of the total channel-forming trimers present may also influence the rate of entry into the cell (Nikaido & Vaara, 1985).

(b) **The Hydrophobic Pathway** :

This is the pathway that allows hydrophobic compounds to penetrate the LPS of the outer membrane. This pathway is the least active one in most Gram-negative bacteria. Many lipid bilayers allow the penetration of hydrophobic and amphiphilic compounds through them (Sha'afi *et al*, 1971 ; Stein, 1967). However, the passage of such compounds is inhibited through *E.coli* and *Salmonella* outer membranes (Nikaido, 1976) and this corresponds to the resistance exhibited by these organisms to hydrophobic antibacterial agents such as actinomycin D, phenol and crystal violet, as well as to detergents and bile salts (Nikaido & Nakae, 1979). The difference between common lipid bilayers ie. the cytoplasmic membrane phospholipid bilayer, and the outer membranes of Gram-negative bacteria is the presence of the anionic LPS molecules dominating the outer leaflet of the lipid bilayer and this confers many unique properties on the outer membrane as compared to the phospholipid bilayer (see section on LPS). Resistance to the hydrophobic agents is mainly attributed to this LPS and the impermeability that it confers on the outer membrane

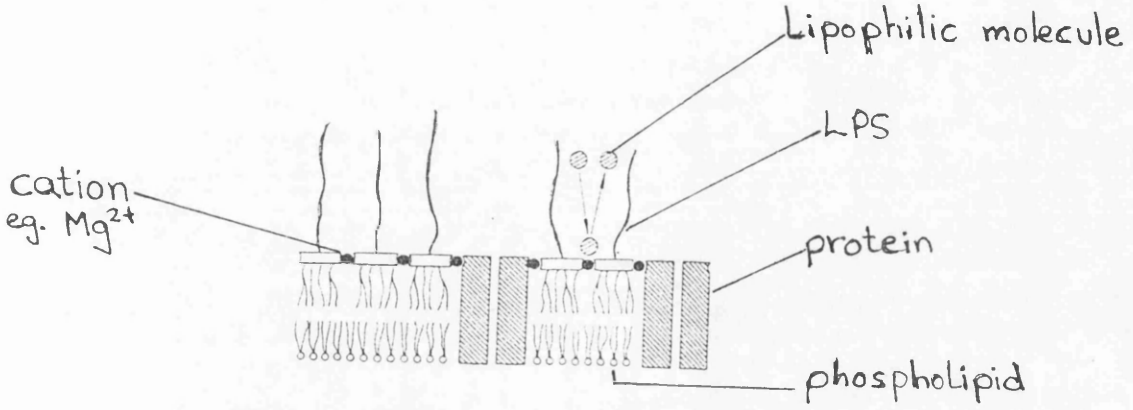
(Hancock, 1984). In support of this is the observed increase in sensitivity to hydrophobic antibacterials if LPS is altered by mutation (Nikaido, 1976), or when LPS is partially removed (Leive, 1974), or when polycations disrupt the LPS packing (Vaara & Vaara, 1983(b)). However, when divalent cations such as magnesium are added, then the increased sensitivity due to the above is reversed. When LPS is partially removed eg. by EDTA, then it is possible that local phospholipid bilayers do form and mimic a lipid bilayer such as the cytoplasmic membrane, and thereby enable the entry of hydrophobic agents by allowing them to partition through the outer membrane (Hancock, 1984). Thus the hydrophobic pathway is normally restricted but can be enhanced by chemical or genetic means.

(c) **The Self-promoted Uptake Pathway** :

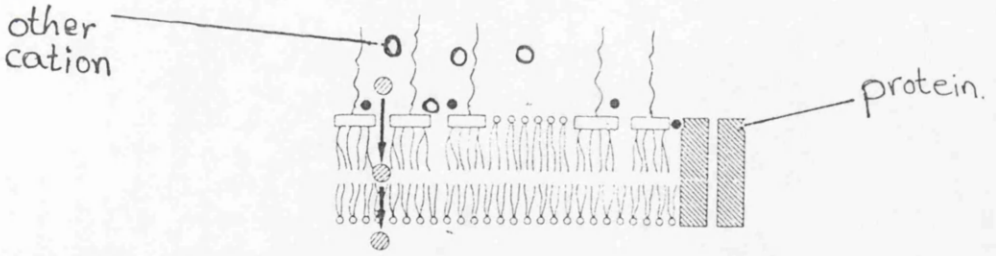
This pathway is postulated for the uptake of polycationic antibiotics like the polymyxins and aminoglycosides in *Pseudomonas aeruginosa* which contains a very impermeable outer membrane (Hancock *et al*, 1981). The proposal here is that these cationic compounds displace the magnesium ions from the LPS and consequently destroy the cross-bridging between LPS molecules, thereby destabilising the outer membrane and thus facilitate their own entry and that of other compounds such as lysozyme, beta-lactam antibiotics and hydrophobic fluorescent dyes (Loh *et al*, 1984). It is not only such cationic antibiotics that are capable of disrupting the outer membrane. In recent years, a wide range of such so called permeabilising agents have come to light and examples of these include EDTA, a non-toxic derivative of polymyxin B ie. polymyxin B nonapeptide (PMBN), certain polyamines, sodium hexametaphosphate, and LPS-binding proteins (Hancock, 1984) ; the mechanisms of action of these agents are discussed below.

Fig. 8 : Model of the self-promoted uptake pathway :

(a) Hypothetical structure of Outer Membrane :



(b) Displacement of Mg^{2+} by cation :



Physical displacement of Mg^{2+} leaves a gap through which other compounds can enter the outer membrane. Removal of Mg^{2+} can also result in repulsion between the charged headgroups of LPS molecules which can contribute to the formation of local disorganisation in the outer leaflet of the outer membrane.
Adapted from Nikaido & Vaara, 1985.

(i) **EDTA** : Ethylenediamine tetraacetic acid is a cation chelator and it is this property that is central to its outer membrane permeabilising effect (Leive, 1974), although except for *Pseudomonas aeruginosa* (Goldschmidt & Wyss, 1967), an organic cation (eg. Tris) and mild alkaline pH are also required. EDTA causes the release of a substantial amount of LPS from the outer membrane in *E.coli* both at 37°C and 4°C (Leive *et al*, 1968). On release of LPS, the outer membrane apparently remains continuous (Gilleland *et al*, 1974; Verkeleij *et al*, 1977) with no holes apparent. LPS released by EDTA appears to be of two types as separated by ultracentrifugation. Fraction F1 contains associated lipid and protein whereas fraction F2 is apparently pure LPS. Mutation analysis (Voll & Leive, 1970) and reconstitution studies suggest that F2 LPS has a greater role to play in the outer membrane permeability as release of F1 LPS (induced in EDTA resistant mutants) does not affect the permeability to a significant extent.

(ii) **Polymyxin B nonapeptide (PMBN) and other permeabilisers :**

PMBN is a nontoxic derivative of the peptide antibiotic polymyxin B. Removal of the fatty acyl chain from the polymyxin B molecule has rendered the antibiotic inactive (Chihara *et al*, 1973). However, the other properties associated with polymyxin B ie. disorganization of the outer membrane and its antiendotoxin activity, although reduced in potency, are still present in this derivative (Danner *et al*, 1989). Presence of PMBN in the medium sensitises *E.coli* to various hydrophobic antibiotics such as erythromycin, clindamycin, rifampicin, fusidic acid, novobiocin and cloxacillin, and also to complement but not to the hydrophilic antibiotic penicillin (Vaara & Vaara, 1983(a)).This antibiotic sensitising effect is only present with polymyxin B susceptible strains but not with polymyxin B resistant strains and so it seems that the PMBN acts at the same site as polymyxin B (Viljanen & Vaara, 1984).

Polylysine (lysine $_{20}$) also has an antibiotic sensitising effect but lysine$_4$, streptomycin, cytochrome *c*, lysozyme and the polyamines cadaverine and spermidine have

neither bactericidal nor sensitising activity (Vaara & Vaara, 1983(b)).

Two types of outer membrane disorganising actions seem to be used by the polycations. The polycationic agents protamine and $lysine_{20}$ release 20-30% of the LPS from the outer membrane and sensitise the bacteria to the anionic detergent SDS but do not make the bacteria sensitive to the hydrophobic probes actinomycin D and fusidic acid. On the other hand, PMBN does not induce the release of LPS or sensitize the bacteria to SDS but makes the outer membrane permeable to the hydrophobic probes. Neither protamine, $lysine_{20}$ or PMBN cause any leakage of periplasmic beta-lactamase. PMBN causes the appearance of long, narrow, finger-like projections on the outer membrane but protamine and $lysine_{20}$ cause only a distinctly wrinkled appearance of the outer membrane and no projections (Vaara & Vaara, 1983(c)). Hence, loosening the outer membrane structure and thereby increasing the overall surface area seems to be common to the action of both types of permeabilising agents.

Gram-negative bacteria which are susceptible to polymyxin B are found to bind PMBN in large amounts, but this binding is subject to competition by low concentrations of other polycations such as octapeptin, polylysine, protamine, and to a lower extent by spermine, calcium ions and magnesium ions. However, no competition was observed with streptomycin, tetralysine, spermidine or cadaverine (Vaara & Viljanen, 1985). This suggests that the PMBN binding site is a general binding site for cations but that only certain polycations had a high affinity for that site.

Moore *et al* (1986) used a fluorescent derivative ie. dansyl-polymyxin, to show that polymyxin B binds to the LPS and the lipid A region of the LPS in the outer membrane, including sites which bind magnesium ions. Many other polycations such as gentamicin, streptomycin, polymyxin B itself and magnesium ions were able to displace dansyl-polymyxin bound to LPS or to lipid A. However, the affinity for LPS of these latter polycations was substantially higher than that of magnesium ions.

Analysis of the relationship between the number of cationic charges

present on the compound and its ability to affect outer membrane permeability shows that cationic agents possessing two (cadaverine), three (spermidine & streptomycin), or four (spermine & tetralysine) net basic charges are totally inactive as outer membrane permeabilising agents, although the polyamines, streptomycin and tetralysine interact with isolated LPS. But five basic charges are sufficient to increase outer membrane permeability if they are in a favourable conformation as they apparently are in PMBN. If the basic charges are in a less proper conformation then more of them are needed and the sensitising effect to some antibiotics (eg. erythromycin and clindamycin) still remains poor (Vaara & Vaara, 1983(b)). Also, an interaction with LPS alone is not enough to increase outer membrane permeability. In fact, cations containing only a few basic charges can actually stabilize the outer membrane and thereby decrease its permeability, as do magnesium and calcium ions (Nikaido *et al*, 1977; Stan-Lotter *et al*, 1979). Spermine, spermidine and streptomycin have been described to stabilize lysozyme-induced *E.coli* spheroplasts against lysis in water, although the molecular mechanism of this stabilization is not known (Tabor, 1962).

At least two mammalian cell proteins are also known to bind LPS of Gram-negative bacteria. Lipopolysaccharide binding protein (LBP ; Tobias *et al*, 1986) and bactericidal/permeability increasing protein (BPI ; Ooi *et al*, 1987) are the first two members of such a protein family to be recognized. Both these proteins have the ability to bind to the lipid A region of LPS, display a high degree of amino-terminal sequence homology, and are immunologically cross-reactive. Rough strains of Gram-negative bacteria are more susceptible than are smooth strains to the permeability increasing action of BPI and like polymyxin B, BPI is inactive against Gram-positive cells (Elsbach *et al*, 1979). In addition, strains of Gram-negative bacteria resistant to polymyxin B, EDTA and aminoglycosides are also resistant to BPI (Finch & Brown, 1979). BPI permeabilizes outer membranes to actinomycin D and rifampicin but its action is antagonized by magnesium and calcium ions (Weiss *et al*, 1983). Permeability induction by BPI also requires the continuous presence of bound BPI as well as of at least two post-binding steps. The proposed mechanism of interaction of

this protein bears many similarities to the self-promoted uptake mechanism (Weiss *et al*, 1984). Other mammalian proteins containing a lipid A binding site, and therefore with potential to be members of this family, are cationic antimicrobial protein (CAP 37, CAP 57), lipid A degrading enzyme, Bactericidal factor, endotoxin inhibitor and LPS binding membrane protein (Tobias *et al*, 1988).

Sodium Hexametaphosphate (HMP) can also act as a permeabilizer of Gram-negative cell outer membrane. In *E.coli, Pseudomonas aeruginosa* and other *Pseudomonas* species HMP increases the outer membrane permeability to hydrophobic antibacterial agents such as rifampicin and fusidic acid as well as to detergents such as triton X100 and SDS (Vaara & Jaakkola, 1989). Polyphosphates are highly anionic condensation products of polyphosphoric acid and form chelate complexes with multivalent cations such as magnesium, calcium and manganese (Van Wazer & Callis, 1958 ; Van Wazer & Campanella, 1950). As antimicrobial agents, they are weak or very weak and mainly active against Gram-positive bacteria (Post *et al*, 1963) and are therefore not generally regarded as antimicrobial agents. The action of HMP as a permeabilizer is proposed to be similar to that by EDTA as its action is reversed by magnesium ions. HMP has also been reported to sensitize *E.coli* to glycerol monocaprate which is a common preservative (Tsutsumi *et al*, 1983). The ability of HMP to sensitize Gram-negative bacteria to hydrophobic compounds may well have some value in food microbiology. Polyphosphates are used in foods and in food processing for emulsification, moisture retention, leavening, sequestering of cations, buffering and improving tenderness (Davidson *et al*, 1982 ; Dziezak, 1982 ; Tompkin, 1983). They are not preservatives themselves, but several antimicrobial food additives or naturally occuring food antimicrobial agents (eg. monoglycerides, sugar esters of fatty acids and free fatty acids) are hydrophobic and therefore do not permeate effectively through the outer membrane.

Yet another group of compounds recently found to increase outer membrane permeability to antibiotics which are normally excluded from entering *Escherichia coli* outer

membranes are local anaesthetics such as procaine, tetracaine and dibucaine, phenothiazine tranquilizers eg. chlorpromazine and antimalarial agents such as quinine, Besides their specific therapeutic use, all these drugs have several common properties, such as blockage of action potential and modification of membrane fluidity (Seeman, 1972). Some of these anaesthetics have also been reported to have various effects on bacterial cells including an inhibitory effect on cell growth (Lazdunski *et al*, 1979; Pugsley *et al*, 1980). The antibiotic sensitising effect by the above anesthetics was found to be independent of firstly, the presence of OmpC porin, as the effect was given by *E.coli* B, a strain which lacks this porin; and secondly, of the nature of the hydrophilic saccharide of LPS molecules (Labedon, 1988).

1.3. Plasmid association of pathogenic E.coli :

A significantly high proportion of strains isolated from clinical samples have been found to contain one or more plasmids. Plasmids are extrachromosomal genetic elements capable of independent replication and stable coexistence within their host strain. Plasmids although not strictly essential for successful growth and metabolism, encode genes for some supplementary activities that allow their host strain to better survive in adverse environmental conditions or permit it to compete more successfully with microorganisms of the same or different species. Many plasmids are self-transmissible and commonly carry a range of other properties including resistance to antibiotics and other antibacterial agents. Plasmid-mediated resistance to antibiotics is usually due to some biochemical modification of the antibiotic eg. phosphorylation, acetylation, etc. by one or more plasmid encoded enzymes before or after antibiotic entry into the cell. Many plasmids also encode proteinaceous toxins which are called bacteriocins. Bacteriocins are produced only by certain strains of bacteria and are lethal only to other closely related strains of the same species (Hardy, 1975). These are mainly produced by Gram-negative bacteria but some Gram-positive bacteria have also been reported to produce them

eg. *Staphylococcus* and *Streptococcus spp.* which produce staphylococcins and streptococcins respectively (Reeves, 1972). The colicins, the most studied group of bacteriocins, are produced by some strains of *E.coli* and *Shigella sonnei.*

The plasmids encoding colicin genes can be divided into two groups according to their molecular weights (Hardy, 1978). Group I plasmids are small in size with an average molecular weight of 5×10^6, and are nonconjugative but can be transferred into other bacteria if present in the same cell with other self-transmissible plasmid(s). These show relaxed mode of replication control which means that they can be present in multicopies eg. 30-50 copies per chromosome equivalent. ColE, ColK and ColA plasmids are examples of this group. Group II plasmids are large, with molecular weights between $40\text{-}100 \times 10^6$, usually conjugative and exhibit stringent replication control such that at any time there are only 1-2 copies of the plasmid genome present in the cell per chromosome equivalent. ColB, ColI and ColV plasmids are examples of this group.

1.4. Colicin V producing Plasmids :

These are large, conjugative plasmids and confer the production of colicin V and immunity to colicin V. As mentioned above, these plasmids have been implicated as the causative factors in serious disorders in man and animals eg. meningitis, septiceamia,etc. One well-studied Col V plasmid is ColV,Ia-K94 which also encodes the production of colicin Ia.

1.4.1. Genome of plasmid ColV,Ia-K94 :

Plasmid ColV,Ia-K94 is 93-95 MDa in size and parts of it are very similar to the well-characterised sex factor F. Both these plasmids belong to the incompatibility group Inc FI (Kahn & Helinski, 1964 ; MacFarren & Clowes, 1967)

1.4.2. Incompatibility of plasmid ColV,Ia-K94 :

Incompatibility is a plasmid-encoded phenomenon which stops two plasmids of the same incompatibility group from coexisting in the same cell (reviewed in Novick & Hoppenstead, 1978).

This is proposed to result from these plasmids having similar replication systems and so if present together then one may prevent the replication of the other. Factor F and ColV,Ia-K94 are unable to coexist in the same cell ; ColV,Ia-K94 is always preferred, unless the F plasmid is present as an Hfr plasmid in which case they both stay as ColV,Ia-K94 switches to a different replicon, and hence their replication systems are presumably very similar.

ColV,Ia-K94 is a chimeric plasmid possessing interesting genetic features of IncFII group as well as IncFI F-like plasmids. It appears to be a cointegrate plasmid containing Rep1, which is homologous to the RepA replicon of IncFII plasmids (Weber *et al*, 1984), and Rep2, which is homologous to the secondary replicon of F and other IncFI plasmids (Mitra & Palchaudhuri, 1984). The advantage of being able to express the IncFII replicon is that it enables ColV,Ia-K94 to exist in a cell at the same time as an IncFI plasmid and not be affected by the replication regulatory mechanisms of the IncFI plasmid.

1.4.3. Structure of ColV,IaK94 :

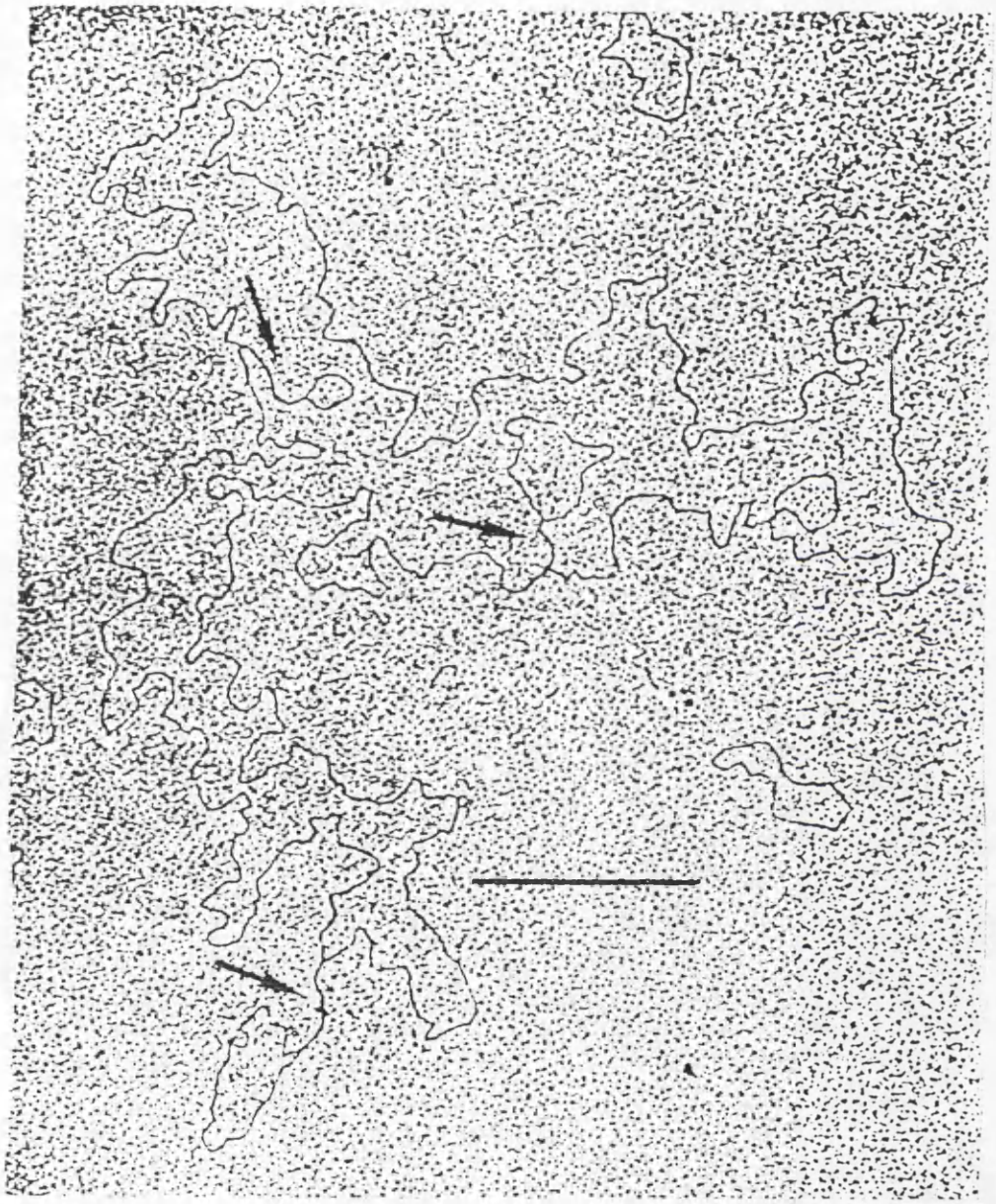
Fig. 9 shows an electron micrograph of single strand of ColV,Ia-K94 (Sharp *et al*, 1973). In the cell, this plasmid exists as a double stranded covalently-closed circular molecule, except when it is replicating or being transferred to another cell by conjugation.

Heteroduplex studies carried out between F and ColV,Ia-K94 have shown extensive DNA sequence homology especially in the transfer operon region. This region is responsible for efficient and successful conjugational transfer of such plasmids. The region of homology extends over at least 45 kb of the F genome (Sharp *et al*, 1973).

1.4.4. Conjugation system of ColV,Ia-K94 :

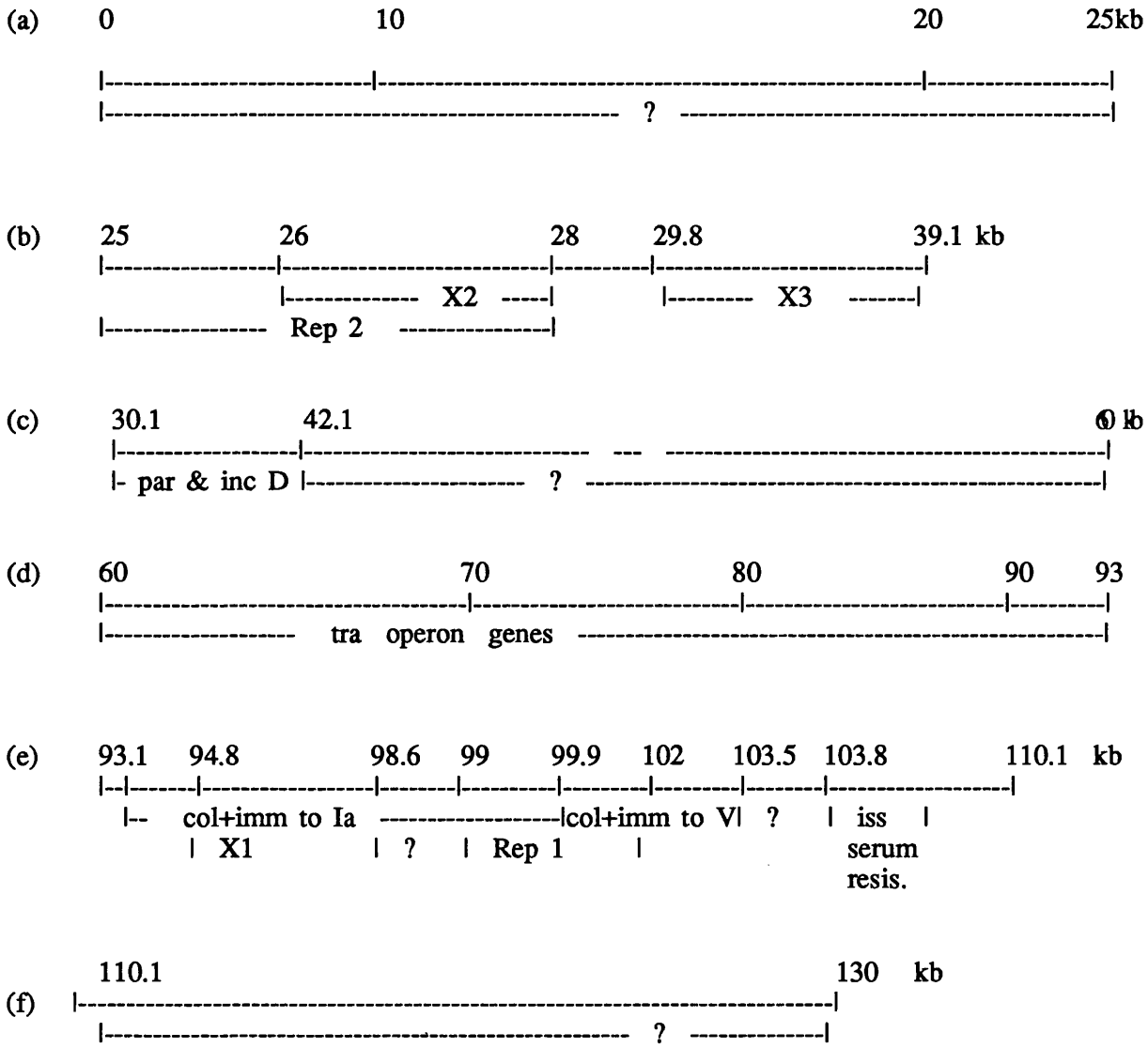
The conjugation system of F, to which that of ColV,Ia-K94 is similar (Sharp *et al*, 1973), presents a successful form of evolution. Such conjugation systems exhibit three common features : firstly, an extracellular filamentous organelle called a pilus is essential for recognition of and mating pair formation with a potential recipient cell (Achtman,

Fig. 9 : Electron micrograph of single stranded ColV, Ia-K94 DNA



Adopted from Sharp et al (1973)
Bar represents 1 μm.

Fig. 10 : Sectional Map of ColV,Ia-K94



This map is not drawn to scale. Compiled from the coordinates from Fig. 11 and the references cited in Fig. 11.

Unmapped regions : 0 - 25 kb
28 - 29.8 kb
42.1 - 60 kb
103.5 - 103.8 kb
110.1 - 130 kb

Fig. 11 : This figure shows the corresponding genetic and physical coordinates of the map of plasmid ColVIa-K94.

Weber & Palchaudhuri (1982, 1984 & 1985) :

Rep 2	25	- 28	kb
X2	26	- 28	kb
X3	29.8	- 39.1	kb
par & inc D	39.1	- 42.1	kb
tra genes	60	- 93	kb
X1	94.8	- 98.6	kb
Rep 1	99	- 102	kb

Binns et al, 1979 :

colicin Ia + immunity to it	93.1	- 99.9	kb
colicin V + immunity to it	99.9	- 103.3	kb
serum resistance *iss* genes	103.8	- 110.1	kb

1975); secondly, a special system for conjugal replication and transfer of DNA is synthesized ; and thirdly, "surface exclusion" proteins in the cell envelope (Achtman *et al*, 1977) prevent the cell from being used as recipient by another donor cell carrying the same or a closely related plasmid. The importance and complexity of conjugation are emphasized by the length of DNA devoted to this (Skurray *et al*, 1978) about one third of the whole F plasmid molecule and about tenfold more than which is required for replication and incompatibility functions.

A brief model of the conjugation process is as follows : firstly, the pilus allows the donor cells to recognize and bind the recipient cell (Ou, 1973) and ultimately the two form a shear-resistant mating pair (Achtman, 1978). Formation of the mating pair triggers conjugal DNA metabolism whereby beginning at a defined site (*ori* T), a specific plasmid strand is transferred to the recipient cell (Rupp & Ihler, 1968). Prior to triggering, a plasmid-determined *ori* T-specific endonuclease-ligase catalyzes the inter-conversion of covalently-closed circular and nicked open circular forms of the plasmid. The transferred strand is replaced in the donor by *de novo* DNA synthesis and its complement is synthesized in the recipient cell. Recircularisation in both cells then gives the plasmid configuration that can be inherited and expressed. The donor and recipient cells can now disaggregate, the expression of the transfer genes in the new cell rapidly convert it to a donor cell that can join its donor cell in reentering the cycle.

1.4.5. The Transfer Operon :

Genetic analysis of the transfer genes has been carried out byisolating and studying *tra* mutants (Achtman et al 1971). The nineteen known transfer genes can be divided into four groups (Miki *et al*, 1978; Achtman *et al*, 1980). The first group includes *tra* A, L, E, K, B, V, W, C, U, F, H and G which are directly required for pilus formation, as well as for infection by F-specific phages. The second group contains *tra* N and *tra* G required for stabilisation of mating pairs and the third includes *tra* M, Y, G, D, I and Z which are

concerned with conjugal DNA metabolism. The sole gene in the fourth group, *tra* J, controls the expression of most if not all of the other transfer genes. There are two other genes, *tra* S and *tra* T, both of which are directly responsible for surface exclusion although not required for transfer as such (Achtman *et al*,1980). The surface exclusion system of ColV,Ia-K94 is distinct from that of factor F (Willets & Maule, 1973). Table 3 shows the *tra* proteins and their molecular weights.

Many of the transfer components are expressed in the outer membrane. This must alter the relative proportions of the normally found outer membrane proteins of plasmid-less cells or if these stay constant then it must be the other components ie. the phospholipids in the inner leaflet or the LPS in the outer leaflet of the outer membrane that alter in quantity if not in quality.

1.4.6. Other Genes of ColV,Ia-K94 :

ColV,Ia-K94 contains three regions on its genetic map called X_1, X_2 and X_3 which are believed to be IS-like elements. X_1 has been mapped at 94.8-98.6 kb, X_2 at 26-28 kb and X_3 at 29.8-39.1 kb. X_1 has been associated with multimerisation of the plasmid copies. This causes plasmid instability as the multimers are less-stably inherited. Thus X_1 seems to be a hot-spot for enhanced recombination when cloned on a separate vector plasmid (Weber & Palchaudhuri, 1986 (b)). However, the plasmid ColV,Ia-K94 is very stable, in fact it is quite difficult to cure and it is a very low copy number plasmid. Therefore, it seems that X_1 must either be under very tight regulation or that it overlaps with some other continuously transcribed gene(s) which may be in a different reading frame such that X_1 is usually inactive.

X_2 and X_3 are located nearer to each other than either is to X_1. X_2 contains the serum resistance and virulence determinant *iss* (for increased survival in serum) of ColV,Ia-K94 (Chuba *et al*, 1986). However, X_3 resembles the aerobactin iron-uptake region of plasmid ColV-K30 in its organisation but ColV,Ia-K94 lacks the ability to synthesise

Table 3 : Products of the F-transfer region

Products of known function		Products of unknown function	
Product	ca. Mol. weight (kDa)	Product	ca. Mol. weight (kDa)
Tra M	13-14	Tra P	21.5-23.5
Tra J	23.5-27	Tra R	9
Tra Y	16	Trb A	14.2
Tra A	13.7-14	Trb B	18.4
Tra L	11	Trb C	21.5
Tra E	19-22	Trb D	23.5
Tra K	24-24.5	Trb E	10
Tra B	55-64		
Tra V	21		
Tra C	85-98		
Tra W	23		
Tra U	20		
Tra N	66		
Tra F	25-26		
Tra Q	12.5		
Tra H	39-45		
Tra G	100-116		
Tra S	18		
Tra T	24-28		
Tra D	77-90		
Tra I	174-180	Tra I*	78-94

Adpted from Ippen-Ihler & Minkley (1986)

either the aerobactin siderophore or its outer membrane receptor (Weber & Palchaudhuri, 1986 (a)).

Like the F factor, ColV,Ia-K94 is derepressed ie. its conjugational functions are fully expressed all the time. Many other F plasmids eg. R100, are repressed for transfer properties and if such a plasmid is present in a cell with ColV,Ia-K94 then the latter becomes repressed. Thus ColV,Ia-K94 seems to have a functional operator site ie. fertility inhibition operator region (*tra* O). *tra* O has been mapped at 93.6-95.3 kb on the ColV,Ia-K94 genome. This is very close to the X_1 region or may even overlap it.

ColV,Ia-K94 is a very versatile plasmid in that it can undergo various rearrangements in structure via loop formation of adjacent inverted repeat sequences (Davies *et al*, 1982). Such rearrangements may sometimes result in increased copy number of certain genes eg. colicin V genes, so that its production is increased in response to the gene dosage (Mitra & Palchaudhuri, 1984). Whether such rearrangements are under some regulation or influenced in response to some external stimuli eg. nutrient deficiency, levels of cAMP in the cell, high/low growth temperature or pH of the medium, in response to toxins present in the medium either environmental or those produced by other microorganisms, remains as yet a topic of speculation.

1.5. Properties of Col V Plasmids :

1.5.1. Colicin Production by Col V plasmids :

Colicin V production (and immunity to it) is only one of many properties associated with ColV plasmids. Many Col V plasmids also encode production of and immunity to other colicin(s).

Colicin V is a small, proteinaceous toxin whose target site is the cytoplasmic membrane where it is thought to prevent the formation of the membrane potential and thereby interferes with energy generation. Colicin V differs from other colicins in its small size and by its constitutive synthesis rather than SOS-inducible synthesis as

is observed with other colicins. Some colicins require cell lysis for their release from the cell, however, colicin V does not use this mode of release.

The genes for production of and immunity to colicin V have been mapped on a 4.4 kb DNA fragment on the plasmid ColV-K30. Genes *cva A* and *cva* B are responsible for the export of colicin V to the outside of the cell and *cva* C is the structural gene for colicin V. Gene *cva* A (850-1450 bp) encodes two proteins of 27 kDa and 43 kDa in size as observed from expression in a minicell system. These two proteins may be the products of two overlapping genes or it may be that the *cva* A gene product exists as a modified dimer that is in equilibrium with the monomer form. No gene product was found associated with *cva* B gene which is 1900-2500 bp in size. The *cva* C gene (150-1050 bp) product is 6 kDa in size but the molecular weight of colicin V has been reported to be 4 kDa therefore, it seems that colicin V undergoes some modification or that its leader peptide which helps it to cross the cytoplasmic membrane is synthesized from the gene itself. The colicin V immunity protein is coded for by the *cvi* gene (700 bp) and is 7 kDa in size (Gilson *et al*,1987). There have been suggestions that colicin V should be classified as a microcin because of its small size and noninducibility by DNA-damaging agents, as these are also low molecular weight antibiotic substances produced by many species of *Enterobacteriaceae.*

Plasmid ColV,Ia-K94 also encodes the colicin Ia and its immunity component in addition to colicin V. Colicin Ia (626 amino acids, 69418 Da) acts by depolarising the energy-transducing cytoplasmic membrane by forming aqueous channels across it. Production of colicin Ia is regulated by the SOS system of DNA repair and by catabolite repression. The C-terminal region of the colicin Ia protein has many hydrophobic amino acid residues. The immunity to colicin Ia protein (111 amino acids, 13824 Da) is large compared to the immunity protein of colicin V, but both are located in the cytoplasmic membrane. One proposal for the mechanism of immunity is that the colicin Ia immunity protein which is

hydrophobic itself sits in the cytoplasmic membrane and interacts with the hydrophobic C-terminal of the colicin Ia protein molecule to prevent it from entering the lipidic cytoplasmic membrane and thereafter forming ion-permeable channels (Mankovich et *al*, 1986).

1.5.2. Col V plasmids and Adhesion :

One property of the bacterial intestinal pathogens of many species and also associated with Col V plasmids is the capacity to adhere to gut epithelial surfaces (Clancy & Savage, 1981). This property usually correlates with the presence of pili or fimbriae (Ottow, 1975) and is assayed by the phenomenon of agglutination, whereby bacterial cells adhere to more than one mammalian erythrocyte simultaneously and thus cause clumping of the erythrocytes. Pili are filamentous structures on the bacterial cell and are composed of a single subunit of protein pilin. Various types of pili have been found to be associated with uropathogenic *E.coli* (Uhlin *et al*, 1985(a))

Common (Type 1) pili are produced by *E.coli* and cause adhesion to monkey kidney cells and to mucus, via mannose-containing receptors (Orskov *et al*, 1980). Hence they are said to show a mannose-sensitive haemagglutination pattern .

Enterotoxigenic *E.coli* from humans and domestic animals often possess fimbrial adhesins such as K88 (in piglets), K99 (in calves) and CFAs I and II (in humans ; Gaastra & Graaf, 1982). These cause mannose-resistant haemagglutination, probably mediated by other receptors eg. glycolipid and/or glycoprotein. Adhesins may be chromosomally encoded but on a separate gene from that for pilin (Uhlin *et al*, 1985(b) ; Minion *et al*, 1986) or they may be plasmid-encoded as K88 (Mooi et al, 1979) and K99 are.

Expression of the conjugational transfer properties of ColV,Ia-K94 appears to be necessary for the above mentioned adherence mediated by the plasmid. This observation is in agreement with the fact that adherence is influenced by the growth temperature of Col V strains as is the expression of the transfer operon genes of this plasmid.

Attachment encoded by Col V not only has direct medical significance, but may also be a factor in environmental survival of *E.coli*. Thus, studies on attached organisms revealed that they are comparatively more resistant to chlorine (Hicks & Rowbury, 1986) and acrylic acid (Hicks & Rowbury, 1987(a)), and also to killing by phages T4, TuIa and K3 (Hicks & Rowbury, 1987(b)). The mechanism of action of these nonspecific resistances to the various agents is not known but the presence of an outer layer of cells in a clump could well protect the cells in the interior of the clump by just forming a physical barrier between the underlying cells and the inhibitory agent. However, the advantage of resistance to environmental polluting agents such as acrylic acid or disinfectants such as chlorine, and bacteriophages which are abundant in nature eg. in the alimentary canal or sewage, can be easily visualised as providing a selective advantage to the organism during unfavourable conditions allowing it to remain viable and then to flourish when exposed to more favourable conditions.

1.5.3. Iron-uptake system of Col V plasmids :

Iron is abundant in nature but its availability is reduced as most of it is complexed eg. with tranferrin in blood, lactoferrin in milk, or is insoluble at biological pH. Enteric bacteria are known to secrete the siderophore enterobactin (enterochelin) which binds iron and the combined complex is then actively transported into the cell via specific receptors.

Enhanced virulence by invasive strains of *E.coli* carrying the Col V plasmids has been shown to be due to the ability of the plasmids to sequester iron under iron-limiting conditions. This ability to obtain iron was independent of the presence of transferrin in the medium (Williams, 1979). Plasmid Col V-K30 in which the iron-uptake system is well-defined, encodes another siderophore which is chemically different from the catechol enterobactin. This is a hydroxamate siderophore called aerobactin. The iron-regulated aerobactin operon of ColV-K30 is 8.3 kb in size and encode five genes : *iuc*ABCD (iron-uptake chelator) and *iut*A (iron-uptake transport). Gene *iuc* A encodes a 6.3 kDa protein

which is the aerobactin synthetase, *iuc* B encodes a 33 kDa acetylase, *iuc* C encodes a 62 kDa protein involved in the last step of aerobactin synthesis (Lorenzo & Neilands, 1986) and *iuc* D encodes a 53 kDa oxygenase enzyme. *The iut* A gene product is 74 kDa in size and is known to be the receptor for ferric-aerobactin complex (Grewal *et al*, 1982; Lorenzo *et al*, 1988).

Many invasive strains of *E.coli* encode the aerobactin siderophore in addition to enterobactin. Catechol siderophores eg. enterobactin, are in general chemically and enzymically less stable than hydroxamates ; in addition they are less soluble and their iron complexes are very pH dependent. Also, the biosynthesis of enterobactin and its hydrolytic cleavage to release complexed iron within the cell consumes much energy as compared to aerobactin complexes. Furthermore, catechols are antigenic and thus render the cells expressing them and taking them up sensitive to the host immune system during infection. Hence, the presence of genes encoding aerobactin is positively advantageous to the cell and thus enhances their virulence (Warner et *a*l, 1981).

Plasmid ColV,Ia-K94 has been studied in great detail and the properties that follow relate to this plasmid although many of them have also been observed in other ColV plasmids.

1.5.4. VmpAp : Col V encoded Major Outer Membrane Protein

Plasmid ColV,Ia-K94 has been found to encode a major outer membrane protein, VmpAp, which is similar to another outer membrane protein OmpAp although these two are quite distinct. VmpAp has an apparent molecular weight of ca. 33 kDa and runs at approximately the same position as OmpAp on SDS polyacrylamide gels. It is trypsin sensitive but not murein-associated just as OmpAp. However, VmpAp cannot replace OmpAp as a functional receptor for phages K3 and TuII*, for colicin L or in efficient conjugation with F-like plasmids. VmpAp is not a transfer component nor is it dependent on colicin V production or immunity to colicin V (Moores & Rowbury,1982).

Production of VmpAp is related to growth temperature of cells containing ColV,Ia-K94, the least amount of VmpAp expression being at 25°C higher at 30°C and even more at 44°C (Reakes *et al*, 1988).

ColV,Ia-K94 containing cells were found to reverse some effects of OmpAp mutants and it is suggested that it is the VmpAp component of this plasmid that is involved in suppressing the lesions eg. an OmpAp mutant was unable to grow at 44°C in minimal salts medium whilst the ColV$^+$ derivative grew (Deeney *et al*, 1986). In a second case, three types of *ompA* mutants had become sensitive to aminoglycosides such as gentamicin, kanamycin and amikacin, and to certain other antibiotics like polymyxin B and spectinomycin as well as to agents such as EDTA, protamine and polylysine. However, introduction of the plasmid ColV,Ia-K94 was found to reverse these phenotypes to those present in normal ompA$^+$ cells (Reakes *et al*, 1988). Both these effects were found to be independent of transfer and colicin components which leaves VmpAp as another known component which may play a role in outer membrane permeability.

1.5.5. Phage resistance caused by Col V plasmids :

Presence of the plasmid ColV,Ia-K94 leads to resistance to phages Me1 and P1. However, the mechanisms of resistance seem to be different in each case. With Me1, the phage attachment is normal but infection and consequent reproduction is inhibited (Reakes *et al*, 1987) whereas with P1 there is greatly reduced phage attachment at the cell-surface of the plasmid containing strain (Goodson & Rowbury, 1987). The receptor for P1 attachment is LPS (Franklin, 1969) and it may therefore be that ColV,Ia-K94 alters the LPS such that it fails to function as an active receptor. Appparently both transfer and colicin components are involved in mediating this resistance to P1 attachment.

1.5.6. Serum resistance conferred by Col V plasmids :

ColV,Ia-K94 encodes a 5300 kbp fragment which is responsible for conferring the serum resistance phenotype on its host. This phenotype has been linked to two separate genes, 50 kb apart on the plasmid genome : the *iss* determinant and *tra* T gene

product (25 kDa in size) which is expressed on the outer membrane (Chuba et *al*, 1986). Colicin V and immunity to colicin V components are not thought to be involved in serum resistance although the genes encoding serum resistance (*iss*) and those encoding colicin production and immunity are located very near to each other (Binns *et al*, 1979)

1.5.7. Col V plasmids and sensitivity to Environmental Agents

ColV,Ia-K94 confers some properties that are detrimental to its host strain. Increased sensitivity to low pH (Cooper & Rowbury, 1986) and high temperatures (Abu Ghazalah *et al*, 1989) are linked with the presence of ColV,Ia-K94. Also increased sensitivity to detergents like deoxycholate and to the lytic action of lysozyme are associated with the presence of this plasmid (De Pacheco *et al*, 1985).

1.5.8. Col V plasmids and sensitivity to Antibiotics :

ColV plasmids are associated with high sensitivity to hydrophobic antibiotics eg. erythromycin (Alfa *et al*, 1987), rifampicin and novobiocin (Davies *et al*, 1986) sensitivities have been associated with this plasmid. No specific component related to the plasmid has yet been identified as responsible for the above mentioned properties except that it must act at the outer membrane of the plasmid containing cell. Another observation relating to the increased hydrophobic antibiotic sensitivity is that the presence of magnesium ions reverses the antibiotic activity as if magnesium ions were strengthening the outer membrane.

1.6. Col V plasmids and Virulence :

Enteroinvasive strains of E.coli are more likely to be haemolytic, produce colicin V, haemagglutinate human red blood cells in presence of D-mannose and kill 13-day old chick embryos. Most of these properties are known to be plasmid-mediated (Elwell & Shipley, 1980). Colicin V producing plasmids (ColVs) have been very extensively studied in recent years in relation to virulence.

Smith (1974) found that 78 % of E.coli strains responsible for generalised

infections in livestocks (calves, chicks and lambs) produced colicin V. Elimination of the ColV plasmid from the infective strain reduced its virulence when tested on experimental animals but virulence was restored upon reintroduction of the ColV plasmid into the same strain. In another study, strains isolated from the blood of septicaemic patients and from urines of patients with urinary tract infections and both their faeces, were tested for colicin production; 31.6 % of strains isolated from blood and 26.2 % of strains isolated from urine samples were found to produce colicin V as compared to 13.6 % of strains isolated from faeces. Hence, the proportion of entero-invasive strains producing colicin V was significantly higher ie. at least twice the proportion of faecal isolates. 63.6 % of the colicin V producing strains also produced at least one other type of colicin (Davies et al, 1981). The colicin V producing phenotype therefore seems to be very closely associated with virulent *E.coli* strains.

1.7. Transposons :

Transposons are defined genetic entities which are capable of inserting themselves as discrete and non-permuted DNA segments at many different sites in procaryotic genomes. Transposons are normal constituents of most bacterial genomes and of many extrachromosomal plasmids and phages. They can alter both the organisation and expression of procaryotic genomes at frequencies comparable to or greater than spontaneous mutation rates.

Transposons in bacteria are widespread, diverse and highly evolved in their mechanism of insertion and its regulation. Two types of evolutionary explanations for the existence of such elements are being debated : (i) these may have evolved as nature's tools for genetic engineering. Their ability to rearrange other DNA sequences would thus be a directly selectable phenotype which could lead to the increased survival of either individual replicons, organisms or a population of organisms harbouring such elements ; (ii) The discovery of transposition as a fundamemtally replicative process has also lead to the suggestion that the existence of transposons is only due to their ability to overreplicate and move as otherwise the normal

DNA replication mechanisms would eliminate DNA sequences for which no direct phenotype selection exists.

The transposons used in this work are Tn1 and Tn7. Tn1 is a TnA group transposon and was derived originally from plasmid RP4. Tn1 carries resistance to the antibiotic ampicillin and is 5 kb in length with 5 bp terminal repeat sequences at each end (Kleckner, 1981). Tn7 is a much larger element, 14 kb in length with a 5 bp terminal repeat and was originally derived from the plasmid R483 (Lichenstein & Brenner, 1982). Tn7 (reviewed by Kleckner, 1981) carries resistance to the antibiotics trimethoprim, streptomycin and spectinomycin.

Most transposons promote rearrangements at a frequency of 10^{-4}-10^{-8} per generation. These low frequencies are probably due to a combination of stringent regulation, inefficient translation /transcription signals or inefficiencies in the transposition process itself in the host strain.

1.8. Antibacterial Agents : Structure and mode of action :

1.8.1. Erythromycin :

Erythromycin belongs to a group of antibiotics called Macrolides. These contain a large lactone ring (12-16 atoms) having a few double bonds and no nitrogen atoms. This ring is substituted with one or more sugar residues, some of which may be amino-sugar. Classification of the antibiotics of this group is based on the nature and the relative arrangement of the sugar moieties and removal of one or more of the normal sugar(s) results in altered activity of the agent. These agents are inhibitors of bacterial protein synthesis both *in vivo* and *in vitro*. *In vitro*, macrolides are found to be bacteriostatic although at high concentrations they may become bactericidal.

Erythromycin binds to the 50S ribosomal subunits in the presence of magnesium and potassium ions. Binding with the 50S subunit is in a 1:1 stoichiometry over a wide range of concentrations but multiple binding sites on both the ribosomal subunits have

been observed at very high concentrations. Erythromycin has been found to bind ribosomes *in vivo* and *in vitro* but not polyribosomes. The exact mechanism of action of erythromycin is not known but it is not involved in the ribosomal GTPase reactions, does not inhibit the binding of aminoacyl-tRNA to ribosomes, with or without the elongation factor EF-Tu and nor does it affect the peptide bond formation between amino acids catalysed by the enzyme peptidyl transferase. Translocation is the other process which may be the target of this antibiotic, but they probably stimulate the dissociation of the peptidyl-tRNA from the ribosome. Fig. 12 shows the structure of erythromycin.

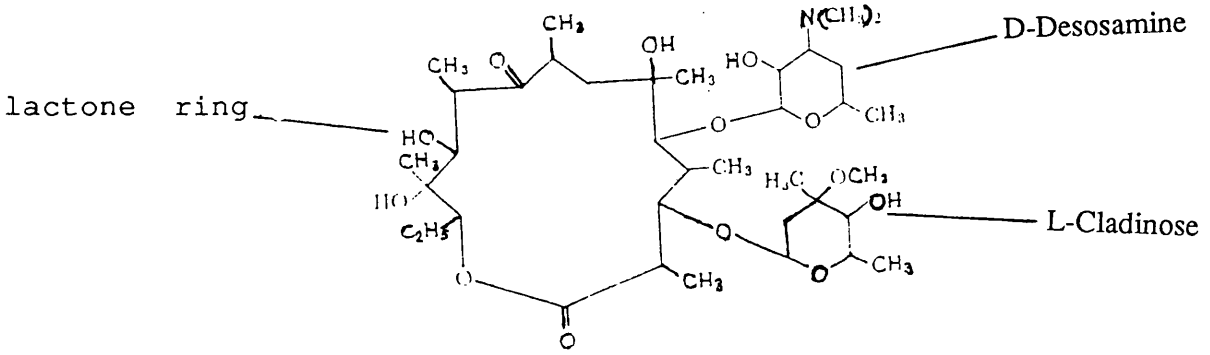
1.8.2. Novobiocin :

Novobiocin is a coumerin antibiotic, hydrophobic in nature and is made of three different chemical groups : a sugar noviose, a coumerin residue and a benzoic group at the end. The subgroup with the sugar and coumerin residues is called novenamine and that containing the coumerin and the benzoic acid is called novobiocic acid (Reusser & Dolak, 1986). Novobiocin itself is a dibasic acid and forms acidic as well as neutral salts with sodium, disodium, calcium and amines (Hoeksema et al, 1956).

Gram-negative bacteria are not very sensitive to novobiocin and this is believed to be as a result of its lack of entry into the cell via the outer membrane. However, the site of action for novobiocin is the enzyme DNA gyrase, and specifically its B subunit. Novobiocin interferes with the DNA-replicative functions of the gyrase and also acts on transcription, especially on those genes whose operons are sensitive to catabolic-repression (Gomez-Eichelmann, 1981). Studies done with toluene-treated cells (to make them permeable), have shown that the minimum part of the novobiocin molecule required to give the same amount of gyrase inhibition is novenamine. However, with intact cells novenamine is not active and this suggests that the benzoic acid moeity of the antibiotic is needed to facilitate its entry through the outer and cytoplasmic membranes (Reusser & Dolak, 1986).

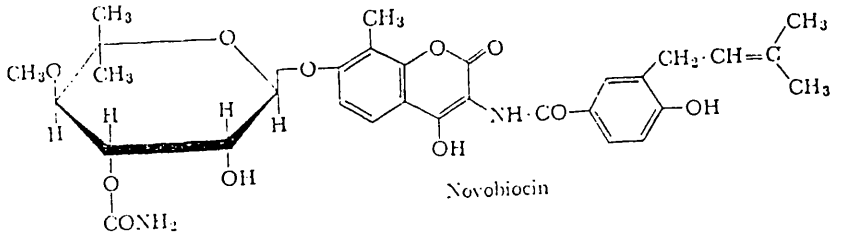
Fig. 12 : Molecular Structures of Erythomycin & Novobiocin

(a) Erythromycin



Adopted from Gale et al (1982).

(b) Novobiocin :



Noviose

Novenamine

Novobiocic acid

Adopted from Reusser & Dolak (1986).

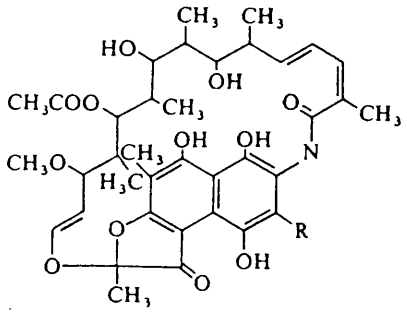
1.8.3. Rifamycins :

Rifamycins are a family of antibiotics produced by *Streptomyces mediterranei*. Rifampicin is a derivative of rifamycin SV. These antibiotics are large, hydrophobic and not very active versus *E.coli* and some other Gram-negative bacteria but rifampicin markedly affects many Gram-positive bacteria and is very effectively used to treat tuberculosis. Rifampicin acts on the DNA-dependent-RNA polymerase and thereby inhibits transcription (Hartmann et *al*, 1967 ; Riva & Silvestri, 1972). Studies on the mechanism of action of rifampicin indicate that it interrupts transcription by preventing elongation of the new RNA chain past a few nucleotides (Johnston & McClure, 1976), probably by binding in the vicinity of the substrate binding site (Jin & Gross, 1988). Entry of rifamycins into *E.coli* is believed to be via the very inefficient hydrophobic pathway; however, a new semi-synthetic derivative CGP4832 has recently been shown to enter via the *Fhu*A-*Ton*B dependent uptake route which is normally used by the ferrichrome complex in the outer membrane. This derivative apparently also competes with ferrichrome and other iron-siderophore complexes for entry into the cell (Pugsley *et al*, 1987).

1.8.4. Gentamicin :

This is a aminoglycoside antibiotic produced by *Micromonospora purpurea* and was isolated in 1963. It is the most active of the aminoglycoside antibiotics and the naturally produced form contains several components such as C_1, C_{1a} and C_2. It has a broad spectrum of antibacterial activity (Garrod *et al*, 1973). Aminoglycosides inhibit protein synthesis and cause significant increase in mRNA mis-reading, they are also known to inhibit ribosomal translocation and to stabilize EFG-GDP-ribosome complexes. They bind to 30S subunits which could lead to mRNA misreading and their binding to the 50S subunit could cause stabilization of the EFG-GDP-ribosome complexes ; inhibition of translocation then could be due to a simultaneous fixation of the antibiotic on both subunits (Campuzano *et al*, 1979). Gentamicin acts cooperatively with ribosomes from a sensitive *E.coli* strain in a multiphasic way with several classes of sites on the ribosomes. Also, this interaction is highly dependent

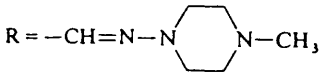
Fig. 13 : Molecular structures of Rifamycins :



(a) Rifamycin SV

R = H

(b) Rifampicin



on the concentrations of magnesium ions and on various endogenous polyamines which are found closely associated with ribosomes (Moukadden *et al*, 1986). Gentamicin has recently been shown to inhibit LPS synthesis in the cell (Cashell & Rudd, 1987) but does not induce the LPS release that some other aminoglycosides do after affecting the strigently controlled regulation of LPS synthesis in the cell (Kusser & Ishiguro, 1988). Gentamicin can enter the *E.coli* cell via the porin pathway quite efficiently (Nakae & Nakae, 1982) but it has also been known to disrupt the outer membrane and thus may also use the self-promoted pathway of entry into the cell (Martin & Beveridge, 1986 ; Hancock, 1984).

1.8.5. Polymyxin B :

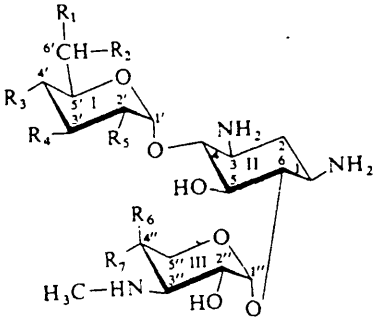
Polymyxin B is a peptide antibiotic with a broad spectrum of activity against Gram-positive and Gram-negative bacteria. Fig. 14 shows the structure of this antibiotic. It has a heptapeptide ring with a high proportion of 2,4-diaminobutyric acid and a fatty acid, 6-methyl octanoic acid, attached to the other end of the peptide (Suzuki *et al*, 1964). Thus it is both cationic and hydrophobic at the same time.

Polymyxin B is bacteriostatic at low concentrations and bactericidal at high concentrations. It affects a wide range of processes in the cell including selective membrane permeability, tranport phenomena, respiration, ATP pool size, nucleic acid and protein synthesis, LPS and peptidoglucan synthesis and specific enzyme activities (Rosenthal *et al*, 1976).

But the primary site of action appears to be the cytoplasmic membrane where it induces rapid changes in the permeability with respect to polar and charged molecules. Divalent cations such as magnesium and calcium inhibit the antibiotic activity of polymyxin B (Storm *et al*, 1977).

Fig. 14 : Molecular structures of Gentamicin & Polymyxin :

(a) Gentamicin :

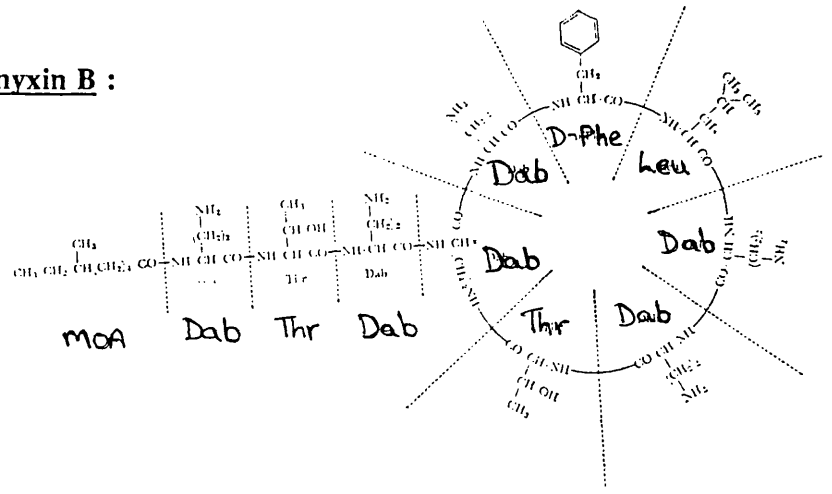


Adopted from Gale *et al* (1982)

	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Gentamicin A	H	OH	OH	OH	NH_2	H	OH
Gentamicin B	H	NH_2	OH	OH	OH	OH	CH_3
Gentamicin C_1	CH_3	$NHCH_3$	H	H	NH_2	OH	CH_3
Gentamicin C_{1a}	H	NH_2	H	H	NH_2	OH	CH_3
Gentamicin C_2	CH_3	NH_2	H	H	NH_2	OH	CH_3

Sisomycin is 4',5'-dehydrogentamicin C_{1a}

(b) Polymyxin B :



Adopted from Storm et al (1977). Abbreviations : MOA, 6-methyloctonate; Dab, diaminobutyric acid; Thr, threonine; Leu, leucine.

1.8.6. 4-Quinolones :

The 4-Quinolones used in this work are nalidixic acid and the five fluorinated 4-quinolones norfloxacin, ciprofloxacin, ofloxacin, pefloxacin and flumequin. Fig. 15 shows the structures of these compounds (Woolfson & Hooper, 1985).

Quinolones are synthetic, very potent and broad-spectrum antibacterial agents whose primary mechanism of action is on the bacterial enzyme DNA gyrase. DNA gyrase is made up of 2A and 2B subunits and quinolones are thought to act on the A subunit. However, ofloxacin has been recently found to have some affinity towards the B subunit (Monks & Campoli-Richards, 1987).

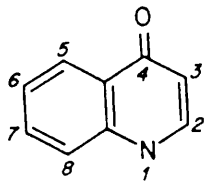
Nalidixic acid is known to inhibit DNA replication reversibly and to also affect the expression of some genes, especially those with catabolic-sensitive operons, more than others (Gomez-Eichelmann, 1981).

The antibacterial activity of ciprofloxacin (Campoli-Richards et *a*l, 1988) and ofloxacin (Monks & Campoli-Richards, 1987) are not affected *in vitro* by inoculum size, growth medium or serum but low pH and presence of urine do lower their activity. Norfloxacin activity is influenced by all of the above mentioned factors (Holmes *et al*,1985). In general, 4-quinolones containing a piperazine group eg. norfloxacin, ofloxacin and ciprofloxacin, are less active at low pH whereas those without a piperazine group are more active. Activity of ciprofloxacin and ofloxacin is antagonised by the presence of magnesium ions in the medium. Quinolones are known to induce LPS release (McConnell & Cohen, 1986), increase cell-surface hydrophobicity and outer membrane permeability to B-lactams (Chapman & Georgopapadakou, 1988), and also to sensitise cells to lysis by detergents. All these effects are antagonised by magnesium ions.

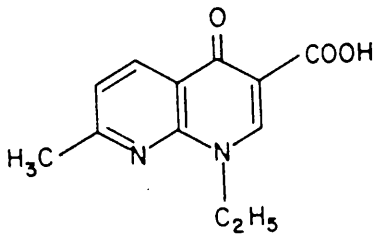
The main route of quinolone entry into the cell is via the the OmpF

Fig. 15 : **Structures of 4-Quinolones**

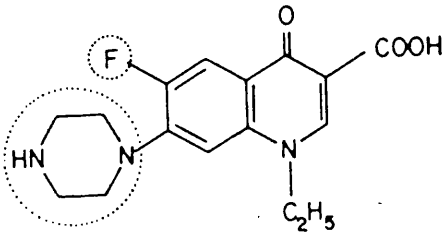
Quinolone ring



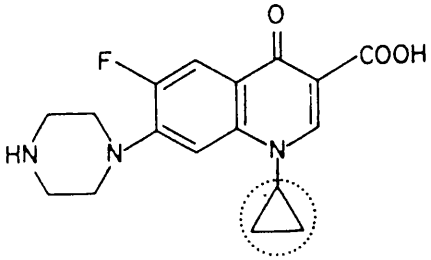
Nalidixic acid



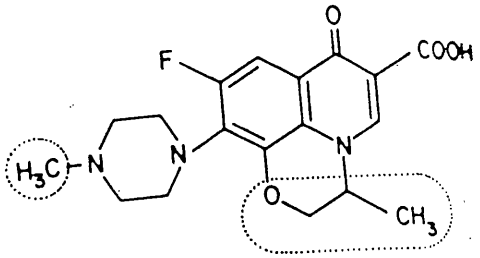
Norfloxacin



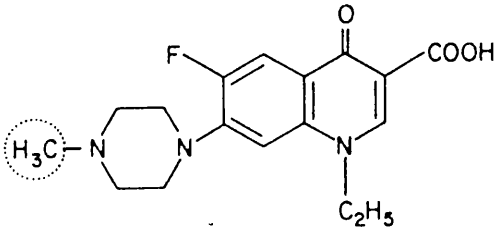
Ciprofloxacin



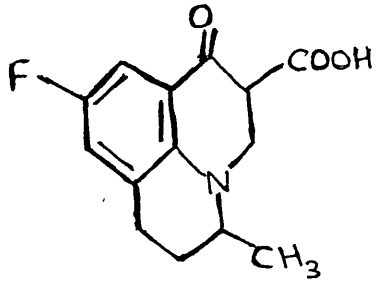
Ofloxacin



Pefloxacin



Flumequin



Adopted from Woolfson & Hooper (1985).

Table 4 : Ionic type and Hydrophobicity of 4-Quinolones

Agent	Hydrophobicity*	Ionic type
Norfloxacin	0.01	Amphoteric
Ciprofloxacin	0.02	Amphoteric
Ofloxacin	0.33	Amphoteric
Pefloxacin	1.32	Amphoteric
Nalidixic Acid	3.90	Acidic
Flumequin	13.0	Acidic

* Hydrophobicity is given as a partition coefficient in n-octanol-0.1 M phosphate buffer (pH 7.2)
(Adopted from Hirai et *al*, 1986(a))

porin channels as demonstrated both by the use of porin deficient strains and by isolation of quinolone-resistant mutants that lack porins. However, the hydrophobicity of the compound also governs, to a certain extent, which pathway of entry is used. Quinolones may also chelate divalent cations as shown by an increase in fluorescence of the quinolone when the two are mixed in solution (Chapman & Georgopapadakou, 1988).

1.9. Aims of the Project :

(1) To investigate whether the antibacterial agent sensitising effect conferred by Col V plasmids extends to 4-quinolones.

(2) To investigate whether colicin (and immunity to) and/or transfer components are involved in conferring the above mentioned antibacterial agent sensitivity.

(3) To determine the effect of Col V plasmids on envelope components eg. outer membrane protein, phospholipid and LPS.

(4) To transposon-mutagenise ColV,Ia-K94 with Tn1 and Tn7 in an attempt to isolate mutations in VmpA protein and other ColV-associated properties.

(5) To test permeability enhancing effect of various cationic compounds with regard to the entry into the cell of hydrophobic antibacterials such as novobiocin.

(6) To investigate the influence of pH on the activity of hydrophobic and cationic antibactertial agents such as novobiocin and gentamicin respectively.

(7) To investigate novobiocin activity of outer membrane porin mutants and some iron-uptake mutants at pH 5 and at pH 7.

2. Methods & Materials

2.1. Strains :

The strains used in this work ED1829 *(trp), P678-54 (thr,leu, thi),* C600 *(thy,thr,leu)*, J53 *(meth, pro), strain 14 (chicken isolate from Dr. Bayan Abu Ghazalah, UCL)*, HB101 *(thy)*, W3110 and PC0479 were obtained from laboratory stocks unless otherwise stated. The plasmids used here (ColVIa-K94, ColVIa-K94 Tn10 (Tetracycline resistance), ColV-K30, ColV-8, ColV-41, ColV-M405, ColV-M501, R483 ColIa, ColB-K98 & R124) were also obtained from laboratory stocks. Plasmids plg338 (tetracycline and kanamycin resistance) is a vector plasmid and pCR612 is a derivative of plg338 containing colined colicin V and immunity to colicin V genes. Plasmid pKF40 also contains cloned colicin V and immunity to colicin V genes (Reakes,1987). Mutants altered in *omp*F (by resistance to phage TuIIa) and *omp*C (by resistance to phage Me1) were isolated during this work from strain PC0479. *Also, omp*C*omp*F (1 & 6) mutants and *omp*C-R124 (1 & 2) derivatives were isolated from the previous *omp*C mutant by conjugational transfer in the case of R124 derivative of PC0479 *omp*C. Iron-uptake mutants 1187(*fep*A), 1619(*fhu*A), H1594(*fiu*E) and Z1379(*fec*A) were a kind gift from Dr.N.Curtis, Pharmaceutical Division ICI, Alderley Park, Cheshire. Strain stocks were kept in glycerol (800µl culture + 200µl sterile glycerol) at -70°C; strains in routine use were subcultured every month and then kept on nutrient agar plates at 4°C.

2.2. Chemicals :

The antibacterial agents erythromycin, rifampicin, rifamycin, nitrofurantoin, gentamicin, polymyxin B sulfate, nalidixic acid, bacitracin, novobiocin, fusidic acid, vancomycin, oxacillin and nafcillin were obtained from Sigma Chemical Co. The fluoro-quinolones : norfloxacin, ciprofloxacin, ofloxacin, pefloxacin and flumequin were a kind gift from Dr.Gruneberg (University College Hospital, London). The cationic agents N-(2-Pyrimidinyl)-piperazine 2HCl, 4,5,6-triaminopyrimidine sulfate H_2O, 1,4-diaminopiperazine HCl, methylglyoxyl bis(guanyl-hydrazone) 2HCl, 1,3-diaminoguanidine HCl, 1,3 diaminoacetone 2HCl H_20, 3,4-diamino-5-hydroxypyrazole sulfate, moroxydine HCl, 3,3'-diaminobenzidine 4HCl $2H_20$, triethylenetetramine 2HCl, triethylenetetramine H_20, tetraethylene pentamine, diethylenetriamine, N,N,N',N'-tetramethyl-p-phenylenediamine, formamidine disulfide 2HCl, 3-aminobenzimidine 2HCL & 1,4-diamino-2-butanone 2HCl were obtained from Aldrich Chemical Co. Ltd. Dorset, England; spermidine, agmatine, pyridoxamine, hexamethylene tetramine & hexamethylenetetramine-mandelate salt were obtained from Sigma Chemical Co. St. Louis, USA. 1,3-diaminoacetone 2HCl H_20 was obtained from Fluka Chemical Co., England.

2.3. Growth Media and Conditions :

Oxoid Nutrient Broth No.2 (broth) was used at 25 g/l for growing the organisms in liquid cultures and broth + 2% Difco Bacto Agar (NA) was used for keeping routine strain stocks, colicin and phage tests as well as for viable count experiments. For minimal medium, Davis minimal salts medium (Davis & Mingoli, 1950) suplemented with 2% glucose was used.

2.4. Growth Curve Analysis :

This was done in waterbaths over 3h, in shaken culture (at UCL) or in microtiter plates over 7 h (at ICI) both at 37°C. Overnight cultures (10 ml)* were used to inoculate 50 ml broth to obtain an exponentially growing culture. Growth analysis in the

presence of the various chemical agents eg. antibacterials, cationics, magnesium sulphate, etc. were done on subcultured exponentially growing cells. 1 ml samples were taken from flask cultures for optical density measurements using a Hilger Photoelectric Colorimeter at 520 nm with broth as blank. When microtitre plates (at ICI) were used then the whole plate was inserted into a Titretek Multiscan linked to an IBM computer so that the optical density data from the whole plate was fed into the computer in a separate file for each plate. Each culture in the microtitre plate was distinct but each was done in duplicate so that the result was an average of two identical test cultures. Hence only two separate repeats were done using this system. When growth curves were done in 20ml cultures 5 repeats were done of each test strain and agent (unless otherwise stated) with the control strain being done at the same time. When viable count experiments were performed then colonies were counted after overnight incubation at the appropriate temperature. The iron-uptake mutants were grown at 30°C. (* 10 mls. was the maximum volume used).

2.5. Colicin Tests :

Test strains were streaked onto NA plates,incubated overnight at 37°C, surface-killed with chloroform vapour for 15 min, and after exposure to air for 20 min the plates were overlaid with 5 ml soft agar containing 100 µl of indicator strain culture. The indicator strains routinely used were either ED1829 or C600 unless a specific colicin immunity test was being done then the corresponding colicin producing strain was used as an indicator. After overlaying with the indicator strain and once the soft agar was set, the plate was then incubated again at 37°C overnight. Zones of growth inhibition were compared with control colicin producing strains.

2.6. Phage Tests :

Phage suspension was streaked onto a NA plate and soft agar containing 100 µl of the indicator strain was overlaid on top. On 6-24 h incubation at 37°C,

plaques were observed only in the streaked area if the indicator strain was sensitive to the phage. When unknown phages were being isolated from sewage, then the same method was applied and phage was picked from the plaques with a sterile toothpick and stored in 1 ml sterile broth and stored at 4°C for further tests and propagation.

2.7. Phage Propagation :

For MS2 propagation ED1829 ColV,Ia-K94 Tn10 was used as this strain was found to give a very high phage yield. For plate propagation, the procedure for phage test (above) was followed except that the phage was added to the soft agar with the indicator strain and then overlaid on an unstreaked NA plate. On incubation at 37°C, 2 ml sterile broth was poured onto the soft agar with confluently lysed culture and the soft agar gently scraped. The suspension produced was then transferred to a sterile universal bottle and centrifuged to pellet unlysed bacterial colonies and the soft agar mass to leave the high titre phage in the suspension. The phage was then stored at 4°C with 1-2 drops of chloroform. For liquid propagation, 1 ml of an overnight strain culture (37°C shaking) was mixed with 100 µl phage suspension in a 1.5 ml sterile eppendorf tube and incubated at 37°C in a static waterbath for 15 min. 0.2 ml of this was then added to 10 ml broth and incubated for 2-3 h at 37°C and shaken slowly. The resulting culture was then centrifuged for 15 min at 3000 rpm and the supernatant stored as above. The MSE Bench Centrifuge was used for both types of propagation and other routine small scale centrifugation. Liquid propagation was found to give a higher yield of phage as compared to plate propagation.

2.8. Plasmid transfer by Filter Conjugation :

Overnight cultures of the recipient and donor strains were diluted into 20 ml broth and allowed to grow up to exponential phase. A 0.2 µm pore-size filter (UV sterilised, 15 min) was used to filter the donor and recipient strains. The order of filtration was as follows : 1 ml donor strain, mixture of 1 ml donor and 2ml recipient followed by

1 ml recipient strain. This layered cake of cells on the filter was then transferred on to a NA plate and incubated at 37°C for 3-5 h. The cells were resuspended in 10 ml 0.75 % saline and dilutions plated onto selective NA plates. After incubation at 37°C, individual colonies were picked, transferred to another plate, labelled and then analysed for the presence of the appropriate marker(s), usually colicin production and MS2 sensitivity for the Col V plasmids.

2.9. Plasmid Isolation :

For routine screening of plasmid DNA, the plasmid extraction procedure of Takahashi & Nagano (1984) was used.

2.10. Plasmid Curing :

1 ml of an overnight strain culture was diluted in to 10 ml broth containing 0.5 %, 1 % & 1.5 % SDS, incubated for 48 h at 37°C and then at 42°C for a further 48 h before plating a dilution series on NA plates and testing for the loss of corresponding plasmid associated marker(s). Attempts to cure ColV plasmids from strains failed and hence the results are not shown here.

2.11. Transposon Mutagenesis of ColV,Ia-K94 :

Strain J53 Tn1 (Ampicillin resistant) was used as a recipient for plasmid ColVIa-K94 from donor ED1829 ColVIa-K94. Filter conjugation was used to transfer the plasmid ColVIa-K94 into strain J53 Tn1 and selection of transconjugants was done on NA + ampicillin (20 µg/ml) plates. The colonies obtained were of J53 Tn1 and J53 Tn1 ColVIa-K94 but mostly the latter as this plasmid is very efficiently transferred. Colicin (V & Ia) and immunity (to both colicins) tests were carried out to select the transconjugants before subculturing these again on NA + ampicillin plates and leaving the plates at room temperature for 14 days so as to eliminate any unstable transconjugants and also to allow the Tn1 tranposon to jump from the J53 chromosomal DNA to the plasmid ColVIa-K94 DNA.

The transconjugants were then retested for the two colicins and immunity to colicins so that the transconjugants selected are not such that the transposon has altered the colicin and immunity regions as these are the only markers easily usable for selection of the plasmid. After MS2 tests, the sensitive isolates were then used for conjugation into strain P678-54 (streptomycin^r, 200 μg/ml) so as to select out the transconjugants which now contain the tranposon on the plasmid. The same procedure was used to mutagenise the plasmid ColVIa-K94 with transposon Tn7 (Trimethoprim^r, Spectinomycin^r, Streptomycin^r).

Selection of Tn7 carrying strains was done on NA plates containing trimethoprim and streptomycin.

2.12. Assessment of accessible phospholipid in whole cells:

Strains ED1829, ED1829 ColVIa-K94 & 1829 ColV-K30 were inoculated into 10 ml broth and incubated for 4 h with shaking at 37°C; and 100 μl of this culture was then used to inoculate 3 X 20 ml broth in (100 ml conical flasks) for each strain. 50 μl of 1 mCi/ml Na_2HPO_4 (^{32}P labelled) was added to two flasks of each strain. All flasks were then incubated for 12-16 h (37°C, shaking 100 rpm). The cells were harvested and washed in cold (ie. unlabelled) Na_2PO_4 buffer pH 7.4 to displace any ^{32}P from the outside of the cells (Sorvall refrigerated centrifuge RT6000B, room temp., 15 min, 3000 rpm). The pellet was resuspended in 15 ml unlabelled broth, transferred to 100 ml flasks and incubated at 37°C, shaken. 1 ml samples were taken at 30 min intervals for measurements of optical density and ^{32}P release in the absence/presence of Phospholipase C enzyme (1 unit/ml) for each strain. The samples from labelled cultures (Phospholipase treated and untreated) were spun down to separate cells and medium so that an estimation of cell-associated and free label could be made. The cells were found to be very heavily labelled and hence the incorporation of ^{32}P was very efficient.

Precautions : All additions, transfers and resuspensions were done in a fume-cupboard behind a perspex shield to minimise exposure to the ^{32}P.

2.13. Lipopolysachharide Extraction Method : (Darveau & Hancock, 1983)

500 mg of cells (lyophilised) were resuspended in 15 ml of buffer A (10 mM TRIS-HCl, pH 8, 2 mM $MgCl_2$, 20 mg/ml pancreatic DNase I, 10 mg/ml pancreatic RNase A) and then passed through a French Pressure Cell twice at 15000 lb/sq inch. The lysate was then sonicated for 30 secs. X 2 at 1.5 Amps (Intensity 75). This sonicate was incubated at 37°C for 2h in the presence of DNase (200 µg/ml) and RNase (50 µg/ml). Then 5 ml of 0.5 M Na_4EDTA, 2.5ml 20% SDS (in 10 mM TRIS-HCl, pH 8.0) and 2.5 ml 10mM TRIS-HCl, pH 8.0 were added to make the final volume 25 ml. This suspension was centrifuged at 50,000 g for 30 min at 20°. The supernatant was transferred to another tube and incubated overnight at 37°C in a shaking waterbath with 200 µg/ml Pronase. To this solution 2 volumes of 0.375 M $MgCl_2$ in 95% ethanol were added and the mixture was left at -20°C for 15 min before spinning at 12000 g for 15 min, at 0-4°C. The pellet was resuspended in 25 ml 2% SDS, 0.1 M EDTA and 10 mM TRIS-HCl, pH 8.0, sonicated 30 sec X 2 and left at 85°C for 15 min before an overnight incubation at 37°C in the presence of 25 µg/ml Pronase. LPS was then precipitated with 2 vols. 0.375 M $MgCl_2$ in 95% ethanol at 0°C and then spun at 12000 g for 15 min at 0-4°C. The pellet was resuspended in 15 ml of 10 mM TRIS-HCl, pH 8.0 and sonicated 30 sec X 2. This suspension was then centrifuged at 20,000 g for 2 h at 15 °C in the presence of 25 mM $MgCl_2$ in 95% ethanol. The LPS pellet was then resuspended in distilled water and lyophilised.

2.13.1. Method for KDO estimation : (Karkhanis *et al*, 1977)

2 mg LPS was added to 1 ml 0.2 N H_2SO_4 and heated to 100°C for 30 min. On cooling, the suspension was centrifuged at maximum* speed in a clinical microfuge for 5 min and 0.5ml of clear supernatant was transferred to another tube containing 0.25 ml 0.04 M periodic acid in 0.125 N H_2SO_4, vortexed and then left at room temperature for 20 min. Then 0.25 ml 2.6% $NaAsO_2$ in 0.5 N HCl was added and the mixture was left until the brown colour disappeared. To this 0.5 ml 0.6% thiobarbituric acid

(TBA) was added, and the mixture vortexed and heated at 100°C for 15 min. While still hot, 1 ml dimethyl sulphoxide (DMSO) was added and upon cooling, the optical density was measured at 548 nm. The standard curve was obtained using KDO from Sigma.

(* ca. 12000 rpm)

2.14. Isolation of the Outer Membrane :

Cells from an overnight 10 ml culture in broth were harvested (15 min, 2500 rpm, MSE Bench Centrifuge) and washed in 5 ml 0.9% saline before resuspension in 1.4 ml 50 mM TRIS-HCl, 2 mM EDTA pH 8.5. This suspension was then frozen for 15 min at -70°C. Upon thawing at room temperature, the cells were sonicated at 1.5 Amp (6 X 30 sec. bursts with 15 sec. cooling interval in between). The sonicate was centrifuged (Eppendorf microfuge, 2 min, 12000 rpm) to pellet unbroken cells and other large debris. The supernatant was then centrifuged again at the same speed for 15 min to pellet the outer membranes, which were then stored at 4°C in 20 µl 2 mM TRIS. Membrane suspension (10 µl) was added to 40 µl sample buffer and denatured (100°C, 5 min or 30°C 15 min) before loading onto SDS PAGE gel. The sample buffer contained 60 mM TRIS-HCl pH 6.8, 2% SDS, 10% Glycerol, 0.001% Bromophenol Blue and 5% Mercaptoethanol.

2.15. Protein assay : (Markwell *et al*, 1978)

This method for protein assay is a modified version of the Lowry Procedure so as to be useful for whole cells as well as purified proteins. 10 ml of cells were used for whole cell protein assay, 10 ml for outer membrane extraction and subsequent protein assay and another 10 ml of the same culture was used for dry weight measurement. When a protein assay was done on any strain containing a plasmid then an exponentially growing culture was used otherwise an overnight culture was used. 100 parts of Reagent A (2 % Sodium Carbonate, 0.4 % Sodium Hydroxide, 0.16% Sodium Tartrate and 1% SDS) were added to 1 part Reagent B (4 % Copper Sulphate) to make reagent C. 1 ml of the sample protein suspension was added to 3 ml Reagent C and left at room

temperature for 1 h. Then 0.3 ml 1:1 Folin-Ciocalteu's Phenol Reagent : Water was added to each sample and vortexed before leaving at room temperature for 45 min and vortexing again. The absorbance was then measured at 660 nm with control (no protein) test tube as blank. The standard curve was done with Bovine Serum Albumin within the range 0-500 µg protein for outer membrane protein and 0-1.5 mg protein for whole cell protein assay .

2.16. Polyacrylamide Gel Electrophoresis : (Laemmli, 1970)

(a) **For Proteins :** The apparatus was set and sealed from the outside with 2% Agar before the gel mixture was poured into it. The separating gel was poured first to fill 3/4 of the total gel space and allowed to set before pouring the stacking gel and placing a comb to form wells. The separating gel mixture contained : 14 ml stock acrylamide (30% Acrylamide & 0.8% bis-Acrylamide), 20 ml 0.75 M TRIS-HCl pH 8.8, 0.4 ml 10% SDS, 3.6 ml distilled water, 2 ml 10 mg/ml Ammonium persulphate (freshly prepared) and 60 µl TEMED. TEMED was added just before pouring the gel to prevent premature polymerisation of the acrylamide. The stacking gel mixture contained 3 ml stock acrylamide (as above), 10 ml 0.25 M TRIS-HCl pH 6.8, 0.2 ml 10% SDS, 5.8 ml distilled water, 1 ml 10 mg/ml Ammonium persulphate and 60µl TEMED. The gel was run in a vertical electrophoresis apparatus at 25 mAmps for 5-6 h (until the marker dye in the sample buffer reached the bottom of the gel) in electrophoresis buffer (25 mM TRIS, 200 mM Glycine, 0.1% SDS). The proteins were stained overnight in 10% glacial Acetic Acid, 50% Methanol and 0.1% Coomassie Blue R250 (Sigma) and then destained in several changes of 10% Acetic Acid & 50% Methanol until the protein bands could easily be distinguished. The gel was then washed and stored in distilled water.

(b) **For LPS :** The gel electrophoresis for LPS was similar to that for proteins except that the stock acrylamide was 40% (20 g Acrylamide & 0.54 g bis-Acrylamide in 50 ml distilled water), SDS was omitted from both the separating and the stacking gel mixtures,

LPS was dissolved directly into the sample buffer for LPS (0.1 M TRIS-HCl pH 6.8, 2% SDS, 10% Sucrose (w/v), 1% 2-Mercaptoethanol (v/v), 0.001% Bromophenol Blue and 40 mM EDTA) before boiling for 5 min. The gel was electrophoresed for 2 h or until the dye front reached 3/4 through the separating gel as the LPS of *E.coli* K12 is very low molecular weight and so would otherwise run off the gel.

2.17. Silver stain for LPS : (Tsai & Frasch, 1982)

The gel was fixed overnight in 200 ml 40% ethanol, 5% acetic acid in distilled water. Then this solution was replaced with 200 ml 0.7% periodic acid in 40% ethanol and 5% acetic acid for 5 min before three 15 min washes. The gel was then stained with 150 ml of the following stain : 2 ml ammonium hydroxide, 28 ml 0.1 N sodium hydroxide and 5 ml 20% silver nitrate and 115 ml water. Staining was for 15 min, the gel being shaken for 10 min at a speed which allowed the reagent to cover the gel most of the time without breaking it. The staining reagent was then discarded as it is explosive when dry. The gel was washed again in water, (3 X 15 min) before pouring on the developer solution (10 mg citric acid and 100 µl 33% formaldehyde) in 200 ml water. The developing solution was discarded as soon as the gel background started to stain brown (5 min). The gel was then quickly washed with several changes of water to prevent any more stain developing and thereby overstaining the gel. The gel was stored in water at room temperature. The water used throughout this staining procedure was purified double distilled water.

2.18. Permeabilisation of E.coli K12 :

A 1 litre culture exponentially growing, but optical density not higher than 0.6 at 620 nm, was harvested (4500 rpm, 20 min, Sorvall Refrigerated centrifuge RC-3B) and then resuspended and washed in 10 ml 50 mM phosphate buffer at pH 7.4 or at pH 5 (Oakridge tube, 9000 rpm, 20°C, Sorvall RC5C centrifuge rotor SS34). The cells were then warmed to 37°C for 10 min before adding 100 µl Toluene and the tubes vortexed for 9 min 30 sec on a Multitube

warmed to 37°C for 10 min before adding 100 µl Toluene and the tubes vortexed for 9 min 30 sec on a Multitube vortexer (SMI model 2601, speed setting 5). The cells were then washed four times in 10 ml buffer to remove toluene. Care was taken to make sure that the supernatant containing the toluene after each wash was discarded immediately after the spin so as not to the damage the cells otherwise the DNA leaks out and makes the suspension very viscous and then it cannot be used for assay. This permeabilised cell suspension (50 µl) was used to assay antibacterial agent sensitivity. The assay was carried out in 96 well U-bottom microtitre plates. Each well contained a separate assay and each assay was replicated three times on the same plate. Incorporation of ^{3}H-methyl-thymidine into the DNA was measured in the presence and absence of ATP to differentiate between DNA synthesis, which is ATP dependent and DNA repair which is not. Each well contained 50 µl toluenised cells, 50 µl substrate/cofactor mix and 2 µl antibacterial agent solution. The substrate/cofactor mix contained deoxyribose Adenosinetriphosphate (dATP; 20 µl of 10 mg/ml stock made in phosphate buffer), deoxyribose Guanosinetriphosphate (dGTP; 20 µl of 10 mg/ml stock), deoxyribose Cytidinetriphosphate (dCTP; 20 µl of 10 mg/ml stock), 1 ml 26 mg/ml magnesium hexahydrate in phosphate buffer, 25 µl ^{3}H methyl-Thymidine (1 mCi/ml) and 8 ml phosphate buffer. 1 ml 24 mg/ml ATP in buffer was added to the substrate/cofactor mix used for the assay with ATP. After loading the samples, the plate was vibrated on a plate vibrator for 2-3 min before incubation at 37°C by floating it on a rack in a waterbath for 40 min. The assay was stopped by adding 100 µl 20% Trichloroacetic acid in 0.2 M tetrasodium pyrophosphate and leaving the plate at 4°C for at least 1 hour or overnight to precipitate the cells. The cells were harvested using a Skatron semiautomatic cell harvester setting 5, on to filter paper. Filter paper discs containing the cell pellets were transferred to minivials and 4 ml PCS liquid scintillation fluid (1 l fluid + 1 ml glacial acetic acid; Amersham Ltd) was added before counting the radioactivity in the cells in a LKB 1218 Rackbeta liquid scintillation counter for 1 min. When pH shift assay was done then the culture was grown at one pH and the cells washed-toluene treated-washed again in

buffer at the same pH as the broth used for growing the culture but the substrate/cofactor mix was made up in buffer at the shift pH.

2.18. Formula used for calculation of standard error :

Standard Error :

$$\frac{\sqrt{\frac{\Sigma \Delta^2}{N - 1}}}{N}$$

Where :

Δ = difference between mean and sample values.

$\Sigma\Delta^2$ = sum of Δ squared.

N = number of samples.

Adopted from Hicks & Rowbury, 1987(b). The value of N was 5 unless otherwise stated.

NB. :

Text in section 3.1. is based on the corresponding tabulated results which are mean values $\pm$ standard errors. Graphs are drawn using optical density readings obtained from an individual experiment.
Comparison of the growth rate of the ColV$^+$ organisms is done with that of the ColV$^-$ isogenic control strain and not with other ColV$^+$ strains.

3. RESULTS

3.1. Col V plasmids and antibacterial agent sensitivity

Because of their medical importance as virulence plasmids, the effects of a range of Col V plasmids on sensitivity to various types of antibacterial agents was investigated.

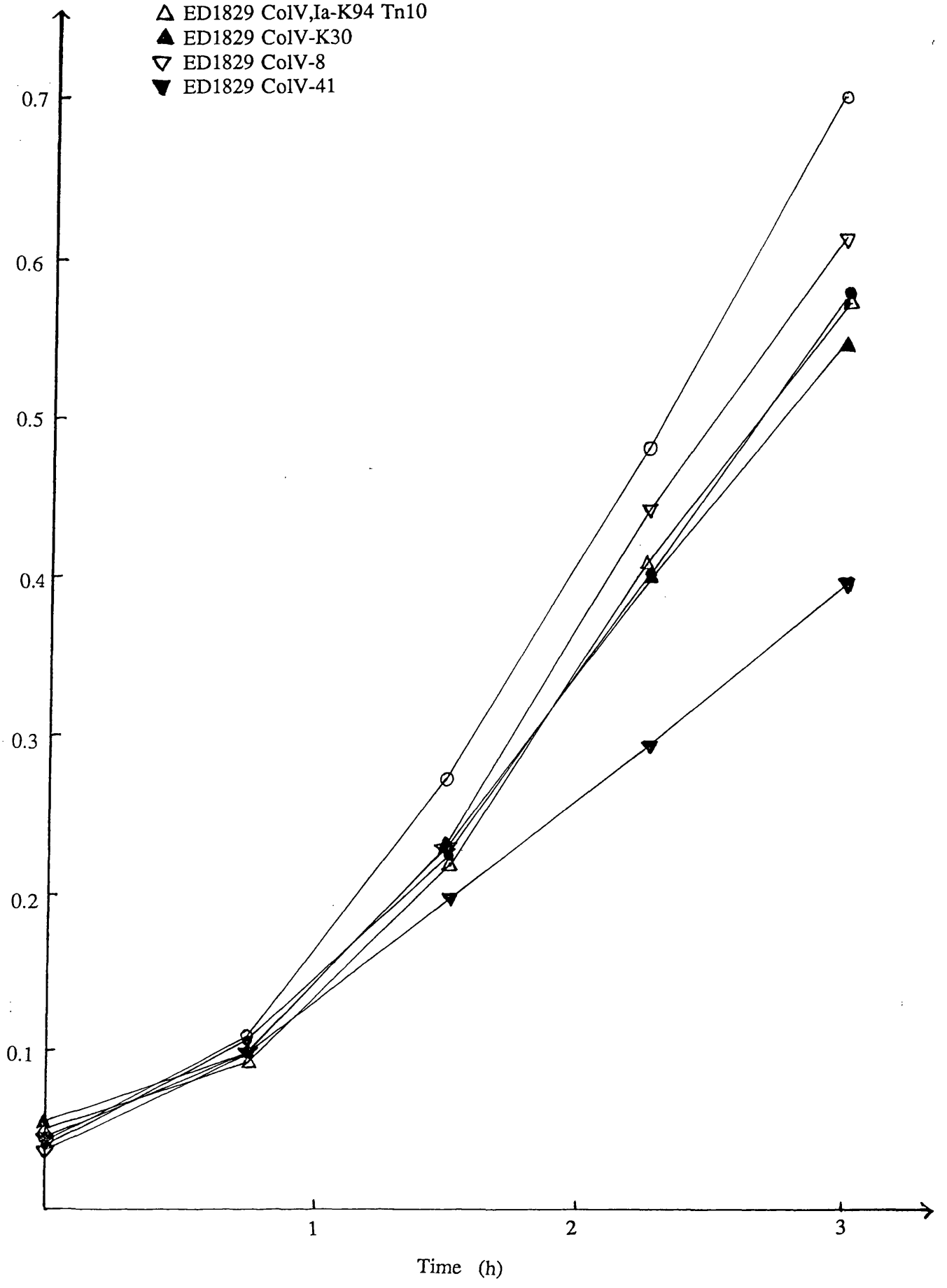
None of the plasmids used in this study except ColV-41, were found to have any appreciable effect on the growth rate of its host strain in broth when grown in small (10-100 ml) cultures. ED1829 ColV-41 was found to grow consistently at a much slower rate (Fig.16) and this property appears to be conferred by the plasmid and not due to the host strain.

3.1.1. Effect of Col V plasmids on Erythromycin Sensitivity

At 5 μg/ml concentration, all the Col V plasmids tested ie. ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-8 and ColV-41, except ColV-K30, were found to significantly increase the sensitivity of strain ED1829 to erythromycin (Table 5, Fig.17). Plasmid ColV-41 (a sewage isolate, Hicks *et al*, 1987), was extremely effective in conferring this sensitising effect as compared to the other Col V plasmids. These results agree with the Col V-associated increased sensitivity to erythromycin found by Reakes *et al* (1988).

Erythromycin is a macrolide antibiotic and its bactericidal target is the protein synthesis system of bacteria. In Gram-negative bacteria, erythromycin is usually not very effective as the outer membrane permeability barrier is very successful in preventing its entry into the cell. The target site of this antibiotic is equally sensitive in Gram-negative bacteria as it is in Gram-positive bacteria. Erythromycin is more active at alkaline pH (Alfa *et al*, 1987) and it may be therefore, that the presence of the Col V plasmids is associated with some changes in the cell metabolism leading to secretion of metabolic products which may increase the pH

Fig. 16 : Effect of Col V plasmids on growth rate



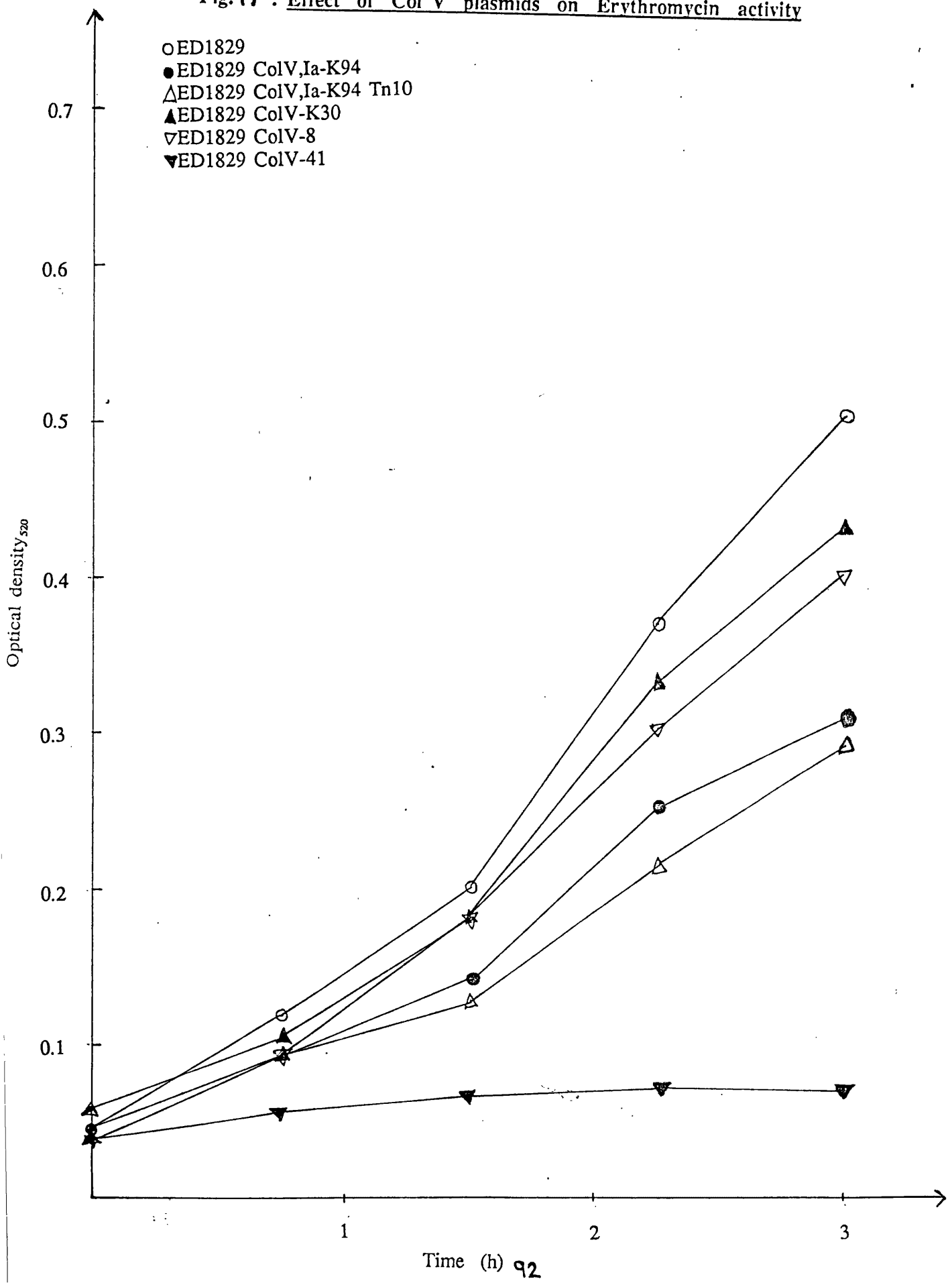
of the medium and thereby contribute towards the increased sensitivity effect. However no such increase in the pH of the medium of cultures of Col V plasmid containing cells was observed. During growth in liquid medium (broth), there is a slight increase in the medium pH, but this is the same for the plasmid-less control strain and the Col V plasmid containing isogenic strain. Another working hypothesis is that some of the Col V plasmid encoded proteins, in particular those expressed in the outer membrane eg. VmpAp, certain transfer proteins such as tra-J, -T, -B, etc., colicin V and immunity to colicin V components, and also colicin Ia and its immunity component in the case of plasmid ColVIa-K94, etc. may affect the uptake of hydrophobic compounds into the cell. This hypothesis is investigated in section 3.2.

Table 5 : Col V plasmid effect on Erythromycin sensitivity

STRAIN	% Growth Inhibition (± Standard Error) with 5 μg/ml Erythromycin
1829	29.94 ± 1.65
1829 Col VIa-K94	56.91 ± 1.80
1829 Col VIa-K94 Tn 10	53.82 ± 2.28
1829 Col V-K30	26.92 ± 3.93
1829 Col V-8	42.64 ± 1.67
1829 Col V-41	94.88 ± 0.94

The conditions of growth are given in the Methods & Materials Section.

Fig. 17 : Effect of Col V plasmids on Erythromycin activity



3.1.2. Effect of Col V plasmids on Rifamycin sensitivity

Two antibiotics from the rifamycin group, rifamycin SV and rifampicin were tested with the Col V plasmid containing derivatives of strain ED1829 (Table 6 & 7). At 15 μg/ml rifamycin, the Col V plasmids ie. ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30 , and Col V-8 had no effect on the sensitivity of their host strain ED1829 to this antibiotic. However, presence of plasmid ColV-41 was extremely effective in conferring a high rifamycin sensitivity.A similar trend was observed with this plasmid for erythromycin sensitivity in section 3.1.1.

When rifampicin was tested (10 μg/ml;Table 7, Fig.18) all the Col V plasmids ie. ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30, ColV-8 and ColV-41, sensitised their host strain ED1829 to rifampicin. These results are in agreement with those found by Davies *et al* (1986), however, they had used the plasmid ColV,Ia-K94 only whereas this study is extended to include four other ColV plasmids.

Rifamycin and rifampicin belong to the same group, in fact, rifampicin is a much more active derivative of rifamycin. Again, like erythromycin, the relative resistance of many Gram-negative pathogens to rifamycin and rifampicin is due to their inability to penetrate the cell through the outer membrane barrier. In the case of rifampicin, all the ColV's tested conferred increased sensitivity whereas with rifamycin , none of the ColV's, except ColV-41, showed any enhanced sensitising effect. The difference in activities and entry mechanism(s) of these two antibiotics must presumably be attributable to the difference in their structures (fig. 13). The involvement of transfer and colicin plus immunity to colicin components for the plasmid ColV,Ia-K94 is investigated in section 3.2.

Table 6 : Col V plasmid effect on Rifamycin SV sensitivity

STRAIN	% Growth Inhibition (± Standard Error) with 15 µg/ml Rifamycin
1829	2.04 ± 1.77
1829 Col VIa-K94	6.59 ± 3.12
1829 Col VIa-K94 Tn 10	5.95 ± 5.64
1829 Col V-K30	6.49 ± 0.94
1829 Col V-8	6.22 ± 4.68
1829 Col V-41	95.40 ± 1.04

The conditions of growth are given in the Methods & Materials section.

Table 7 : Col V plasmid effect on Rifampicin sensitivity

STRAIN	% Growth Inhibition (± Standard Error) with 10 μg/ml Rifampicin
1829	34.56 ± 3.03
1829 Col VIa-K94	79.09 ± 2.77
1829 Col VIa-K94 Tn 10	88.20 ± 2.53
1829 Col V-K30	51.21 ± 11.01
1829 Col V-8	46.15 ± 1.78
1829 Col V-41	97.75 ± 1.66

The conditions of growth are given in the Methods & Materials section.

3.1.3. Effect of Col V plasmids on Novobiocin sensitivity

At 30 μg/ml concentration, all the Col V plasmids tested ie. ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30, ColV-8 and ColV-41, conferred increased sensitivity on their host strain ED1829 to novobiocin (Table 8, Fig 19). These results are in agreement with those of Davies *et al* (1986) but this study has been extended to include four more Col V plasmids.

Again, like erythromycin and rifampicin, novobiocin is poorly effective on Gram-negative bacteria due to its poor penetrability into the cell. The intracellular target of novobiocin is the bacterial enzyme DNA gyrase and to bind the B-subunit of this gyrase and cause its subsequent inactivation, novobiocin needs to gain access to the cell. Novobiocin is very active at low pH and one way to increase the sensitivity to this antibiotic is by lowering the medium pH. Accordingly such a pH fall could have occured in the medium of ColV$^+$-strain but not in that with ColV$^-$-strain. However, no such alteration in pH is found to be associated with growth of strains carrying these Col V plasmids. On the contrary, Col V plasmid-containing strains are sensitive to low pH themselves (Cooper & Rowbury,1986) and hence would not grow under such conditions regardless of whether there is any antibiotic present in the medium or not. Hence, the increased sensitivity seems more likely to be the result of increased permeation of the antibiotic into the cell. Davies *et al* (1986) found that transfer components expressed by these large conjugative Col V plasmids contribute towards this novobiocin activity enhancing effect. As mentioned before, many of the proteins involved in the conjugational transfer of such plasmids are expressed in the outer membrane. However, whether it is the physical presence of these proteins or the active involvement of one or more of these proteins eg. activity as a receptor, in the outer membrane which causes the effect remains to be investigated. The outer membrane is very rich in its protein content and the presence of any extra proteins, followed by the consequent inevitable change in the proportion of the normal constituents of the outer membrane, especially in the outer leaflet, may alter the penetrability of certain types of

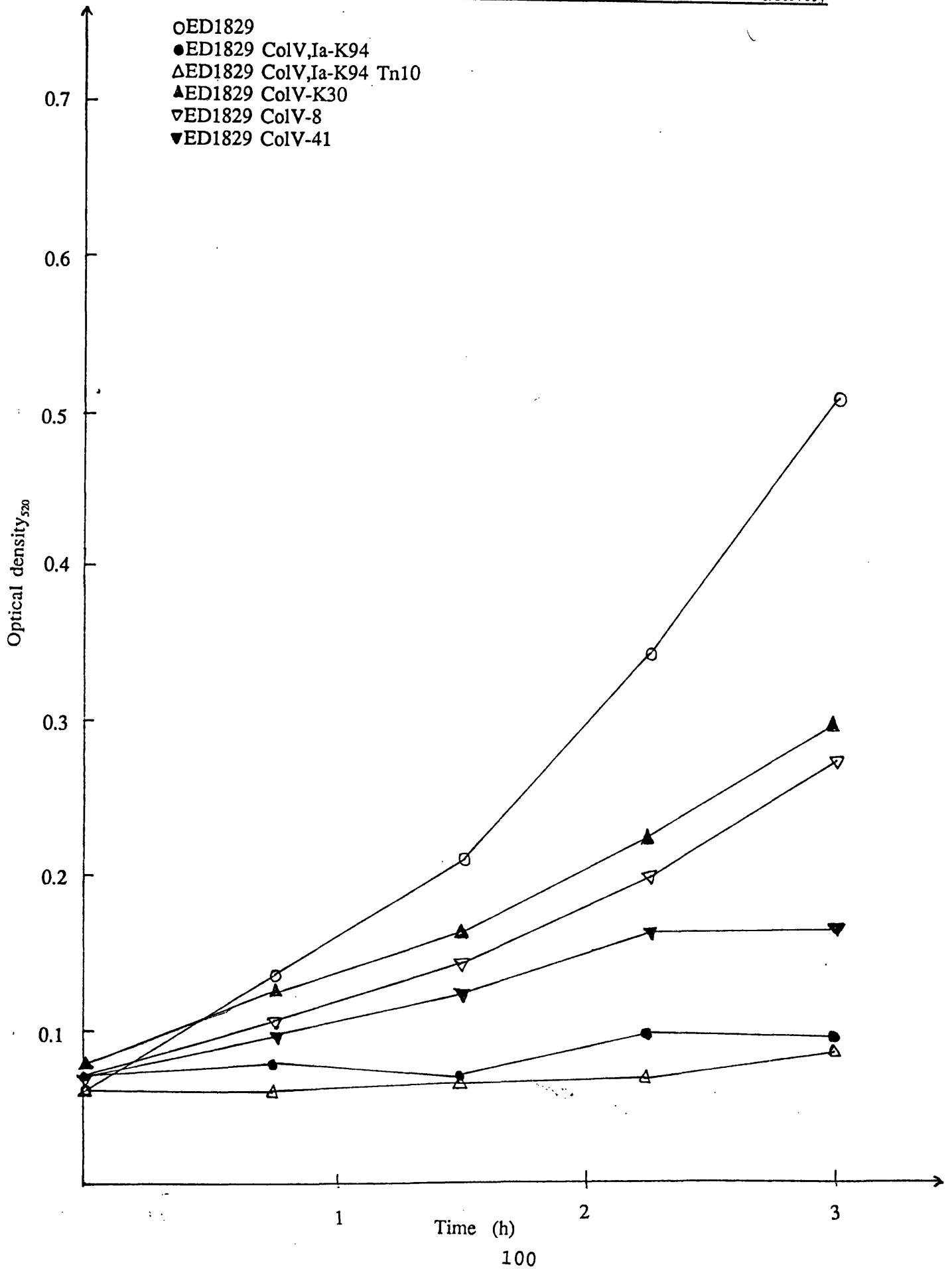
compounds eg. hydrophobic agents, into the cell. This aspect of the effects of the outer membrane components is investigated in section 2. On the other hand Col V plasmids have been known to encode at least one protein, VmpAp (Moores & Rowbury, 1982), which is expressed in the outer membrane in quantities similar to the major proteins such as OmpAp, OmpFp and OmpCp. These latter proteins act as receptors for various bacteriophages and in non-specific uptake of various hydrophilic charged compounds (see section 1.2.3.(a)). Hence, it is possible that one or more of the Col V plasmid associated proteins are involved more directly in the increased uptake of hydrophobic agents.

Table 8 : Col V plasmid effect on Novobiocin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) with 30 μg/ml Novobiocin
1829	29.82 ± 3.47
1829 Col VIa-K94	91.48 ± 2.30
1829 Col VIa-K94 Tn 10	93.87 ± 0.72
1829 Col V-K30	50.23 ± 2.37
1829 Col V-8	65.51 ± 3.72
1829 Col V-41	72.92 ± 2.24

The conditions of growth are given in the Methods & Materials section.

Fig. 9 : Effect of Col V plasmids on Novobiocin activity



3.1.4. Effect of Col V plasmids on Nitrofurantoin sensitivity

At 10 μg/ml concentration in broth, the sensitivity of ED1829 was significantly increased by the presence of plasmids ColV,Ia-K94 and ColV,Ia-K94 Tn10, but not significantly so by the plasmids ColV-K30, ColV-8 and ColV-41 (Table 9, Fig. 20).

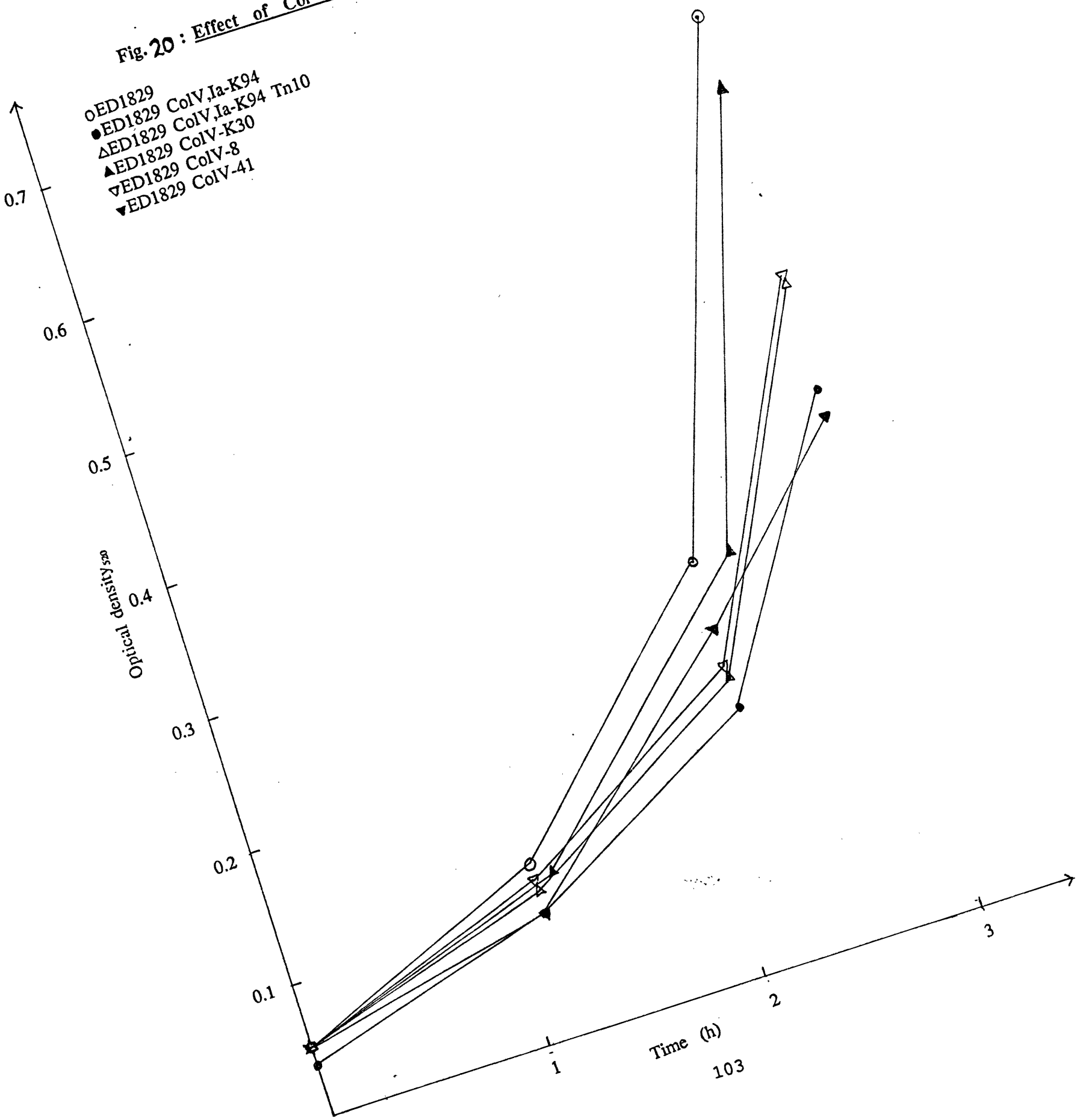
Nitrofurantoin is a synthetic antibacterial agent clinically used to treat urinary tract infections. A significant proportion of Col V plasmids have been isolated from extraintestinal infections, including UTI's, by various groups (Davies *et al*, 1981; Williams Smith & Huggins, 1976). The increased susceptibility of Col V containing organisms in such infections to nitrofurantoin would be clinically useful as then lower doses of the drug could be administered to treat the infection. However, three of the Col V's tested showed no effect on the host organism.

Table 9 : Col V plasmid effect on Nitrofurantoin sensitivity

STRAIN	% Growth Inhibition (± Standard Error) by 10 µg/ml Nitrofurantoin
1829	25.18 ± 3.99
1829 Col VIa-K94	57.98 ± 3.54
1829 Col VIa-K94 Tn 10	52.47 ± 2.50
1829 Col V-K30	32.06 ± 2.80
1829 Col V-8	38.96 ± 5.17
1829 Col V-41	32.41 ± 3.75

The conditions of growth are given in the Methods & Materials section.

Fig. 20 : Effect of Col V plasmids on Nitrofurantoin activity



3.1.5. Effect of Col V plasmids on Gentamicin sensitivity

Table 10 shows the gentamicin sensitivity of ED1829 containing the various Col V plasmids. The pattern of sensitivity to this antibiotic is not consistent with each Col V plasmid. ColV,Ia-K94 and its derivative ColV,Ia-K94 Tn10 as well as ColV-41 were very effective in conferring increased sensitivity to gentamicin in broth (Fig. 21). Plasmids ColV-K30 and ColV-8 showed no significant effect on their host ED1829.

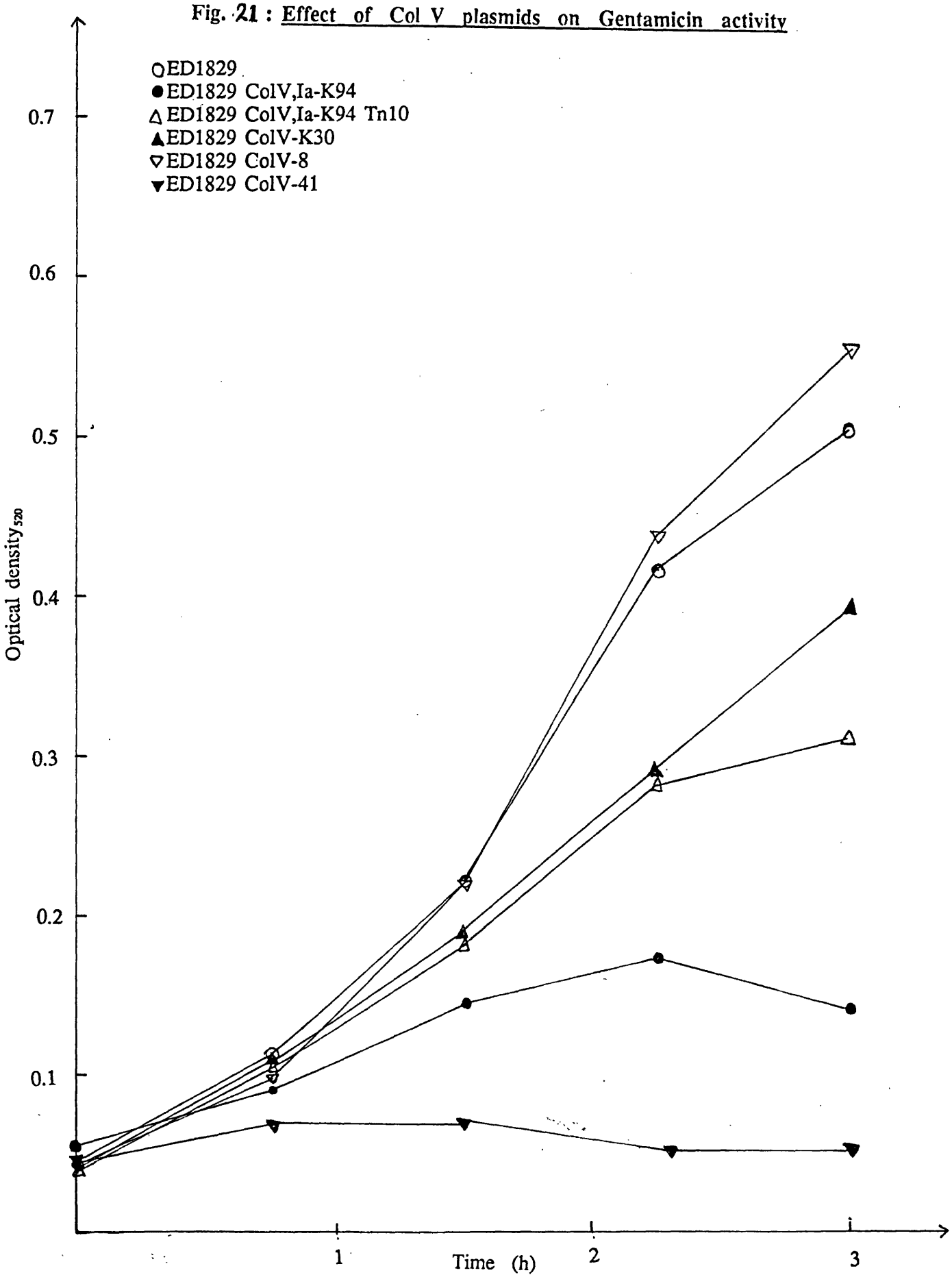
Gentamicin is a cationic aminoglycoside antibiotic whose major route of entry into the Gram-negative cell is by using the porins (Nakae & Nakae, 1982). However, such cationic agents have also been proposed to use the self-promoted uptake pathway (Hancock, 1984). In ColV-containing organisms, it is posssible that either pathway could be effective for gentamicin uptake as the hydrophobic permeability barrier is apparently weaker as implied by the increased sensitivity to the many hydrophobic antibiotics tested in the preceding sections. However, by comparison with the plasmid-less control isogenic strain ED1829, the selfpromoted uptake pathway seems more efficiently utilised in the ColV-plus strains, at least for the plasmids ColV,Ia-K94, ColV,Ia-K94 Tn10 and ColV-41.

Table 10 : Col V plasmid effect on Gentamicin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 0.15 µg/ml Gentamicin
1829	25.86 ± 4.04
1829 Col VIa-K94	83.58 ± 2.88
1829 Col VIa-K94 Tn 10	59.17 ± 2.78
1829 Col V-K30	36.34 ± 9.98
1829 Col V-8	16.03 ± 1.78
1829 Col V-41	99.94 ± 0.87

The conditions of growth are given in the Methods & Materials secion.

Fig. 21 : Effect of Col V plasmids on Gentamicin activity



3.1.6. Effect of Col V plasmids on Polymyxin B sensitivity

Table 11 shows the polymyxin B sulfate sensitivity of strains carrying the Col V plasmids ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30, ColV-8 and ColV-41. At 0.15 μg/ml, polymyxin B sensitivity was found to be markedly enhanced by ColV,Ia-K94, ColV,Ia-K94 Tn10 and ColV-8 but not at all affected by the plasmids ColV-K30 and ColV-41 (Fig.22).

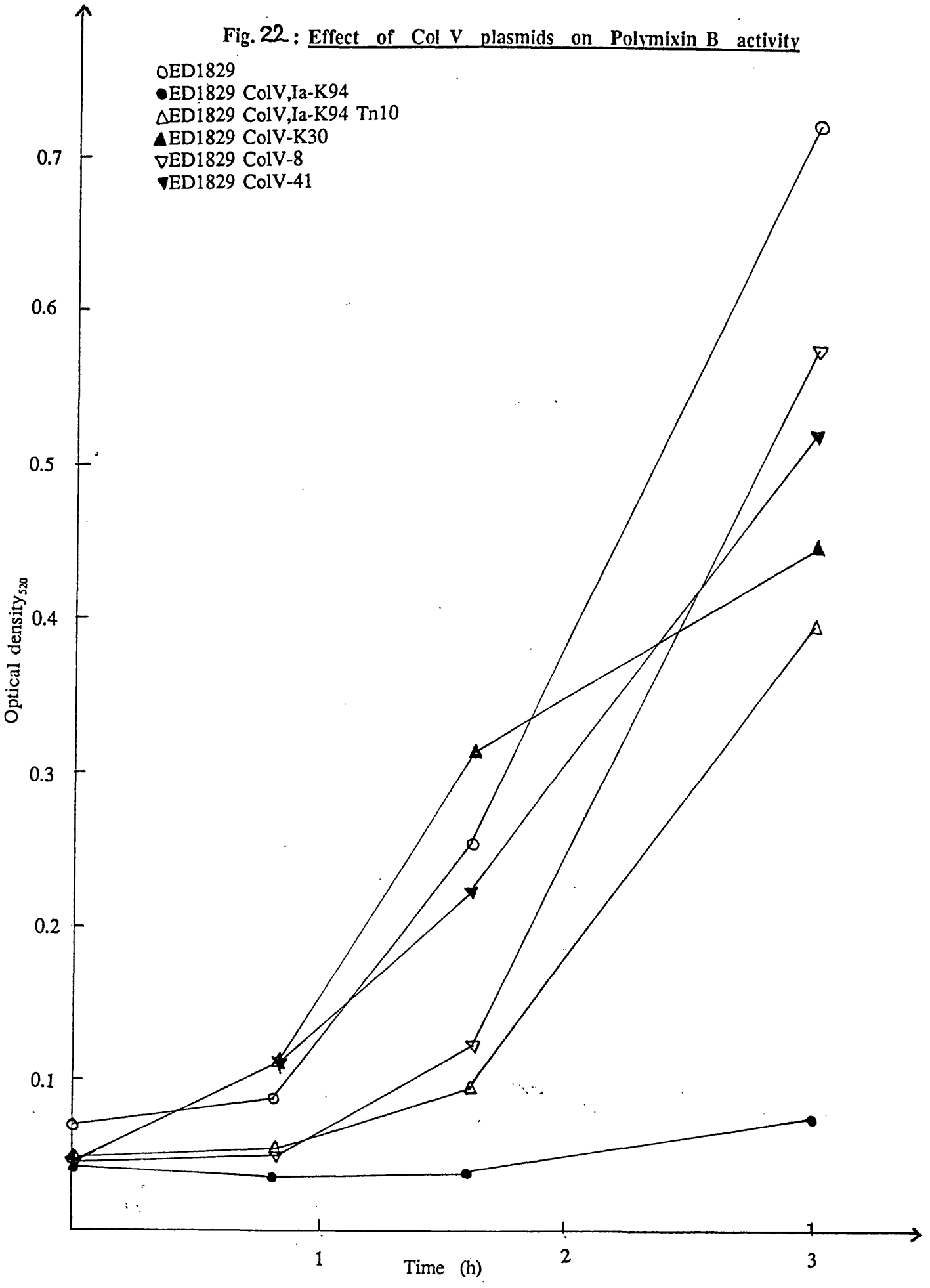
The inability of ColV-41 to enhance polymyxin B sensitivity was surprising as with most of the other agents tested so far ie. erythromycin, rifampicin, rifamycin, novobiocin and gentamicin, ColV41 has consistently conferred high sensitivity on its host strain. ColV-K30 has consistently conferred no effect on its host strain except in the case of novobiocin and rifampicin. Polymyxin B is known to bind the lipid A part of LPS and then to disrupt the outer membrane, thus gain access to the cytoplasmic membrane where its disrupting action leads to leakage of the cytoplasm and consequent cell death (Teuber, 1974; Storm *et al*, 1977). It seems possible that some property conferred by ColV-41 interferes with polymyxin B binding to lipid A. It is striking that with both the cationic agents used (gentamicin and polymyxin B), ColV,Ia-K94 confers markedly greater sensitivity than its transposon mutagenised derivative.

Table 11 : Col V plasmid effect on Polymyxin B sensitivity

STRAIN	% Inhibition (± Standard Error) by 0.15 μg/ml Polymyxin B
1829	10.53 ± 2.66
1829 Col VIa-K94	91.55 ± 8.72
1829 Col VIa-K94 Tn 10	59.07 ± 9.08
1829 Col V-K30	2.08 ± 2.38
1829 Col V-8	30.67 ± 6.12
1829 Col V-41	6.88 ± 1.67

The conditions of growth are given in the Methods and Materials section.

Fig. 22 : Effect of Col V plasmids on Polymixin B activity



3.1.7. Effect of Col V plasmids on Nalidixic acid sensitivity :

At 1.5 μg/ml concentration, plasmids ColV,Ia-K94 Tn10 and ColV-8 conferred a significant increase in nalidixic acid sensitivity whereas the plasmids ColV,Ia-K94, ColV-K30 and ColV-41 had no effect. At 2 μg/ml, plasmids ColV,Ia-K94 Tn10 and ColV-8 maintained the increased sensitivity effect that they conferred on their host ED1829 but plasmids ColV,Ia-K94, ColV-K30 and ColV-41 conferred resistance on their host to nalidixic acid (Table 12; Fig. 23).

At 1.5 μg/ml, the difference in the response to nalidixic acid sensitivity given by ColV,Ia-K94 and its derivative ColV,Ia-K94 Tn10 is interesting as otherwise with all the other antibacterials tested, these two plasmids have behaved in a similar fashion (but see response to gentamicin and polymyxin B). Nalidixic acid is an acidic and fairly hydrophobic 4-quinolone which most probably enters the cell via the hydrophobic or the self-promoted pathway. The insertion position of Tn10 transposon on ColV,Ia-K94 has not been determined yet but from this difference in response to nalidixic acid, it may be that Tn10 has inserted itself within or next to a region on ColV,Ia-K94 which influences the LPS component of the outer membrane as this component is involved in the working of the above mentioned pathway. Alternatively, the Tn10 element itself might alter the envelope so as to sensitise the strain to nalidixic acid.

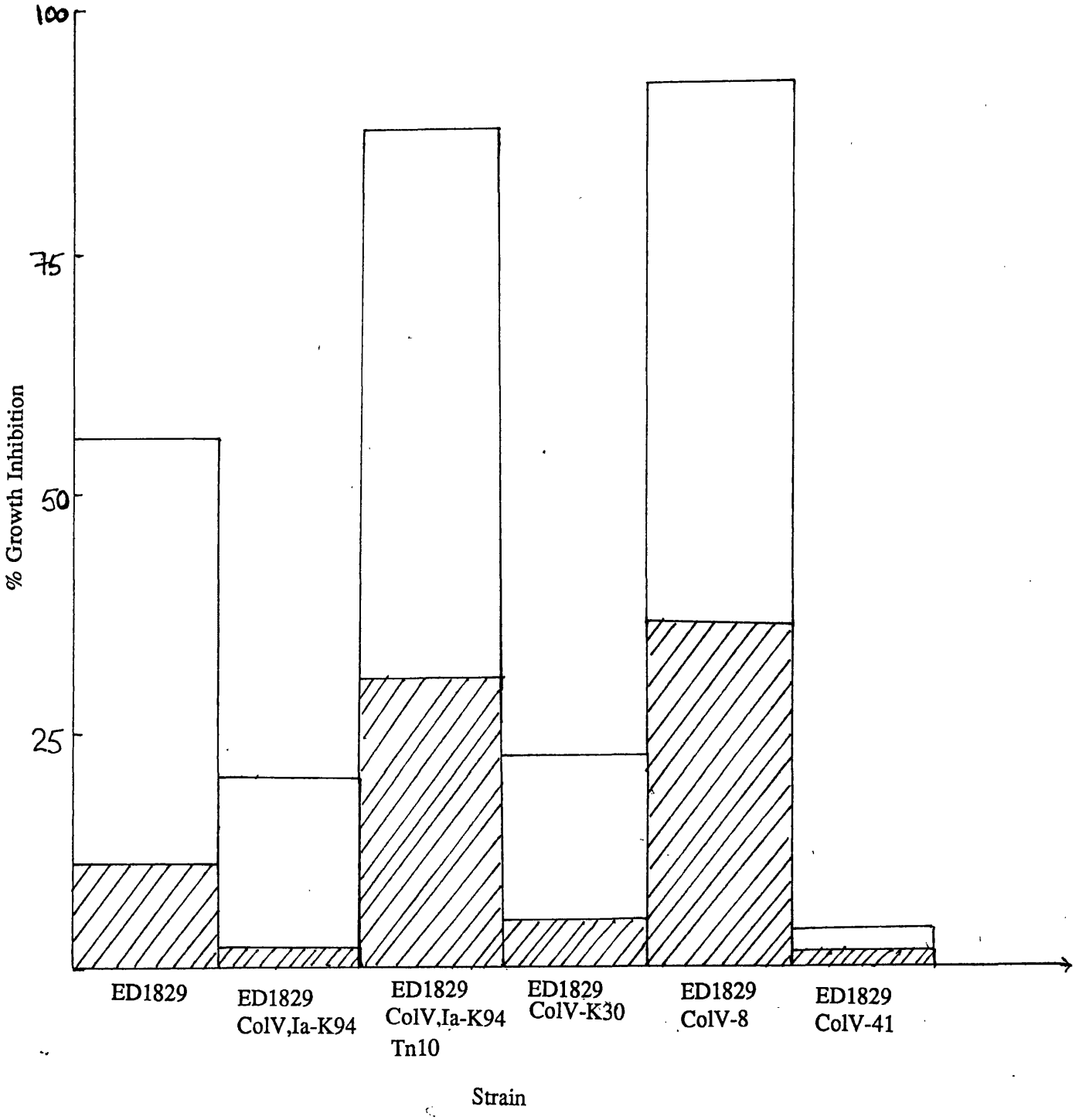
Table 12 : Col V plasmid effect on Nalidixic acid sensitivity in broth

STRAIN	% Inhibition (± Standard Error)	
	1.5 µg/ml	2 µg/ml
1829	18.73 ± 4.30	71.70 ± 1.61
1829 Col VIa-K94	3.08 ± 2.04	28.01 ± 3.08
1829 Col VIa-K94 Tn 10	46.85 ± 5.93	90.10 ± 2.00
1829 Col V-K30	7.36 ± 2.88	27.73 ± 3.81
1829 Col V-8	50.76 ± 1.61	89.05 ± 2.95
1829 Col V-41	2.35 ± 2.93	2.86 ± 5.67

The conditions of growth are given in the Methods & Materials section.

Fig. 23 : Effect of Col V plasmids on Nalidixic acid activity

▨ at 1.5 µg/ml Nalidixic acid.
▢ at 2.0 µg/ml Nalidixic acid.



3.1.8. Effect of Col V plasmids on Norfloxacin sensitivity :

None of the Col V plasmids tested (Table. 13;Fig. 24) had any effect on norfloxacin sensitivity of their host strain ED1829. norfloxacin is the most hydrophilic of the 4-quinolones used in the present work and it uses the porin pathway of entry into the cell. Apparently, it seems that the Col V plasmids do not affect the functioning of this pathway at least with respect to this agent.

3.1.9. Effect of Col V plasmids on Ciprofloxacin sensitivity :

Table 14 and Fig. 25 show ciprofloxacin sensitivity of ED1829 ± ColV plasmids. ColV-K30 and ColV-8 increase the sensitivity of ED1829 to ciprofloxacin significantly whereas ColV,Ia-K94, ColV,Ia-K94 Tn10 and ColV-41 had no effect. Ciprofloxacin is an amphoteric compound and may well use the porin pathway for entry as a major route. It is striking that the two plasmids which have least effect on the sensitivity to hydrophobic agents, sensitize the cell to ciprofloxacin. Perhaps these two plasmids encode a porin protein, or they could enhance the expression of an already existing one and thereby facilitate the entry of ciprofloxacin and consequently its inhibitory activity by just providing a larger number of sites for entry.

3.1.10. Effect of Col V plasmids on Ofloxacin sensitivity :

Only plasmid ColV-8 showed a marked increase in ofloxacin sensitivity (Table 15;Fig. 26). Ofloxacin is also an amphoteric compound but is slightly more hydrophobic than ciprofloxacin. Lack of any effect on ofloxacin sensitivity by the other four plasmids implies that ofloxacin may well not use the hydrophobic pathway at all, and cells containing ColV-8 appear to enhance ofloxacin uptake.

Table 13 : Col V plasmid effect on Norfloxacin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 0.01 µg/ml Norfloxacin
1829	6.86 ± 1.78
1829 Col VIa-K94	7.83 ± 2.46
1829 Col VIa-K94 Tn 10	10.80 ± 1.80
1829 Col V-K30	8.34 ± 1.60
1829 Col V-8	8.78 ± 2.45
1829 Col V-41	5.23 ± 2.48

The conditions of growth are given in the Methods & Materials section.

Fig. 24 : Effect of Col V plasmids on Norfloxacin activity

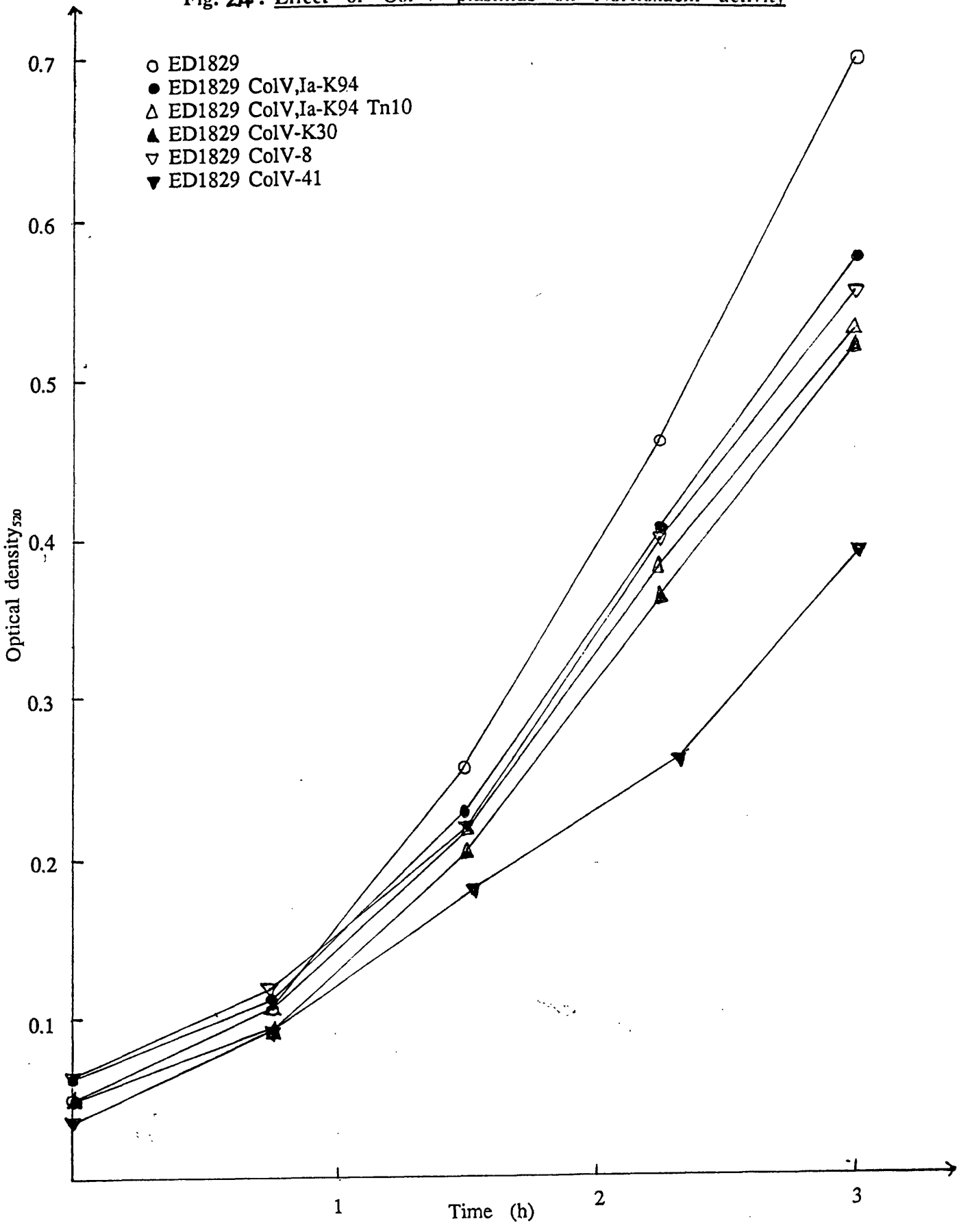


Table 14 : Col V plasmid effect on Ciprofloxacin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 0.005 µg/ml Ciprofloxacin
1829	16.61 ± 4.49
1829 Col VIa-K94	31.42 ± 4.20
1829 Col VIa-K94 Tn 10	27.51 ± 6.94
1829 Col V-K30	40.64 ± 3.63
1829 Col V-8	56.77 ± 7.86
1829 Col V-41	5.06 ± 1.80

The conditions of growth are given in the Methods & Materials section.

Fig. 25: Effect of Col V plasmids on Ciprofloxacin activity

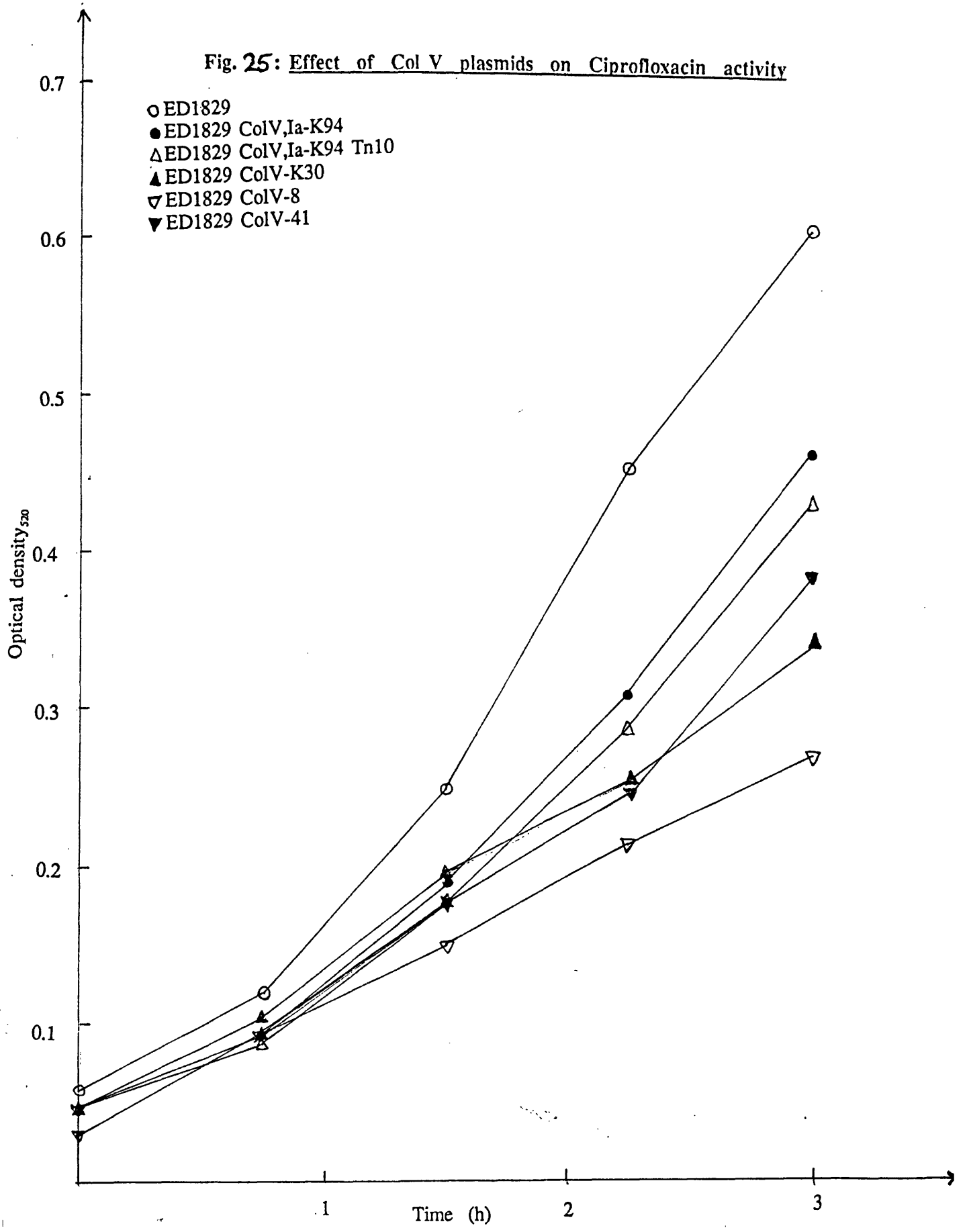
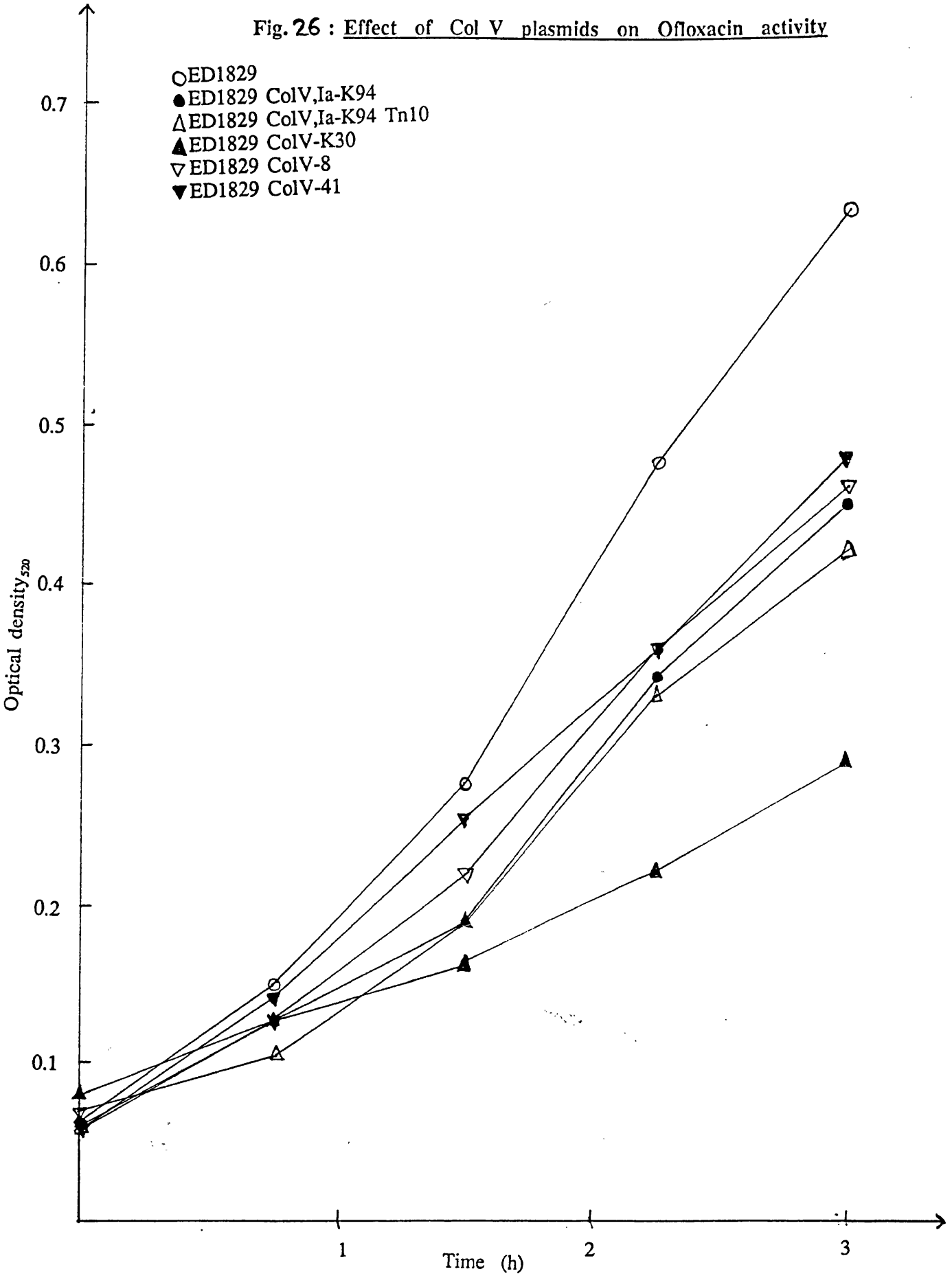


Table 15 : Col V plasmid effect on Ofloxacin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 0.02 µg/ml Ofloxacin
1829	9.14 ± 3.57
1829 Col VIa-K94	24.77 ± 2.33
1829 Col VIa-K94 Tn 10	28.86 ± 2.21
1829 Col V-K30	24.21 ± 2.70
1829 Col V-8	38.01 ± 1.83
1829 Col V-41	1.05 ± 1.83

The conditions of growth are given in the Methods & Materials section.

Fig. 26 : Effect of Col V plasmids on Ofloxacin activity



3.1.11. Effect of Col V plasmids on Pefloxacin sensitivity :

The pattern of pefloxacin sensitivity of the various Col V plasmids is identical to that for ofloxacin. Only ColV-8 was successful in conferring sensitivity on its host to pefloxacin (Table 16; Fig. 27). Plasmids ColV,Ia-K94, ColV,Ia-K94 Tn10 and ColV-K30 conferred a slight increase in sensitivity but this is not statistically significant. ColV-41 seems to confer resistance but again this is not statistically significant and higher concentrations of pefloxacin need to be tested to verify this potential effect.

3.1.12. Effect of Col V plasmids on Flumequin sensitivity :

ColV,Ia-K94, ColV,Ia-K94 Tn10 and ColV-8 conferred a significant increase in sensitivity of their host ED1829 to flumequin, the effect given by ColV-K30 is not statistically significant and ColV-41 conferred no effect at all (Table 17; Fig. 28). The outer membrane permeability differences caused by ColV,Ia-K94 and its derivative ColV,Ia-K94 Tn10 seem to be large enough for this hydrophobic quinolone to use to gain entry.

3.1.13. Summary of effects of ColV plasmids on 4-Quinolone sensitivity :

Table 18 shows a summary of 4-quinolone sensitivity of the five ColV plasmids tested. All plasmids except ColV-41 conferred sensitivity to a significant extent to ofloxacin and flumequin. These differences in sensitivity cannot be explained by proposing that the hydrophilic/amphoteric quinolones use the porin pathway and that the greater the hydrophobicity of the quinolone, the greater the extent to which it uses the hydrophobic pathway for entry into the cell. These compounds are not cationic, in fact nalidixic acid and flumequin are acidic, and therefore cannot be visualised as using the self-promoted pathway as it is proposed for the uptake of the cationic antibacterials such as gentamicin and polymyxin B.

Another possibility is that there is a novel mechanism for the entry of 4-quinolones associated with ColV plasmids, which may operate either specifically or non-

specifically. A possible non-specific loosening of the outer membrane due to the presence of the additional ColV-associated outer membrane-expressed proteins does not explain the indifference in nalidixic acid, norfloxacin, ciprofloxacin or pefloxacin sensitivities. However, a specific uptake route for ofloxacin and flumequin (probably not relating to their hydrophobicity;Table 4) may be present and if so, it appears to be well illustrated by the plasmid ColV-8. ColV-8 has shown consistent sensitivity to all of the quinolones except norfloxacin, and the recognition factor for the proposed pathway for these agents may be the 4-quinolone nucleus that all these agents have in common; however, norfloxacin would be an exception to this proposal.

Plasmid ColV-41 has shown consistent inability to sensitize ED1829 to all the 4-quinolones tested and this is in complete contrast to the severe sensitivity that it conferred on ED1829 to the larger hydrophobic agents erythromycin (Table 5), rifamycin (Table 6), rifampicin (Table 7) and novobiocin (Table 8), and to the cationic compounds gentamicin (Table 10) and bacitracin (Table 19). Of these latter antibacterials, plasmid ColV-8 succeeded in sensitising its host ED1829 to erythromycin (Table 5) and novobiocin (Table 8) only. From the effects of these two ColV plasmids on the sensitivity to the antibacterials tested here, there is evidence for the existence of at least one novel pathway in addition to the three already proposed ie. hydrophilic, hydrophobic and self-promoted pathways, which is very operational in ED1829 ColV-8 for the uptake of 4-quinolones but is absent from ED1829 ColV-41. Plasmids ColV-8 and ColV-41 were isolated originally from sewage water, and the responses to antibacterials observed here for strains harbouring these plasmids may be of significance in that environment but the fact these are seen in laboratory cultivated strains and in a rich medium (broth) suggests that they are not induced just by the presence of the agent in the medium.

Table 16 : Col V plasmid effect on Pefloxacin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 0.03 µg/ml Pefloxacin
1829	15.45 ± 3.32
1829 Col VIa-K94	28.19 ± 2.93
1829 Col VIa-K94 Tn 10	29.45 ± 6.83
1829 Col V-K30	27.31 ± 7.44
1829 Col V-8	50.38 ± 6.24
1829 Col V-41	8.32 ± 2.72

The conditions of growth are given in the Methods & Materials section.

Fig. 27 : Effect of Col V plasmids on Pefloxacin activity

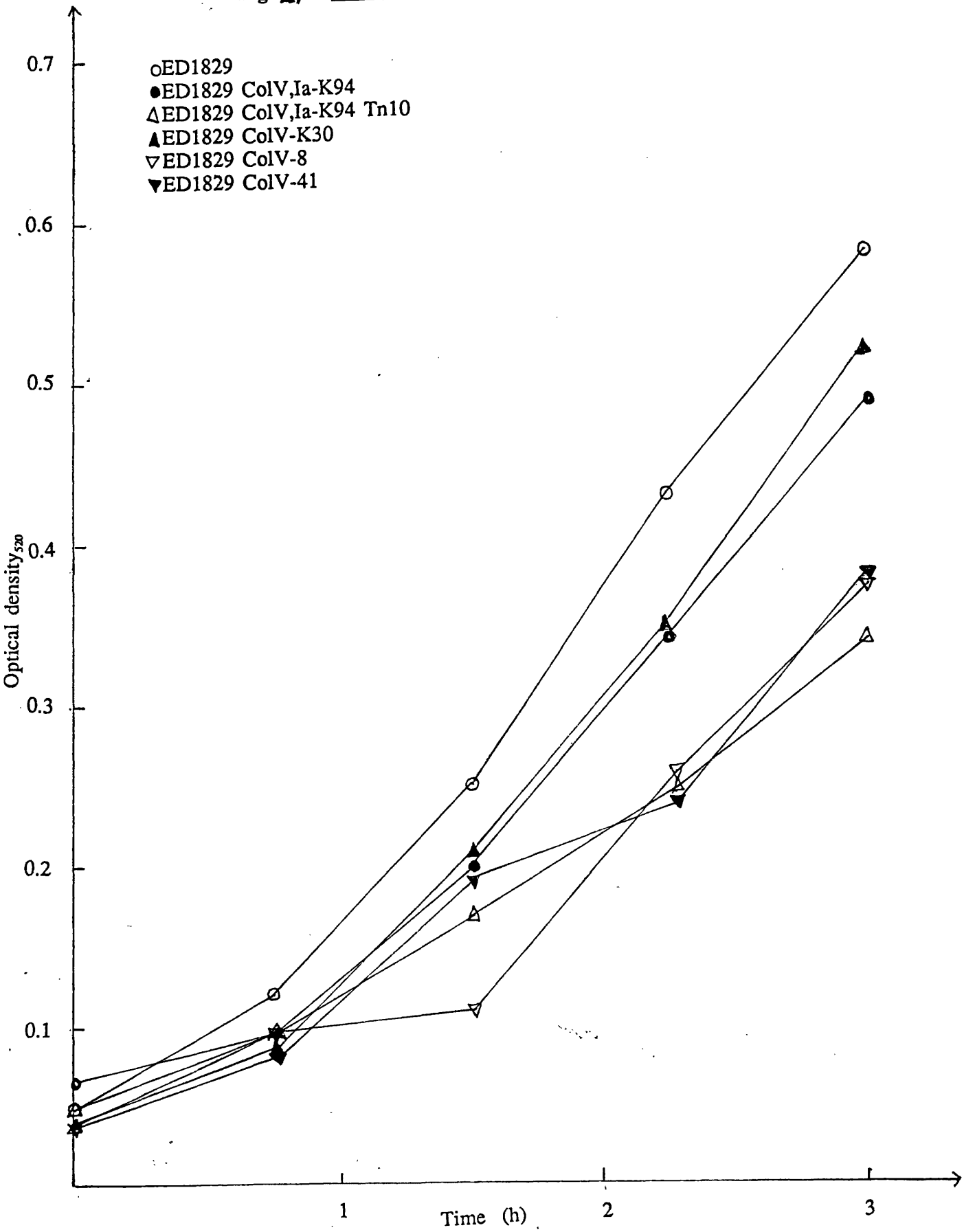


Table 17 : Col V plasmid effect on Flumequin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 0.03 µg/ml Flumequin
1829	7.85 ± 1.38
1829 Col VIa-K94	42.71 ± 6.96
1829 Col VIa-K94 Tn 10	42.51 ± 3.63
1829 Col V-K30	20.62 ± 7.29
1829 Col V-8	60.42 ± 7.69
1829 Col V-41	6.92 ± 3.01

The conditions of growth are given in the Methods & Materials section.

Fig. 28 : Effect of Col V plasmids on Flumequin activity

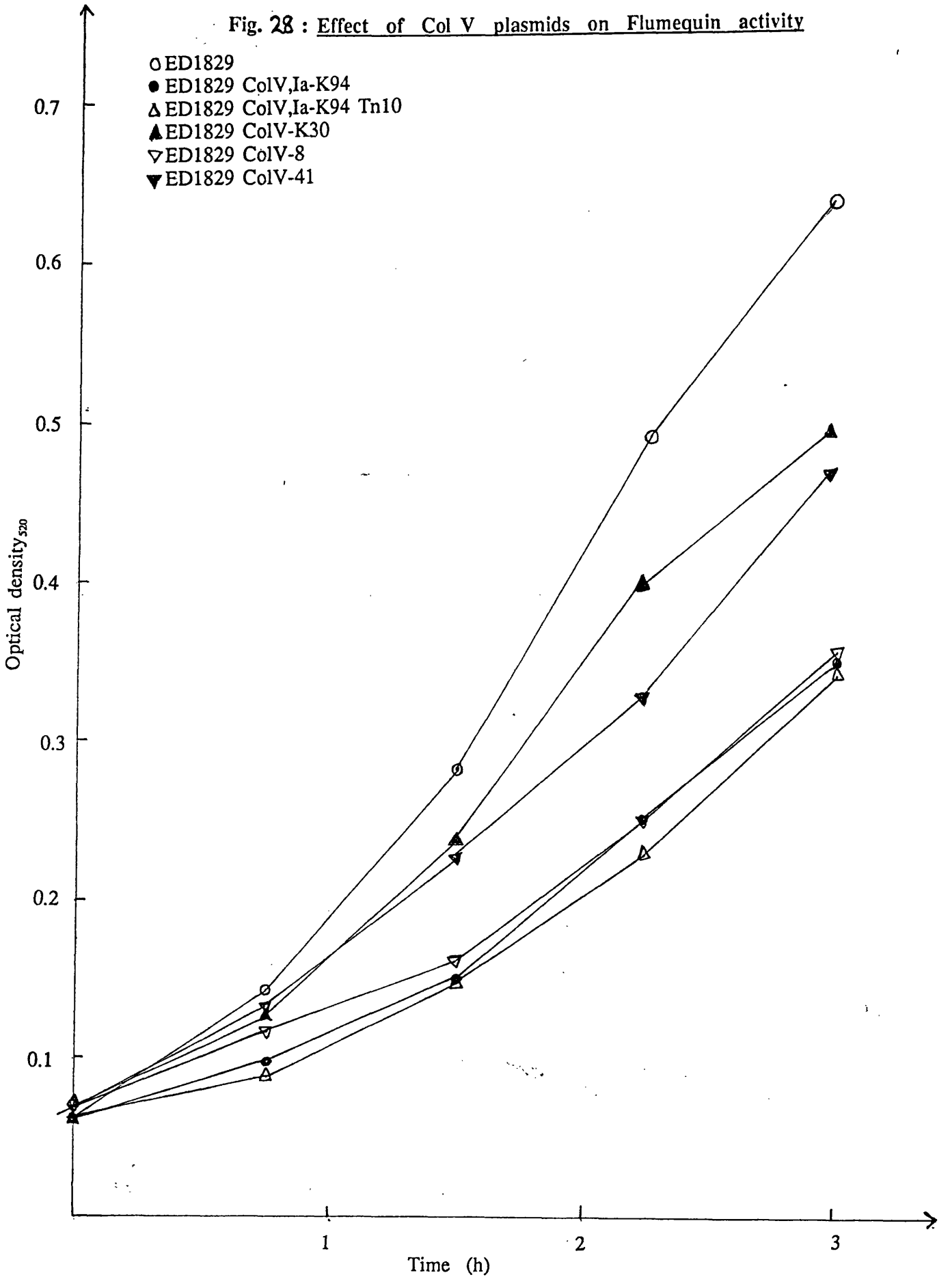


Table 18 : Effect of Col V plasmids on 4-Quinolone sensitivity

4-Quinolone (concentration) (µg/ml)	Plasmid				
	ColV,Ia-K94	ColVIa-K94 Tn10	ColV-K30	ColV-8	ColV-41
Nalidixic Acid (1.5)	-	+	-	+	-
(2.0)	R	+	R	+	R
Norfloxacin (0.03)	-	-	-	-	-
Ciprofloxacin (0.005)	-	-	+	+	-
Ofloxacin (0.02)	+	+	+	+	-
Pefloxacin (0.03)	-	-	-	+	-
Flumequin (0.3)	++	++	+	+++	-

The inhibition of growth is given as compared to that for the control strain ED1829 in broth by the factor of 1 (-), 2-4 (++) or 6(+++). R indicates resistance as compared to the control strain which does present some basal level of sensitivity to the agent.

3.1.14. Effect of Col V plasmids on Bacitracin sensitivity

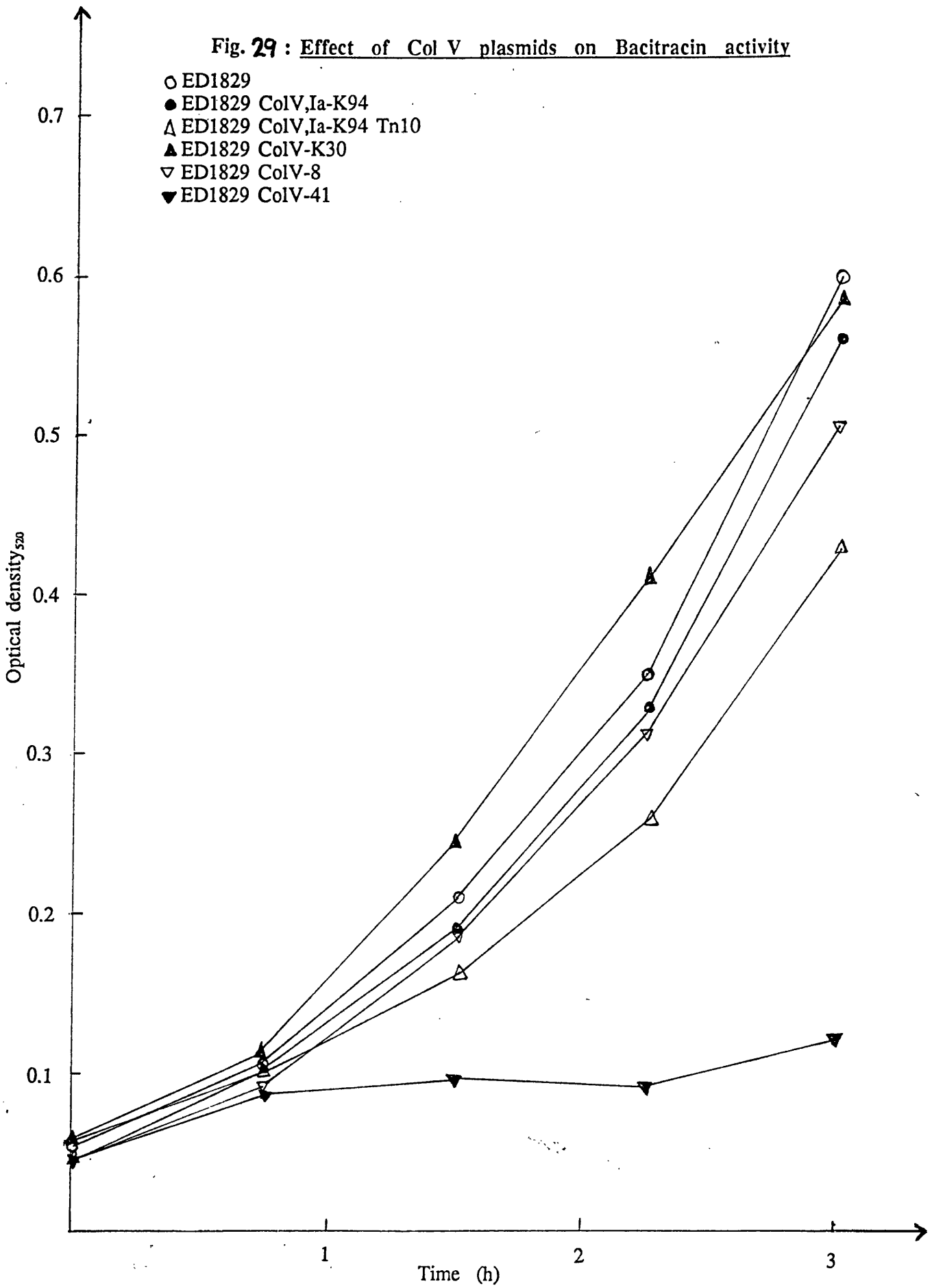
ColV-41 is the only plasmid that succeeded in conferring a high sensitivity on ED1829 to bacitracin, the plasmids tested ie. ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30 and ColV-8 conferred no effect on ED1829 with respect to bacitracin sensitivity. Bacitracin is a polypeptide antibiotic which inhibits cell-wall synthesis. It is most effective versus Gram-positive bacteria but usually not at all versus Gram-negative ones (Table 19; Fig. 29). The effect of ColV-41 confirms that this plasmid actively sensitises strain ED1829 to hydrophobic agents except for hydrophobic 4-quinolones.

Table 19 : Col V plasmid effect on Bacitracin sensitivity in broth

STRAIN	% Growth Inhibition (± Standard Error) by 250 μg/ml Bacitracin
1829	29.76 ± 0.81
1829 Col VIa-K94	28.07 ± 6.52
1829 Col VIa-K94 Tn 10	31.77 ± 3.25
1829 Col V-K30	20.69 ± 5.04
1829 Col V-8	27.78 ± 6.40
1829 Col V-41	89.82 ± 7.06

The conditions of growth are given in the Methods & Materials section.

Fig. 29 : Effect of Col V plasmids on Bacitracin activity



3.2. Basis of increased antibiotic sensitivity by ColV,Ia-K94

As seen from section 3.1. ColV,Ia-K94 makes its host strain ED1829 more sensitive to many hydrophobic antibacterial agents. To examine the basis of this increased sensitivity to antibiotics ie. to locate which plasmid-determined components are involved, the following experiments were performed. Firstly strains carrying pooled Col V-components cloned on vector plasmids were tested ie. carrying pCR612 or pKF40 with ColV-M405, which is a mutant derivative of ColV,Ia-K94 unable to produce colicin but capable of undergoing conjugational transfer and therefore able to confer transfer components. Secondly, by studying the response of derivatives of ED1829 containing ColV-M405 or ColV-M501 (another mutant derivative of ColV,Ia-K94 but one which produces colicin and is transfer deficient). ColV-M405 and ColV-M501 are incompatible and so cannot be pooled together in the same cell.

3.2.1. Involvement of colicin and transfer components :

(a) Using cloned colicin components :

The antibiotics tested were erythromycin and rifampicin. The presence of colicin V and immunity to colicin components (on pCR612 & pKF40) was found to confer no effect on erythromycin sensitivity (Tables 20 & 22) or on rifampicin sensitivity (Tables 21 & 23) of the sets of strains tested. However, the presence of transfer components (on M405) correlated with enhanced rifampicin sensitivity of the various host strains harbouring this plasmid but conferred essentially no effect on erythromycin sensitivity of the host strains in either set.

When the sensitivity of the strains containing both colicin V (& immunity) and transfer components was tested to inhibition by erythromycin and rifampicin, the response was at a similar level to that given by the isogenic host strain harbouring M40 which confers only the transfer components. The exception to this is the strain W3110 (in set 2) which appears to be inherently more sensitive to erythromycin and the presence of any of the plasmids used conferred no significant effect on its sensitivity to this antibiotic. Lastly, the level

of enhancement of rifampicin sensitivity by transfer components in both sets of strains does not reach that exhibited by the whole intact plasmid ColV,Ia-K94.

The failure of ColV-M405 plus pKF40 or pCR612 to enhance erythomycin seniivity might suggest that transfer and colicin components are not needed for enhancement. Since ColV-M405, however, contains nearly all the ColV,Ia-K94 genome except for the colicin region, it is more likely that for sensitivity, either the colicin and transfer components need to be together consequtively on the same replicon as they are on ColV,Ia-K94 (Fig. 9), or that production of the ColV-M405 derivative also deleted DNA encoding for another component (other than colicin components) essential for sensitivity which may act on its own to sensitize or with transfer (and, possibly colicin) components. The study of rifampicin sensitivity implicates transfer components but another factor (other than transfer or colicin components) may be implicated.

The nature of the third factor can be either specific eg. it may be a receptor which would be required to be present on the outer membrane , the prime candidate for this being the ColV,Ia-K94-associated major outer membrane protein VmpA; or, it may be non-specific but apparently succeeds in destabilising the outer membrane such that large molecules such as erythromycin and rifampicin can gain their way into the envelope. The possible involvement of VmpA protein in this context is investigated in the following section as it is expressed by one of the ColV,Ia-K94 derivatives used to provide colicin V components. The non-specific destabilisation of the outer membrane suggested above is investigated in section 3.2.2.

Table 20 : Erythromycin sensitivity of Set 1 strains in broth

STRAIN	% Growth Inhibition (± Standard Error) by 15 µg/ml Erythromycin
P678-54	41.30 ± 2.02
P678-54 M405	54.88 ± 4.16
P678-54 pCR612	40.87 ± 2.37
P678-54 ColVIa-K94	70.00 ± 4.79
ED1829	52.38 ± 2.03
ED1829 M405	60.60 ± 3.31
15-P678-54-pCR612 M405	54.45 ± 2.68
ED1829 ColVIa-K94	77.29 ± 4.42
ED1829 R483 colIa	57.26

The conditions of growth are given in the Methods & Materials section.

Table 21 : Rifampicin sensitivity of Set 1 strains in broth

STRAIN	% Growth Inhibition (± Standard Error) by 15 μg/ml Rifampicin
P678-54	25.62 ± 1.06
P678-54 M405	47.34 ± 2.32
P678-54 pCR612	26.61 ± 0.75
P678-54 ColVIa-K94	81.80 ± 4.55
ED1829	28.97 ± 2.03
ED1829 M405	46.53 ± 2.34
15-P678-54-pCR612 M405	43.90 ± 3.45
ED1829 ColVIa-K94	51.48 ± 5.56
ED1829 R483 colIa	26.92

The conditions of growth are given in the Methods & Materials section.

Table 22 : Erythromycin sensitivity of Set 2 strains in broth

STRAIN	% Growth Inhibition (± Standard Error) by 15 μg/ml Erythromycin
P678-54	32.58 ± 2.43
P678-54 M405	48.63 ± 3.92
P678-54 ColVIa-K94	73.30 ± 0.85
W3110	61.72 ± 2.27
42-W3110 M405	69.95 ± 1.47
24-W3110 ColVIa-K94	61.49 ± 4.30
1401 pKF40	53.03 ± 3.57
37-1401 pKF40 M405	64.58 ± 2.48

The conditions of growth are given in the Methods & Materials section.

Table 23 : Rifampicin sensitivity of Set 2 strains in broth

STRAIN	% Growth Inhibition (± Standard Error) by 15 µg/ml Rifampicin
P678-54	29.64 ± 2.69
P678-54 M405	65.97 ± 6.83
P678-54 ColVIa-K94	82.35 ± 5.00
W3110	40.86 ± 6.52
42-W3110 M405	73.78 ± 7.32
24-W3110 ColVIa-K94	84.02 ± 5.81
1401 pKF40	32.59 ± 3.10
37-1401 pKF40 M405	67.36 ± 7.31

The conditions of growth are given in the Methods & Materials section.

3.2.1(b). Using two mutant derivatives of ColV,Ia-K94 :

Plasmids ColV-M405 and ColV-M501 are two derivatives of ColV,Ia-K94. ColV-M405 is able to undergo conjugational transfer but does not produce colicin or immunity components. ColV-M501 however, is transfer deficient but produces colicin and immunity components. The growth response of strains carrying these two mutant derivatives to the presence of a range of hydrophobic and other antibacterials was tested. Sensitivity to erythromycin, rifampicin, nitrofurantoin, gentamicin, novobiocin and nalidixic acid is considerably enhanced almost equally well by both plasmids (Table 24). This response is similar to that given by the parent plasmid ColV,Ia-K94 (section 3.1.).

However, the response to the fluorinated 4-Quinolones is variable as compared to that given by the parent plasmid. ColV,Ia-K94 increased the sensitivity of its host strain ED1829 to flumequin but not significantly to either norfloxacin, ciprofloxacin, ofloxacin or pefloxacin, and in addition conferred resistance to Nalidixic Acid. Both ColV-M405 and ColV-M501 sensitised their host ED1829 to pefloxacin but only ColV-M405 succeeded in increasing sensitivity to norfloxacin and ofloxacin; both conferred no effect on either ciprofloxacin or flumequin sensitivity.

The route of entry used by the 4-quinolones has been found to be linked to their hydrophobicity and charge (Table 4; Hiarai *et al*, 1986(a)), with the hydrophilic ones using the porins as a major route but the hydrophobic ones opting more for one of the other two routes for entry into the cell. A threshold effect is demonstrated by the plasmid ColV-M501 and pefloxacin appears to be at the critical stage in its hydrophilicity and charge properties so as to gain excess into the cell. Also, colicin V and immunity components may also be involved in the uptake of pefloxacin. Clearly, the transfer components are involved in the uptake of the less hydrophobic agents ofloxacin and norfloxacin. The factor influencing flumequin sensitivity in the parent plasmid appears to be absent from both of these derivatives. From the results

obtained in this section, it seems that the plasmid ColV,Ia-K94 has a rather integrated genome regulatory machanism as separation of its genotypic characters does not reflect its phenotype. Or it could be that the antibacterial agent sensitivity changes associated with its presence are due to a general metabolic or growth change in the cell, or that the component influencing the antibacterial agent sensitivities overlaps with the transfer and the colicin genes and hence its expression is disrupted in any attempt to separate the two. To investigate this latter possibility it may require the contruction of a gene library of the whole plasmid, its genetic mapping in terms of restriction enzyme maps, and scanning of the fragments with respect to the various phenotypes exhibited by the plasmid. The genes for colicin V and Ia, their respective immunity components and those responsible for serum resistance have been located on the plasmid however a whole area, excluding the transfer operon has not been looked at yet.

Table 24 : Effect of M405 and M501 on antibacterial agent sensitivity of ED1829 in broth

Antibacterial Agent (concentration)	% Growth Inhibition (7 h)			
	ED1829	ED1829 ColV,Ia-K94	ED1829 ColV-M405	ED1829 ColV-M501
Erythromycin (5 µg/ml)	45.2	68.9	68.0	70.1
Rifampicin (5 µg/ml)	57.4	77.0	82.7	88.0
Nitrofurantoin (10 µg/ml)	34.0	55.1	59.3	51.4
Gentamicin (0.25 µg/ml)	25.0	80.8	68.8	77.7
Novobiocin (50 µg/ml)	22.3	87.7	100	100
Nalidixic Acid (3 µg/ml)	41.7	50.5	94.7	71.0
Norfloxacin (0.03 µg/ml)	60.8	43.4	87.6	60.4
Ciprofloxacin (0.01 µg/ml)	21.5	50.8	27.4	22.5
Ofloxacin (0.04 µg/ml)	29.5	32.3	87.1	24.4
Pefloxacin (0.04 µg/ml)	22.1	45.5	79.4	51.6
Flumequin (0.3 µg/ml)	9.3	17.8	13.1	4.1

The conditions of growth are given in the Materials & Methods section.

3.2.1. Col V associated envelope changes in ED1829 ColV,Ia-K94 :

Increased antibacterial agent sensitivity conferred by ColV,Ia-K94 is apparently by a change in the outer membrane permeability. To examine whether the plasmid affects the distribution of the normal outer membrane components and thereby renders the structure of the outer membrane or its components defective, the following experiments were carried out. Col V plasmids may affect the quality or the quantity of any of the three major constituents of the outer membrane ie. proteins, LPS or the phospholipids. Some types of LPS lesions appear to alter the outer membrane such that appreciable amounts of phospholipids are present in the outer leaflet. In this situation, phospholipid should be accessible to externally added phospholipase and the amount of accessible phospholipid can be used as a measure of the difference in the outer leaflet composition of plasmidless control strain and the plasmid-containing derivative.

(a) Quantitation of accessible Phospholipid in whole cells

Release of label ^{32}P from cultures of 1829 $\pm$ ColV-Ia-K94 & ColV-K30 were compared (Tables 25-27, Fig. 31) in the presence and absence of the enzyme Phospolipase C. Over a period of 3 h in the absence of enzyme, release of ^{32}P was found to be greater and at a higher rate for 1829 ColVIa-K94 and 1829 ColV-K30 than for 1829. When Phospholipase C was added to the cultures, the release of ^{32}P was found to increase slightly for 1829 but very little for 1829 ColV-k30 and not at all for 1829 ColVIa-k94. These results suggest that the presence of the ColV plasmids alters the outer leaflet of the outer membrane of strain 1829 such that the phospholipid from the inner leaflet leaks out into the outer leaflet and is released. Normally, the outer leaflet contains LPS as its major component and outer membrane proteins which may either be exclusively in the outer leaflet or may span both the leaflets . The leakage of the phospholipid to the outer leaflet implies a defect in the structure of the outer leaflet and this may be due to an alteration in either the quality or the quantity of the outer leaflet

components. The ColV plasmids are known to encode a lot of proteins some of which are present in the outer membrane eg. colicin and immunity to colicin components, transfer components and some others whose function(s) is(are) not yet determined. Hence, LPS and proteins of the outer membrane need to be further investigated (see following sections).

Table 25 : Release of ^{32}P from ED1829

Time (h)	% label released /ml cells	
	No Phospholipase	+ Phospholipase
0.0	3.53	4.11
1.0	4.64	5.56
1.5	4.78	5.75
2.5	5.02	5.66
3.0	5.98	6.23

Conditions of growth are given in the Material & Methods section.

Table 26 : Release of ^{32}P from ED1829 ColV,Ia-K94

Time (h)	% label released / ml cells	
	No Phospholipase	+ Phospholipase
0.0	4.50	4.35
0.5	5.94	6.04
1.0	6.37	6.43
1.5	6.80	7.02
2.5	7.39	7.11
3.0	8.42	8.31

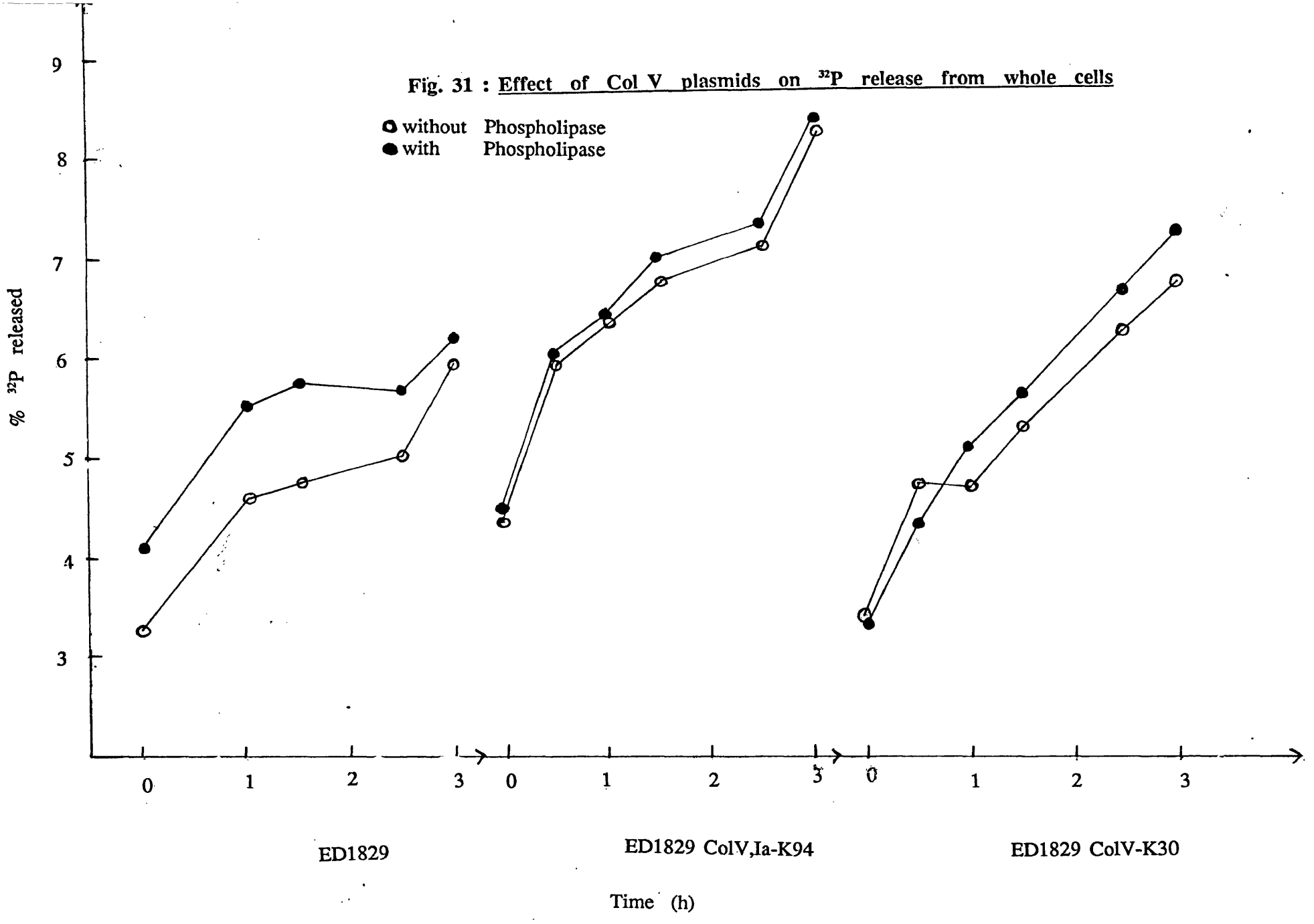
Conditions of growth are given in the Materials & Methods section.

Table 27 : Release of ^{32}P from ED1829ColV-K30

Time (h)	% label released / ml cells	
	No phospholipase	+ Phospholipase
0.0	3.38	3.33
0.5	4.74	4.35
1.0	4.67	5.11
1.5	5.30	5.65
2.5	6.25	6.67
3.0	6.67	7.28

Conditions of growth are given in the Materials & Methods section.

Fig. 31 : Effect of Col V plasmids on ^{32}P release from whole cells



3.2.2.(b). Effect of Col V plasmids on Outer membrane protein content

To determine whether any of the Col V plasmid associated changes in cell permeability are due to a change in the protein content of the ColV-harbouring cells, the outer membrane protein content was compared (Table 28).

Presence of plasmids ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30 and ColV-8 was found to correlate with an increase in the outer membrane protein content by a factor of at least 7 and reached 10 for ColV,Ia-K94 Tn10. The results presented in Table 28 are from one experiment but this trend has been observed at least on three independent observations. Plasmid ColV-41 was found to be the exception as it increased the protein content by a factor of 2, which when statistically analysed is not significant. This effect was unexpected as ColV-41 was found to consistently sensitise its host ED1829 to a range of large hydrophobic antibacterials tested in section 3.1. eg. erythromycin, rifampicin,etc. that ColV,Ia-K94 also conferred sensitivity to, and in addition ColV-41 also conferred sensitivity to agents like rifamycin and bacitracin towards which none of the other plasmids tested had any effect.

Hence, ColV-41 would be an exception to any proposal put forward to account for ColV-associated increased outer membrane permeability mediated due to increased outer membrane protein content. ColV-41 mediated increased permeability may involve an alteration in the LPS and/or phospholipid structure/composition or distribution or it may confer a specific receptor or pore. As seen from section 3.2.2.(a), the rate of labelled phosphate (which is presumably outer membrane phospholipid) release from ColV-harbouring cells was higher than that from the isogenic plasmid-minus control strain, and hence the outer membranes of $ColV^+$ cells appears to be leaky. Any changes in the LPS of ColV-harbouring strain are investigated in the following section (3.2.2.(c)).

Table 28 : Effect of Col V plasmids on Protein content

STRAIN	Protein in outer membrane as % of total cell protein
1829	2.14
1829 ColVIa-K94	14.88
1829 ColVIa-K94 Tn10	21.01
1829 ColV-K30	16.40
1829 ColV-8	14.95
1829 ColV-41	5.93

Protein was extracted from exponentially growing cells in the case of outer membranes and the assay for both outer membrane and whole cells was carried out by the method of Markwell *et al*, 1978. Outer membrane protein is expressed as % of whole cell protein.

3.2.2.(c) Effect of Col V plasmids on LPS :

From the preceding sections (3.2.2.(a & b)), ColV-plasmid associated increased phospholipid release and outer membrane protein content (except ColV-41) was observed. To investgate whether these plasmids induce any changes in the LPS structure or composition, pure LPS from ED1829, ED1829 ColV,Ia-K94 and ED1829 ColV-K30 was prepared.

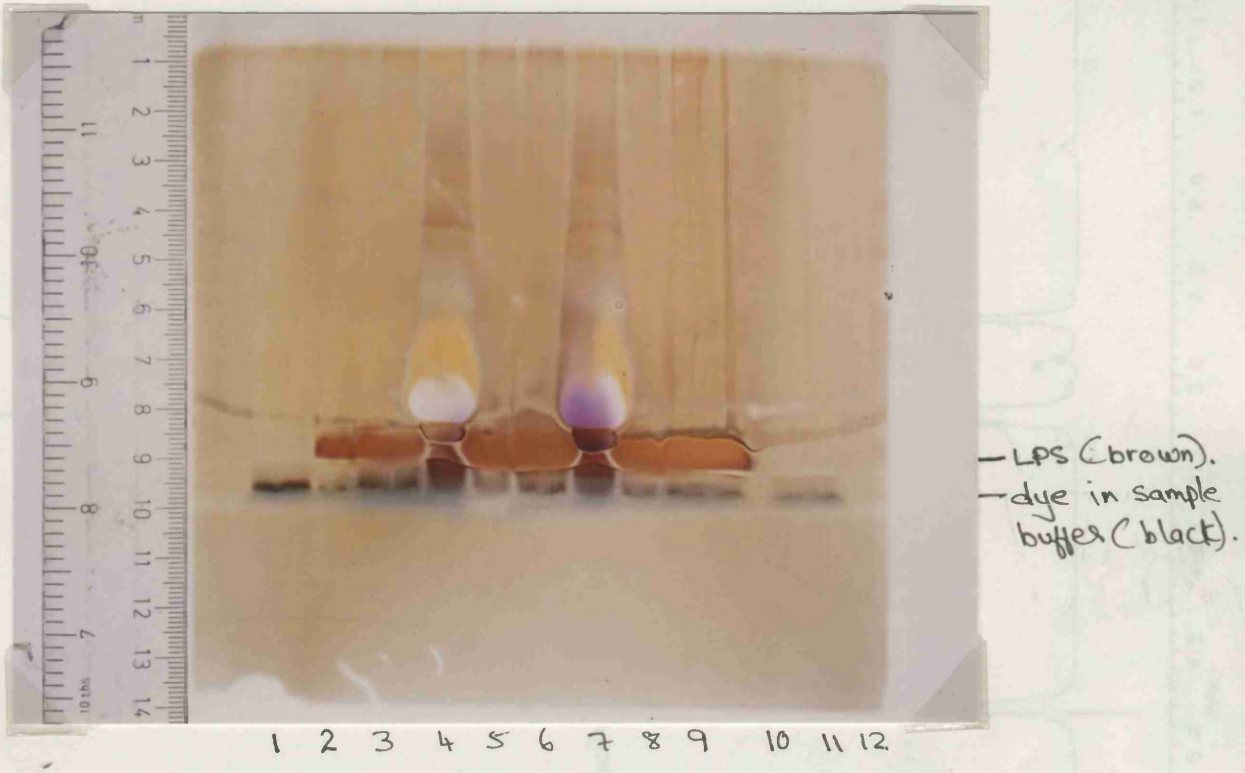
A simple but very significant observation made of the purified lyophilysed LPS from the three strains was that the LPS from ED1829 was very white and fluffy whereas that from ED1829 ColVIa-K94 although originally white and fluffy, upon storage at room temperature within 1 hour shrunk in volume, became grey and settled down to the bottom of the universal bottle containing it. LPS from ED1829 ColV-K30 behaved in an intermediate way. The only obvious explanation for this behaviour is that the ability of LPS to absorb moisture from the surrounding atmosphere was different. This shows that the presence of the colicin V producing plasmids does alter the LPS of its host strain ED1829 such that at least its hygroscopic properties are changed. This may be due either to a change in the conformation such that some additional charged groups are now exposed and so are available to form non-covalent bonds with other ionic molecules, or to a change in structure eg.increase/decrease in the number of KDO residues, Heptose content or the content of other sugars. If the change is structural then it may be reflected in the molecular weight of the LPS, although no major difference in the molecular weight of the three LPSs was observed when polyacrylamide gel electrophoresis (no SDS, except in the sample loading buffer) was carried out. The LPS was only visualised upon silver staining the gel (Fig. 32).

However, when the KDO content* of the pure LPS preparation was assayed then LPS from ED1829 ColV,Ia-K94 and ED1829 ColV-K30 was found to contain increased KDO by factors of 4 & 3 respectively. This is in accordance with the enhanced moisture absorbing ability of the LPS preparations from these two ColV-containing strains as each

additional KDO residue would contribute phosphate and carboxyl groups to the others already present on the LPS macromolecule, and therefore increase the total number of groups available for forming hydrogen bonds or ionic interactions. (* KDO content is shown in Table 29).

An attempt was also made to analyse the LPS preparations by 1HNMR spectroscopy to help elucidate any minor changes in the number or nature of chemical groups present on the LPS's from ED1829 and ED1829 ColV$^+$ strains. Fig. 32(a,b &c) shows the NMR spectrum of ED1829 LPS. LPS extracted from ED1829 ColV,Ia-K94 and ED1829 ColV-K30 failed to dissolve in any of the solvents (ethanol, methanol or DMSO) routinely used for sample preparation for this technique and could not be used for analysis. Some peaks on the chemical shift scale (parts per minute; ppm) can be attributed to certain chemical groups on the LPS : three peaks at 7.8-8.4 represent acidic protons ie. reflecting the presence of one hydrogen and one carbon atom between two oxygen atoms eg. a hydroxyl group; one peak at 2.8-3.0 indicates a possible phosphate group eg. $-CH_2-CH_2-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-OH$; peak at 2.6-2.4 indicates a possible acetyl group $CH_3\overset{\overset{X}{|}}{C}{=}{=}O$ eg. on N-acetylglucoseamine; peaks at 5.8-6.2 indicate six anomeric sugars, some of which are duplet signals representing two hydrogen atoms on two adjacent carbon atoms eg. on glucose, and one triplet signal which represents three hydrogens on two adjacent carbon atoms ie. two hydrogens are on one of the two carbons (instead of the carbon atom having one hydrogen atom and one hydroxyl group). This LPS preparation appears to be free from protein contamination as otherwise any aromatic groups of aromatic amino acids in the protein would be reflected by the presence of peaks in the region 6.8-7.8. The main peak at 3.0-3.4 may be due to ethanol which was used repeatedly during the final LPS purification steps (Edgar Anderson, UCL, personal communication).

Figure 32 : Silver stained LPS of ED1829, 1829 ColVIa-k94 & 1829 ColV-k30.



Wells are numbered left to right, 1 to 12 respectively.

(1) Protein standard
(2) 2 ug purified LPS strain ED1829
(3) 5 ug purified LPS strain ED1829
(4) sample buffer only
(5) 2 ug purified LPS strain ED1829 ColV,Ia-k94
(6) 5 ug purified LPS strain ED1829 ColV,Ia-k94
(7) sample buffer only
(8) 2 ug purified LPS strain ED1829 ColV-k30
(9) 5 ug purified LPS strain ED1829 ColV-k30
(10) Blank
(11) Protein standard
(12) Blank

Fig. 31(a) : 2HNMR spectrum of ED1829 LPS

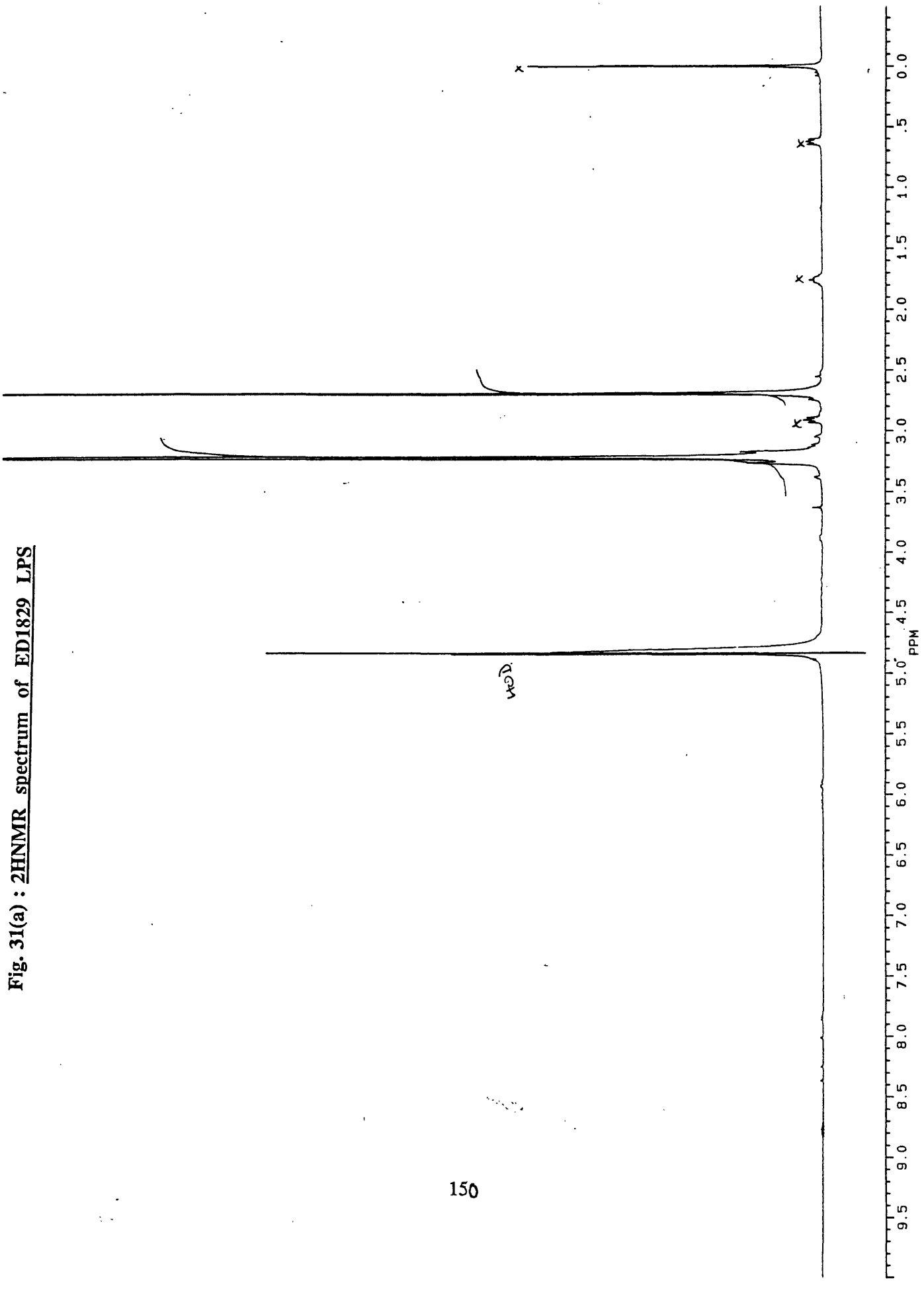


Fig. 31(b) : 2HNMR spectrum of ED1829 LPS

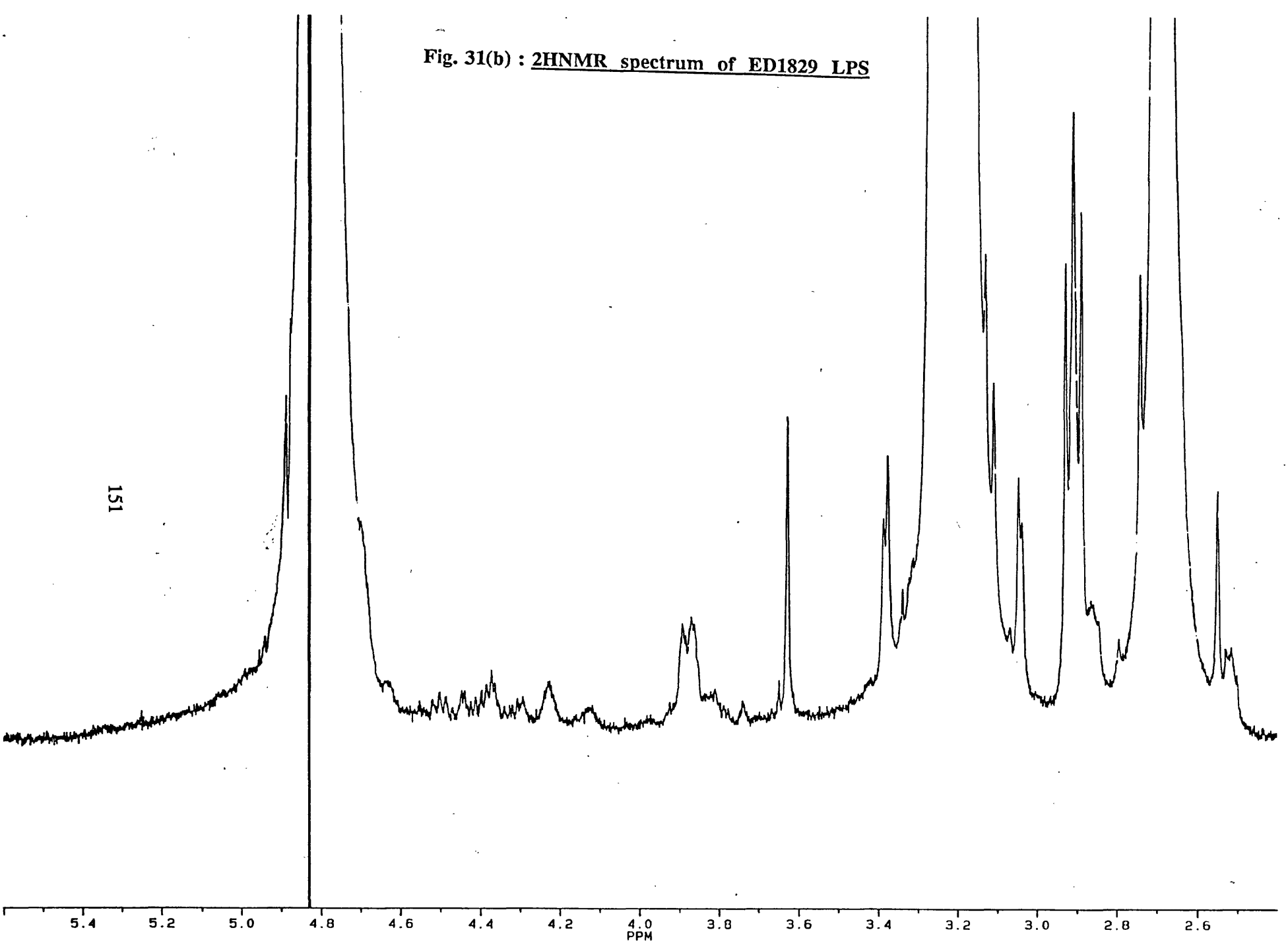


Fig. 31(c): NMR spectrum of ED829 LPS.

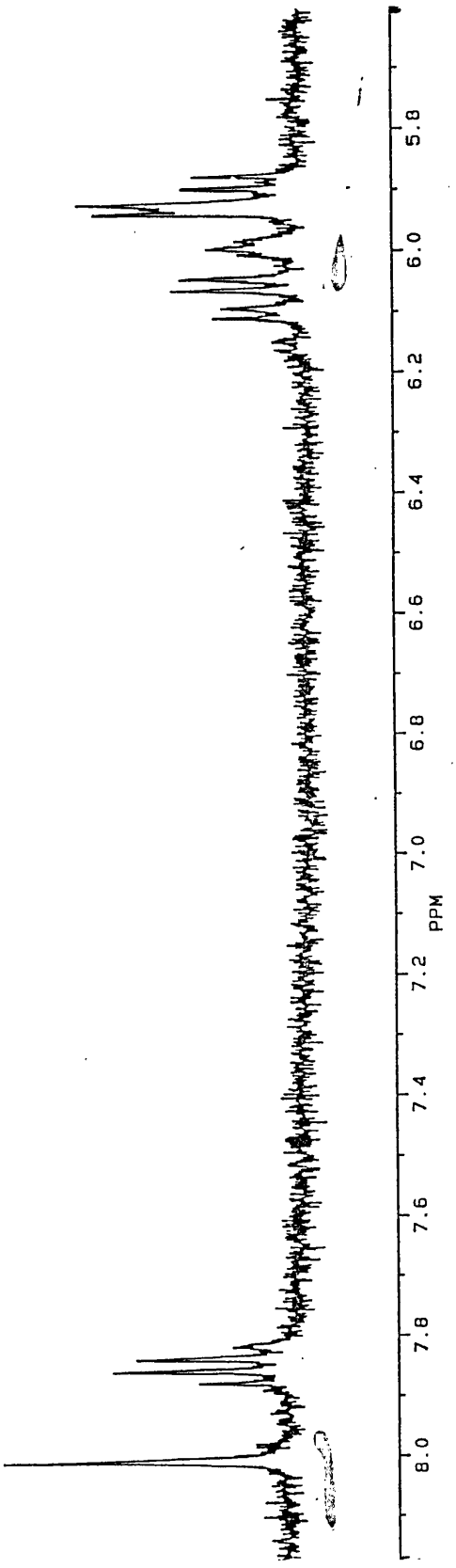


Table 29 : Effect of Col V plasmids on KDO content

Strain	Amount of KDO (μg/mg purified LPS)
ED1829	5.75
ED1829 ColV,Ia-K94	20.5
ED1829 ColV-K30	17.0

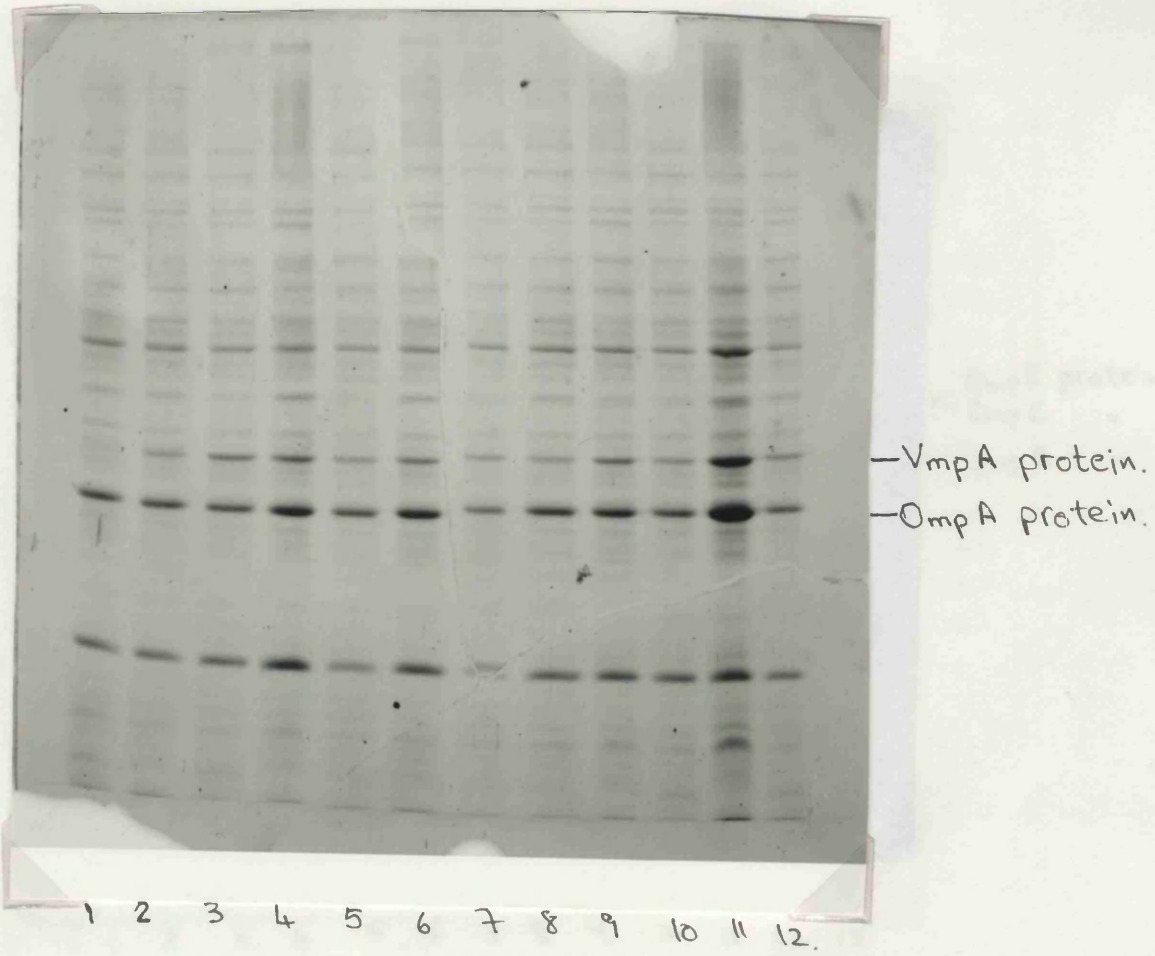
The conditions of assay are given in the Methods & Materials section.

3.3. Transposon Mutagenesis of ColV,Ia-K94 :

An attempt was made to mutagenise ColV,Ia-K94 using the transposons Tn1 and Tn7 in order to isolate mutants altered in the expression of primarily the VmpA protein and other ColV-associated properties.

Fig. 33 & 34 show SDS PAGE gels of the outer membrane proteins of Tn7 and Tn1 mutagenised strains. No obvious differences appear to be present as a result of either Tn1 or Tn7 insertion into the genome of ColV,Ia-K94. Table 30 shows the MS2 sensitivity profile of the various Tn1- and Tn7-mutant plasmid containing isolates. Loss of MS2 sensitivity for two Tn1-ColV,Ia-K94 isolates and three Tn7-ColV,Ia-K94 isolates was observed and this indicates at least a deficiency in the production of F-pili by the these isolates. Several genes are involved in the production of F-pili and further detailed analysis by complementation and restriction mapping should give more information on the sites of insertion of these two transposons in the ColV,Ia-K94-Tn1 and -Tn7 derivatives concerned. Further work relating to the inhibitor sensitivity profiles of these transposon derivatives may yield some linkage data between the various phenotypes associated with ColV,Ia-K94 eg. sensitivity to hydrophobic antibacterials, increased adherence, and serum resistance, although the genes for serum resistance and colicin production have already been mapped, they may serve as useful markers for the genome of ColV,Ia-K94.

Fig.33 : Outer membrane proteins of ColV,Ia-K94 Tn7 mutants



Wells are numbered from left to right, well number 1 to 12 respectively. (Samples were treated at 30°C for 15 min. before loading on gel)

(1) J53 Tn7
(2) J53 Tn7 ColV,Ia-k94 ::2
(3) J53 Tn7 ColV,Ia-k94 ::6
(4) J53 Tn7 ColV,Ia-k94 ::10
(5) J53 Tn7 ColV,Ia-k94 ::11
(6) J53 Tn7 ColV,Ia-k94 ::9
(7) J53 Tn7 ColV,Ia-k94::16
(8) J53 Tn7 ColV,Ia-k94::20
(9) J53 Tn7 ColV,Ia-k94::26
(10) J53 Tn7 ColV,Ia-k94::29
(11) J53 Tn7 ColV,Ia-k94::36
(12) J53 Tn7 ColV,Ia-k94::38

Fig. 34 : Outer Membrane Protein Composition

Wells are labelled left to right, 1-13 respectively.

(1) ED1829	30°C	(8) ED1829	100°C
(2) ED1829 ColV,Ia-K94	30°C	(9) ED1829 ColV,Ia-K94	100°C
(3) J53-47-Tn1-ColV,Ia-K94	30°C	(10) J53-47-Tn1-ColV,Ia-K94	100°C
(4) P678-54	30°C	(11) P678-54	100°C
(5) P678-54-47-1-Tn1-ColV,Ia-K94	30°C	(12) P678-54-47-1-Tn1-ColV,Ia-K94	100°C
(6) P678-54-47-2-Tn1-ColV,Ia-K94	30°C	(13) P678-54-47-2-Tn1-ColV,Ia-K94	100°C

Table 30 : MS2 sensitivity profile of Tn1 & Tn7 mutagenised ColV,Ia-K94 derivatives

Strain	MS2 sensitivity	Strain	MS2 sensitivity
J53 ColV,Ia-K94::Tn1-7	sens.	J53 ColV,Ia-K94::Tn7-2	resis.
J53 ColV,Ia-K94::Tn1-10	resis.	J53 ColV,Ia-K94::Tn7-6	sens.
J53 ColV,Ia-K94::Tn1-11	sens	J53 ColV,Ia-K94::Tn7-9	sens.
J53 ColV,Ia-K94::Tn1-12	sens.	J53 ColV,Ia-K94::Tn7-10	sens.
J53 ColV,Ia-K94::Tn1-13	sens.	J53 ColV,Ia-K94::Tn7-11	sens.
J53 ColV,Ia-K94::Tn1-14	sens.	J53 ColV,Ia-K94::Tn7-16	sens.
J53 ColV,Ia-K94::Tn1-15	sens.	J53 ColV,Ia-K94::Tn7-20	resis.
J53 ColV,Ia-K94::Tn1-16	sens.	J53 ColV,Ia-K94::Tn7-26	sens.
J53 ColV,Ia-K94::Tn1-17	sens.	J53 ColV,Ia-K94::Tn7-29	resis.
J53 ColV,Ia-K94::Tn1-18	sens.	J53 ColV,Ia-K94::Tn7-36	sens.
J53 ColV,Ia-K94::Tn1-25	sens.	J53 ColV,Ia-K94::Tn7-38	sens.
J53 ColV,Ia-K94::Tn1-27	sens.		
J53 ColV,Ia-K94::Tn1-30	sens.		
J53 ColV,Ia-K94::Tn1-33	sens.		
J53 ColV,Ia-K94::Tn1-34	sens.		
J53 ColV,Ia-K94::Tn1-37	sens.		
J53 ColV,Ia-K94::Tn1-38	sens.		
J53 ColV,Ia-K94::Tn1-41	sens.		
J53 ColV,Ia-K94::Tn1-42	resis.		
J53 ColV,Ia-K94::Tn1-47	sens.		

The conditions of growth and phage test are given in the Methods & Materials section. Abbreviations : sens., sensitivity; resis., resistance.

3.4. Effect of wildtype Col V plasmid on antibacterial agent sensitivity of laboratory E.coli strain HB101.

Presence of this wildtype plasmid Col-V2/5656 conferred resistance to the antibacterial agents erythromycin, rifampicin, gentamicin, novobiocin, ciprofloxacin and flumequin; but conferred sensitivity to the agents nitrofurantoin, norfloxacin, ofloxacin and pefloxacin (Tables 31 & 32). No difference in sensitivity to nalidixic acid was observed between the plasmid-less control strain HB101 and its Col-V2/5656^{+} derivative.

From the above antibacterial agents sensitivity profiles, it seems that the wildtype ColV plasmid makes its host strain resistant to hydrophobic agents but sensitises it to hydrophilic or amphoteric agents. This pattern is opposite to that obtained with the laboratory and sewage-isolated ColV plasmids. Increased sensitivity to hydrophilic compounds implies that the wild-type plasmid Col-V2/5656 confers a porin protein or that it may enhance the production or activity of an existing one such that the hydophilic compounds are taken up by the cell at a higher rate. The ability to take up more nutrients faster would obviously be advantageous to the pathogenic strain cell and if this is associated with a Col V plasmid, then the ColV-derivatives will be selected out at a faster rate. This selection may occur regardless of whether any other pathogenic characters such as increased adhesion to eukaryotic cells, serum resistance, iron-uptake system, etc. are also associated with the plasmid or not.

Resistance to the hydrophobic antibacterials may result from repressed transfer properties and would fall in line with the observation (this work & C.Reakes PhD thesis UCL) that the ColV plasmids from clinical samples and environmental sources eg. sewage water (S.Hicks PhD thesis, UCL) are very difficult to transfer into other strains by conjugation and when they do transfer then it is only at a very low frequency. This could result from the transfer operon being repressed as seen by the low or absent MS2 sensitivity in the presence of the plasmid (this work). Increased sensitivity to hydrophobic/cationic antibacterial agents is associated

with the presence of and full expression of the transfer operon eg. for ColV,Ia-K94, colicin V and its immunity components as well as some undefined factor and if these parts of the plasmid genome are repressed then so will the phenotypes associated with their expression.

3.5. Effect of laboratory plasmid ColV,Ia-K94 on wildtype E.coli strain 14.

The effect of plasmid ColV,Ia-K94 on the antibiotic sensitivity of a wild type strain 14 was investigated by growth curve analysis in microtitre plates for 7 h. Strain 14 is *E.coli* strain isolated from chickens. No difference in antibiotic sensitivity (Table 33) was observed in the control strain 14 and strain 14 ColV,Ia-K94 for the antibiotics erythromycin, rifampicin, nitrofurantoin, gentamicin or novobiocin at the concentrations used. Under similar conditions and at the given concentrations sensitivity of strain ED1829 to all the above mentioned antibiotics was significantly increased if it harboured the plasmid ColV,Ia-K94 (Section 3.1.).

From these results, the wild type strain 14 seems to be immune to the permeability changes induced by ColV,Ia-K94 in strain ED1829. This difference may reflect the difference(s) of the expression of plasmid ColV,Ia-K94 in the two strains. From section 3.2. it appears that the colicin V and Ia components as well as their corresponding immunity components are not substantially involved in conferring the antibiotic sensitising phenotype on the host strain of ColV,Ia-K94. Transfer components alone do increase the sensitivity of the host strain but not to the full extent of the whole intact plasmid. In strain 14, further investigation of how much of the plasmid genome is expressed needs to be determined so that by elimination of genes not involved in conferring this antibiotic sensitising effect, we can perhaps pinpoint which areas of the genome may be involved in strain ED1829. One proposal for the future would be to examine whether the transfer components are expressed eg. formation of pili and sensitivity to phage MS2.

Table 31 : Effect of a wildtype Col V on antibacterial agent sensitivity of laboratory strain HB101 in broth.

Antibiotic (concentration)	% Growth Inhibition (7 h)		
	HB101	HB101 plg338	HB101 plg338 Col V2/5656
Erythromycin (5 µg/ml)	51.30	53.10	23.70
Rifampicin (5 µg/ml)	40.80	31.00	-0.30
Nitrofurantoin (10 µg/ml)	11.6	10.50	40.70
Gentamicin (0.25 µg/ml)	25.90	27.40	8.40
Novobiocin (50 µg/ml)	11.20	9.50	1.10

The conditions of growth are given in the Methods and Materials section.

Table 32 : Effect of a wildtype Col V on 4-Quinolone sensitivity of strain HB101 in broth.

4-Quinolone (concentration)	% Growth Inhibition (7 h)		
	HB101	HB101 plg338	HB101 plg338 ColV2/5656
Nalidixic acid (3 µg/ml)	32.60	50.60	29.90
Norfloxacin (0.03 µg/ml)	37.60	43.80	75.50
Ciprofloxacin (0.01 µg/ml)	36.20	43.10	12.00
Ofloxacin (0.04 µg/ml)	42.90	32.00	72.00
Pefloxacin (0.04 µg/ml)	23.50	41.00	69.30
Flumequin (0.3 µg/ml)	28.00	22.50	3.00

The conditions of growth are given in the Methods and Materials section.

Alternatively, however strain 14 may have an altered envelope which is not affected by full Col V expression. Plasmid ColV,Ia-K94 expresses almost all the properties seen in Col V plasmids isolated from clinical infections such as increased adhesion to cell and other surfaces, cell surface hydrophobocity, serum resistance, and production of and immunity to colicin V. The above mentioned properties are related to increasing the pathogenicity of the host bacterial cell. However, ColV,Ia-K94 also confers increased sensitivity on its host strain to various environmental agents such as detergents like deoxycholate, low pH, high temperatures; and antibiotics such as erythromycin, rifampicin, novobiocin,etc. By testing the sensitivity to these antibiotics in a wild type strain containing ColV,Ia-K94, the aim was to see if these medically useful properties of ColV plasmids can be used to treat infections associated with their presence in the pathogenic strains of E.coli. However, it seems that although the sensitising effects have been demonstrated in many laboratory E.coli K12 strains they may not occur in clinical ones.

Table 33 : Effect of ColV,Ia-K94 on antibacterial agent sensitivity of strain 14 in broth.

Antibiotic (concentration)	% Growth Inhibition (7 h)	
	Strain 14	Strain 14 ColV,Ia-K94
Erythromycin (5 µg/ml)	55.50	56.10
Rifampicin (5 µg/ml)	94.90	91.30
Nitrofurantoin (10 µg/ml)	1.80	6.60
Gentamicin (0.25 µg/ml)	41.80	39.70
Novobiocin (50 µg/ml)	100	100

The conditions of growth are given in the Methods and Materials section.

3.6. Effect of cationic agents on Novobiocin sensitivity of strain ED1829.

(a) In Nutrient Broth :

Agents N-(2-pyrimidinyl)piperazine 2HCl (250 µg/ml), 4,5,6-triaminopyrimidine sulfate hydrate (600 µg/ml), 1,4-diaminopiperazine HCl (500 µg/ml), methylglyoxyl bis(guanyl-hydrazone) 2HCl (100 µg/ml; Table 34, Fig. 35,36), spermidine (250 µg/ml), agmatine sulfate (300 µg/ml), 1,3-diaminoacetone 2HCl hydrate (150 µg/ml), 3,3'-diaminobenzidine 4HCl dihydrate (200 µg/ml), pyridoxamine (450 µg/ml), 3-aminobenzimidine 2HCl (350 µg/ml; Table 35) and 1,4-diamino-2-butanone 2HCl (600 µg/ml; Table 36) were found to greatly enhance the inhibitory effect of novobiocin. For example, two of the most active of these agents, methylglyoxyl bis(guanyl-hydrazone) 2HCl and 1,4-diamino-2-butanone 2HCl produced no growth inhibitory effect alone but even a slight enhancement of growth whereas a very marked growth inhibition when added with novobiocin.

On the other hand the cationic agents, 1,3-diamino-guanidine 2HCl (300 µg/ml), triethylenetetramine 2HCl (400 µg/ml), triethylenetetramine hydrate (350 µg/ml; Table 36, Fig. 37), tetraethylenepentamine (200 µg/ml), diethylenetriamine (500 µg/ml), moroxydine HCl (450 µg/ml), N,N,N',N'-tetramethyl-p-phenylenediamine (200 µg/ml) and formamidine disulfide 2HCl (75 µg/ml;Table 37) were found not to enhance the inhibitory effect of novobiocin.

The enhancers of novobiocin activity were found to have no significant growth inhibitory activity at all on the strain ED1829. Compared to this, most of the non-enhancers, except moroxydine , did exhibit a basal level of growth inhibition even in the absence of novobiocin.

When cell viability was tested, cationic agents N(-2-pyrimidinyl)piperazine, triaminopyrimidine, diaminopiperazine, methylglyoxyl bis(guanyl-hydrazone), spermidine (Table 38), agmatine sulfate, diaminoacetone and diaminobenzamidine (Table 39) were found to significantly enhance inhibition by novobiocin of ED1829 on nutrient agar; and the cationic agents diaminoguanidine, moroxydine and triethylenetetramine failed to significantly affect novobiocin inhibition (Table 39).

Table 34 : Effect of cationic agents on Novobiocin (30µg/ml) sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	26.68 ± 1.57
N-(2-Pyrimidinyl) piperazine 2HCl (250)	7.58 ± 2.71	75.90 ± 3.17
4,5,6-Triamino- -pyrimidine sulfate hydrate (600)	9.05 ± 2.26	95.07 ± 1.88
1,4-Diaminopiperazine HCl (500)	6.99 ± 1.41	94.34 ± 2.77
Methylglyoxyl bis- (guanyl-hydrazone) 2HCl (100)	-2.81 ± 3.16	95.87 ± 1.56

The conditions of growth are given in the Methods & Materials section. Growth enhancement by agent is indicated by a minus sign before % Growth Inhibition.

Fig. 35: Effect of N(-2-pyrimidinyl)piperazine on Novobiocin activity on ED1829 in broth.

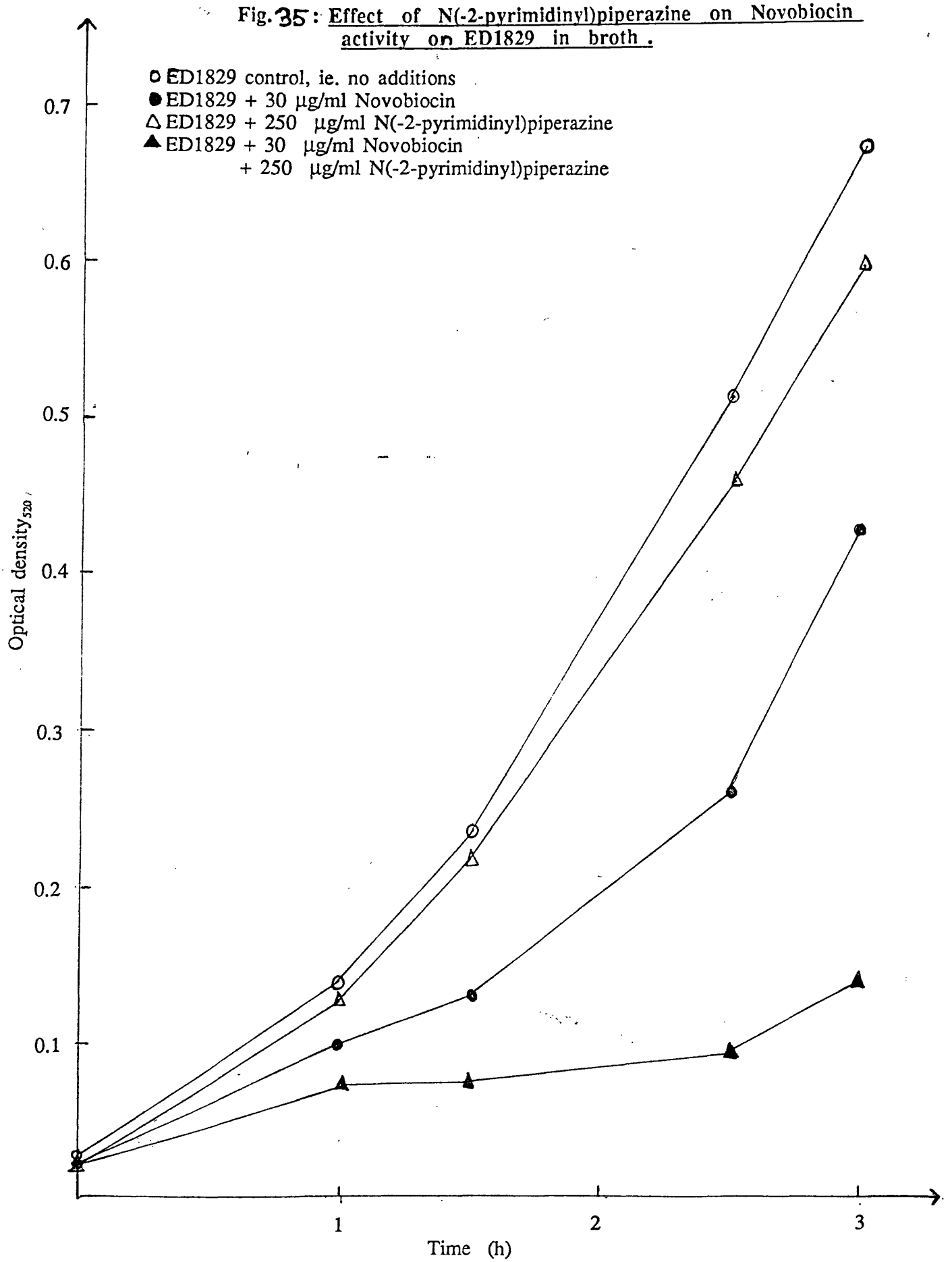


Fig. 36 : Effect of Methylglyoxyl bis(guanyl-hydrazone) on Novobiocin activity on ED1829 in broth .

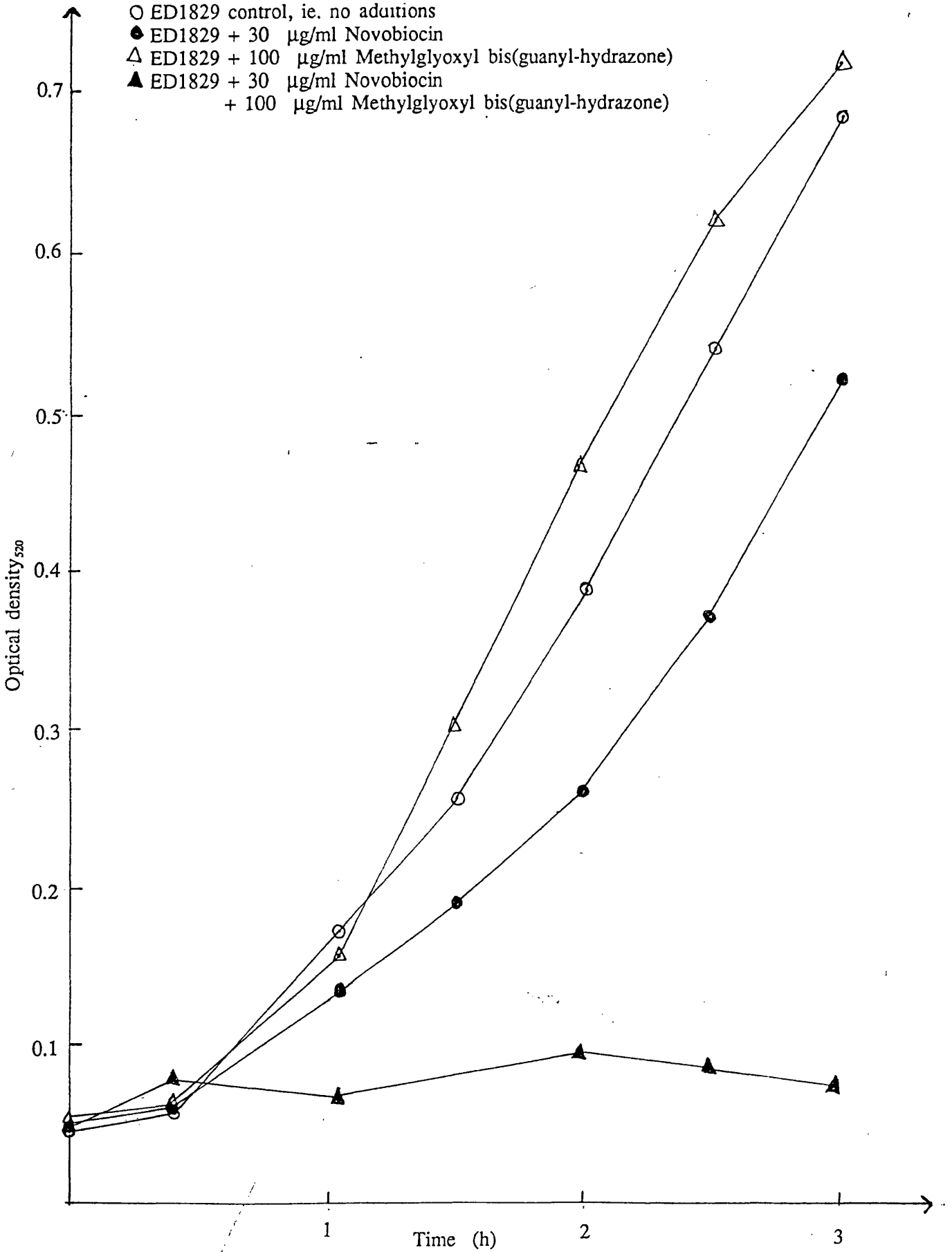


Table 35 : Effect of cationic agents on Novobiocin (30µg/ml) sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	26.68 ± 1.57
Spermidine (250)	6.17 ± 1.17	89.56 ± 1.83
Agmatine sulfate (300)	5.16 ± 1.63	82.26 ± 4.32
1,3-Diaminoacetone 2HCl hydrate (150)	2.31 ± 1.22	93.64 ± 1.20
3,3'-Diaminobenzidine 4HCl dihydrate (200)	1.76 ± 0.97	83.43 ± 2.15
Pyridoxamine(450)	2.57 ± 2.07	85.96 ± 2.80
3-Aminobenzamidine 2HCl (350)	2.35 ± 0.77	88.89 ± 2.32

The conditions of growth are given in the Methods & Materials section.

Table 36 : Effect of cationic agents on Novobiocin (30µg/ml) sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	26.68 ± 1.57
1,4 Diamino-2-butanone 2HCl (600)	-1.89 ± 2.20	63.04 ± 1.14
1,3 Diaminoguanidine HCl (300)	26.67 ± 0.98	41.99 ± 2.55
Triethylenetetra--amine 2HCl (400)	15.65 ± 2.07	53.12 ± 4.35
Triethylenetetra--amine hydrate (350)	30.16 ± 5.91	34.13 ± 3.60

The conditions of growth are given in the Methods & Materials section. Growth enhancement by agent is indicated by a minus sign before the % Growth Inhibition.

Fig. 37: Effect of Triethylene tetramine on Novobiocin activity of ED1829 in broth.

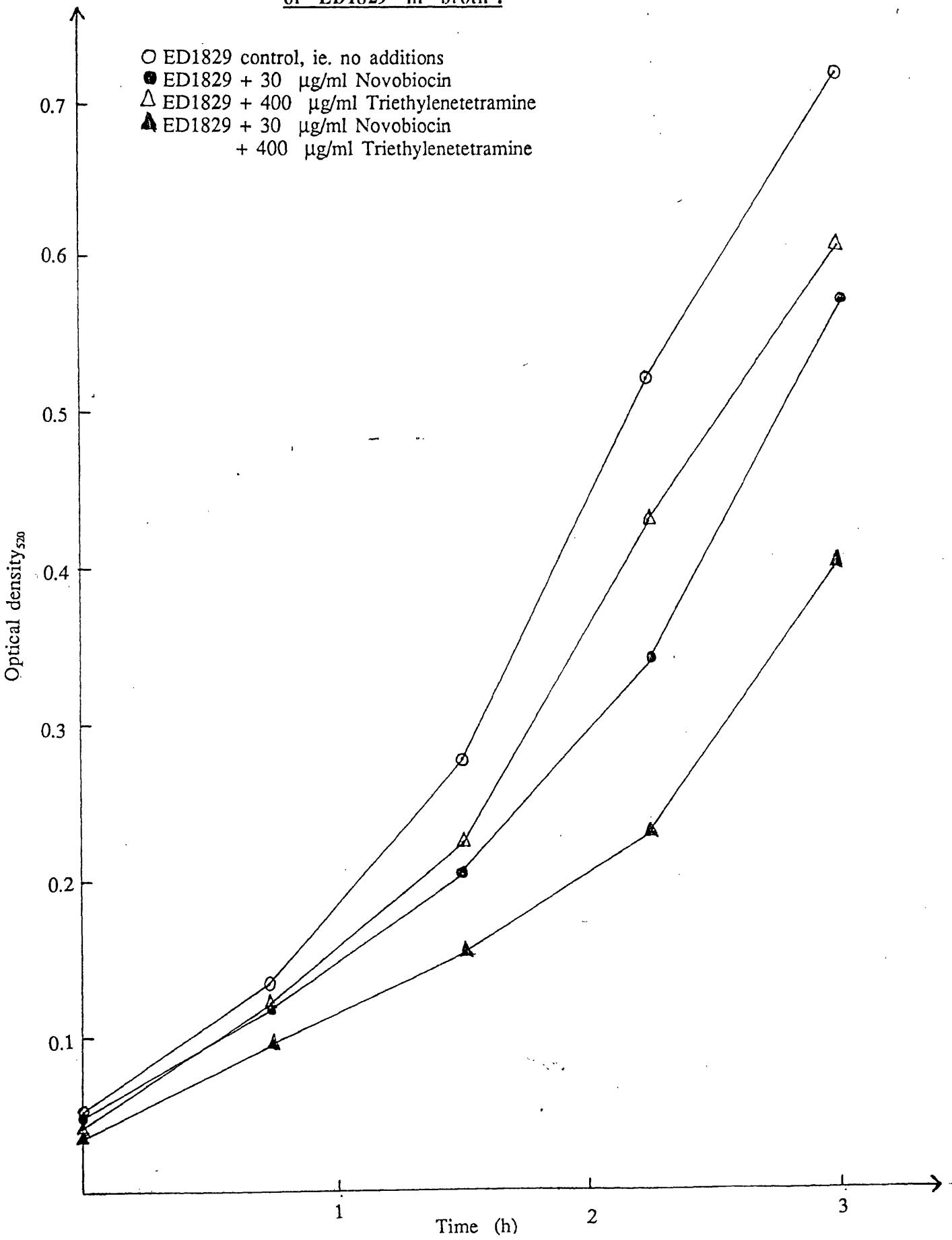


Table 37 : Effect of cationic agents on Novobiocin (30µg/ml) sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	26.68 ± 1.57
Tetraethylene pentamine (200)	25.68 ± 6.23	54.34 ± 6.17
Diethylenetriamine (500)	26.01 ± 3.35	28.29 ± 1.83
Moroxydine HCl (450)	4.89 ± 2.76	28.97 ± 1.34
N,N,N',N'-Tetramethyl -p-phenylenediamine (200)	25.44 ± 6.55	50.96 ± 19.59
Formamidine disulfide 2HCl (75)	11.21 ± 4.34	29.19 ± 3.48

The conditions of growth are given in the Methods & Materials section.

Table 38 : Effect of cationic agents on cell viability in presence of Novobiocin

Agent (μg/ml)	% Inhibition in the presence of : Agent	Agent + Novobiocin
-	-	24.65
N(-2-pyrimidinyl) piperazine 2HCl (250)	17.27	68.60
4,5,6-Triaminopyrimidine sulfate hydrate (350)	0.0	74.45
1,4-Diaminopiperazine (150)	7.45	62.04
Methylglyoxyl bis(guanyl-hydrazone; 100)	0.0	95.92
Spermidine (250)	2.98	88.70

Viable counts were performed on NA plates containing the given concentrations of Novobiocin ± cationic agent. Colonies were counted after overnight incubation at 37°C.

Table 39 : Effect of cationic agents on cell viability in presence of Novobiocin

Agent	% Inhibition in the presence of :	
(µg/ml)	Agent	Agent + Novobiocin
-	-	24.65
Agmatine sulfate (300)	3.45	77.24
1,3-Diaminoacetone (150)	1.10	93.41
3,3'-Diaminobenzidine (200)	0.0	82.73
1,3-Diaminoguanidine (300)	40.22	65.94
Moroxydine HCl (350)	0.83	13.0
Triethylenetetramine (250)	7.17	22.33

Viable counts were performed on NA plates containing the given concentrations of Novobiocin ± cationic agent. Colonies were counted after overnight incubation at 37°C.

(b) In Davies Minimal Medium :

Strain ED1829 was less sensitive to novobiocin in minimal medium as compared to broth. When novobiocin sensitivity (45 μg/ml) was tested in the presence of the cationic agents, then of those tested only agents methylglyoxyl bis(guanyl-hydrazone) and spermidine were found to enhance novobiocin sensitivity (Tables 40 & 41). The other agents N-(2-pyrimidinyl)-piperazine 2HCL, 4,5,6-triaminopyrimidine sulfate, 1,4-diaminopiperazine, 1,3-diaminoacetone and 3,3'-diaminobenzidine 4HCL which acted as very good enhancers of novobiocin activity for strain ED1829 in broth showed no such effect in minimal medium. Preliminary studies with non-enhancers (for ED1829 in broth) triethylenetetramine 2HCL, tetraethylenepentamine and diethylenetriamine, as well as the enhancer (for ED1829 in broth) pyridoxamine also showed no enhancement of novobiocin activity in minimal medium.

Hence, it seems that the type of growth medium used and possibly the changes in the cell properties or in cell metabolism induced as a result, play a big role in sensitization to novobiocin.

Table 40 : Effect of cationic agents on Novobiocin (45 µg/ml) sensitivity of ED1829 in minimal medium.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	32.18 ± 1.61
N-(2-Pyrimidinyl) piperazine 2HCl (250)	6.00 ± 2.86	49.37 ± 3.08
4,5,6-Triamino- -pyrimidine sulfate hydrate (600)	10.17 ± 2.61	43.69 ± 3.34
1,4-Diaminopiperazine HCl (500)	7.87 ± 1.82	37.57 ± 2.28
Methylglyoxyl bis- (guanyl-hydrazone) 2HCl (100)	1.63 ± 2.28	91.54 ± 2.27

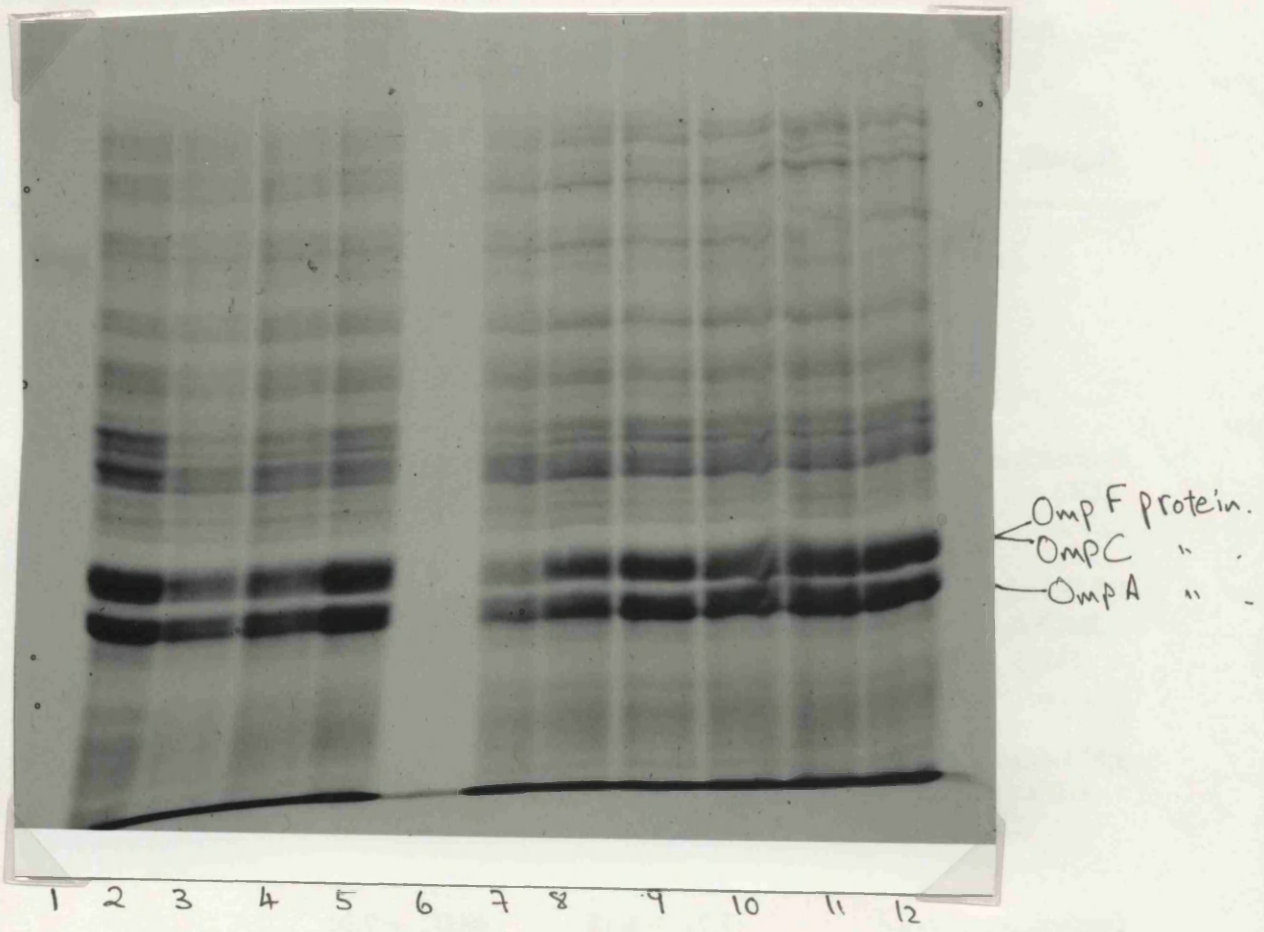
The conditions of growth are given in the Methods & Materials section.

Table 41 : Effect of cationic agent on Novobiocin (45µg/ml) sensitivity of ED1829 in minimal medium.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	32.18 ± 1.61
Spermidine (250)	1.32 ± 3.06	77.05 ± 2.58
1,3-Diaminoacetone 2HCl hydrate (150)	12.29 ± 2.37	41.37 ± 2.40
3,3'-Diaminobenzidine 4HCl dihydrate (200)	11.54 ± 4.01	52.03 ± 2.28
Triethylenetetramine 2HCl (400)	27.71 ± 4.13	43.02 ± 9.96
Pyridoxamine	15.42 ± 4.06	44.18 ± 0.20
Tetraethylene pentamine	24.12 ± 10.22	44.52 ± 10.93
Diethylene triamine	11.97 ± 1.07	33.50 ± 7.61

The conditions of growth are given in the Methods & Materials section.

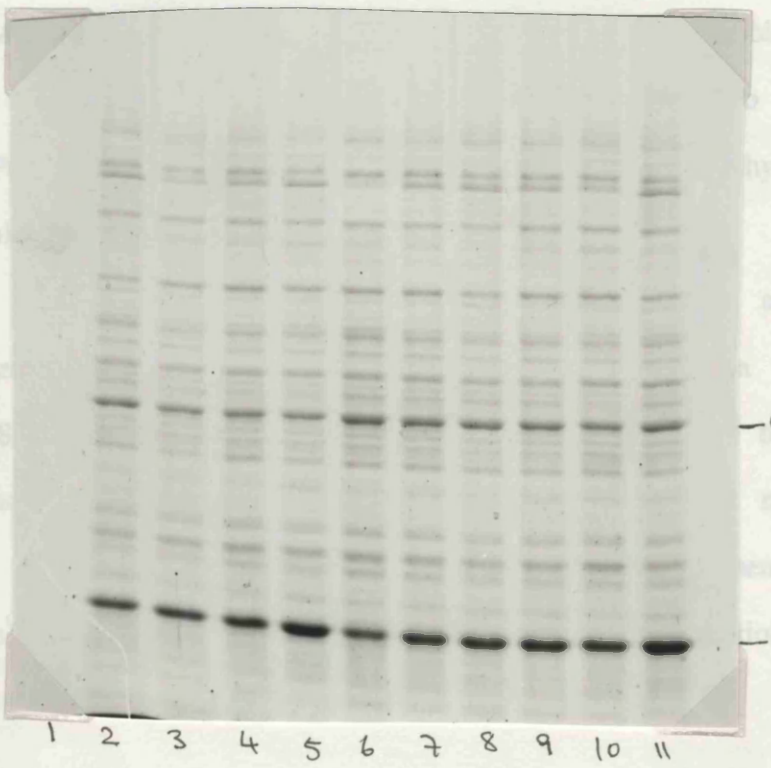
Fig. : Outer Membrane protein composition of ED1829 after growth with cationic agents.



Wells are labelled left to right, 1-13 respectively.
Samples were treated at 100°C for 5 min before loading on gel.

(1) Bovine serum albumin
(2) Control ie. no additions
(3) + 250 µg/ml N-(2-pyrimidinyl)piperazine
(4) + 400 µg/ml 4,5,6-Triaminopyrimidine sulfate hydrate
(5) + 500 µg/ml 1,4-Diamino piperazine
(6) Blank
(7) + 100 µg/ml Methylglyoxyl bis(guanyl-hydrazone)
(8) + 250 µg/ml Spermidine
(9) + 300 µg/ml Agmatine sulphate
(10) + 200 µg/ml 3,3'-Diaminobenzidine
(11) + 450 µg/ml Pyridoxamine
(12) + 350 µg/ml 3-Aminobenzamidine

Fig. 38b: **Outer Membrane protein composition of ED1829 after growth with cationic agents.**



Wells are labelled left to right, 1-11 respectively.
Samples were treated at 30°C for 15 min before loading on gel.

(1) Bovine serum albumin
(2) Control ie. no additions
(3) + 250 µg/ml N-(2-pyrimidinyl)piperazine
(4) + 400 µg/ml 4,5,6-Triaminopyrimidine sulfate hydrate
(5) + 500 µg/ml 1,4-Diamino piperazine
(6) + 100 µg/ml Methylglyoxyl bis(guanyl-hydrazone)
(7) + 250 µg/ml Spermidine
(8) + 300 µg/ml Agmatine sulphate
(9) + 200 µg/ml 3,3'- Diaminobenzidine.
(10) + 450 µg/ml Pyridoxamine
(11) + 350 µg/ml 3- Amino benzidine.

3.7. Effect of cationic agents on Novobiocin sensitivity of strain P678-54 in broth.

Strain P678-54 is rather resistant to novobiocin but at very high concentrations, there is a basal level of growth inhibition. When novobiocin sensitivity (200 μg/ml) was tested in the presence of the agents 4,5,6-triaminopyrimidine sulfate, 1,4-diaminopiperazine, methyl-glyoxyl bis(guanyl-hydrazone), spermidine, 1,3-diaminoacetone, 3,3'-diaminobenzidine and pyridoxamine, there was a great enhancement of novobiocin activity (Tables 42 & 43). These agents were also effective as novobiocin activity enhancers for ED1829 in broth. However, N-(2-pyrimidinyl)-piperazine failed to enhance novobiocin activity for strain P678-54. A non-enhancer (for ED1829 in broth) triethylenetetramine also failed to alter novobiocin activity for strain P789-54.

These results show that the novobiocin activity enhancing effect by cationic agents is not strain specific nor does it occur as a result of some mutation in strain ED1829. The fact that novobiocin activity is increased by the cationic agents in a relatively novobiocin resistant strain shows that these agents may well act on the entry process of novobiocin. These agents may act at the outer membrane in a way so as to permeabilise the cell to novobiocin so that a higher proportion of the antibacterial agent can enter the cell and thereby overcome the resistance.

Table 42 : Effect of cationic agents on Novobiocin (200µg/ml) sensitivity of P678-54 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	15.96 ± 2.19
N-(2-Pyrimidinyl) piperazine 2HCl (250)	8.72 ± 2.29	30.14 ± 3.37
4,5,6-Triamino- -pyrimidine sulfate hydrate (600)	14.04 ± 2.27	76.79 ± 3.43
1,4-Diaminopiperazine HCl (500)	5.53 ± 1.52	40.65 ± 1.71
Methylglyoxyl bis- (guanyl-hydrazone) 2HCl (100)	17.05 ± 1.80	62.39 ± 2.25

The conditions of growth are given in the Materials & Methods section.

Table 43 : Effect of cationic agents on Novobiocin (200µg/ml) sensitivity of P678-54 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	15.69 ± 2.19
Spermidine (250)	9.76 ± 2.37	50.70 ± 3.34
1,3 Diaminoacetone 2HCl hydrate (150)	7.87 ± 41.77	62.01 ± 2.48
3,3'-Diaminobenzidine 4HCl dihydrate (200)	-5.16 ± 1.69	36.57 ± 3.16
Triethylenetetramine 2HCl (400)	17.68 ± 2.31	30.05 ± 1.91
Pyridoxamine (450)	3.11 ± 0.60	34.32 ± 4.00

The conditions of growth are given in the Methods & Materials section. A minus sign before % Growth Inhibition indicates growth enhancement.

3.8. Effect of cationic agents on Novobiocin sensitivity of strain ED1829 ColV,Ia-K94 in broth.

Plasmid ColV,Ia-K94 confers a very high sensitivity to novobiocin on its host strain ED1829 and hence a very low concentration of novobiocin (5 μg/ml) was used to give a basal level of growth inhibition. Tables 44, 45 & 46 show the novobiocin sensitivity of ED1829 ColV,Ia-K94 in the presence of the various cationic agents.

In broth, agents 4,5,6-triaminopyrimidine sulfate, 1,4-diaminopiperazine, methylglyoxyl bis(guanyl-hydrazone), spermidine, 1,3-diaminoacetone, 3,3'-diaminobenzidine and pyridoxamine were found to be successful novobiocin activity enhancers for strain ED1829 ColV,Ia-K94. The plasmid carrying strain however, did not show an appreciable enhancing effect of N-(2-pyrimidinyl)piperazine and 1,4-diamino-2-butanone on novobiocin inhibition.

The non-enhancers (for ED1829 in broth) triethylene tetramine, tetraethylene pentamine and diethylenetriamine had no effect on novobiocin sensitivity of ED1829 ColV,Ia-K94. The above results show that the presence of the ColV,Ia-K94 plasmid does not interfere with either the uptake or activity of novobiocin as far as the enhancing/non-enhancing effect of the cationic agents are concerned for strain ED1829. However, as mentioned above, the concentration of novobiocin had to be dropped to one/sixth for the Col V^+ strain as compared to the isogenic control to obtain the same results. Col V plasmids are known to sensitise their host strain to hydrophobic antibacterial agents such as novobiocin (Davies et al,1986) and this is believed to be as a result of some change in the permeation of the antibiotic into the cell as opposed to an alteration in the site of action of the agent induced by the Col V plasmid. Hence, once the permeability change induced by the Col V plasmid is taken account of, ie. by decreasing the concentration of novobiocin, the consistency of the novobiocin sensitivity enhancement observed for both the plasmid-minus control and the plasmid-containing strain , implies that the mechanism used by the cationic agents for increasing novobiocin activity is the same in both cases, and that the envelope change in the $ColV^+$ strain does not appreciably interefere with the enhancing effect.

Table 44 : Effect of cationic agents on Novobiocin (5µg/ml) sensitivity of ED1829 ColV,Ia-K94 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	27.01 ± 3.11
N-(2-Pyrimidinyl) piperazine 2HCl (250)	5.31 ± 2.70	42.72 ± 3.88
4,5,6-Triamino- -pyrimidine sulfate hydrate (600)	12.57 ± 3.74	93.72 ± 2.96
1,4-Diaminopiperazine HCl (500)	7.84 ± 3.04	62.82 ± 2.25
Methylglyoxyl bis- (guanyl-hydrazone) 2HCl (100)	2.66 ± 2.90	54.12 ± 3.68

The conditions of growth are given in the Methods & Materials section.

Table 45 : Effects of cationic agents on Novobiocin (5µg/ml) sensitivity of ED1829 ColV,Ia-K94 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
–	–	27.01 ± 3.11
Spermidine (250)	3.39 ± 1.82	52.76 ± 2.79
1,3-Diaminoacetone 2HCl hydrate (150)	5.63 ± 3.08	61.40 ± 4.29
3,3'-Diaminobenzidine 4HCl dihydrate (200)	3.27 ± 2.39	66.21 ± 3.46
Pyridoxamine (450)	9.71 ± 7.60	60.85 ± 2.13

The conditions of growth are given in the Methods & Materials section.

Table 46 : Effect of cationic agents on Novobiocin (5µg/ml) sensitivity on ED1829 ColV,Ia-K94 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Novobiocin
-	-	27.01 ± 3.11
Triethylene tetramine 2HCl (400)	4.53 ± 1.64	38.27 ± 3.65
Tetraethylene pentamine (200)	13.92 ± 5.21	27.51 ± 1.70
Diethylene triamine (500)	22.86 ± 1.83	27.70 ± 2.81
1,4-Diamino--2-butanone 2HCl (600)	-4.54 ± 1.83	28.43 ± 4.67

The conditions of growth are given in the Methods & Materials section. A minus sign before % Growth Inhibition indicates growth enhancement.

3.9. Effect of cationic agents on 4-Quinolone sensitivity of ED1829 in broth.

Norfloxacin is a fluorinated 4-Quinolone whose site of action is the enzyme DNA gyrase, the same enzyme inhibited by novobiocin. To establish whether the cationic agents enhance novobiocin sensitivity by interacting with the DNA or the gyrase, the effect of these agents on norfoxacin activity was tested.

Norfloxacin sensitivity in strain ED1829 in broth was unaffected by the cationic agents methylglyoxyl bis(guanyl-hydrazone), spermidine, 1,3-diaminoacetone and 3,3'-diaminobenzidine (Table 47). All of the above agents enhanced novobiocin sensitivity in this strain. This suggests that the DNA or the gyrase may not be the site of action of the cationic agents. Cations and cationic agents such as polyamines, similar to those used here, are known to bind to DNA during replication to facilitate the process . If the mechanism of action of these cationic agents involved in enhancing novobiocin activity involved such interaction then we would expect that norfoxacin activity might also be somehow influenced as well, although norfloxacin acts on the A subunit of DNA gyrase whereas novobiocin acts on the B subunit and this difference at the molecular level between the sites of action of the two antibacterials might be enough to abolish the effects of the cationic agents.

Another difference between the two agents is their route of entry into the cell. Norfloxacin is a small hydrophilic molecule which enters the cell mainly via the OmpF porin channels in the outer membrane (Chapman and Georgopapadakou, 1988). Novobiocin is a hydrophobic antibiotic, larger than norfloxacin and is believed to enter the cells of Gram-negative bacteria by the hydrophobic pathway ie. through any phospholipid bilayers that may form in the outer membrane. The reason for the lack of enhancement of norfloxacin activity may well be that the cationic agents act at the cell surface to influence the entry mechanism. Cations such as Mn^{2+} and Mg^{2+} are known to bind LPS and help maintain the outer membrane as a rigid and impermeable structure, hence the

cationic agents used here may compete with the normally found cations such as magnesium and disrupt the tightly packed LPS in the outer leaflet of the outer membrane, thus allowing local phospholipid bilayers to form whereby novobiocin can penetrate better and reach its site of action. Such an effect of cationic agents would not influence norfloxacin sensitivity.

Preliminary tests with two other fluorinated 4-quinolones ofloxacin and flumequin (Table. 48) showed that diaminoacetone and methylgyloxyl bis(guanyl-hydrazone) had no effect on ofloxacin sensitivity but there was a considerable enhancement of flumequin sensitivity. This response supports the concept that cationic agents used here act at the outer membrane as the difference between ofloxacin and flumequin is in their hydrophobicity but not in their site and mode of action. The hydrophobicity and the extent to which a 4-quinolone uses the porin pathway for entry into the Gram-negative cell has been shown to be inversely related (Hirai *et al*, 1986). The results obtained here support this relationship between hydrophobicity of the 4-quinolone and its route of entry into the cell.

Table 47 : Effect of cationic agents on Norfloxacin (0.01µg/ml) sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : + Standard Error	
(µg/ml)	Agent	Agent + Norfloxacin
-	-	23.80 ± 1.97
Methylglyoxyl bis-(guanyl-hydrazone) 2HCl (100)	2.97 ± 3.31	17.90 ± 2.83
Spermidine (250)	1.88 ± 1.68	18.84 ± 3.14
1,3-Diaminoacetone 2HCl hydrate (150)	0.50 ± 3.20	21.09 ± 3.18
3,3'-Diaminobenzidine 4HCl dihydrate (200)	1.94 ± 3.56	18.56 ± 2.71

The conditions of growth are given in the Methods & Materials section.

Table 48 : Effect of cationic agents on Ofloxacin and Flumequin sensitivities of ED1829 in broth.

Agent (concentration)	% Growth Inhibition (3h) in presence of :		
		1,3-Diaminoacetone (150µg/ml)	Methylglyoxyl bis(guanyl-hydrazone) (100 µg/ml)
-	-	1.89	0.94
Ofloxacin :			
0.01 µg/ml	49.43	41.51	32.08
0.02 µg/ml	79.62	72.64	65.09
Flumequin :			
0.3 µg/ml	27.45	64.31	45.10
0.5 µg/ml	78.43	96.08	86.27

The conditions of growth are given in the Methods & Materials section.

3.10. Effect of 1,3-Diaminoacetone on antibacterial agent sensitivity of ED1829 in broth.

If the cationic agents which enhance novobiocin sensitivity do so by activating the hydrophobic permeation pathway, the the sensitivity of *E.coli* to other hydrophobic agents should be increased as well. This has been tested here using a range of hydrophobic and cationic antibacterial agents as well as the detergent deoxycholate.

1,3-Diaminoacetone is a very good enhancer of novobiocin activity for strain ED1829 in broth. To establish whether the enhancing effect by this cationic agent is specific to novobiocin, various other antibacterial agents were tested with this cationic agent (Tables 49-52).

Only erythromycin, rifampicin, nalidixic acid, bacitracin, polymyxin B, serum, hydrogen peroxide and hexanoic acid at pH 4 sensitivities were enhanced by 1,3-Diaminoacetone to a significant level. The inhibitory activities of rifamycin, fusidic acid, vancomycin, oxacillin, nafcillin, deoxycholate, nitrofurantoin and hexanoic acid at pH 7 were not at all affected by this cationic agent even when in some cases the concentration of diaminoacetone was increased to 250 μg/ml. EDTA sensitivity was partially reversed by diaminoacetone and methylglyoxyl bis(guanyl-hydrazone).

Erythromycin (a macrolide antibiotic), rifampicin (a rifamycin derivative) and nalidixic acid (a 4-Quinolone) are all hydrophobic antibacterials and Gram-negative bacteria are rather resistant to these. This resistance is because of their inability to permeate the cells due to their hydrophobicity. The ability of diaminoacetone to enhance erythromycin, rifampicin and nalidixic acid inhibition suggests that the cationic agents may partially activate the hydrophobic pathway. The enhancement of the effect of these hydrophobic antibacterials is relatively small in the cases of erythromycin and rifampicin , it is however significant and may occur as a result of a general partial loosening of the outer leaflet of the outer membrane enabling these antibacterials to elbow their way through the outer membrane. This mechanism of entry would fall under the self-promoted pathway of entry

through the outer membrane. High enhancement of nalidixic acid sensitivity by diaminoacetone is interesting as both nalidixic acid and novobiocin are hydrophobic, acidic and have the same target enzyme ie. DNA gyrase as the site of action. Preliminary tests with coumeromycin A1 (at 20 μg/ml concentration) and diaminoacetone showed no enhancement of coumeromycin A1 activity even at 250 μg/ml diaminoacetone.

Enhancement of serum sensitivity by diaminoacetone suggests that interaction of the cationic agent at the cell surface results in exposure of certain sites at which activated complement components can be inserted. The idea that interaction of cationic agents with the cell surface exposes certain types of sites is supported by the observed enhancement of polymyxin B sensitivity which is known to bind the lipid A portion of LPS as well as to other regions of the LPS. However, there appears be no or little leakage of LPS in the presence of the cationic agents since EDTA inhibition is reversed and EDTA is known to chelate divalent cations such as magnesium ions and thereby disrupt the outer membrane as a result of the induced instability within the LPS layer. If the cationic agents acted in a similar way ie. disrupted the LPS layer and thereby cause LPS release then an enhancement of EDTA sensitivity would be expected but this does not appear to be the case. Possibly, the replacement of Mg^{++}, etc. by the cationic agents used here prevents EDTA from removing LPS.

Table 49 : Effect of 1,3-Diaminoacetone (150µg/ml) on antibacterial agent sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Diaminoacetone
-	-	2.67 ± 3.33
Erythromycin (5 µg/ml)	22.85 ± 1.73	44.19 ± 1.68
Rifampicin (10 µg/ml)	24.87 ± 1.58	47.89 ± 8.76
Rifamycin (15 µg/ml)	1.84 ± 2.83	12.70 ± 3.48
Fusidic Acid (35 µg/ml)	7.78 ± 4.82	17.48 ± 4.28
Vancomycin (40 µg/ml)	4.64 ± 3.10	9.81 ± 2.28

The conditions of growth are given in the Methods & Materials section.

Table 50 : Effect of 1,3-Diaminoacetone (150µg/ml) on antibacterial agent sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Diaminoacetone
-	-	2.67 ± 3.33
Oxacillin (150 µg/ml)	25.16 ± 1.75	27.64 ± 2.12
Nafcillin (150 µg/ml)	20.15 ± 2.38	26.41 ± 1.94
Nalidixic Acid (1.5 µg/ml)	19.12 ± 1.67	81.30 ± 3.27
Polymyxin B :		
0.2 µg/ml	33.70	64.13
0.25 µg/ml	67.37	83.24
Bacitracin (250 µg/ml)	29.76 ± 0.81	35.74 ± 2.45

The conditions of growth are given in the Methods & Materials section.

Fig. 39. : Effect of Diaminoacetone on Nalidixic Acid activity

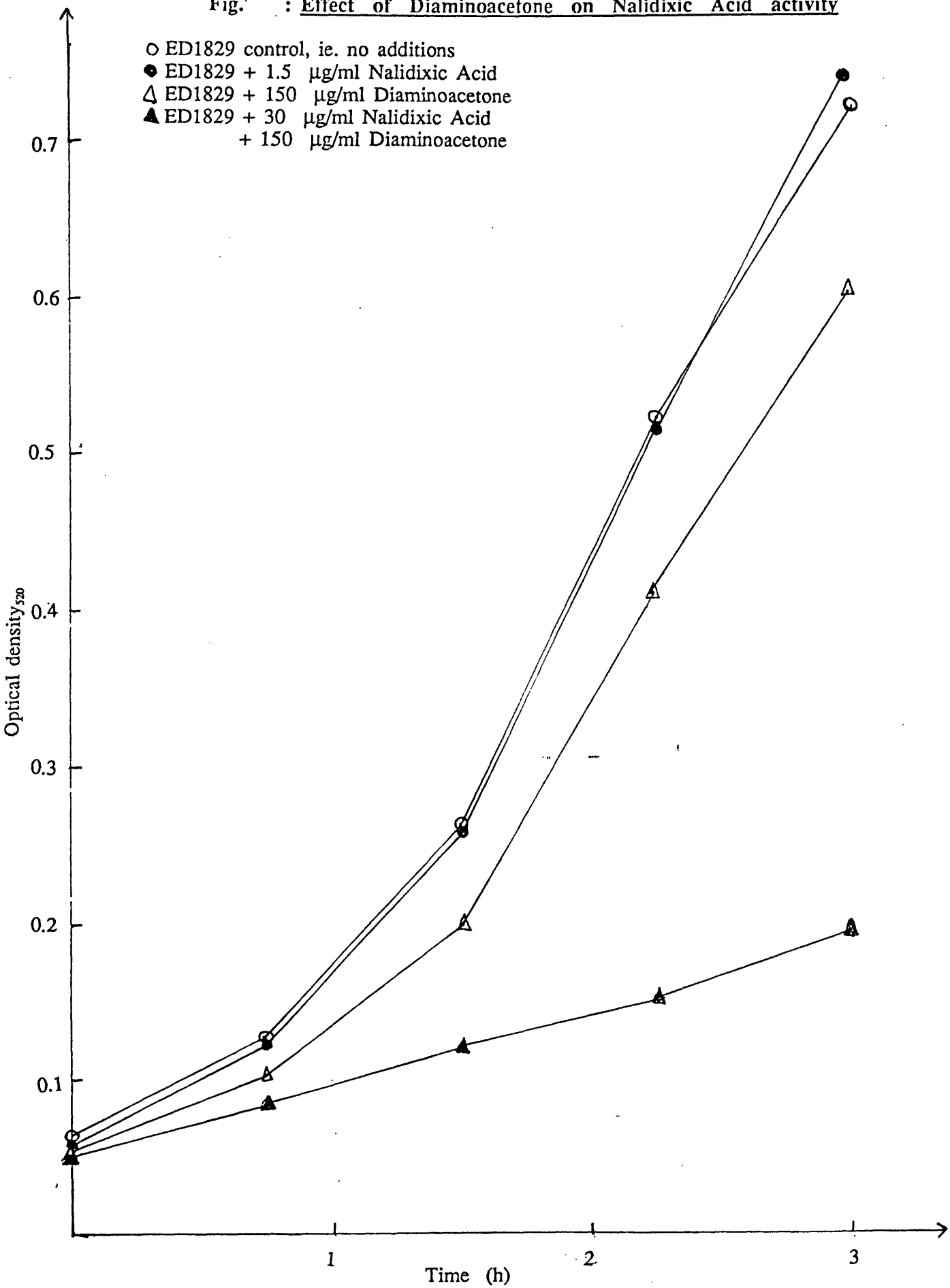


Table 51 : Effect of 1,3-Diaminoacetone (250µg/ml) on antibacterial agent sensitivity of ED1829 in broth.

Agent	% Growth Inhibition (after 3h) in presence of : ± Standard Error	
(µg/ml)	Agent	Agent + Diaminoacetone
-	-	2.48 ± 1.14
Deoxycholate (5 %)	49.71 ± 1.62	47.33 ± 1.21
Nitrofurantoin	37.85 ± 4.01	45.42 ± 3.60
EDTA :		
25 µg/ml	30.77	2.20
50 µg/ml	72.53	48.35

Agent	Agent	+ Methylglyoxyl* bis(guanyl-hydrazone)
EDTA		
25 µg/ml	30.77	15.38
50 µg/ml	72.53	80.22

The conditions of growth are given in the Methods & Materials section.
* concentration of methylgyloxyl bis(guanyl-hydrazone) is 100 µg/ml

Table 52 : Effect of 1,3-Diaminoacetone on antibacterial agent sensitivity of ED1829 in broth.

Agent (concentration)	% Survival	
	Agent	Agent + Diaminoacetone
-	-	100
Serum (30%)	53.33	32.04
Hydrogen peroxide :		
2.5 mM	44.10	29.77
5 mM	20	5.25
Hexanoic acid pH 7 (70mM)	8.30	33.33
Hexanoic acid pH 4 (5mM)	100	0.012

Growth inhibitions in the presence of the above agents were determined by viable counts on Nutrient agar after treatment in the presence of the agent for the given time period broth.

3.11. Effect of Magnesium on Novobiocin sensitivity of of ED1829 in broth.

Sensitivity to novobiocin was found to be reversed by the presence of magnesium ions in the growth medium (Table 53). Magnesium ions are very important in maintaining impermeability of the outer membrane structure as they are believed to crossbridge the highly charged hydrophilic domains of LPS.

Antagonism by magnesium ions of antibacterial agents is commonly found eg. of erythromycin (Alfa et al, 1987), of rifampicin (Davies *et al*, 1986), of ciprofloxacin (Campoli-Richards *et al*, 1988) and of ofloxacin (Monk and Campoli-Richards, 1987). The inhibitory sites of action of the above mentioned agents are all different and hence interference by magnesium ions at all these sites seems unlikely. Accordingly, Mg^{2+} may interfere with the entry pathways of these inhibitory agents and/or interact directly by binding to the agents and affecting their uptake and/or uptake. But the mechanisms of entry of the above mentioned agents may be similar especially for erythromycin and rifampicin. For the 4-quinolones, ciprofloxacin and ofloxacin, the main route of entry is proposed to be the OmpF porin but other routes are also implicated by the relatively small increase in MIC observed for these agents in OmpF mutants (Hirai *et al*, 1986). If Mg^{2+} does not directly interact with the agents, then the reason for this observed antagonism by magnesium ions may be the strengthening effect conferred on the outer membrane by Mg^{2+} enhancing the tight packing of LPS molecules and hence hindering the passage of any compound unless a specific pathway for its uptake already exists. Accordingly, magnesium ions probably reduce permeation but novobiocin and other hydrophobic antibacterial agents may however, also directly interact with magnesium, or the divalent cations may affect the site of action of such agents.

When novobiocin sensitivity was tested in the presence of magnesium ions and some of the cationic agents, then Mg^{2+} reversed the enhanced inhibition (Table 54). The enhancement of novobiocin sensitivity given by N-(2-pyrimidinyl)piperazine, methylglyoxyl

bis(guanyl-hydrazone) and 1,3-diaminoacetone was found to be reversed from approximately 90% to 4-32%. When various concentrations (350-550 μg/ml) of 3-Aminobenzimidine were used versus various concentrations of magnesium ions (20-50 mM) and novobiocin sensitivity was tested, then 20 mM magnesium was found to give a very significant reversal of the novobiocin enhancement effect given by each concentration of 3-aminobenzimidine (Table 55). However as higher levels of magnesium were used ie. 40 mM and 50 mM, the reversal of enhancement decreased slightly. This was unexpected and it seems likely that 20 mM magnesium is the optimum concentration under these conditions for reversal of novobiocin activity enhancement. Increasing the level of 3-aminobenzamidine indicated that there was competition between Mg^{2+} and the cationic agents (Table 54).

Table 53 : Effect of Magnesium on Novobiocin sensitivity of ED1829 in broth.

	% Growth Inhibition (after 3h) in presence of :	
	+ 0 mM Magnesium	+ 5 mM Magnesium
Control	0.00	1.86 ± 7.55
+ Novobiocin (30 µg/ml)	40.37 ± 0.85	8.79 ± 0.99
+ Novobiocin (50 µg/ml)	91.12 ± 3.41	27.56 ± 3.73

The conditions of growth are given in the Methods & Materials section.

Table 54 : Effect of magnesium on cationic agent enhanced Novobiocin sensitivity of ED1829 in broth.

Agent (concentration)	% Growth Inhibition in presence of : Agent	Agent + 25 mM Magnesium	Agent +20 µg/ml Novobiocin	Agent +25 mM Magnesium + 20 µg/ml Novobiocin
-	-	3.61	19.34	3.78
N-(2-pyrimidinyl) piperazine (250 µg/ml)	1.64	4.10	55.74	25.41
Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	8.52	3.28	91.80	4.10
1,3-Diaminoacetone (150 µg/ml)	2.46	4.92	91.80	32.62

The conditions of growth are given in the Methods and Materials section.

Table 55 : Reversal of enhanced Novobiocin (30µg/ml) sensitivity of ED1829 by Magnesium in broth.

3-Aminobenzamidine concentration	% Growth Inhibition (after 3h) in presence of : Magnesium sulphate concentration			
	0	20 mM	40 mM	50 mM
-	35.11	20.74	21.78	14.81
350 µg/ml	96.51	15.70	29.65	36.63
400 µg/ml	97.67	21.51	34.30	41.28
450 µg/ml	99.19	20.35	36.63	40.12
500 µg/ml	98.46	39.05	42.60	56.80
550 µg/ml	100.00	31.95	43.79	55.62

The conditions of growth are given in the Methods and Materials section.

3.12. Effect of prior growth with cationic agents and/or magnesium on antibacterial agent sensitivity of ED1829 in broth.

Magnesium is a common antagonist of antibiotic action and it has been found to antagonise not only novobiocin activity but also the novobiocin acitivity enhancement by cationic agents N-(2-pyrimidinyl)piperazine, methylgyloxyl bis(guanyl-hydrazone), diaminoacetone and aminobenzamidine. Failure to enhance the activities of the 4-quinolones norfloxacin and ofloxacin suggests that the cationic agents affect the entry mechanism of novobiocin (also supported by increased flumequin and nalidixic acid sensitivities induced by diaminoacetone) rather than interfere with DNA or the gyrase. Magnesium ions are associated with both DNA and LPS and their role in this association has been proposed to be as non-specific neutralisers of the numerous anionic charges present on both these macromolecules. However, competition observed between magnesium and the cationic agents with regard to hydrophobic antibacterial agent activity (such as for novobiocin) suggests that these two competitors are acting either at the cell surface or that they may interact with the novobiocin molecule so as to increase its permeability. To examine which of these associations ie. interaction with cell surface or novobiocin, is the prominent factor involved in enhanced novobiocin activity, the following prior growth experiments in the presence of cations were carried out.

If magnesium ions and the cationic agents both interacted with the novobiocin molecule and thereby altered its permeation through the outer membrane, then prior growth of cells in the presence of either magnesium or the cationic agent or both, followed by the removal of excess unattached cations from the cell surface should have no effect on novobiocin activity. Interaction of cations with novobiocin can occur in at least two ways : (a) cations may bind to the novobiocin molecule via Van der Waal's or ionic bonds (covalent bond-formation needs energy and so is the least likely in the growth medium), and thereby alter its conformation such that it can better (or worse in the case of magnesium) permeate the outer membrane either through the hydrophobic pathway or

possibly through the porin pathway; or (b) both cations may form a salt with novobiocin which in the case of magnesium would be a neutral salt and therefore not be able to use the porin pathway or the self-promoted uptake pathway which involves the highly charged LPS; but the salt in the case of the cationic agent such as diaminoacetone would be an amine salt with one amino group remaining free to act as a cation, thereby giving the whole salt molecule a cationic charge, which may then become a candidate for the self-promoted uptake pathway. However, on the other hand if magnesium and the cationic agents interacted only with the cell surface, the outer membrane in particular, then prior growth with either magnesium or the cationic agent or both and their removal prior to novobiocin sensitivity testing, should still show altered novobiocin sensitivity with respect to the control cells which are grown in broth alone.

(a) Effect of prior growth with cations on Novobiocin sensitivity

Table 56 shows the novobiocin sensitivity profile of cells grown in the presence of either diaminocetone, methylglyoxyl bis(guanyl-hydrazone), magnesium, magnesium and diaminoacetone, magnesium and methylglyoxyl bis(guanyl-hydrazone) or with no additions ie. control. Prior growth with diaminoacetone resulted in a significantly enhanced novobiocin sensitivity of ED1829 at 30 μg/ml novobiocin concentration, but there was no effect at the lower novobiocin concentration. Prior growth with methylglyoxyl bis(guanyl-hydrazone) enhanced novobiocin sensitivity at both 20 μg/ml and 30 μg/ml concentrations to such a high extent that it almost appears as if methylglyoxyl bis(guanyl-hydrazone) is still present in the medium. Hence, methylglyoxyl bis(guanyl-hydrazone) must either be strongly associated with the cell surface or the outer membrane, the prime target molecule for such an association being the LPS, or that growth in the presence of this cationic agent has produced cells with altered outer membranes which remained so even on removal of any excess unattached methyglyoxyl bis(guanyl-hydrazone) from the cells. Methyglyoxyl bis(guanyl-hydrazone) has four amino groups in close proximity and these have the potential to bind to the anionic

charges on LPS by displacing magnesium from such sites, but due to the bigger size of methylglyoxyl bis(guanyl-hydrazone) as compared to magnesium ions, the physical tight packing of LPS may get disrupted even though the charge repulsion between LPS molecules has been reduced after neutralisation by the amino groups. However, this neutralisation of the anionic groups may occur only in patches and in the remaining area of the outer leaflet efflux of LPS may occur due to repulsion within the LPS molecules, and this released LPS may get replaced by the phospholipids from the inner leaflet resulting in local phospholipid bilayers through which hydrophobic compounds can enter after partitioning into it; release of LPS may at the same time lead to release of some of the outer membrane proteins if their interaction with the LPS in the outer membrane due to reduced amounts of LPS is not enough to maintain them there. All these changes in the relative proportions of the three major constituents may have major consequences on the overall integrity of the outer membrane leading to its altered permeability properties.

Prior growth with magnesium was found to give a little effect at 20 μg/ml novobiocin but no effect on sensitivity to 30 μg/ml. It appears that the magnesium content of broth is enough to saturate all the non-specific magnesium binding sites on exponentially growing cells and that washing twice with broth removes any excess unattached magnesium from the cell surface. Prior growth in the presence of magnesium and diaminoacetone results in enhanced novobiocin sensitivity at 20 μg/ml novobiocin concentration. At 30 μg/ml novobiocin, the sensitivity is the same as that for the control strain. Prior growth with magnesium and methylglyoxyl bis(guanyl-hydrazone) shows a partial reversal of the greatly enhanced novobiocin observed with prior growth in the presence of methylglyoxyl bis(guanyl-hydrazone). It appears that magnesium ions and methylglyoxy bis(guanyl-hydrazone) compete for cation binding sites during prior growth and this proportionality in the amount of each type of cation bound remains so even after washing, and hence leaving the outer membrane with a heterogenous cation content. Table 57 shows the novobiocin sensitivity profile of ED1829 grown in the presence of a range of

aminobenzimidine and magnesium ion concentrations prior to novobiocin testing. Competition is observed between these two cationic agents during prior growth as far as novobiocin sensitivity is concerned.Interaction between the cations and the cell surface appears to be the major factor governing novobiocin sensitivity.

Table 56 : Effect of prior growth with cationic agents and magnesium on Novobiocin sensitivity in broth.

Agent (concentration)	% Growth Inhibition (after 3h) in presence of : + 20 µg/ml Novobiocin	+ 30 µg/ml Novobiocin
Control	15.56	26.67
1,3-Diaminoacetone (150 µg/ml)	12.59	48.95
Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	95.35	103.10
Magnesium sulphate (20 mM)	2.80	25.87
Magnesium sulphate (20 mM) and 1,3-Diaminoacetone (150 µg/ml)	27.91	30.23
Magnesium sulphate (20mM) and Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	68.75	82.29

Cells were grown in broth ± the cationic agents Diaminoacetone and Methylglyoxyl bis(guanyl-hydrazone) ± Magnesium sulphate all the given concentrations overnight and then with similar addtions to mid -exponential phase, washed and then used to assay novobiocin sensitivity.

Fig. 40 : Effect of prior growth in presence of Methylglyoxyl bis(guanyl-hydrazone) on Novobiocin sensitivity of ED1829 in broth.

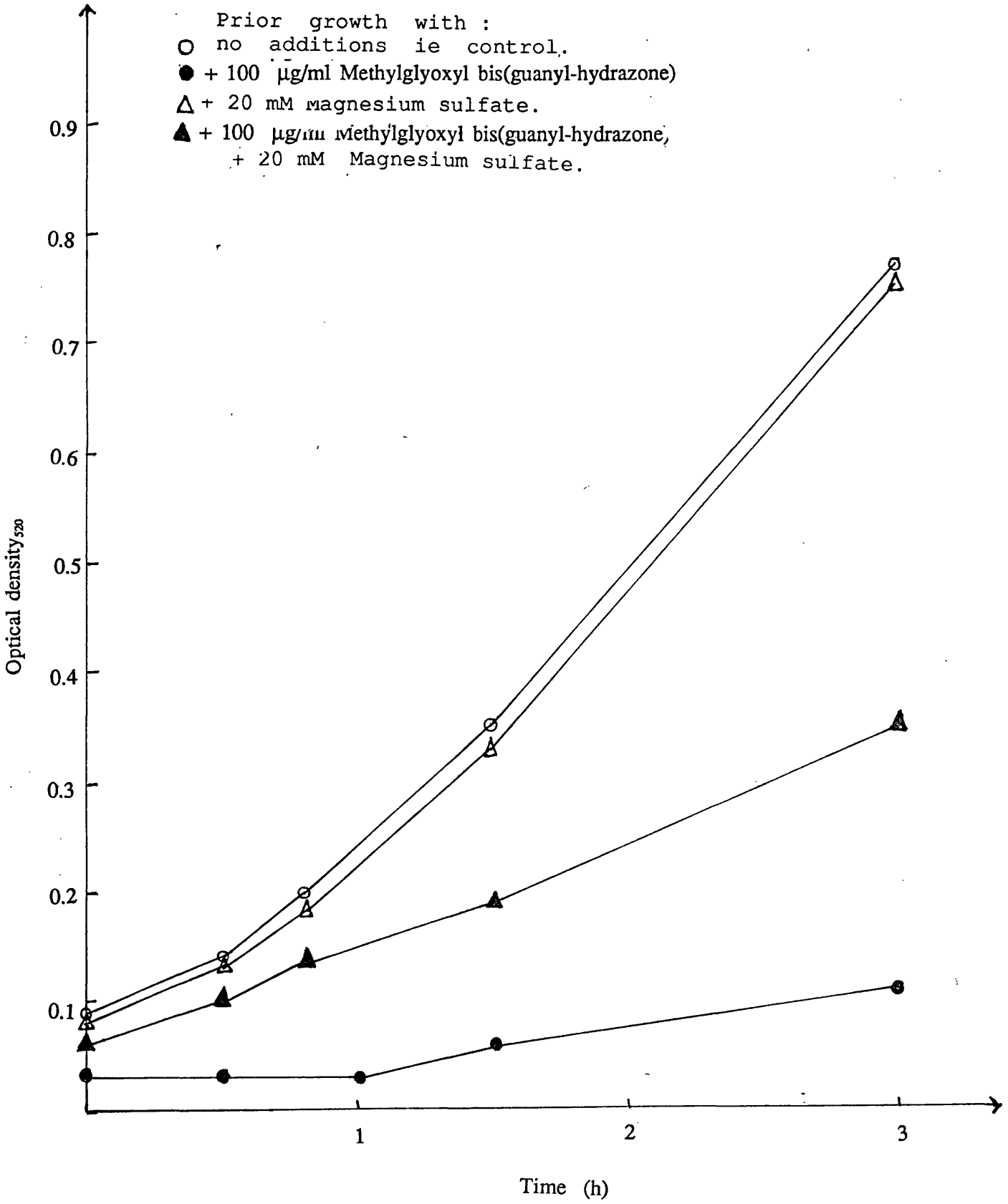


Table 57 : Competition between 3-Aminobenzamidine and Magnesium during growth prior to Novobiocin sensitivity testing

3-Aminobenzamidine (concentration)	% Growth Inhibition (after 3h) in presence of : Magnesium sulphate concentration			
	0 mM	20 mM	40 mM	50 mM
-	18.97	27.24	25.86	29.31
250 µg/ml	19.83	23.28	26.72	24.14
350 µg/ml	31.02	35.43	34.65	36.69
400 µg/ml	60.63	37.01	41.73	35.12

Cells were grown in broth ± 3-aminobenzimidine ± magnesium sulphate at the given concentrations overnight and similarly to mid-exponential phase and washed before assaying novobiocin sensitivity.

(b) **Effect of prior growth with cations on Polymyxin B sensitivity**

Under similar experimental conditions ie. prior growth with cationic agents and/or magnesium, polymyxin B sensitivity shows a quite different pattern to that given by novobiocin sensitivity of ED1829 (Table 58). Prior growth with diaminoacetone partially reverses polymyxin B sensitivity at 0.15 μg/ml polymyxin B, but this effect is abolished at 0.2 μg/ml concentration. This response is interesting as when polymyxin B and diaminoacetone were both present in the medium (section 3.1) diaminoacetone significantly enhanced polymyxin B activity. Prior growth with methylglyoxyl bis(guanyl-hydrazone) completely reverses polymyxin B sensitivity at both concentrations of polymyxin. Polymyxin B is known to bind to the LPS and also to the lipid A region of the LPS and disrupt the outer membrane as a result of this association. Methylglyoxyl bis(guanyl-hydrazone) and to a much lesser extent diaminoacetone, appear to change polymyxin B binding site by either masking it or possibly binding to it themselves, such that polymyxin B cannot gain excess to it or cause outer membrane disruption. This alteration in the polymyxin B binding site during prior growth with methylglyoxyl bis(guanyl-hydrazone) may be permanently induced or methylglyoxyl bis(guanyl-hydrazone) may be required to be present to maintain the alteration. The latter is possible as washing of the cells may not remove the cationic agent if it is bound or closely associated with the binding site.

Prior growth in the presence of magnesium considerably reduces polymyxin B sensitivity, and this is in accordance with the observation that magnesium ions reverse polymyxin B activity when both are present in the medium, however, in relation to polymyxin B sensitivity the magnesium content of broth appears to be non-saturating compared to that for novobiocin sensitivity. Prior growth with diaminoacetone and magnesium also reduces polymyxin B sensitivity at 0.2 μg/ml concentration. However, at 0.15 μg/ml polymyxin B sensitivity of cells grown in the presence of diaminoacetone and magnesium is similar to that of control cells (grown in broth) and this is difficult to explain as the sensitivity of cells grown with either of these two cations alone is less than that given

by the control cells; unless the competition between magnesium and diaminoacetone is at such a stage that the overall effect on the outer membrane is similar to that of cells grown in broth.

Prior growth in the presence of magnesium and methylglyoxyl bis(guanyl-hydrazone) showed no significant effect on polymyxin B sensitivtiy as compared to the control, but all the reversal of polymyxin B sensitivity exhibited by cells grown in the presence of methylglyoxyl bis(guanyl-hydrazone) appears to be counteracted by the presence of magnesium ions in the prior growth medium and this can only be explained by proposing competition between magnesium and methylglyoxyl bis(guanyl-hydrazone) for polymyxin B binding sites on LPS.

Table 58 : Effect of prior growth with cationic agents and magnesium on Polymyxin B sensitivity in broth.

Agent (concentration)	% Growth Inhibition in presence of : + 0.15 µg/ml Polymyxin B	+ 0.20 µg/ml Polymyxin B
Control	43.08	76.15
1,3-Diaminoacetone (150 µg/ml)	26.15	83.85
Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	0.0	5.30
Magnesium sulphate (20 mM)	6.25	52.08
Magnesium sulphate (20 mM) and 1,3-Diaminoacetone (150 µg/ml)	39.68	52.38
Magnesium sulphate (20mM) and Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	35.29	84.03

Cells were grown in broth + the cationic agents diaminoacetone and methylglyoxyl bis(guanyl-hydrazone) + magnesium sulphate all the given concentrations overnight and then with similar addtions to mid -exponential phase, washed and then used to assay polymyxin B sensitivity.

(c) **Effect of prior growth with cations on EDTA sensitivity**

Prior growth in the presence of diaminoacetone, methyglyoxyl bis(guanyl-hydrazone), magnesium, magnesium and diaminoacetone and magnesium and methylglyoxyl bis(guanyl-hydrazone) has little or no effect on EDTA sensitivity of ED1829 (Table. 59). When both EDTA and diaminoacetone are present in the growth medium, EDTA sensitivity of ED1829 is reversed by diaminoacetone. The mechanism of action of EDTA is chelation of divalent cations such as magnesium ions and then cause the release of LPS and any associated proteins thereby causing a major disruption of the outer membrane. EDTA also permeabilises the outer membrane to many hydrophobic agents and this property is similar to that exhibited by the cationic enhancers of novobiocin sensitivity. However, the lack of enhancement of EDTA sensitivity and the partial reversal when the two are present together, suggests that the cationic agents do not cause LPS to be released from the outer membrane but may partially strengthen it by perhaps acting to neutralise the anionic charges on LPS which would otherwise be neutralised by magnesium ions when the magnesium ions are removed by a chelating agent such as EDTA.

Table 59 : Effect of prior growth with cationic agents and magnesium on EDTA sensitivity in broth.

Agent (concentration)	% Growth Inhibition (after 3h) in presence of : + 25 µg/ml EDTA	+ 50 µg/ml EDTA
Control	43.08	80.00
1,3-Diaminoacetone (150 µg/ml)	41.54	72.31
Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	46.61	79.45
Magnesium sulphate (20 mM)	41.67	69.79
Magnesium sulphate (20 mM) and 1,3-Diaminoacetone (150 µg/ml)	44.44	73.02
Magnesium sulphate (20mM) and Methylglyoxyl bis (guanyl-hydrazone) (100 µg/ml)	50.42	72.27

Cells were grown in broth ± the cationic agents diaminoacetone and methylglyoxyl bis(guanyl-hydrazone) ± magnesium sulphate all the given concentrations overnight and then with similar addtions to mid -exponential phase, washed and then used to assay EDTA sensitivity.

3.13. Effect of pH on antibacterial agent activity in broth.

Tables 60-67, show the % Growth Inhibitions of strain ED1829 and ED1829 containing Col V plasmids ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30, ColV-8, ColV-41, ColV-M405 and ColV-M501 in the presence of the agents erythromycin, rifampicin, nitrofurantoin, gentamicin and novobiocin at pH's 5, 7.4, 8.5 and 9. Erythromycin and gentamicin were found to be more active at neutral to alkaline pH whereas rifampicin, novobiocin and nitrofurantoin were found to be more active at acid pH in broth.

The increased erythromycin sensitivity associated with Col V plasmids was maintained at every pH although it was optimum at pH 8.5 with most of the Col V's tested. The differences in sensitivities of the Col V plasmids found at pH 7.4 (section 3.1) were also maintained at the optimum pH for each antibacterial agent used. The enhanced activity associated with novobiocin is investigated further in the following sections.

Table 60 : Effect of pH on antibacterial agent activity in broth for ED1829.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	2.20	45.20	52.80	41.20
Rifampicin 5 µg/ml	73.00	57.40	38.10	17.60
Nitrofurantoin 10 µg/ml	49.10	34.00	13.80	0.0
Gentamicin :				
0.1 µg/ml	-	-	38.10	-
0.25 µg/ml	-	5.10	-	-
0.5 µg/ml	0.0	-	-	-
Novobiocin :				
10 µg/ml	90.30	-	-	-
50 µg/ml	-	22.30	-	-
100 µg/ml	-	-	11.80	11.80

The conditions of growth are given in the Methods & Materials section; - = not done.

Table 61 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV,Ia-K94.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	33.60	68.90	89.20	82.20
Rifampicin 5 µg/ml	90.00	77.00	49.40	20.80
Nitrofurantoin 10 µg/ml	66.60	24.10	25.40	0.0
Gentamicin :				
0.1 µg/ml	-	-	72.50	-
0.25 µg/ml	-	80.00	-	-
0.5 µg/ml	17.20	-	-	-
Novobiocin :				
10 µg/ml	89.30	-	-	-
50 µg/ml	-	87.70	-	-
100 µg/ml	-	-	31.90	31.90

The conditions of growth are given in the Methods & Materials section. - = not done.

Table 62 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV,Ia-K94 Tn10.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	17.30	89.90	100.00	65.00
Rifampicin 5 µg/ml	88.50	80.10	100.00	27.80
Nitrofurantoin 10 µg/ml	66.4	42.30	6.5	24.10
Gentamicin :				
0.1 µg/ml	–	–	18.40	–
0.25 µg/ml	–	27.90	–	–
0.5 µg/ml	10.10	–	–	–
Novobiocin :				
10 µg/ml	97.50	–	–	–
50 µg/ml	–	100.00	–	–
100 µg/ml	–	–	46.50	19.10

The conditions of growth are given in the Methods & Materials section. – = not done.

Table 63 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV-K30.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	0.0	52.40	62.80	57.90
Rifampicin 5 µg/ml	91.30	58.00	40.40	13.00
Nitrofurantoin 10 µg/ml	66.70	37.90	22.40	0.0
Gentamicin :				
0.1 µg/ml	–	–	76.40	–
0.25 µg/ml	–	85.10	–	–
0.5 µg/ml	9.30	–	–	–
Novobiocin :				
10 µg/ml	93.50	–	–	–
50 µg/ml	–	34.20	–	–
100 µg/ml	–	–	24.80	21.60

The conditions of growth are given in the Methods & Materials section.
– = not done.

Table 64 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV-8.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	25.80	57.20	51.40	52.10
Rifampicin 5 µg/ml	95.90	69.20	50.00	9.70
Nitrofurantoin 10 µg/ml	63.30	37.40	13.50	0.0
Gentamicin :				
0.1 µg/ml	-	-	48.90	-
0.25 µg/ml	-	65.00	-	63.10
0.5 µg/ml	27.40	-	-	-
Novobiocin :				
10 µg/ml	97.90	-	-	-
50 µg/ml	-	59.40	-	-
100 µg/ml	-	-	19.30	-

The conditions of growth are given in the Methods & Materials section.
- = not done.

Table 65 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV-41.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	32.00	57.50	56.10	51.80
Rifampicin 5 µg/ml	98.70	84.90	34.50	15.60
Nitrofurantoin 10 µg/ml	63.30	38.80	7.50	0.0
Gentamicin :				
0.1 µg/ml	—	—	53.70	—
0.25 µg/ml	—	59.90	—	62.80
0.5 µg/ml	29.80	—	—	—
Novobiocin :				
10 µg/ml	96.90	—	—	—
50 µg/ml	—	47.10	—	—
100 µg/ml	—	—	26.30	—

The conditions of growth are given in the Methods & Materials section.
— = not done.

Table 66 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV-M405.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	26.60	68.00	42.70	61.40
Rifampicin 5 µg/ml	100.00	82.70	73.70	18.00
Nitrofurantoin 10 µg/ml	64.90	59.30	0.0	10.30
Gentamicin :				
0.25 µg/ml	-	68.80	-	61.20
0.5 µg/ml	31.10	-	–	–
Novobiocin :				
10 µg/ml	99.50	-	–	–
50 µg/ml	-	100.00	–	–
100 µg/ml	-	-	–	13.10

The conditions of growth are given in the Methods & Materials section.
– = not done.

Table 67 : Effect of pH on antibacterial agent activity in broth for ED1829 ColV-M501.

Antibacterial Agent (concentration)	% Growth Inhibition (7 h) at pH :			
	5.0	7.4	8.5	9.0
Erythromycin 5 µg/ml	29.20	70.10	83.10	61.50
Rifampicin 5 µg/ml	100.00	88.00	69.40	24.20
Nitrofurantoin 10 µg/ml	57.10	51.40	38.20	15.70
Gentamicin :				
0.25 µg/ml	–	77.70	–	52.20
0.5 µg/ml	35.50	–	–	–
Novobiocin :				
10 µg/ml	100.00	–	–	–
50 µg/ml	–	100.00	–	–
100 µg/ml	–	–	–	3.4

The conditions of growth are given in the Methods & Materials section.
– = not done.

3.14. Effect of pH on Novobiocin activity :

Novobiocin is one of the antibacterial agents that is more active at low pH. To determine whether it is the prior growth pH or the test pH of the growth medium which is significant to novobiocin sensitivity, the following experiments involving a shift in growth pH either from pH 7 to pH 5 (or vice versa) of the medium that novobiocin sensitivity was tested in, were carried out.

Cells of ED1829 grown at pH 7 in broth and then tested for novobiocin sensitivity at pH 7 (Table 68) were found to give 31.75 % growth inhibition at 30 μg/ml, however if the same cells were tested at pH 5, then the concentration of novobiocin which gives a similar (slightly higher at 48.73 %) growth inhibition drops by a factor of 120 (ie. at 0.25 μg/ml). It appears that the pH of the medium when novobiocin is present determines its inhibitory activity.

When ED1829 cells were grown at pH 5 and then tested for novobiocin sensitivity at the same pH, then the growth inhibition observed at 0.5 μg/ml novobiocin was 52.70 %. When the same cells ie. grown at pH 5, were tested for novobiocin sensitivity at pH 7 then the concentration of novobiocin required to give 33.54 % growth inhibition was 17.5 μg/ml ie. the concentration increased by a factor of 35. Again, the pH of the test medium and not that of the prior growth medium was found to be the factor responsible for novobiocin activity. A shift in the pH of the medium, either from pH 7 to pH 5 or from pH 5 to pH 7, then a difference in the concentration of novobiocin required to give a similar growth inhibition was different by a factor of 2 for the shift pH. This may be explained by the fact that during the pH 5 shift from pH 7 in the growth medium upon subculturing the cells are undergoing an adaptation process to adjust to the new pH and that under such conditions they may be more susceptible to harmful agents such as antibiotics. Plasmid ColV,Ia-K94 sensitises its host ED1829 to novobiocin at both pH 5 and pH 7 as well as when the cells are shifted from one pH to

another.

One possible explanation for the greatly increased sensitivity at pH 5 is that the uptake mechanism is altered. Furthermore, there is a possible change in the conformation of the novobiocin molecule at pH 5 from that at pH 7. Two peaks, one at 334nm and 238nm are lost when the solvent is at pH 5 (Fig.41). This supports the idea that it is the novobiocin molecule that is more active at low pH.

The reason for increased novobiocin activity may be that it uses a different pathway for entry into the cell. Novobiocin is believed to enter the Gram-negative cell at neutral pH via the hydrophobic uptake pathway. However, with a change in conformation as happens as pH 5, it may be better able to use either the porin pathway, or the hydrophobic pathway or the self-promoted pathway. Novobiocin is a weak acid and at low pH it may be fully protonated, thereby becoming neutral or even transiently be cationic due to the formation of hydrogen bonds between the the increased quantity of available protons at low pH and its own electronegative nitrogen and oxygen atoms. As a neutral molecule it may be able to use the hydrophobic pathway better or it may that the hydrophobic pathway operates better at low pH and thus aids the entry of novobiocin through the outer membrane; or as a cationic molecule, it may enter via the self-promoted pathway. Or due to its possible altered conformation novobiocin may become a candidate for one of the specific uptake channels present in the outer membrane such those present for the uptake of iron, phosphate, maltose and malodextrins, vitamin B_{12}, etc.

The possible uses of the non-specific porin pathway provided by the OmpF protein and the OmpC protein, and the iron-uptake pathway by novobiocin at pH 5 are examined in the following sections.

Table 68: Effect of prior growth pH on Novobiocin sensitivity

Strain	Test pH		% Growth Inhibition (3h)	
	pH 5		pH 7	
	Novobiocin conc. 0.25µg/ml	0.5µg/ml	Novobiocin conc. 17.5µg/ml	30µg/ml
Prior growth at pH 5 :				
ED1829	-	52.70	33.52	-
ED1829 ColV,Ia-K94	-	85.31	66.49	-
Prior growth at pH 7 :				
ED1829	48.73	-	-	31.75
ED1829 ColV,Ia-K94	91.01	-	-	95.86

Cells were grown at either pH 5 or pH 7 overnight and then grown to exponential phase in medium at the same pH, but subcultured into same or slope pH medium as given for the test cultures.

3.15. Effect of pH on Novobiocin sensitivity in permeabilised cells :

Novobiocin is more active at pH 5 and to determine whether it is the entry mechanism or the site of action of novobiocin that is affected at pH 5, the following experiments were carried out using toluene-treated permeabilised cells of strains ED1829 and P678-54.

DNA synthesis in cells made permeable to macromolecules can occur if they are supplied with the triphosphate nucleosides and this process requires ATP, however, repair of DNA in such cells does not require ATP (Moses & Richardson, 1970). Novobiocin is a strong inhibitor of DNA replication but ATP-independent DNA repair, RNA and protein synthesis are not affected by novobiocin (Staudenbaur, 1975).

Strain ED1829 appears to have a very strong DNA repair mechanism as seen by the high incorporation of ^{3}HThymine triphosphate in the absence of ATP, and there appears to be no DNA synthesis occuring on addition of ATP to the same cells tested at either pH 5 (of the medium, not necessarily of the cytoplasm of these cells at pH 5) or at pH 7 (Table 69). A similar response is seen when the cells (of ED1829) were grown at pH 5 and then tested at both pH 5 and at pH 7 (Table 70), except that the incorporation of the label at pH 5 is very low. This may be due to the DNA repair enzymes being inactivated altogether at this low pH. Strain P678-54 also shows the same trend in label incorporation when grown at pH 7 and then tested at both pH 5 and at pH 7 (Table 71). This system does not appear to work for these two strains but may work for strains containing DNA polymerase mutants which are deficient in carrying out DNA repair and hence only DNA synthesis occurs when ATP is added to the test solution.

Table 69 : Effect of prior growth pH on DNA repair in permeabilised cells of strain ED1829.

Novobiocin (concentration) (μg/ml)	Incorporation of ^{3}H (cpm) (~~proir~~ prior growth pH 7)			
	Test pH 5		Test pH 7	
	-ATP	+ATP	-ATP	+ATP
-	4869	4048	3316	2646
0.01	5137	5972	-	-
0.025	4968	5769	-	-
0.05	5289	5196	-	-
1.0	5103	4549	3271	2694
2.5	-	-	3112	2526
5.0	-	-	2941	2387

The conditions of growth and treatment are given in the Methods & Materials section.

Table 70 : Effect of prior growth pH on DNA repair in permeabilised cells of strain ED1829.

Novobiocin (concentration) (μg/ml)	Incorporation of ^{3}H (cpm) (prior growth pH 5)			
	Test pH 5		Test pH 7	
	-ATP	+ATP	-ATP	+ATP
-	139	170	2796	2305
0.001	179	200	-	-
0.01	-	-	2748	2143
0.025	399	403	3002	2394
0.10	421	435	2756	2224
0.25	-	-	2963	2588
0.50	-	-	3132	2226
1.00	343	324	2994	2480
5.00	-	-	3098	2242

The conditions of growth and treatment are given in the Methods & Materials section.

Table 71 : Effect of prior growth pH on DNA repair in permeabilised cells of strain P678-54.

Novobiocin (concentration) (μg/ml)	Incorporation of ^3H (cpm) (prior growth pH 7)			
	Test pH 5		Test pH 7	
	-ATP	+ATP	-ATP	+ATP
-	7660	5687	4007	4104
1.00	7175	5624	-	-
5.00	7154	5540	-	-
10.00	6633	5547	4004	4054
50.00	-	-	3859	3263

The conditions of growth and treatment are given in the Methods & Materials section.

3.16. Novobiocin sensitivity of porin mutants :

At pH 7, both OmpF and OmpC porin mutants were more sensitive to novobiocin than the isogenic control strain, as were two double porin mutants OmpC-OmpF(1) & OmpC-OmpF(6), and two derivatives of an OmpC mutant containing the plasmid R124 : OmpC-R124(1) & OmpC-R124(2) (Table 72). It appears that the absence of one or both porins, or a reduction in the amount of OmpF combined with the absence of OmpC sensitise the cells to novobiocin. These results support the idea that novobiocin does not use the porin poathway at neutral pH and thus may well use the hydrophobic pathway, as otherwise if the porin pathway was being used then the porin mutants would be at least as resistant (if not more) as the control to novobiocin at this pH. Loss of porins or a reduction in the number of these major outer membrane proteins may have a drastic effect on the composition of the outer membrane ie. there may be more LPS in the outer leaflet (which would facilitate the self-promoted uptake pathway), or there could be leakage of phospholipid into the outer leaflet resulting in phospholipid bilayers (which would facilitate the hydrophobic pathway).

At pH 5 the sensitivity of the porin mutants to novobiocin is different for each type of mutant. Mutation in the OmpF protein renders the strain extremely sensitive to novobiocin, mutation on the OmpC protein only slightly sensitises the strain to novobiocin whereas both the double OmpC-OmpF mutants (1 & 6) are unaltered. Derivatives of OmpC mutant containing R124 show some sensitivity (30-60%) to novobiocin at pH 5. Plasmid R124 is associated with conferring reduced levels of the OmpF porin in its host strain, and so the outer membrane porin profile of the double mutants OmpC-OmpF and the R124 containing derivatives of OmpC mutant would be expected to be similar. From these results there appears to be no evidence for the uptake of novobiocin by porins OmpF and/or OmpC either at pH 5 or at pH 7.

3.17. Novobiocin sensitivity of Iron-uptake mutants :

At pH 7, mutants in the genes fe*pA gene (enterochelin* uptake), *fiuE gene* (copragen uptake) and *fec*A gene (ferric-citrate uptake) had the same sensitivities to novobiocin as their respective isogenic control strain (Table 73).

At pH 5, the novobiocin sensitivity pattern is similar to that at pH 7, not significant enhancement or reversal of novobiocin sensitivity. Hence, the outer membrane proteins involved in enterochelin uptake or ferric citrate uptake do not seem to influence novobiocin uptake at either pH 7 or pH 5 in *E.coli K12*.

Table 72 : Novobiocin sensitivity of Porin mutants

Mutation (in PC0479)	% Survival	
	pH 5 0.75 µg/ml Novobiocin	pH 7 70 µg/ml Novobiocin
Control	100	89.67
ompF	0	8.69
ompC	81.33	29.42
ompCompF(1)	100	63.32
ompCompF(6)	100	42.86
ompC-R124(1)	31.34	14.86
ompC-R124(2)	59.39	33.33

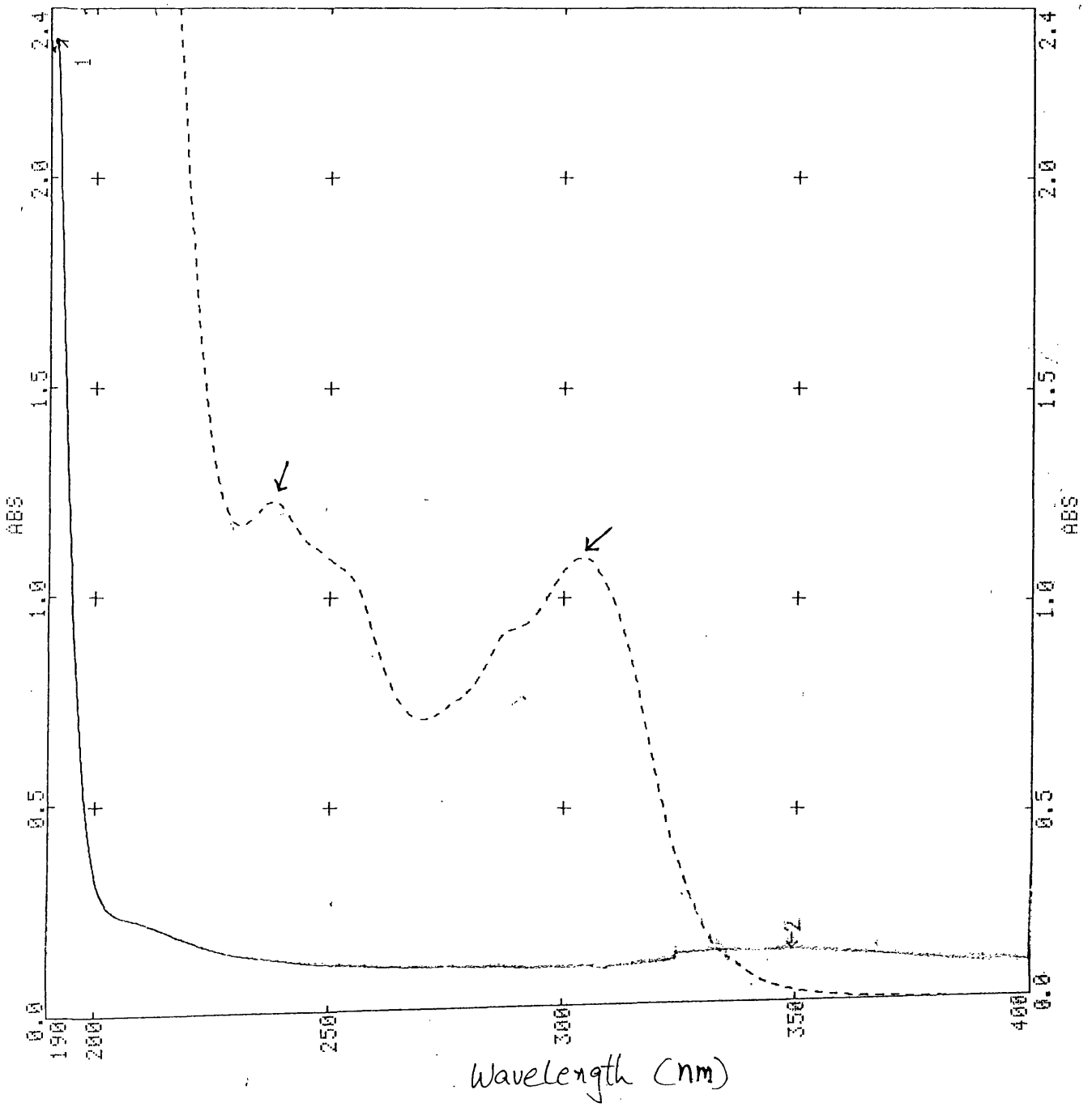
The conditions of growth are given in the Methods & Materials section.

Table 73 : Novobiocin sensitivity of Iron-uptake mutants

Strain	% Growth Inhibition with Novobiocin at pH :		
	pH 5	pH 7	
	5 µg/ml	70 µg/ml	90 µg/ml
H455 (control)	50.33	37.98	-
H455-1187 (fepA)	59.57	37.14	-
H1443 (control)	46.39	-	30.82
H1443-1619 (fhuA)	46.60	-	13.04
H1443-1594 (fiuE)	29.41	-	-
H1443-Z (fecA)	12.37	-	7.14

Cells were grown and tested at 30°C in broth. Other conditions are given in the Materials & Methods section.

Fig.41 : Effect of pH on Novobiocin spectrum .



: at pH 5 in distilled water.
: at pH 7 in distilled water, 2 peaks at 238 nm and 304 nm.

4. Discussion

Col V and antibacterial agent sensitivity :

ColV,Ia-K94 sensitises its host to many hydrophobic antibacterials (eg. erythromycin, rifampicin, novobiocin, nitrofurantoin), to cationic antibacterials (gentamicin and polymyxin B) and to acidic hydrophobic quinolones (nalidixic acid and flumequin) but not to hydrophilic/amphoteric quinolones (norfloxacin, ciprofloxacin, ofloxacin and pefloxacin). The effect of ColV,Ia-K94 on sensitivities to the antibacterial agents erythromycin, rifampicin and novobiocin found here were in agreement with those found by Davies et al (1986) and Alfa et al (1987). ColV,Ia-K94 has also been shown to sensitise its host strain to a variety of environmental factors (Ghazaleh et al, 1989; Cooper & Rowbury, 1986; Rowbury, 1986;de Pacheco et al, 1985) and the presence of the conjugational transfer functions encoded on the plasmid has been partly implicated in promoting this increased sensitivity. The ColV,Ia-K94 plasmid appears to be fully expressed in the laboratory strain ED1829 as suggested by the consistent exhibition of its phenotypic characteristics such as a high frequency of conjugational transfer, consistent colicin production, increased sensitivities to various antibacterials, etc. This is not always the case with large conjugational plasmids; those present in strains isolated from the environment eg. from sewage water, or from clinical samples often show poor expression of for example the transfer properties. The possible involvement of the transfer functions and colicin components of ColV,Ia-K94 in altering the antibacterial agent sensitivity profiles have been investigated in this study. Transfer components and some other components were found to be necessary to confer the increased sensitivities to the hydrophobic antibiotics erythromycin and rifampicin in one system used to determine this, whereas in another system used to give a comparable effect a plasmid which carries the transfer components or one which carries the colicin components were each

found to be sufficient to confer increased antibacterial agent sensitivity associated with ColV,Ia-K94 even when each type of plasmid was present alone. In the first system, the colicin components were cloned on vector plasmids (pCR612, pKF40) and the transfer components were on a mutant derivative of ColV,Ia-K94 which was deficient in colicin production and immunity. In the second case, both types of components were on mutant derivatives of ColV,Ia-K94 ie. colicin and immunity components on transfer deficient ColV-M50(1) and the transfer components on ColV-M40(5). Hence in the latter case, ColV-M40(5) may confer sensitivity to antibacterials due to transfer components plus some other Col V component(s) whilst ColV-M50(1) may confer some sensitivity due to the latter, assuming that all transfer components are completely absent from this ColV,Ia-K94 derivative. This conclusion that an additional unknown component(s) is(are) needed for conferring full sensitivity is supported by the results obtained from the first system which implied the operation of a factor other than transfer components in conferring the increased antibacterial agent sensitivity phenotype exhibited by the intact ColV,Ia-K94. This additional factor may be a specific permeation factor such as a major plasmid-encoded outer membrane protein capable of facilitating the entry of hydrophobic agents through the normally relatively impermeable outer membrane, or it may be a component which alters envelope composition and/or has a general destabilising effect on the outer membrane which is reflected by the increased uptake of compounds normally unable to enter.

The only specific component of the type suggested above that has yet been found is the major outer membrane protein VmpAp; the attempted cloning of and preliminary transposon-mutagenesis (by Tn1) of this component to determine its function were not successful (Reakes, 1987). An attempt was made here to transposon mutagenise the ColV,Ia-K94 by transposons Tn1 and Tn7, and then to create a library of its genome by which to help elucidate the areas of ColV,Ia-K94 concerned in conferring the various (above mentioned) phenotypes. Outer membrane protein profiles on SDS-PAGE gels of Tn1 and Tn7 transconjugants

of ColV,Ia-K94 were found to be similar to those of strains carrying the original parent plasmid ColV,Ia-K94, with no obvious changes in the major protein content. Thus it appears that in the mutants isolated, the transposons may have inserted into regions not affecting the synthesis of major envelope components, however only further detailed studies of these mutagenised derivatives with respect to the phenotypes associated with ColV,Ia-K94 will enable us to define specific areas of the genome associated with the corresponding phenotype if the phenotypes tested are due to the presence of a specific ColV-encoded permeation factor.

Investigations into the second possibility ie. that ColV,Ia-K94 may affect the overall stability of its host cell outer membrane have shown some interesting results. Changes in the outer membrane may occur in either the structures of the components eg. LPS structure, or in the relative proportion of each component with respect to the others or in their interactions. Any of these changes might contribute towards altered outer membrane permeability via changes in one or more of the entry pathways across the outer membrane eg. hydrophilic (eg. due to a change in the porin content or activity), hydrophobic (due to increased amounts of phospholipid bilayer) or self-promoted uptake (lower amounts of LPS or weakly-bonded LPS) pathway.

Changes in envelope composition of ColV$^+$ strains :

Protein Content :

Four of the five Col V plasmids tested here (ColV,Ia-K94, ColV,Ia-K94 Tn10, ColV-K30 and ColV-8), the exception being ColV-41, for their effect on the outer membrane protein content were found to confer significant increases in this parameter* (Table 28). This increase may be attributed to the presence of some transfer proteins which are inserted into the outer membrane, to colicin or immunity proteins, VmpA protein, iron-uptake proteins associated with ColV-K30 and possibly to some other proteins not yet defined. In any case, the

increase in the outer membrane protein content must also affect the amount of the LPS content in the outer leaflet and the phospholipid content in the inner and (possibly also in the outer leaflet) as the overall surface area of the cell is assumed to be the same in cells containing these Col V plasmids and the isogenic cells lacking them. Also the extent of the hydrophobic or self-promoted pathways might be altered by the changed protein-LPS interactions that are likely due to the increased protein content. (↑ protein content of outer membranes).

Phospholipid Content :

Increased permeability to hydrophobic agents such as certain antibacterial agents as well as various dyes, may involve the partition of these molecules into phospholipid bilayer regions that form in the defective outer membrane of the ColV,Ia-K94$^+$ strain, hence implying a facilitation of the hydrophobic pathway. If there is an increase in the relative amount of phospholipid bilayer then it should be reflected in a corresponding increase in the amount of phospholipid accessible to an externally added phospholipase. Investigation into the amount of accessible phospholipid in the outer membrane of Col V containing cells showed that the background rate of phosphate (probably phospholipid) release into the culture medium was higher for cells containing ColV,Ia-K94 and ColV-K30 compared to that for the isogenic plasmid-less control strain. This difference in the background phosphate or phospholipid release rate could suggest that there may be more phospholipid in the outer leaflet of the outer membrane of ColV$^+$ cells, ie. that there could be a greater area of the cell surface with phospholipid bilayers and hence support the above proposal that increased permeability to hydrophobic agents (as observed with Col V,Ia-K94) is partly due to the formation of such bilayer regions. Alternatively, the ColV$^+$ strain outer membrane may be less stable ie. components may be lost during growth. This could be due to discontinuities in the outer membrane, and such discontinuities could allow entry of normally non-penetrating agents. Phospholipase addition released very little more ^{32}P from ColV$^+$ strains compared to the amount released in the absence of this enzyme. This could result

from weak enzyme activity but it is difficult to explain.

Altered LPS structure or Content :

Increased sensitivity to cationic antibacterial agents such as gentamicin and polymyxin B associated with ColV,Ia-K94 suggests that the Col V may affect the operation of the self-promoted uptake pathway possibly by altering the LPS content in the outer membrane or its structure. The former may be true as the excess protein content may mean a lower LPS content in the outer membrane. An attempt was made to investigate a possible change in LPS structure. The LPS of the ColV,Ia-K94+ strain differs in its hygroscopic properties from the ColV- one (with ColV-K30+ LPS in between). It may be the same altered properties that affect hygroscopic behaviour that render the stored molecules difficult to dissolve in solvents such as water, ethanol, methanol and DMSO. However, no apparent difference in the molecular weight of the three LPS's was seen from their migration properties on polyacrylamide gel. Altered ability to interact with water ie. moisture from the atmosphere, suggests that the Col V has altered some groups on the LPS which are involved in ionic interactions eg. phosphate groups or amino groups on one or more sugars of the LPS core region, carboxyl groups on the KDO or the fatty acid tails of lipid A region, or both. There appears to be more KDO in the purified LPS from ED1829 ColV,Ia-K94 and ED1829 ColV-K30 (Table 29) as compared to the LPS from the control strain and this may in part account for the proposed increase in the number of ionic groups on the LPS which may alter the LPS-LPS or LPS-protein interactions. It should be interesting to see whether these extra ionic groups are cationic or anionic in nature. NMR spectroscopy of Col V LPS should also aid the pinpointing of the type of molecule that the altered charged groups are present on and exactly how many are altered. Inability to dissolve the purified LPSs from ColV strains hindered this aspect of investigation but future preparations of purified LPS from Col V strains should be made as a salt of a cation such as sodium or

a small organic amine such triethylamine which may facilitate its solubility properties to suit its analysis. Also, mass spectroscopy (suitable for macromolecules) may highlight any possible small changes in the LPS molecular weight from the different strains which may be too small to be detected by polyacrylamide eletrophoresis.

Inhibitor sensitivities conferred by other Col V plasmids :

A Tn10 derivative of ColV,Ia-K94 responded in an almost identical fashion to its parent plasmid with regards to the antibacterial agent sensitivities except that ColV,Ia-K94 Tn10 sensitised its host to nalidixic acid whereas ColV,Ia-K94 conferred resistance to this agent. ColV-K30 showed a mixed response to the antibacterials tested in this study. ColV-K30 sensitised its host to rifampicin and novobiocin but not to the other hydrophobic antibacterials tested ie. erythromycin, rifamycin, or nitrofurantoin, or to the cationic ones gentamicin and polymyxin B. A mixed response was observed to 4-quinolone sensitivity by ColV-K30 as it conferred no effect on norfloxacin, pefloxacin or flumequin sensitivities but conferred an increase in sensitivities to ciprofloxacin and ofloxacin. It appears that the hydrophobic pathway in ColV-K30+ strain is not enhanced to the same extent as it is in ColV,Ia-K94$^+$ strain, and that the self-promoted pathway is not significantly affected by plasmid ColV-K30. Possibly, if LPS lesions are responsible for altered sensitivity, then ColV-K30$^+$ strain has lesions which are smaller than those in ColV,Ia-K94$^+$ strain. The data obtained from the phospholipid release study and also from the KDO content data are in accord with this as values for both these parameters for ED1829 ColV-K30 were found to be intermediate when compared to those from ED1829 and ED1829 ColV,Ia-K94. Some of the known factors which distinguish ColV-K30 from ColV,Ia-K94 and which might influence the envelope, include the presence and expression of the aerobactin-mediated iron uptake system and the absence (on ColV-K30) of the colicin Ia and its immunity component.

ColV-8 and ColV-41 are plasmids originally present in sewage-water isolates

of *E.coli*, but there seem to be major differences in the antibacterial agent sensitivities that each confers on its laboratory host, strain ED1829. ColV-8 sensitises ED1829 to erythromycin, novobiocin and nalidixic acid (at 1.5 ug/ml, but not at 2 ug/ml), and to the 4-quinolones ciprofloxacin, ofloxacin, pefloxacin and flumequin, but it confers no effect on sensitivity to rifamycin, rifampicin, nitrofurantoin, norfloxacin or to the cationic antibacterials gentamicin and polymyxin B. The consistency observed in the increased sensitivity to most of the 4-quinolones, except norfloxacin, by ColV-8 implies that this Col V may either encode a new pathway via possibly a new porin, or that it may enhance the expression of an existing porin which would then account for the relatively higher permeability to these agents which use the hydrophilic pathway as a major route of entry across the outer membrane. The hydrophobic pathway in cells harbouring ColV-8 appears to be enhanced to a similar level as it is by the plasmid ColV-K30. The self-promoted uptake pathway however, appears not at all influenced by ColV-8.

Another isolate from the same environmental source as ColV-8, ie. ColV-41, appears to be extremely active in enhancing the hydrophobic and self-promoted uptake pathways across the outer membrane. ColV-41 conferred sensitivity to the hydrophobic inhibitors erythromycin, rifampicin, novobiocin, as well as to the cationic inhibitor gentamicin, but not to the hydrophilic/amphoteric 4-quinolones norfloxacin, ciprofloxacin, ofloxacin, pefloxacin or to the acidic hydrophobic flumequin. ColV-41 was the only Col V plasmid found to confer an increased sensitivity to the two rather inefficient Gram-negative inhibitors rifamycin and bacitracin. Although this plasmid might affect the sites of action of these inhibitors ie. transcription mediated by the DNA-dependent-RNA polymerase, and peptidoglycan synthesis respectively, it is more likely that it is extreme penetrability of the envelope that allows sensitivity. Indifference to polymyxin B sensitivity of ED1829 ColV-41 suggests that the lipid A region and possibly the core region of the LPS may be altered in ED1829 ColV-41 cells such that the region concerned is either not accessible to polymyxin B or that its ability to bind polymyxin B is lost. ColV-41 noticeably

slows down the growth rate of its host strain ED1829. Plasmids ColV-8 and ColV-41 are difficult to transfer by conjugation which might imply repression of the transfer operon in these plasmids as compared to that in ColV,Ia-K94 and ColV,K30 and hence the differences observed in the ability of these two plasmids to confer antibacterial agent sensitivities might suggest that the transfer components may well not have any specific role to play in altering outer membrane permeability. However, in fact, although ColV-41 confers poor transfer ability, it makes *E.coli* sensitive to the male-specific MS2 phage (M.Goodson, unpublished observations). Accordingly, ColV-41 must confer certain transfer components and presumably these may be linked to sensitivity to antibacterials. Also, ColV-8 and ColV-41 are both very good colicin V producers ie. better than ColV,Ia-K94, as seen by the relatively larger inhibition zones obtained on colicin testing, and this common property of the Col V plasmids may have some role to play in altering outer membrane permeability. Up till now, ColV,Ia-K94 has been the Col V plasmid used to test the various Col V associated phenotypes as it apparently is a better exhibitor of these properties. However, the discovery of increased enhancement of antibacterial agent sensitivity, with ColV-41 has provided us with an extra tool.

Comparisons between the effects conferred on their host strain by plasmids ColV,Ia-K94 and ColV-41 may be made regarding eg. the outer membrane protein profiles of the isogenic host strain carrying each plasmid, either on one dimension SDS-polyacrylamide gels, or on two dimension gels to help resolve the presence of any extra major proteins associated with ColV-41 which may be masked by the normally found major proteins as these migrate to the same position on one dimension SDS-PAGE gels eg. one or more transfer components may be present at excessive levels in ColV-41$^+$ strain. Also, sensitivity profiles to various colicins and phages may show some differences with respect to any specific outer membrane receptors which may be associated with ColV-41, including sensitivity to cloacin DF/13 which is a receptor for the iron-aerobactin siderophore complex which has been found to be used by a

rifampicin derivative for entry across the outer membrane, and hence may partly account for the increased sensitivity to rifamycin which is a very weak inhibitor of Gram-negative cells regardless of the presence of the Col V plasmids. Analysis of purified LPS eg. molecular weight determination, homogeneity/heterogeneity of LPS bands on polyacrylamide gels and ^{1}HNMR, ^{13}CNMR or ^{31}PNMR spectrosopy, from ColV-41 harbouring cells may also shed some light on the possible LPS alterations associated with ColV-41. Effects of magnesium ions on the possible reversal of enhanced antibacterial agent sensitivity associated with ColV-41 may also indicate whether the self-promoted pathway is affected by this plasmid although magnesium ions are involved in many cellular processes other than outer membrane permeability. Regions of ColV-41 genome may be cloned on small vector plasmids to determine the involvement of at least one important region ie. the transfer operon, in the expression of the various ColV-associated phenotypes.

Properties of ColV from a Clinical isolate :

A Col V plasmid (Col-V2/5656) originally isolated from a clinical strain failed to confer enhancement of sensitivity to hydrophobic antibacterial agents on its laboratory host strain. Col-V2/5656 was transferred by conjugation from the clinical strain to a laboratory strain. In general, conjugational transfer of clinical plasmids is at a very low frequency if it occurs at all (Reakes, 1987 PhD thesis & this study). It may be that the transfer functions are suppressed under most conditions and/or that their expression is facilitated only when the cell is inside the host organism. The wild-type Col V plasmid Col-V2/5656, encodes the production of the VmpA protein (Rowbury *et al*, 1985) which ColV,Ia-K94 has also been shown to express. However, many other properties associated with ColV,Ia-K94 such as effects on motility and on the ability to clump or agglutinate in static culture at 37°C (Tewari *et al*, 1986), mucoid phenotype, sensitivity to phages MS2, T4 and Mel (de Pacheco *et al*, 1985;Reakes *et al*, 1987) and

derepressed transfer functions are absent when Col-V2/5656 is in a laboratory host HB101. A similarity between ColV,Ia-K94 and Col-V2/5656 is the ability to suppress an increased sensitivity associated with *ompA* mutants to gentamicin, EDTA and polymyxin B. This similarity can be correlated with the presence of the major outer membrane VmpA protein which is associated with both plasmids, and its presence being able to complement the lesion caused by the *ompA* mutation. In ColV,Ia-K94, there is a directly proportional relationship between temperature and the expression of certain plasmid-associated phenotypes such as the production of VmpA protein and colicin and transfer components (Tewari *et al*, 1985), which suggests that there may be one or more groups of operons on ColV,Ia-K94 which are only fully switched on in the animal body ie. at temperature of 37°C or higher. However, with Col-V2/5656 such factors alone do not lead to expression of most Col V properties.

Properties conferred by ColV,Ia-K94 on a wild E.coli strain :

ColV,Ia-K94 sensitises its host laboratory strain to acid pH (Cooper & Rowbury, 1986), alkaline pH (Rowbury, 1986), high temperature (Ghazaleh *et al*, 1989) and to inhibition by some other putative environmental agents (de Pacheco *et al*, 1985). When the laboratory plasmid ColV,Ia-K94 is transferred by conjugation into a wildtype *E.coli* strain, 14 (isolated from a chicken processing plant), the antibacterial agent sensitivities associated with this plasmid when it is present in the laboratory strain ED1829 are abolished. It appears therefore that ColV,Ia-K94 properties are also repressed in a wildtype strain eg. in strain 14. Sensitivity increases associated with ColV,Ia-K94 to hydrophobic inhibitors such as erythromycin (Alfa *et al*, 1987), rifampicin and novobiocin (Davies *et al*,1986) have been proposed to involve the transfer functions of ColV,Ia-K94 and therefore 14 ColV,Ia-K94 may be repressed in transfer properties; this could be easily checked by assessing the frequncy of conjugational transfer or by testing for sensitivity to MS2 phage. It would be interesting to see whether expression of other ColV,Ia-K94 phenotypes such as serum resistance,

increased adherence, resistance to phages P1 and Me1, are also supressed in the new host strain background. It may simply be, however, that the envelope of strain 14 does not allow integration of some ColV,Ia-K94 components and therefore does not show inhibitor sensitivity.

Effect of Polycations on Permeability :

A major part of the present study showed that polycationic agents enhanced the inhibitory activity of a number of antibacterial agents. Because of the role of cations in preserving the integrity of the outer membrane permeability barrier to hydrophobic antibacterials, one major possible mechanism for this effect of polycations is by permeabilising the outer membrane. Alternatively, cationic agents may act internally eg. on the active sites of enzyme or enzyme-substrate complexes, or on other macromolecules eg. nucleic acids. This is particularly likely since a number of the active cationic agents are polyamines and naturally occurring members of this group have important cytoplasmic effects.

Polyamines are widely distributed in nature, being present in animal tissues, fungi and bacteria. They have a role as growth factors but their action may in part be related to nucleic acid metabolism as evidenced by their high affinity for nucleic acid *in vitro* (Razin & Rozansky, 1959) and their association with phage DNA (Ames & Dubin, 1960). Polyamines such as spermidine, are intrinsically involved in cellular metabolism especially in keeping the cation balance via acetylation and deacetylation reactions. At alkaline pH, spermidine has bacteriostatic properties (Rozansky *et al*, 1954) and it is in this pH range that spermidine is most actively acetylated whereupon it loses its toxicity (Dubin & Rosenthal, 1960). Polyamines act as nonspecific polyvalent cations to neutralize DNA and can be replaced by other cations (Ames & Dubin, 1960). In addition, a role for polyamines such as spermidine in maintaining the fidelity of protein synthesis has also been reported (Abraham *et al*, 1972; Atkins *et al*, 1975). The mechanism influencing the fidelity of mRNA translation may involve an alteration of the tRNA conformation

(Sakai & Cohen, 1973) followed by an influence on the codon-anticodon recognition process (Revel & Littauer, 1966). Recent work with polyamines has highlighted their involvement in enhancing the rate of DNA replication, transcription and translation (Geiger & Morris, 1980; Kurland, 1982;Russell, 1983; Moussatche, 1985). It may be because of this that an disruption of polyamine synthesis due to either mutation (Hafner *et al*, 1979) or by using inhibitors (Pegg, 1986) results in a reduction or absence of cell growth. If polyamines which do not occur naturally can have this effect, this may explain that growth in the presence of some of the polyamine compounds, especially the enhancers of novobiocin activity, resulted in an increase in the growth rate of the control strain. *In vitro* interaction of polyamines also influences the phase transition of DNA structure from an extended conformation to compact structures, which then undergo higher transcriptional activity than naked DNA (Baeza *et al*, 1987). However, polyamines do not bind to nucleases (Shishido, 1985) or to DNA polymerases although because of its interaction with nucleic acids spermidine makes plasmid DNA resistant to restriction endonuclease cleavage (Pingoud *et al*, 1984; Kousmanen & Posa, 1985) and stimulates bacterial polymerases (Russel, 1983). Accordingly, natural polyamines can influence reactions taking place on nucleic acids and, presumably, if they can enter the cell, other cationic agents might have similar effects. Novobiocin is a DNA gyrase inhibitor and binds to the B subunit to stop ATP-dependent DNA replication. One explanation for the enhancing effect of the polycations on novobiocin activity could be that they bind to DNA and cause an alteration in its conformation such as to alter the affinity of the gyrase for DNA. If this were so then these polyamines would cause an inhibitory effect themselves which most of the enhancers do not appear to. Alternatively, polycation binding to the DNA might alter the gyrase conformation so as to influence novobiocin binding without influencing overall activity. Such possible binding to the gyrase (to either A or B subunit) and causing a possible induction of a change in its conformation to make it more susceptible to its inhibitors is probably eliminated by the lack of enhancement observed with norfloxacin and ofloxacin

activity. The enhancement of the activity of the very hydrophobic 4-quinolone flumequin by cationic agents suggests that enhancement of inhibition by cationic agents depends on the hydrophobicity of the inhibitor, which in turn implicates a difference in the route of entry of the compound into the cell, rather than any change (induced by the cationic agent) in the interaction of the inhibitor at its site of action inside the cell. In addition, the fact that only the effects of erythromycin, rifampicin, nalidixic acid and bacitracin are enhanced by cationic agents and not the effects of nafcillin, oxacillin, deoxycholate, nitrofurantoin, rifamycin and fusidic acid, suggests the presence of a certain upper and lower limit in terms of charge and hydrophobicity with respect to entry enhanced by the cationic agents. Erythromycin and rifampicin are large and hydrophobic and may be able to penetrate due to any transient displacement of LPS. Polycations can compete with magnesium ions and displace them as shown by the prior growth experiments where the presence of both magnesium ions and cationic agents was followed by increased sensitivity to novobiocin even upon the removal of any unattached cations from the cells. It is very likely that the cationic agents do bind to LPS and neutralize the anionic charges, and may even cross-link LPS molecules in the outer leaflet but their larger size (as compared to magnesium ions) does not reproduce the tightpacking of LPS that magnesium ions allow.

All the above experiments measured optical density. Inhibition of DNA replication leads indirectly to inhibition of growth. Here, it has been shown however, that at lower novobiocin concentrations (10-12.5 μg/ml), cell division, which is more directly affected by inhibition of DNA replication, is inhibited more in the presence of the cationic agents than in their absence. Filamentation in cultures containing either novobiocin alone or diaminoacetone alone is similar to that in the control ie. very low. This suggests that the cationic agents facilitate the entry of novobiocin such that it reaches its target where its concentration is high enough

to inhibit DNA replication but not high enough to inhibit the duplication of the cell components thus allowing the cell mass to increase, followed by partition of the cell components into daughter cells but the lack of chromosome duplication does not allow the full cell division.

It is interesting to note that nalidixic acid sensitivity is enhanced to the same great extent as novobiocin sensitivity by diaminoacetone, which is higher than the enhancement produced for either erythromycin, rifampicin or bacitracin sensitivity. This may suggest some possible involvement of the acidic properties of both novobiocin and nalidixic acid in their facilitated entry.

Piperazines are able to complex cations and the cationic agents N-(2-pyrimidinyl)piperazine and 1,4-diaminopiperazine may act to simply displace magnesium ions as above or may act as chelators in a similar way to EDTA or polyphosphates to remove magnesium ions from their proposed normal sites between the LPS molecules. This would make the outer leaflet of the outer membrane unstable due to the increased repulsion between the LPS molecules and the possible release of LPS due to its efflux from the unstable outer leaflet, thus facilitating the entry of hydrophobic compounds such as novobiocin and others. Further experiments to determine the effect of these piperazine cationic agents on sensitivity to antibacterial agents such as EDTA, polymyxin and novobiocin, when present together and during growth prior to inhibition testing need to be carried out to determine whether the chelating property of these piperazines is involved to any extent in the novobiocin activity enhancing effect given by them.

For the cationic agents spermidine, agmatine, diaminoacetone, 3,3'-diaminobenzidiine, pyridoxamine, 3-aminobenzamidine and 4,5,6-triaminopyrimidine, the magnesium displacement theory and thereafter the entry of novobiocin ie. the self-promoted uptake pathway, seems adequate to explain the enhancement in novobiocin sensitivity. However, the lack of any enhancing effect on the sensitivities to the agents nafcillin, oxacillin, vancomycin, deoxycholate and

nitrofurantoin cannot be explained by simply displacement of magnesium ions by diaminoacetone and concomitant proposed instability of the outer membrane followed by entry of any hydrophobic inhibitor to reach their various different sites of action. A dependence on the extent of hydrophobicity, and possibly also on the acidity of the entering compound, in combination with the membrane destabilising action by diaminoacetone is implied by the responses seen to the sensitivities given by these latter inhibitors.

Serum sensitivity was significantly enhanced by diaminoacetone. The extent of serum sensitivity has been found to be dependent on the structure of many of the envelope components of Gram-negative bacteria such as O and K antigens as well as on the protein and lipid profiles including any changes in LPS in the outer membrane (Binns *et al*, 1982). Another polycation shown to enhance serum sensitivity of bacteria is PMBN (Vaara & Vaara, 1983(a)). Enhancement of polymyxin B sensitivity by diaminoacetone suggests that diaminoacetone is not a competitor but a facilitator of polymyxin B binding to its site in the outer membrane, thus if both diaminoacetone and PMBN enhanced serum sensitivity by exposing the same specific site for the formation of the complement cascade reactions then competition between the two would be expected when they are both present in the medium. Alternatively, both polymyxin B and diaminoacetone may just have a general destabilising effect on the outer membrane which exposes the area of the cell envelope sensitive to the complement system. Further work on the effect of diaminoacetone on the sensitivity of heat treated serum ie. inactive complement, and with specific antibodies should throw some light on which immunity pathway this cationic compound facilitates.

Enhancement of hydrogen peroxide activity towards *E.coli* by diaminoacetone suggests that cationic agents might enhance phagocytic killing of Gram-negative bacteria even when no specific immune response to the organism has been initiated.

The nonenhancers of novobiocin activity differ in structure and the number

of positive charges from the enhancers (Figs.41 & 42). It appears that to be able to act as a novobiocin activity enhancer, it is necessary to have at least two amino groups in close vicinity to each other on the molecule eg. on diaminobenzidine and agmatine ; or that two or more amino groups should be adjacent to each other due to the orientation of the other atoms in the molecule eg. in diaminoacetone, methylglyoxyl bis(guanyl-hydrazone), spermidine and diamino butanone. With the cationic piperazines the striking common feature is the presence of one or more nitrogen atoms in the ring eg. with N-(2-pyrimidinyl)piperazine, or supplementation of these nitrogen atoms by additional amino groups branching out from the ring.

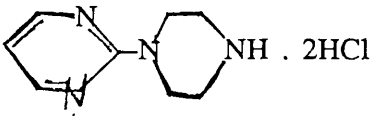
When the structures of the nonenhancers of novobiocin activity are compared then the presence of interrupting carbon atoms or methylene groups between amino groups is very noticeable eg. on triethylenetetramine, tetraethylenepentamine and diethylenetriamine; or that the amino groups are located at each end of the molecule with a short aliphatic chain of methylene groups separating them thus allowing the cationic charges to be dispersed on the molecule eg. on formamidine disulfide. Hence it appears that the conformation of the cationic compound and the resulting orientation of the amino groups is crucial in conferring the ability to displace magnesium ions as is also the number of amino groups. Viljanen & Vaara (1984) found that for activity in their system at least five positive charges on the cationic agent (as there are on PMBN) are required, and that any other cationic compounds having a lower number of cationic charges are not effective permeabilisers for hydrophobic antibacterials. Spermidine was found (by them) to be ineffective in sensitising *E.coli* to the hydrophobic antibiotics such as erythromycin. Although spermidine was not tested in this study for its possible effect on erythromycin activity, the enhancing effect of diaminoacetone on erythromycin sensitivity (in this study) suggests that as low as two positive charges, if they are in the right conformation are enough to confer an outer membrane destabilising effect. Triethylenetetramine (both as hydrochloride and hydrate) was unsuccessful in permeabilising *E.coli* to novobiocin. Other workers

have found this cationic compound to enhance the inhibitory activities of carbenicillin (Smith, 1975) and gentamicin (Light & Briggs, 1979). However, in both these studies serum was present during the tests : the medium contains 25% serum *in vitro* in one case; and in the other case the tests were carried out *in vivo*. In both these cases, the observed enhancement of antibiotic activity may well be a combination of the possible enhancing effect of triethylenetetramine on serum activity which reflected as synergism between triethylene tetramine and the action of the antibiotics. In this study, triethylenetetramine has fallen under the nonenhancer (of novobiocin activity) category but this compound and others in this category may have significant clinical activity *in vivo* if they acted synergistically with serum even though they may fail to act as permeabilisers of antibacterial agents.

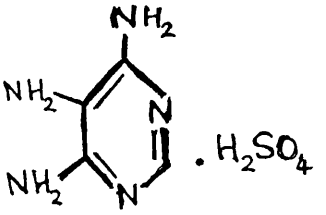
The novobiocin activity enhancing effect by cationic agents appears to be dependent on the growth medium as N-(2-pyridimidinyl)piperazine, 4,5,6-triaminopyrimidine, 1,4-diaminopiperazine, 1,3-diaminoacetone, 3,3'-diaminobenzidine and pyridoxamine lost their ability to enhance novobiocin activity in minimal medium. The growth rate of ED1829 in minimal medium is much slower than that in broth and any associated changes in the outer membrane composition eg. in the porin content and type, or an increase in LPS (Gilbert & Brown, 1978) may well affect the sensitivity of the cells towards antibiotics such as novobiocin. Also, the role of the cationic agents as permeabilisers in broth due to nonspecific binding to LPS may not occur in minimal medium possibly due to the strengthening of the outer membrane by the presence of more LPS. However, under these conditions, specific and possibly strongly associating cationic agents, such as methylglyoxyl bis(guanyl-hydrazone), (its ability to associate more strongly with LPS is suggested by enhancement of novobiocin activity even when excess unattached agent is removed from the cell surface by washing), may still function in the same manner as they do in cells grown in broth as the structure of the LPS in cells grown in either medium is presumably the same. Another possible factor influencing the novobiocin activity enhancing effect

Fig. 41 : <u>Structures of Novobiocin-activity enhancers</u> :

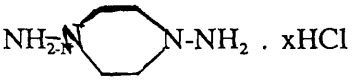
N(2-pyrimidinyl)-piperazine :



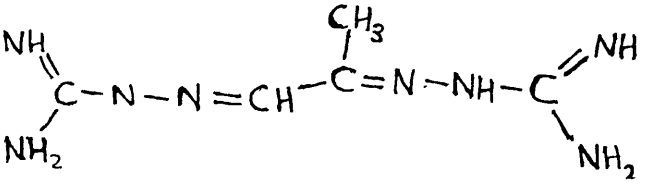
4,5,6-Triaminopyrimidine :



1,4-Diaminopiperazine :



Methylglyoxyl bis :
(guanyl-hydrazone)



Spermidine :

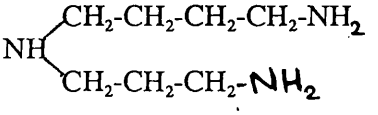
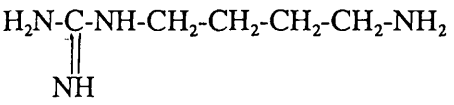
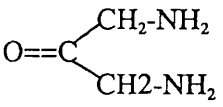


Fig. 41 continued

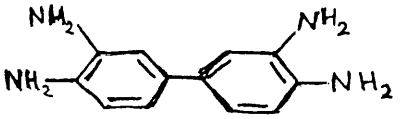
Agmatine sulfate :



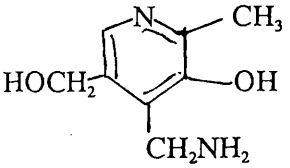
1,3-Diaminoacetone :



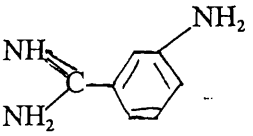
3,3'Diaminobenzidine :



Pyridoxamine :



3-Aminobenzamidine :



1,4-Diamino-2-butanone :

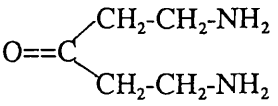
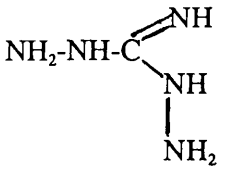
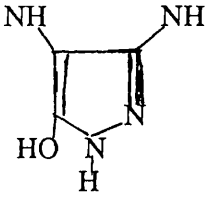


Fig 42 : Structures of non-enhancers of Novobiocin activity.

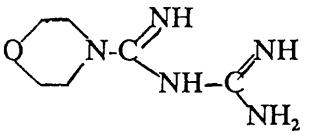
Diaminoguanidine :



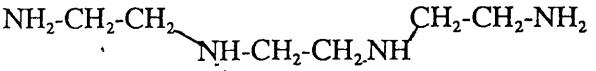
3,4-Diamino-5-hydroxy pyrazole sulfate :



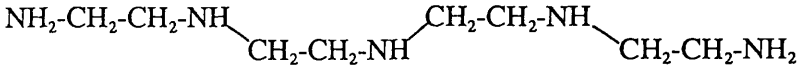
Moroxydine :



Triethylenetetramine (trien) :



Tetraethylene pentamine :



Diethylene triamine :

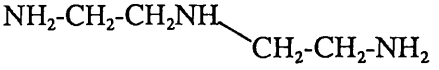
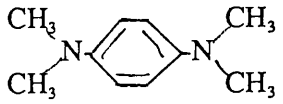


Fig. 42 continued

N,N,N',N'-Tetramethyl-p-phenylene Diamine :



Formamidine disulphide :

[-SC (=NH) NH_2]$_2$

of the cationic agents in either growth medium may be the quantity of divalent cations such as magnesium, which has been shown in this study to antagonise both novobiocin activity and also the novobiocin effect enhancing effect of the cationic agents in broth. The magnesium content of minimal medium used here was approximately 0.4 mM which may be different to that in broth.

The novobiocin inhibition enhancing effect however, is not strain-specific, as the response of another laboratory strain P678-54 was similar to that of ED1829 to the enhancers and nonenhancers of novobiocin activity except for N-(2-pyrimidinyl)piperazine which failed to enhance novobiocin activity towards P678-54. When tested on ED1829 ColV,Ia-K94, all the enhancers of novobiocin activity for ED1829 in broth conferred a similar effect except N-(2-pyrimidinyl)piperazine and 1,4-diamino-2-butanone both of which failed to enhance novobiocin activity towards the Col V^+ derivative of ED1829.

Growth inhibitory effects of various antibacterials including erythromycin, novobiocin, rifampicin, nalidixic acid, nafcillin, vancomycin and fusidic acid have been found to be enhanced by local anaesthetics such as tetracaine but those of the cationic antibacterials such as gentamicin, kanamycin and neomycin were slightly reversed by tetracaine (Labedon, 1988). In this study, 1,3-diaminoacetone succeeded in enhancing the inhibitory effects of erythromycin, rifampicin and nalidixic acid only as compared with the other inhibitors used in both cases, but further studies on the effects of some of the other cationic agents used in this study eg. methylglyoxyl bis(guanyl-hydrazone), etc. on activities of inhibitors other than novobiocin need to be done to determine the spectrum of these cationic permeabilisers. Another difference between this study and that by Labedon (1988) is that in this study the growth inhibitions were determined on exponentially growing cultures over a three hour period under well-aerated conditions ie. shaking, whereas the other study used MIC's determined as growth (or lack of) in tubes containing the antibacterial agent and the permeabiliser over a 24h period

during which the culture may well be in the stationary phase. Hence the data from these two studies cannot be directly compared as the phase of cell growth and the growth rate are both crucial factors in determining the outer membrane composition and therefore its permeability, either of which cannot be closely determined in the Labedon study due to the method used.

Prior growth with magnesium and the cationic agents diaminoacetone or methylglyoxyl bis(guanyl-hydrazone) showed no effect on EDTA sensitivity. However, EDTA sensitivity was reversed if both EDTA and either of the cationic agents were present in the medium at the same time. The implication from these results is that the cationic agents (diaminoacetone and methylglyoxyl bis(guanyl-hydrazone) protect the cell against LPS release which is induced by the divalent cation chelator EDTA; this is presumably because EDTA cannot remove cationic agent bonded to LPS. Another deduction from these responses to EDTA sensitivity is that the enhancement of novobiocin sensitivity by the two cationic agents tested does not involve LPS release to any great extent. However, release of small amounts of LPS or just the local displacement of LPS and the consequent formation of local phospholipid bilayers which hydrophobic antibacterials can partition into, seems a further feasible explanation for the enhancing effect given by the cationic agents.

Prior growth experiments with diaminoacetone or methylglyoxyl bis(guanyl-hydrazone) and magnesium, and the subsequent response of the cells to polymyxin B suggests that diaminoacetone may bind only poorly to LPS but that methyglyoxyl bis(guanyl-hydrazone) may well bind very strongly to LPS and to the lipid A component of LPS and to any other polymyxin B binding site such that it is no longer available or accessible to polymyxin B. Diaminoacetone enhanced polymyxin B sensitivity when both were present in the medium together suggesting that diaminoacetone does not bind or binds poorly to the polymyxin B binding site but its competition with magnesium ions results in actually helping polymyxin B to gain access to its binding site.

The medical importance of this enhancement of antibacterial agent activity by cationic permeabilisers might be further increased by either finding or developing non-toxic derivatives of the enhancers already studied, or by modifying the existing antibacterial agents (eg. examining whether a straight forward amination reaction increases its activity by altering its uptake) by placing additional cationic charges in a favourable conformation on the molecule. Most of the cationic agents used in this study are toxic which reduces their use in *in vivo* studies but they can still have a great potential use as permeabilisers of outer membranes in *in vitro* studies.

pH and antibacterial agent activity :

Results from the effect of pH on antibacterial agent sensitivity suggest that the level of protonation of the agent before its entry into the cell is responsible for its uptake and activity. Novobiocin, rifampicin and nitrofurantoin are most active versus *E.coli* K12 at low pH whereas erythromycin and gentamicin are most active at alkaline pH. Col V plasmids were found to confer no interference on the effect of pH on antibacterial agent activtities.

Another possibility is that these antibacterial agents use a different uptake pathway at the different pH's. Results from novobiocin sensitivity of porin mutants at pH 5 and pH 7 suggest that novobiocin mainly uses the the hydrophobic pathway at neutral pH, or the self-promoted pathway if any permeabilisers such as polycations are present, but at pH 5, it may use a combination of the hydrophobic pathway and a new pathway to enter the outer membrane. The response of the OmpF mutant to novobiocin sensitivity suggests that the OmpF porin is not used by novobiocin at either pH, but the consequences suffered by the outer membrane as a result of the absence of large amounts of the OmpF porin protein in the outer membrane considerably changes the proportions of the other remaining components, and this altered outer membrane composition is favourable for novobiocin entry.

Mutations in the iron-uptake system do not affect novobiocin uptake at either pH 5 or at pH 7. However, the siderophore-iron complex uptake porin must be sensitive to the cationic charge on the complex and on this assumption, the effect of the cationic charges on novobiocin uptake in these mutants should be interesting. The cationic agents may succeed in opening the large iron-siderophore uptake channels.

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Appendix

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