THE MODE OF ACTION OF COLICIN E2 WITH REGARD TO THE
STRUCTURE OF THE \textit{Escherichia coli} CELL ENVELOPE

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List of Abbreviations used in this Text

c = centi (as a prefix)
m = metre, milli (as a prefix)
\(\mu\) = micron, micro (as a prefix)
\(\AA\) = Ångstrom unit (= 0.1 \(\mu\) = \(10^{-8}\) cm)
g = gram (mass) or 980 cm sec\(^{-2}\) (centrifugation)
l = litre
C = Curie
A = Ampère
sec = second
min = minute
hr = hour
fig = figure
u = unit of colicin activity (spot assay)
w/v = weight in grams per final volume in mls
v/v = volume per final volume (aqueous solution unless otherwise specified)
pfu = phage forming unit
dia. = diameter
i.d. = internal diameter
e.d. = external diameter
\(x^\circ\) = \(x\) degrees Centigrade
Tris = Tris (hydroxymethyl) amino methane
S = Svedberg unit
NB = Nutrient Broth
O.D. = Optical Density (1 cm light path)
DEAE = Diethylaminoethyl
NT = Not Tested
BDB = Bisdiazotized benzidine
SNLS, MNLS, CNLS = Sodium, Magnesium, Cadmium N-lauroyl sarcosinate respectively
I = Ionic strength (in buffers)
HEPES = N-2-Hydroxyethylpiperazine N'-2-ethane sulphonic acid
BSA = Bovine serum albumin
CM = Carboxymethyl
R = Resistant
S = Sensitive
K = 1000 fold diameter magnification
M = Molar or Mole
CPM = counts per minute (radioactivity)
EDTA = ethylenediamine tetraacetate (disodium salt)
SDS = Sodium dodecyl (Lauryl) sulphate
UV = Ultraviolet
TCA = trichloroacetic acid
DOC = deoxycholate (sodium salt)

sodium phosphate buffer = equimolar mixture of Na$_2$HPO$_4$ and NaH$_2$PO$_4$ salts to give the desired pH

potassium phosphate buffer = ditto potassium salts
# INDEX

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>MATERIALS AND GENERAL METHODS</strong></td>
<td>31</td>
</tr>
<tr>
<td><strong>SECTION I</strong></td>
<td></td>
</tr>
<tr>
<td>The Preparation of Colicin E2 suitably</td>
<td></td>
</tr>
<tr>
<td>labelled for microscopic study.</td>
<td>46</td>
</tr>
<tr>
<td>Introduction</td>
<td>46</td>
</tr>
<tr>
<td>Methods</td>
<td>55</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td></td>
</tr>
<tr>
<td>The use of fluorescein labelled colicin E2.</td>
<td>87</td>
</tr>
<tr>
<td>Slide agglutination assay for anti-E2 immune serum.</td>
<td>93</td>
</tr>
<tr>
<td>Heterogeneity of 6.6s Y globulin and horse ferritin.</td>
<td>94</td>
</tr>
<tr>
<td>Ferritin conjugation to anti-E2 6.6s Y globulin</td>
<td>104</td>
</tr>
<tr>
<td>Separation of conjugate, reactant and products.</td>
<td>107</td>
</tr>
<tr>
<td>The preparation and assay of bisdiazo­-nized benzidine.</td>
<td>124</td>
</tr>
<tr>
<td>Characterization of the ferritin 6.6s Y globulin conjugate.</td>
<td>124</td>
</tr>
<tr>
<td>Ferritin Conjugation: Conclusion.</td>
<td>128</td>
</tr>
</tbody>
</table>
## SECTION II Electron Microscopy of Phage BF23 and Ferritin Anti-E2 Gamma-Globulin Adsorbed to Bacterial Cells

- **Introduction**
- **Methods**
- **Results and Discussion**
  - Structure of BF23 bacteriophage and plasmolysed *E.coli* K12 cells.
  - Examination of colicin refractory mutants and ferritin anti-colicin E2 treated cells.
- **Conclusions**

## SECTION III The Interaction of Colicin E2 with the Bacterial Cell Surface

- **Introduction**
- **Methods**
- **Results and Discussion**
  - Effect of colicin E2 on the release of surface-located enzymes.
  - Effect of colicin E2 on the composition of envelope proteins.
  - Effect of plasmolysis upon colicin E2 induced DNA degradation.
  - Effect of osmotic shock upon colicin E2 induced DNA degradation.
  - (A) Effect of colicin E2 concentration on induced DNA degradation.
Effect of colicin E2 on DNA degradation in osmotically shocked cells. 205

Protein synthesis in osmotically shocked cells. 209

Effect of osmotic shock on the continuation of DNA degradation induced by mitomycin C and colicin E2. 212

(A) High magnesium shock. 212

(B) Low magnesium shock 217

Conclusions 220

SECTION IV Fractionation of the Bacterial Cell Envelope 224

Introduction 224

Methods 225

Results and Conclusions. 237

Colicin E2 refractory mutants. 237

SDS acrylamide gel electrophoresis 239

(A) General properties of envelope protein profiles. 239

(B) Envelope protein analysis of CetA, CetB and CetC mutants. 253

(C) Envelope protein analysis of CetC revertants. 256

(D) Location of polypeptide e in the bacterial cell envelope. 259

Cadmium N-lauroyl sarcosinate elution. 262

(A) Radioactive protein-lipid profiles. 265
(B) Qualitative analysis of different protein fractions eluted from CNLS complexes. 268

Discussion 271

DISCUSSION 288

APPENDIX I Calculation of the number of colicin molecules that can be close-packed on a typical E.coli cell surface 295

APPENDIX II Benzidine: Its efficiency of bisdiazotization. 297

APPENDIX III Estimation of H^3 and C^{14} isotopes in double labelling experiments. 300

REFERENCES i

ACKNOWLEDGEMENTS xxviii
INTRODUCTION

Preface

It is the intention of the first part of this Introduction to present the challenging problem of bacterial cell organization as it exists in the bacterial cell envelope (cell wall plus cytoplasmic membrane). Emphasis will be laid upon the structure of the organism of this study, *Escherichia coli* in so far as it is known, with occasional reference to the structure of other bacterial envelopes. The second part of this Introduction outlines the phenomenon of colicinogeny and the antibacterial effects of colicins. The object of the present work was to try and understand the mode of action of colicins in terms of the cell envelope structure and function.

A. The Bacterial Cell Envelope

The purpose of this part of the Introduction is not to dwell at length upon the numerous chemical studies on the cell wall, nor to present in depth the mechanism of cell wall or cytoplasmic membrane growth, rather an outline of envelope structure and a presentation of its associated functions will be given. Several papers, reviews and texts cover the chemical aspect of cell wall structure, viz: Weidel, Frank and Martin (1960), Weidel and Pelzer (1964), Martin (1963, 1966), Salton (1956), Rogers and
Perkins (1968), Nikaido (1968), Ghuysen (1968) and Osborn (1969).

(1) **General Features of Bacterial Cell Envelopes.** Cell envelopes of most bacteria exhibit two fundamental characteristics. They are (a) rigidity under a variety of environmental conditions, and (b) cell outer surface location. The exceptions to this statement are the Halobacteria (Brown & Shorey 1963), Mycoplasma (Razin, 1969), and L-forms (Weibull, 1965). The first of these has a rigid cell envelope which is only retained in high salt concentrations, the latter two groups appear to lack any rigidity and are more or less plastic in form.

Against the normal bacterial cell wall, which owes its rigidity to the R or mucopeptide layer (Weidel et al, 1960), is apposed a semi-permeable bag-like membrane maintained in this position by the high osmotic pressure of its contents relative to that of the external environment. Fracturing of the cell wall of a bacterium leads to explosive disruption of the mechanically weak cytoplasmic membrane and consequent loss of cytoplasmic cell contents. The outer surface location of the cell wall allows maximum use of the volume of the bacterial cell as a protected semi-permeable container.

A consequence of the envelope's surface location is that it is the first part of the cell to interact with the environment.
One of the main functions of the envelope is therefore to act as an astute doorkeeper to the cell.

The existence of non-rigid bacterial forms capable of reproduction in liquid media demonstrates that cell rigidity is not essential for cell metabolism and division, however rigidity clearly gives a cell the advantage of surviving in a changeable and osmotically unprotected environment. In addition, since the Mycoplasma constitute the smallest and metabolically simplest known group of cells capable of autonomous existence (Razin, 1969), the often faster growing, rigid bacterial forms may well owe their greater metabolic activity and sophistication to the greater spatial and temporal organisation that is possible with rigid three-dimensional cells.

(2) **Cell Envelope Structure.** Combined chemical dissection and electron microscopic studies on *E. coli* B point to a complex layering pattern for the cell envelope. Martin (1963) suggested the order lipoprotein, lipopolysaccharide protein granulae, mucopentide and cytoplasmic membrane, from outside to inside the cell. This picture is not quite compatible with the observation that lipopolysaccharide antigens are freely accessible to ferritin-labelled antibody (Beachy & Cole, 1966). More recent discussions upon *E. coli* cell wall structure do not appear to commit themselves to a
definite layering of lipoprotein and lipopolysaccharide, but it is established that these species lie outside the mucopeptide layer (Osborn, 1969; Braun & Rehn, 1969; Glauert & Thornley, 1969).

(a) Lipopolysaccharide. The lipopolysaccharide of the Enterobacteriaceae, some of which can be readily released from the bacterial cell with EDTA (Lieve, 1965; Voll & Leive, 1970), has powerful toxic activity against higher animals (Landy & Braun, 1964) and is the chemical basis for somatic antigen specificity (Kauffman, 1954). The lipid moiety of the lipopolysaccharide, Lipid A, is a complex phosphorylated lipid containing glucosamine and myristic acid (Burton & Carter, 1964). The polysaccharide portion which determines the somatic antigen specificity is very complex. Some twenty-five monosaccharides have been isolated from various organisms, and up to eight of these may occur in one organism (Nikaido, 1968; Osborn, Rosen, Rothfield, Zeleznick & Horecker, 1964). As well as contributing to a cell’s antigenicity and toxicity, the lipopolysaccharide affords protection from phagocytes (Morgan, 1937).

(b) Lipoprotein. The lipoprotein comprises the two membranous layers of the cell wall (Rogers, 1970). Part of the lipoprotein is thought to be covalently linked to the mucopeptide layer in a regular fashion (Braun & Rehn, 1969; Braun & Seiglin, 1970).
The majority of the protein of the outer layer lipoprotein has been shown to consist of a single protein on the basis of sodium dodecyl sulphate and urea acrylamide gel electrophoresis (Rothfield & Perlman-Kothencz, 1969; Schnaitman, 1970a,b).

(c) **Mucoprotein Layer.** Approximately 10% of the dry weight of the Gram-negative cell wall and 40-90% of the Gram-positive cell wall is mucopolypeptide (Rogers, 1970). The basic structure of the mucoprotein repeat unit consists of a polysaccharide backbone of 1:4 β linked alternating N acetyl glucosamine and N acetyl muramic acid residues cross linked by a polypeptide attached to each muramic acid residue. The polypeptide in *E. coli* being L-alanine, D glutamic acid, meso diaminopimelic acid (DAP), D alanine; the cross link occurring between meso DAP and the terminal D-alanine of a different polypeptide (Osborn, 1969).

(d) **Cytoplasmic Membrane.** Numerous models of membrane structure exist, some of which will be discussed in the general Discussion, the location and composition will be summarized here. Chemically the cytoplasmic membrane consists of lipid and protein. Recently studies have been made on the protein content of envelopes of *E. coli* which appear to consist of a complex set of proteins, not one major species, as demonstrated for the cell wall lipoprotein layer (Schnaitman, 1969; 1970a,b; Jones & Kennedy, 1969, Inouye &
Guthrie 1969; Weinbaum, Fischman & Okuda, 1970; Shapiro, Siccardi, Hirota & Jacob, 1970; Samson & Holland, 1970). So far these proteins have been characterized by their behaviour in SDS acrylamide gel electrophoresis, quantitative differences amongst mutants allowing some identification of individual proteins.

The *E. coli* cell contains about 7% of its dry weight as phospholipid, of which 90% is found in the envelope (Kanfer & Kennedy, 1963; 1964; Kanesma, Akamatsu & Nojima, 1967; Ames, 1968). For *Salmonella typhimurium* and *E. coli*, Ames showed that approximately 75% of the phospholipid was phosphatidylethanolamine, 18% phosphatidylglycerol and 5-10% cardiolipin, minor groups included phosphatidic acid and phosphatidyl serine. Slow metabolic turnover was found to be a characteristic of the major groups.

(3) **Envelope Functions.**

(a) **DNA Attachment and Cell Division.** The mechanism of bacterial cell division proposed by Jacob, Brenner and Cuzin (1963) requires that the cell's chromosomes are joined to the cytoplasmic membrane, and that separation of the sister genomes into sister cells is via localised extension of the membrane between these genomes.

The membrane area(s) of contact with the genome(s) in *Bacillus* species appear to be associated with the mesosomes (Van Iterson, 1961; Ryter & Jacob, 1964; Ryter, Hirota & Jacob, 1968) whereas *E. coli* does
not appear to have any extensive intracytoplasmic membraneous structure (Ryter, 1968; but see Pontefract, Bergeron & Thatcher, 1969). Nevertheless, area(s) of contact between the cell membrane of *E. coli* and the genome have been inferred from several different types of study: (1) electron microscopic (Ryter & Jacob, 1966; Bayer, 1968b); (2) sucrose density gradient analysis of DNA-membrane complexes (Tremblay, Daniels & Schaechter, 1969; Earhart, Tremblay, Daniels & Schaechter, 1968; Hanawalt & Ray, 1964; Smith & Hanawalt, 1967); (3) shear sensitivity of chromosomal DNA (Rosenberg & Cavalieri, 1968).

Linear membrane growth from a central zone as required by the hypothesis of Jacob *et al* (1963) has been less well demonstrated, and most of the studies have been made with *Bacillus* species (Ryter, 1968). However, Daniels (1969) has shown that for *B. megaterium* and *E. coli* lipid synthesis is accelerated during the cell division period in synchronized cells. Location of sites of membrane synthesis in *Bacillus* species has been attempted by Jacob, Ryter and Cuzin (1966) using potassium tellurite, and by Morrison and Morowitz (1970) using autoradiography of radioactively labelled membranes.

Recently Donachie and Begg (1970) have shown that cell extension in *E. coli* proceeds in one direction from a fixed point
in the cell. They propose a 'unit cell' concept as a model for chromosome segregation which is consistent with the hypothesis of Jacob et al (1963).

Other membrane associated systems have been found which have an intimate relation to the DNA, it is not clear whether these are directly attached to the membrane or are attached indirectly via the chromosome's attachment to the membrane. The studies of Hanawalt & Ray (1964), Smith and Hanawalt (1967), Smith, Schaller and Bonhoeffer (1970) and Knippers & Sträling (1970) indicate the intimate association of the DNA synthesizing machinery with the membrane. Tremblay et al (1969) and Rouvière, Lederberg, Granboulan and Gros (1970) have demonstrated the association of RNA synthesis sites with the membrane DNA fraction in vitro.

(b) Selective Permeability. Recently the presence of surface located proteins (permeases) capable of binding and transferring specific substrates have been demonstrated in E.coli (Fox & Kennedy, 1960, β-galactosidases; Schleif, 1969, L-arabinose; Pardee, Prestidge, Whipple & Dreyfus, 1966, sulphate; Penrose, Nichoalds, Piperno & Oxender, 1968, leucine; Anraku, 1968, galactose and leucine). The surface location of some of these proteins has been demonstrated by their behaviour in osmotic shock (Nossal & Heppel, 1966) or more directly by analysing envelope proteins
Wilson, Rose and Fox (1970) have recently studied the effect of unsaturated fatty acid incorporation upon β-galactosidase and β-glucoside transport. They find that the biphasic Arrhenius plots for transport change slope at 15° and 7° for cells grown with oleate and linoleate respectively. Wilson et al (1970) conclude that membranes can occur in two physical states and that proteins synthesized de novo associate preferentially with lipids synthesized simultaneously.

Besides allowing only specific substances to enter a cell, some control of permease activity is necessary since some substances are preferentially transported. The identification of the β-galactosidase M protein with the y gene product (Fox & Kennedy, 1965) demonstrated the possibility of operon control in permease production, it is not clear whether permease activity once membrane bound, is also subject to control.

It appears from the work of Hennaut, Hilger and Grenson (1970) using Saccharomyces cerevisiae that surface area can limit constitutive permease activity upon increasing ploidy, but that inducible permease activity is not so limited. This work suggests that different permeases have their specific areas of location in the cell envelope and that saturation of one permease's location
does not necessarily affect the location of other permeases.

(c) **Respiratory Activity.** The cell envelope of *E. coli* has been shown to contain 90% of the total cytochromes of the cell and to contain many enzymes known to be specific for the electron transport chain in other organisms (Hendler & Nanninga, 1970; Bishop & King, 1962). Pleiotropic mutants that affect respiratory particles have also had their location in the cytoplasmic envelope fraction determined (Azoulay, Puig & Pichinoty, 1967; Schnaitman, 1969). The active transport of proline into membraneous vesicles derived from *E. coli* that are devoid of most cytoplasmic contents has been demonstrated by Kaback and Stadtman (1966). Furthermore this active uptake was inhibited by known uncouplers of oxidative phosphorylation.

(d) **Motility.** The studies of Weibull (1953) and Lederberg (1956) on the dissolution of cell walls of motile bacteria, and of Vaituzis & Doetsch (1965; 1966) on flagellated sphaeroplasts, demonstrated the necessity of intact wall fragments for translational mobility. Following a recent electron microscopic study of *Vibrio metchnikovii* and *Pseudomonas aeruginosa*, Vaituzis and Doetsch (1969) suggested that polar flagella, which were attached to the cell membrane, caused bacterial mobility by the rowing action of the flagella derived from the relative movement of the
cell membrane and cell wall. The loss of mobility upon cell wall destruction being analogous to dissociating the rower and his oar from the boat. Peritrichous flagellation and the occurrence of Chemotaxis in *E. coli* suggests the necessity of a complicated stimulus transmission system to govern translational mobility.

(e) Chemoreceptor Activity. The fact that many motile bacteria appear to be attracted to certain chemical stimuli has been known for some time; it is still not certain whether this attraction is by chemotaxis or phobotaxis (Weibull, 1960). A recent paper by Adler (1969) demonstrated that metabolism of a substrate was neither necessary nor sufficient for it to act as an attractant for motile *E. coli*. Moreover, chemotaxis was found to occur in strains that were permease-negative for the particular attracting substrate. The existence of specific chemoreceptors was also shown, but their surface location has not yet been demonstrated. That chemotaxis can occur with no demonstrable uptake of an attractant, is however consistent with such a location. Contrariwise, if their location was not in or on the cell envelope, it would be difficult to understand how information about the spatial properties of the environment could be relayed from the disorientated milieu of the cell's cytoplasmic contents. The way in which chemoreceptors relay instructions to organize the flagella for
orientated motility is quite unknown, but it appears that some complex stimulus assessment and transmission would be necessary (Adler, 1969).

(f) **Cell-Cell Interactions.** The fact that the *Enterobacteriaceae* do not grow as cell-associated colonies in their normal habitat, probably accounts for the lack of any sophisticated cell recognition and aggregation phenomena characteristic of higher animal cell surfaces. Bacteria that do naturally grow in colonies (for example the Myxobacteria (Dworkin, 1966)) may well show some cell-cell recognition.

Some intra-species cell-cell interactions do however occur during gene transfer in bacterial conjugation. The presence of a functional F pilus or other conjugal pilus seems sufficient for effective pair formation between piliated and non-piliated bacteria (Brinton, 1965). The significance of the selective advantage of chromosomal gene transfer in the *Enterobacteriaceae* 's normal habitat is uncertain, but the advantage of drug resistance transfer in conjugation is well documented (Watanabe, 1963).

It is not clear whether the pilus serves as a conjugation tube for the transfer of DNA, but transfer is highly specific since negligible amounts of non-DNA cell contents are transferred during mating (Silver, Moody & Clowes, 1965; Rosner, Adelberg &
Yarmolinsky, 1967).

The location of the F pilus on the cell envelope may derive from a membrane location close to the membrane-DNA replication complex; in any case transport of the chromosome towards the F- cell is necessary for chromosome transfer.

B. Colicins

(1) Definition and History. The name 'colicin' was originally intended by Fredericq (1946) to characterize antibiotic substances produced by E.coli. (For an historical review see Fredericq, 1957). The earliest study on such antibiotics was that of Gratia (1925) who reported the growth inhibition of one strain, E.coli φ by another strain, E.coli φV. This substance 'Principle V' was inhibitory to E.coli φ at 1/100 fold dilution, furthermore the absence of plaques indicated that the principle did not contain bacteriophage. Similar, and sometimes identical, antibiotics were later found to be produced by other members of the Enterobacteriaceae such as Shigella (Fredericq, 1948), Salmonella (Fredericq, 1952) and E.freundii (Fredericq & Betz-Bareau, 1948).

The general phenomenon of bacteriocinogeny, whereby certain species of bacteria produce antibiotics acting upon the same or closely related species, is not confined to the Enterobacteriaceae.
Table 1 lists the genera of bacteria known to produce bacteriocins (taken from Hamon, 1964).

Bacterial strains capable of elaborating colicins are termed 'colicinogenic'. They carry a Col factor as the genetic determinant of the colicin, and are denoted Colx* for a given colicin - x. Depending on the col factors involved, several col factors can be maintained in the same bacterial strain. Colicinogenic strains are resistant (immune) to the effect of the colicins they produce.

(2) Terminology and Classification. As pointed out by Nomura, (1967) in his review on colicins, not enough is known about the nature or sites of action of the various bacteriocins to construct a useful classification system based upon these criteria. Although the present system of classification has its anomalies, it seems wise to accept Nomura's (1967) suggestion not to introduce a new system until substantially more is known about colicins. The system used by Fredericq (1957) is still therefore the basis for colicin classification.

Colicins are first assigned to groups depending upon their sensitivity to sets of colicin receptor-negative strains. They are further subdivided by the pattern of immunity displayed by strains colicinogenic for colicins in the same group. For example, colicins E1, E2 and E3 are said to belong to a common group - E
**TABLE 1**

Gram +ve and Gram -ve genera of bacteria known to form bacteriocins*

<table>
<thead>
<tr>
<th>Gram Negative</th>
<th>Gram Positive</th>
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<tbody>
<tr>
<td>Pasteurella, Brucella,</td>
<td>Streptococcus</td>
</tr>
<tr>
<td><em>Escherichia, Shigella, Citrobacter</em></td>
<td>Micrococcus</td>
</tr>
<tr>
<td>Hafnia, Salmonella, Klebsiella,</td>
<td>Listeria</td>
</tr>
<tr>
<td>Aerobacter, Serratia, Enterobacter,</td>
<td>Bacillus</td>
</tr>
<tr>
<td>Erwinia, Achromobacter,</td>
<td>Lactobacillus</td>
</tr>
<tr>
<td>Pseudomonas, Xanthomonas,</td>
<td>Welchia</td>
</tr>
<tr>
<td>Vibrio, Chondrococcus</td>
<td>Mycobacterium</td>
</tr>
</tbody>
</table>

* Taken from Hamon (1964).
because a receptor negative strain can be isolated which is unaffected by all three colicins, but is sensitive to colicins of other groups. Colicinogenic cells carrying only the Col E1 factor are immune to colicin E1 but sensitive to colicins E2 or E3. Justification for this further subdivision is manifested by the fact that these three colicins have quite different biochemical effects upon sensitive cells. Approximately twenty different colicins have been classified in this way (Nomura, 1967).

Some of the difficulties inherent in such a classification system can be illustrated with reference to the E group of colicins. Hill and Holland (1967) have demonstrated the existence of mutants of E.coli that are receptor-negative for colicin E1 but retain receptor activity for colicins E2 and E3. In addition, several workers (Hill & Holland, 1967; Nomura & Witten, 1967; Nagel de Zwaig & Luria, 1967) have demonstrated the existence of E.coli mutants that adsorb colicins but are insensitive to them.

Nomura (1967), following a suggestion by Reeves (1965) proposed that colicins should be referred to by the name of the original producer strain, preceded by the previously assigned conventional name. Thus colicin E2-P9 is a colicin of type E2 produced by Shigella sonnei P9 or any other strain deriving its Col E2 factor from Shigella sonnei P9. The colicinogenic strains
used in this study are given in the Materials and General Methods section. For brevity, and because only a few colicins are used in this study, they will be abbreviated from E1-M1, E2-P9 and E3-CA38 to E1, E2 and E3 respectively when so required.

(3) Colicinogeny. The demonstration that Col factors could be transferred from colicinogenic to non-colicinogenic cells by cell contact was made early on by Fredericq (1954). Fredericq (1954) also showed that no linkage of Col factors existed to any known chromosomal markers. Subsequent work has generally confirmed this extra-chromosomal location for colicinogenic factors (Nagel de Zwaig & Puig, 1964).

Although many Col factors are capable of mediating their own and chromosomal gene transfer by elaboration of a specific surface pilus, Col factors are not thought to be capable of true integration into the cell chromosome, as are F particles (Nagel de Zwaig & Puig, 1964). Col factors are therefore better described as plasmids rather than episomes (Nomura, 1967). Col factors that are not capable of mediating their own transfer can be transferred from cell to cell either by resident F particles or by Col factors that are capable of self-mediated transfer (Smith, Ozeki & Stocker, 1963).

Col factors can therefore be divided into two groups (1) those
mediating their own and chromosomal gene transfer, (2) those mediating no transfer. Examples of group (1) include Col Ib-P9, Col V-K94, Col V-K30, and of group (2) Col E2-P9, Col E1-K30, Col K-K49.

(4) Colicin formation. Colicins are spontaneously liberated from a small fraction of a population of colicinogenic cells, and this colicin synthesis is lethal to the producing cell (Ozeki, Stocker & De Margerie, 1959). Higher levels of colicin production can be achieved by induction of colicinogenic cells with a variety of agents such as: UV light (Jacob, Siminovitch & Wollman, 1952), Mitomycin C (Iijima, 1962) and thymine deprivation (Luzzati & Chevalier, 1964).

Using the inducing agent mitomycin C, De Witt and Helinsky (1965) showed that the amount of satellite DNA corresponding to Col E1-K30 could be increased 30 to 100 fold above the non-induced level. This unregulated replication may account for the effect of mitomycin C upon colicin induction, but temperature induction of Col E2-P9 was found to occur in temperature sensitive DNA synthesis mutant of E.coli (Kohiyama & Nomura, 1965). Bazaral & Helinsky (1968) later showed that isolated Col factor DNA from various colicinogenic cells had a molecular weight of $5 \times 10^6$ daltons for both E2 and E3. They further demonstrated that the DNA was in
the form of closed loops and that in uninduced cells a minimum of four copies of this DNA was present per $E. coli$ chromosome.

(5) **Colicin Structure.** A given Col factor determines the same colicin structure independent of the bacterial host. However, whether or not the colicin is associated with additional components is determined by the Col factor's host. Thus Hinsdill and Goebel (1966) compared colicin produced from $E. coli$ Col K-K235 to colicin produced from *Shigella sonnei*, carrying the same Col K factor as the $E. coli$ strain. They found that the colicin resembled the particular host's somatic antigen when non-colicinogenic, nevertheless both colicins showed the same bactericidal specificity. Upon dissociation of Col K-K235 with 90% phenol, the colicin bactericidal activity was found to be associated with the protein moiety of the original lipopolysaccharide-protein complex (Goebel & Barry, 1958).

Colicins E2-P9 and E3-CA38 have recently been purified and characterized by Herschman and Helinsky (1967). Both colicins appeared to be simple proteins with a molecular weight of about 60,000 daltons. No contaminating carbohydrate was found in either preparation. Amino acid analysis of these two proteins revealed great overall similarity, moreover the colicins had some immunological features in common. One peculiarity of colicin E2 but not
of E3, brought to light in the above study was that colicin E2 could exist in two electrophoretically distinct forms, these forms being interconvertable. The significance of this fact is still unclear. In addition to the two previously mentioned types of colicins, those associated and those not associated with host somatic antigens, more complex particles have been found which resemble defective bacteriophages. A pyocin from Pseudomonas aeruginosa has been studied in the electron microscope and shown by Higerd, Baechler and Berk (1969) to consist of a contractile sheath and an inner core reminiscent of T-even coliphage tails. They also showed that uncontracted, but not contracted forms, could adsorb to sensitive bacteria, these particles subsequently contracting.

(6) Mode of Action.

(a) Adsorption. Indirect evidence that colicins adsorbed to, rather than passively mixed with, bacterial cells was obtained by demonstrating the decrease in colicin titre after mixing with sensitive but not with resistant bacteria. In addition, Bordet and Beumer (1948) showed that extracts of sensitive but not resistant bacteria protected sensitive bacteria from colicin action. Direct evidence for colicin adsorption to sensitive but
not resistant cells had to await the use of radioactively labelled colicin molecules by Maeda and Nomura (1966).

The observations that colicins are fixed by specific receptors and the single hit killing kinetics of colicins (Jacob et al., 1952), both suggested that colicin fixation was analogous to that of bacteriophages (Fredericq, 1949). The finding that certain bacteriophage resistant strains were also colicin resistant supported this interpretation. (Phage BF23 and the E group of colicins (Fredericq, 1949); phages T1, T5 and T7 and colicin M (Fredericq & Gratia, 1950); and phage T6 and colicin K (Fredericq, 1950)).

The analogy with bacteriophage receptors should not be taken too literally as this implies that colicin receptors lie on or very close to the cell wall's outer surface. (For example the T5 phage receptor (Weidel & Kellenberger, 1955)). Several workers, notably Smarda (1966a,b and 1968) and Bhattacharyya, Wendt, Whitney & Silver (1970) maintain that the adsorption site, at least for some E group colicins, lies on the cytoplasmic membrane.

Studies on the reversibility of colicin induced biochemical lesions by the proteolytic enzyme trypsin adds further support for the cell-surface locations of active colicin molecules. Removal of adsorbed colicin by trypsin was demonstrated by Maeda & Nomura (1966) using radioactive colicin E2. Depending upon the nature of
the colicin, the efficiency of trypsin reversal of colicin activity can be quite varied. Thus Nomura and Nakamura (1962) demonstrated that the inhibitory effect of colicin K upon oxidative phosphorylation could be reversed by trypsin up to 30 minutes after colicin contact, whereas rescue from colicin E2 induced DNA degradation could only be made within a 5 minute period after colicin contact (Ringrose, 1970a).

Few quantitative determinations on the maximum number of colicin molecules that can be adsorbed on a bacterial cell have been made. This is because prior to the use of radioactively labelled colicin E2 by Maeda and Nomura (1966), estimates of adsorption had to be made with 'killing units' rather than molecules of colicin (Mayr-Harting 1964; Reeves, 1965). Estimates of the amount of colicin E2 that can be adsorbed to a sensitive bacterial cell range from 30-90 molecules (Reeves, 1965) to 2000-3000 molecules (Maeda & Nomura, 1966).

(b) Biochemical Effects. All colicins eventually kill sensitive cells if their action is allowed to continue to its conclusion. The nature of the colicin induced biochemical lesions depends on the colicin studied. The longer the time given for a colicin to act upon a sensitive cell, the more varied the biochemical lesions become. The first demonstrable biochemical effect a given colicin
has upon a cell has been defined by Nomura (1964) and Nomura and Maeda (1965) as the alteration of the biochemical target for that colicin. The biochemical targets for the more commonly studied colicins are given below.

<table>
<thead>
<tr>
<th>Colicin</th>
<th>Biochemical Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Sites of oxidative phosphorylation</td>
</tr>
<tr>
<td>E2</td>
<td>DNA (which is rapidly degraded)</td>
</tr>
<tr>
<td>E3</td>
<td>Ribosomes (whose modification halts protein synthesis)</td>
</tr>
<tr>
<td>K</td>
<td>Sites of oxidative phosphorylation</td>
</tr>
<tr>
<td>I</td>
<td>Sites of oxidative phosphorylation</td>
</tr>
</tbody>
</table>

No evidence that colicins directly interact and effect their respective targets has yet been found. Colicin E3 has a specific effect on ribosomes when acting in vivo, but mixing of colicin E3 with ribosomes in vitro under a variety of conditions has no demonstrable effect (Konisky & Nomura, 1967). Similarly colicin E2 which induces rapid DNA breakdown in vivo has no demonstrable effect upon DNA when mixed in vitro (Nomura & Maeda, 1965; Ringrose, 1970b).

Although colicins may exert their effects indirectly via the cell membrane as suggested by Nomura (1964) and Nomura and Maeda
no demonstrable effect upon leakage of enzymes or $^3$H

(1965), no demonstrable effect upon leakage of enzymes or $^3$H
cell-labelled substances (Nomura, 1963) or actively concentrated

K$^{42}$ has been found for colicin E2 or E3. Colicin K and E1 have
been shown to have an effect both upon K$^{42}$ uptake and its release
but this was interpreted by its effect on energy metabolism

(c) Killing Kinetics. The consequence of prolonged unperturbed
action of colicins on sensitive cells is cell death. Cells mixed
with excess colicin loose their colony forming ability immediately
after adsorption. Survivors represent either resistant bacteria
or cells that have not adsorbed a lethal dose of colicin.

Jacob et al (1952) were the first to demonstrate that the
killing activity of colicins is probably a single hit process.
Other workers, notably Reeves (1965), Shannon and Hedges (1967) and
Reynolds and Reeves (1969) have confirmed that the killing of
E. coli cells by colicin E2 is consistent with one hit killing.
This means that one active particle of colicin is sufficient to
kill a cell, a cooperative effect with other particles not being
necessary. Although one particle is sufficient for cell death, the
probability of cell death may be very low (Maeda & Nomura, 1966).
(For a discussion of single- versus multi-hit hypotheses for colicin
adsorption and killing see Hedges, 1966)
A dosage effect of colicin upon biochemical target inactivation does however occur in that several colicins adsorbed to a sensitive cell affect more rapidly and more extensively the biochemical target. It is possible that cooperative effects amongst several colicins may make more efficient the destructive effects upon the target, or alternatively, that not all colicin receptors are equally effective at a given time. (For a discussion of possible mechanisms see Holland and Holland, 1970).

Changeux and Thiery (1967) have suggested that colicins, since they contribute to the cell 'membranes' of colicinogenic bacteria and have a high affinity for sensitive cell 'membrances', act cooperatively by invoking conformational changes in the membrane, which in turn affect the colicin's biochemical target.

(d) Transmission System. The evidence outlined above suggests that colicins, unlike conventional antibiotics, do not interact directly with their specific biochemical targets. This consideration and the apparently disperse nature of some colicin targets affected by low multiplicities of colicin molecules, led Nomura and Maeda (1965) to propose that colicins remained at the cell surface and transmitted their effects across and along the cytoplasmic membrane.

The fact that different colicins exert different biochemical effects required these transmissions to be quite specific in nature.
The finding that colicins E2 and E3 competed for adsorption sites suggested that a given receptor might effect different transmissions depending on the nature of the adsorbed colicin (Maeda & Nomura, 1966). Mutual exclusion of one colicin by another could be interpreted as steric hindrance of closely situated receptors by colicin adsorption to one of the receptors. However, the inability of Hill and Holland (1967) to genetically separate the colicin E2 and E3 receptors does not support this interpretation.

The change in the receptor following colicin adsorption is transmitted to the killing or biochemical target by some specific mechanism, presumably along the cell membrane, and causes eventual death and/or observable biochemical change. In support of this model it has been suggested (Nomura, 1964; Nomura & Maeda, 1965; Nomura, 1967) that the biochemical targets have some physical connection to the cell membrane or part of it. Thus DNA, the target for colicin E2, may have a physical connection with the membrane (Ryter, 1968; Ryter, Hirota, & Jacob, 1968). The ribosomes, the target for E3 may have some physical connection to the membrane.
(Schlessinger, 1963; Abrams, Neilson & Thaemert, 1964; Rouvière et al., 1969). Finally the oxidative phosphorylation system affected by colicin E1 and K is known to reside in the bacterial cell membrane (Lascelles, 1965).

(7) **Resistance to Colicins**

(a) **Resistant and Refractory (tolerant) Mutants.** Mention has previously been made of bacterial strains that lack receptors for certain colicins, these non-adsorbing mutants being useful in colicin classification. Colicin adsorbing mutants have also been found which are resistant to the action of colicin and such mutants have been termed 'refractory' by Hill and Holland (1967) and 'tolerant' by Nomura & Witten (1967) and Nagel de Zwaig & Luria (1967). Some of these mutants have been genetically mapped by these workers and also by Holland and Threlfall (1969) (see Fig. 1). Refractory (this term will be used in preference to tolerant in this study) mutants would be expected to fall into several classes depending upon which part of the colicin mechanism they interfered with. One could therefore expect: receptor mutants capable of adsorbing colicin but incapable of transmitting its effect, mutants affecting
Fig. 1. *E. coli K12* Linkage Map Showing In Particular Various Colicin Refractory Mutants

Ref II

Ref I

Ref V, VI, VIII.
the hypothetical transmission system, mutants affecting the specific biochemistry of target lesions, and mutants affecting the target's susceptibility to colicin action.

(b) **Immunity.** If colicinogenic cells were not in some way resistant to the action of their colicins, the phenomenon of colicinogenicity could not exist. This resistance to colicin is specific to the colicin(s) produced by the strain, resistance to other colicins not being shown (Fredericq, 1958). This resistance is not due to any lack of colicin adsorption to colicinogenic cells as demonstrated in Maeda and Nomura's (1967) study with radioactive colicin E2. Neither is immunity due to any obvious change in the biochemical target (Nomura, 1963).

Colicinogenic cells may become susceptible to their own colicins when the latter are applied at very high concentrations (Fredericq, 1958). A study of the phenomenon of immunity and immunity breakdown with colicins Ia and Ib has recently been made by Levisohn, Konisky and Nomura (1967).

**Conclusions**

The multifarious functions associated with the bacterial cell envelope have been outlined. The necessarily coordinated growth of the cell envelope has been implied. A very complex role of temporal and spatial organizer for the cell envelope has been put forward.
The mode of action of colicins appears to be very much involved with the organizational properties of the cell envelope. Study of the transmission systems for colicins, and of refractory mutants with altered transmission systems, offers a powerful tool in understanding the workings of the bacterial cell envelope as a whole.
MATERIALS AND GENERAL METHODS

Culture Conditions

Bacterial strains were stored in duplicate on Nutrient Broth Agar + thymine. One set being kept in sealed stab tubes at 25°, the other (working) set being kept as streaks in petri dishes at 4°.

Unless otherwise stated, bacterial cultures were grown with aeration at 37° in a Gyrotory shaker (New Brunswick Scientific Co. New Jersey, U.S.A.).

Total Cell Count. Samples, 0.05 ml or 0.1 ml, of a suitable dilution of the bacterial culture were taken into 25 ml of particle-free infusion saline (Polyfusor, Boots Pure Drug Co. Ltd., Nottingham) and counted in duplicate in a Model F Coulter counter (Coulter Electronics Ltd., Bedfordshire, England). Coincidence corrections were made where required.

Viable Cell Count. Aliquots, 0.1 ml, of a bacterial culture after suitable dilution in dilution phosphate buffer were spread on nutrient broth or minimal agar plates with the aid of a sterile glass spreader. After appropriate incubation at 37° or 25°, colonies were counted and the original titre determined.

Plaque Assay for Bacteriophage. Aliquots, 0.1 ml of a bacteriophage suspension after required dilution in dilution phosphate medium, were
mixed with 1 ml of $10^9$ sensitive bacteria per ml for 5 minutes at 37°. 4 ml of 1% molten nutrient broth agar was rapidly mixed with the phage and bacteria, and poured on to 2% nutrient broth agar plates. After the soft agar overlayer had gelled the plates were incubated at 37° overnight and plaques counted.

**Colicin Assay: Spot Method.** Serial two- and ten-fold dilutions of the colicin preparation were made in nutrient broth. A loopful of each dilution was spotted on to a previously sown lawn of sensitive bacteria (usually E. coli) containing $10^9$ cells per 5 ml soft nutrient broth agar overlayer. After 14 hours incubation at 37°, plates were examined for areas of growth inhibition. The reciprocal of the highest dilution producing visible inhibition was taken to be the colicin titre in units per ml (u/ml).

**Colicin Sensitivity: Streak Test.** Bacterial strains to be tested for their sensitivity to various colicins were grown to ca. $5 \times 10^8$ cells/ml in NB at 37° or 25°. The colicin preparations usually at $10^4$ u/ml were streaked on to dried NB agar plates in duplicate. After the colicins had been adsorbed into the agar, the agar was overlaided with 4 ml of NB agar and allowed to solidify and dry. Suspensions of the various bacteria were then cross-streaked on the agar overlayer in duplicate. Plates were incubated at 37° and 25° for 16 hr prior to observation for growth inhibition.
Selection of BF23 Resistant Mutants. Approximately $10^7$ bacterial cells were plated on to nutrient broth agar plates previously seeded with 0.5 ml of $10^9$ BF23 pfu/ml. The plates were allowed to dry, and then incubated for 14 hr at 37°. Colonies arising were picked and purified by streaking on nutrient broth plates. Purified colonies were then cross-streaked against BF23, colicin E2 and colicin E3 preparations. All colonies that showed resistance to high levels of BF23, E2 and E3 were denoted BF23$^R$ and $E^R$. 
1. Some additional derivatives of strains 56-3, 56-3I, 563J and 56-3W are described in Section IV.

2. All strains are of *Escherichia coli* K12, except strain 906 which is *Salmonella typhimurium* LT2 carrying the colicinogenic factor for E2 derived from *Shigella sonnei* P9.

3. Strains ML, CA38, ROW and ROW/E were obtained from P. Fredericq. Strain 906 was obtained from H. Ozeki. The remaining strains were from the collections of R.H. Pritchard and I.B. Holland.

4. Abbreviations used are explained in Table 2.

5. Resistant phenotypes only are given, response is otherwise sensitive.

6. Sensitive phenotypes only are given, response is otherwise resistant.
### Bacterial Strains Used in this Study

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Colicin response</th>
<th>UV response</th>
</tr>
</thead>
<tbody>
<tr>
<td>906</td>
<td>E2&lt;sup&gt;+&lt;/sup&gt;, cys&lt;sup&gt;-&lt;/sup&gt;, met&lt;sup&gt;-&lt;/sup&gt;</td>
<td>I&lt;sup&gt;R&lt;/sup&gt;, E&lt;sup&gt;R&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>ML</td>
<td>E1&lt;sup&gt;+&lt;/sup&gt;, BF23&lt;sup&gt;R&lt;/sup&gt;</td>
<td>E&lt;sup&gt;R&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>CA38</td>
<td>E3&lt;sup&gt;+&lt;/sup&gt;, I&lt;sup&gt;+&lt;/sup&gt;, BF23&lt;sup&gt;R&lt;/sup&gt;, str&lt;sup&gt;r&lt;/sup&gt;</td>
<td>I&lt;sup&gt;R&lt;/sup&gt;, E&lt;sup&gt;R&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>ROW</td>
<td>met&lt;sup&gt;-&lt;/sup&gt;, str&lt;sup&gt;r&lt;/sup&gt;</td>
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</tr>
<tr>
<td>ROW/E</td>
<td>met&lt;sup&gt;-&lt;/sup&gt;, str&lt;sup&gt;r&lt;/sup&gt;, BF23&lt;sup&gt;R&lt;/sup&gt;</td>
<td>E&lt;sup&gt;R&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>C600</td>
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<tr>
<td>CR34</td>
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<tr>
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<td>206</td>
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<td></td>
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<td>56-3/E</td>
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<td>56-3I</td>
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<td>E&lt;sup&gt;2&lt;/sup&gt;R&lt;sup&gt;30&lt;/sup&gt;</td>
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<td>E&lt;sup&gt;2&lt;/sup&gt;R&lt;sup&gt;30&lt;/sup&gt;</td>
<td>UV&lt;sup&gt;S&lt;/sup&gt;</td>
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<td>UV&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Characteristic</td>
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<td></td>
</tr>
<tr>
<td>--------------</td>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_1^+$, $E_2^+$, $E_3^+$ or $I^+$</td>
<td>Colicinogenic for $E_1$, $E_2$, $E_3$ or $I$ respectively.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E^R$</td>
<td>Resistant to colicins $E_1$, $E_2$ and $E_3$.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I^R$</td>
<td>Resistant to colicin $I$.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_2 R^{30}$</td>
<td>Resistant to colicin $E_2$ at $30^\circ C$.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BF23$^R$</td>
<td>Resistant to phage BF23.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>str$^R$</td>
<td>Resistant to streptomycin.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cys$^-$</td>
<td>Requiring cysteine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>met$^-$</td>
<td>Requiring methionine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thr$^-$</td>
<td>Requiring threonine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>leu$^-$</td>
<td>Requiring leucine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>thi$^-$</td>
<td>Requiring thiamine.</td>
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<td></td>
</tr>
<tr>
<td>thy$^-$</td>
<td>Requiring thymine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dra$^-$</td>
<td>Deoxyriboaldolase negative.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>drm$^-$</td>
<td>Deoxyribomutase negative.</td>
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<td></td>
</tr>
<tr>
<td>UV$^S$</td>
<td>Sensitive to ultraviolet light.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Estimation of Macromolecules

Proteins

UV absorption. Proteins were measured by determining the O.D. at 260 m\(\mu\) and 280 m\(\mu\) for protein solutions and solvent, and estimating the protein concentrations in dry weight per unit volume from either a standard curve for the protein, or from a standard curve for Bovine Serum Albumin.

Folin-Ciocalteu Reagent. Proteins were measured by determining the O.D. 750 m\(\mu\) or O.D. 500 m\(\mu\) of protein solutions after treating with Folin-Ciocalteu Reagent (Folin & Ciocalteu, 1927). The method described in Methods in Enzymology, Volume III (ed. S.P. Colowick & N.O. Kaplan, Academic Press, N.Y.) page 448 was used throughout.

Ribose Nucleic Acid. Samples and standard RNA solutions were made 5% w/v with TCA and kept overnight in centrifuge tubes at 4°C. Precipitates were collected by centrifugation, and the supernatant discarded, the pellet being washed with 5% TCA and centrifuged. Lipid was extracted from the pellet by shaking with 2 lots of 95% v/v ethanol followed by centrifugation. The pellet was resuspended in 5% v/v TCA and heated for 30 minutes at 100°C in a water bath. The tubes were again centrifuged, and the supernatant kept for the estimation of RNA.

A 0.4 ml sample of the hot TCA extract was mixed with 1.1 ml
H₂O, 1.5 ml FeCl₃ (0.1% w/v in concentrated HCl) and 0.5 ml orcinol (10% w/v in ethanol) and the mixture heated at 100°C for 40 minutes. After cooling, the O.D. at 650 mµ was determined. Standardization was with sodium nucleate.

Cell Wall Mucoprotein. Glucosamine was measured colorimetrically by the Indole method as described in Methods in Enzymology, Volume III, page 98.

Media

Oxoid No.2 nutrient broth (NB), supplemented with 80 µg/ml thymine where required, was used throughout. Nutrient broth agar consisted of NB + 2% Davis agar for pouring plates, soft NB agar being made 1%. M9 minimal salts medium was that described by Anderson (1946) made 2% with Davis agar for pouring plates. This medium was supplemented with sugars, amino acids and salts as required.

Formulae

Ca/Mg Salts

\[
\begin{align*}
\text{CaCl}_2 & \quad 1.1 \text{ g} \\
\text{MgSO}_4 & \quad 24.6 \text{ g} \\
\text{H}_2\text{O} & \quad \text{to 1 L}
\end{align*}
\]

1 ml of Ca/Mg was used per 100 ml final minimal medium.
**M9 x 10 Medium**

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad 60 \text{ g} \\
\text{KH}_2\text{PO}_4 & \quad 30 \text{ g} \\
\text{NaCl} & \quad 5 \text{ g} \\
\text{NH}_4\text{Cl} & \quad 10 \text{ g} \\
\text{H}_2\text{O} & \quad \text{to 1 L}
\end{align*}
\]

10 ml of M9 salts were used per 100 ml final minimal medium. Required sugars were usually added to give a 0.4% w/v final solution. Required amino acids were usually added to give 20 μg/final ml. Thiamine (vitamin B1) when required was added to give 1 μg/final ml.

**Dilution Phosphate Buffer pH 6.9**

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 3 \text{ g} \\
\text{Na}_2\text{HPO}_4 & \quad 7 \text{ g} \\
\text{NaCl} & \quad 4 \text{ g} \\
\text{MgSO}_4\cdot\text{7H}_2\text{O} & \quad 0.1 \text{ g} \\
\text{H}_2\text{O} & \quad \text{to 1 L}
\end{align*}
\]

**Buffer Molarity.** Fractions eluted from various chromatographic columns had their refractive indices measured on an Abbe refractometer (Bellingham & Stanley Ltd., London). The buffer molarity was computed from this refractive index using a standard curve for the appropriate buffer vs refractive index.
Cell Disruption

Sonication was carried out at 0° using an M.S.E. sonic disintegrator (Measuring and Scientific Equipment Ltd., London) fitted with the appropriate capacity probe.

French Press disruption was carried out using an automatic Amico French Press (American Instrument Co. Ltd., Maryland, U.S.A.) operated at 12,000 p.s.i. pressure.

Centrifugation

Low Speed Centrifugation was carried out in a Sorval superspeed RC-2-B refrigerated centrifuge (Ivan Sorval Inc. Norwalk, Connecticut, U.S.A.) using an appropriate capacity centrifuge head. Glass or plastic centrifuge tubes were used throughout.

High Speed Centrifugation was carried out in a Beckman L-2 or L-2-65B ultracentrifuge (Beckman Instruments Ltd., Glenrothes, Fife, Scotland) using an appropriate capacity centrifuge head. Cellulose nitrate centrifuge tubes being used throughout.

Continuous Sucrose Density Gradients were formed in 5 ml cellulose nitrate tubes by mixing 2.4 ml aliquots of high and low density sucrose solutions in a gradient maker (Buchler Instruments Inc., New Jersey, U.S.A.). Gradients were analysed at 260 mμ on an ISCO Model 180 gradient analyser and Model 220 UV analyser. O.D. 260 mμ
was recorded on a Metrohm Labograph E428 pen recorder (Shandon Scientific Co. Ltd., Willesden, London).

**Ultraviolet Irradiation.** Bacteria either streaked, or spread on NB agar plates, were irradiated with ultraviolet light from a low pressure mercury lamp (Hanovia Ltd., Slough) at a distance that gave a dosage of 6 ergs/mm²/sec.

**Spectrometry.** Optical densities of samples between the wavelengths 190 mµ and 750 mµ were measured manually on a Carl Zeiss Spectrophotometer M4 Q III using 1 ml quartz cells internal path length 1 cm.

Infra red spectroscopy was carried out on a Perkin-Elmer 237 Grating Infrared Spectrophotometer, samples being applied as a paste between two rock salt discs.

**Column Chromatography.** The makers instructions for preparation and use of column materials were followed throughout. (Sephadex gel filtration in Theory and Practice, a booklet published by Pharmacia Fine Chemicals, Uppsala, Sweden, was found to be invaluable in this respect.) Eluted fractions were collected in 25 ml glass test-tubes that had been chromic acid cleaned. Usually an LKB Ultrorac 7000 automatic fraction collector was used (LKB-Produckter, Stockholm, Sweden). All chromatographic separations were carried out in the cold room at 4°.
**Acrylamide Gel Electrophoresis.** The particular chemical composition and subsequent staining of the gels is described in the appropriate chapter. Electrophoresis was carried out in a Shandon Electrophoresis Unit with platinum wire electrodes (Shandon Scientific Co. Ltd., Willesden, London). A direct voltage constant current power pack was used as the source of electricity. Gels were made in 9 cm long, 0.6 mm i.d. thin-walled glass tubes cut from a single glass tube. Electrophoretic staining was carried out in 10 cm, 0.8 mm i.d. tubes cut from a single glass tube. Scanning of stained gels was carried out in a Joyce-Loebl Microdensitometer with or without a red filter. A gel holder was designed for this scanning which held the gels immersed in 'scanning' acrylamide, mounted on an optical glass plate.

**Preparation and Measurement of Radioactive Samples**

**TCA Precipitation.** Aliquots (usually 0.1 ml or 0.5 ml) of radioactive material were mixed with 0.9 ml of ice cold 5% w/v TCA or 0.5 ml of ice cold 10% TCA respectively in plastic 2 ml centrifuge tubes. After 60 min at 0° the samples were either centrifuged at 10,000 g for 10 min and the supernatant assayed for radioactivity, or the tube contents were filtered on to 0.45 μm pore dia., 2.5 cm e.d. Millipore filters and washed with five 5 ml lots of 5% w/v cold TCA.
Whole cells. Aliquots of cells (usually 0.5 ml – 1.0 ml) were rapidly filtered on to 0.45 μm pore, 2.5 cm e.d. Millipore filters and washed with 10 ml of non-radioactive growth medium at 37°C.

Sample Counting. Samples on Millipore filters (TCA precipitates or whole cells) were dried for 15 min under an Infra-red lamp and transferred to 3 ml thin-walled glass tubes. The tubes were filled with non aqueous scintillation fluid, stoppered and put in glass screw-topped carrying vials. Their radioactivity was determined in the scintillation counter (Packard Tricarb Scintillation Spectrometer Model 3320) at the appropriate isotope settings. Aqueous samples (0.1 ml) were similarly measured after mixing with 10 ml of aqueous scintillation fluid in plastic or glass carrying vials. Typically, tritium was counted between the 50 and 1000 gate settings using a 65% gain. Carbon 14 was counted between 50 and 1000 using a 10% gain. Counting was usually 10 min per sample. All counts were corrected for background contamination. (Conditions for double labelling are described in Appendix III).

**Formulae**

**Non-aqueous Scintillation Fluid:**

- PPO* 5 g
- POPOP** 33 mg
- Toluene 1 L
Aqueous Scintillation Fluid:  

- PPO* 2.7 g  
- POPOP** 33 mg  
- Ethanol 330 ml  
- Toluene 670 ml

*PPO = 2,5-Diphenyloxazole  
**POPOP = 1,4-Bis-2(4-methyl-5-phenyloxazolyl)-benzene  
(Both obtained from Nuclear Enterprises Ltd., Edinburgh.)

Reagents. All chemicals were Analytical Grade and usually obtained from Sigma Ltd. (London) or B.D.H. (Warwickshire). Sodium N-laurorylsarcosinate was obtained from Koch-Light Ltd. (Buckinghamshire). Uranyl Acetate was obtained from Fisons Ltd. (Loughborough). Enzymes were usually obtained from Worthington Biochemical Corp. (New Jersey, U.S.A.) or Sigma Ltd. (London). All stains and fluorochromes were obtained from G.T. Gurr Ltd. (London). Horse ferritin was obtained from Calbiochem Ltd. (Los Angeles, U.S.A.).

Materials

Glass fibre 2.5 cm filter discs GB/F were obtained from Whatman (Reeve Angel, London). Hydroxyapatite was obtained from Calbiochem (Los Angeles, U.S.A.). CM cellulose, DEAE cellulose, CM sephadex, DEAE sephadex, sephadex G75 to G200 were obtained through Sigma (London). Sepharose 6B was obtained from Pharmacia
(Uppsala, Sweden). Goat anti-rabbit serum, antiserum was obtained from Wellcome Research Laboratories (Beckenham, England). Arlacell A was obtained from Honeywell & Atlas (Chadwell Heath, Essex). Bayol F was obtained from Esso (Queen Anne's Gate, London). Diatomaceous Earth (Celite) was obtained from B.D.H. (Warwickshire). All glassware except for bacterial culture growth, was chromic acid cleaned, distilled water washed and hot air dried. All radioactive materials were obtained from the Radiochemical Centre, Amersham.
SECTI0N I
THE PREPARATION OF COLICIN E2 SUITABLY LABELLED FOR
MICROSCOPIC STUDY

Introduction

As outlined in the general Introduction, the transmission of the colicin induced stimulus affecting the biochemical target is still little understood, whereas the biochemical effect(s) upon the target(s) has been better worked out (Nomura, 1963; 1964). Two extreme theories of colicin action can be entertained that are consistent with present data; one being that the active colicin molecules remain at the bacterial cell's outer surface and affect the intracellular target(s) by some hypothetical transmission system; the other being that the active colicin molecules reach and directly interact with the biochemical target.

The fact that some colicin treated cells may be rescued from cell death by treatment with the proteolytic enzyme, trypsin, was suggestive of a cell surface location for the active colicin molecules (Nomura, 1967; Reynolds & Reeves, 1969). In a thorough study, Maeda and Nomura (1966) using radioactively labelled colicin E2 showed that trypsin did release 60% of radioactive colicin E2 adsorbed to sensitive cells. They also showed that when cells, treated with radioactive colicin E2, were subsequently disintegrated and crudely
fractionated, 90% of the radioactivity was found in the cell envelope (cell wall plus cell membrane), 9% in the ribosome fraction and the remainder in the cytoplasmic moiety. Very little radioactivity was found associated with cellular DNA, a primary biochemical target for colicin E2.

This preliminary evidence that colicins (at least colicin E2) do in fact remain distant from their targets was encouraging because a principal reason for the interest shown in colicin action by workers in the field lies in their apparently unique mode of 'action at a distance'.

The data of Maeda and Nomura (1966) can however, be interpreted in an alternative way. For example, the 1% of radioactively labelled colicin E2 in the cytoplasmic fraction could represent the minority of colicin molecules that succeeded in remaining and interacting with their intracellular target. This suggestion is consistent with the known low probability of killing that colicin molecules have (Nomura, 1967). Also one could argue that since E.coli DNA is probably physically associated with the cell membrane at one or many points (Ryter, 1968; Ryter, Hirota & Jacob, 1968; Tremblay, Daniels & Schaechter, 1969; Knippers & Strütling, 1970), 90% of the radioactive colicin E2 found associated with the cell envelope had in fact reached its target (the DNA-membrane attachment area(s))
even though the bulk of the DNA was only slightly labelled.

Using a different approach, evidence for a direct interaction between colicins and their biochemical targets has been sought by mixing colicins with such targets in vitro. Thus mixing of colicin E2 and E.coli DNA under a variety of conditions does not lead to DNA breakdown nor to any demonstrable structural modification of the DNA (Nomura, 1967; Ringrose, 1970b). Similarly colicin E3 has no effect upon bacterial ribosomes when the two are mixed in vitro under various conditions, although colicin E3 has been shown to alter ribosomes of sensitive cells when acting in vivo (Konisky & Nomura, 1967).

This negative evidence is again compatible with an indirect mechanism of colicin action mediated through cell surface changes. Nevertheless, it could still be argued that the special conditions for the colicin's direct interaction and alteration of the target in vitro exist, but have not been found. Thus Holland (personal communication) has found that there is an ATP requirement for E2-induced DNA breakdown in vivo, but that in the aforementioned studies on E2's in vitro activity no ATP additions were tested.

These preliminary in vitro studies are, however, essential to confirm that colicins are not working by any simple conventional antibiotic means. If colicin E2 had demonstrated DNAase activity
for example, much greater credence would be given to the direct interaction mechanism, and interest in colicins would no doubt swiftly wane. Clearly an unambiguous demonstration of the in vivo location of colicins adsorbed to sensitive cells is necessary to distinguish between the extreme theories of its mode of action, and to thereby act as a pointer to subsequent, more fruitful study.

Experimental Approaches

Extension of the use of isotopically labelled colicins by autoradiographic examination of colicin treated cells was first considered as a means of discerning the in vivo location of colicins in treated cells. Autoradiographic studies with radioactively labelled colicin were nevertheless decided against on several grounds. (1) The preparation of labelled colicin molecules having the high radioactive specific activity necessary for autoradiography is extremely difficult and was not achieved by Maeda & Nomura (1966). (2) If such a radioactive preparation could be made, its high specific activity might well lead to its self-inactivation. (3) The resolution (≥ 0.1 μ) afforded by the difficult technique of autoradiography in electron microscopy is insufficient to discriminate amongst the possible colicin adsorption sites on the cell surface (Caro & Forro, 1961; Ped & Walton, 1968).
In view of the limitations of the autoradiographic technique, an alternative method of determining the distribution of colicin molecules in treated cells was investigated. This involved post-labelling of the colicin subsequent to its adsorption to sensitive cells by a high resolution electron dense specific stain, and studying of such cells in the electron microscope. One such stain, which can be made specific for colicin by conjugating it with anti-colicin immunoglobulin, is ferritin. Ferritin is a biological macromolecule with iron storage function composed of a protein (apoferitin) plus a variable amount of hydrated ferric oxide located as a micelle in the protein's central cavity (Fischbach, Harrison & Hoy, 1969). The high electron density afforded by the mineral micelle enclosed in a protein shell allows its ready identification in the electron microscope when the ferritin is in a milieu of conventional bacterial cell contents.

Direct linkage of colicin to ferritin was rejected since the large (122 Å) diameter of the latter compared with that calculated for colicin E2 or E3 (about 50 Å diameter based on a molecular weight of 60,000 daltons) would be expected to seriously interfere with the colicin's in vivo activity, by, for example, steric hindrance. It was therefore decided to conjugate a specific anti-colicin immunoglobulin to ferritin and to use this material to stain sensitive cells.
subsequent to adsorption of colicin. The advantages of this method are that resolution in the electron microscope is very good, and unlike autoradiography, the colicin molecule is not necessarily obscured by the electron dense particle. This theoretically allows precise location of colicin by tracing its position back from the ferritin molecule via the immunoglobulin molecule. (Dimensions of colicin E2, E3 and ferritin have previously been given; Fig. 1a shows the dimensions of the rabbit 6.6s gamma globulin molecule according to Green (1969)). Possible disadvantages of this method are that the colicin molecule may change its antigenic structure from the free to the cell bound form, and the technical difficulty of preparing fixed embedded sections of bacteria retaining antigenic specificity.

Colicin E2 was chosen for this and further study as it is the best characterised colicin with regard to structure, adsorption data and mode of killing data (Herschman & Helinsky, 1967; Maeda & Nomura, 1966; Nomura, 1967; Holland, 1968). In addition the biochemical target for colicin E2 is more likely to be discrete in location (the DNA-membrane attachment site(s)) than for colicin E3 (the ribosomes).

**Fluorescently Labelled Colicin E2**

Prior to preparation of ferritin stains for high resolution study in the electron microscope, it was decided to determine the
Fig. la. A Scale Diagram of the Rabbit 6.6s γ globulin molecule

L and H signify the Light and Heavy chains of the antibody molecule respectively. The $\equiv S$ (sulphur - sulphur) bond is shown linking the two upper arms of the molecule to the double H chains.
RABBIT GLOBULIN MOLECULE
SCALE DIAGRAM
general pattern of cell surface adsorption of colicin E2. Maeda and Nomura (1966) had previously shown that approximately 2,000-3,000 molecules of colicin E2 per cell were capable of being adsorbed. Assuming colicin E2 is a spherical protein molecule of diameter 50 Å and its area of steric exclusion approximately that of a square, side 50 Å, then it can be calculated (see Appendix I) that approximately $4.4 \times 10^5$ such molecules could be close-packed on a typical *E. coli* K12 bacterium. This value exceeds that of 2,000-3,000 molecules actually adsorbed by approximately 150 fold. This discrepancy is even greater when estimates of the maximum amount of colicin E2 adsorbed found by other workers (Mayr-Harting, 1964; Reeves, 1965a) are taken into account. When, however, the number of molecules of colicin that could be packed one molecule thick round the girth of an *E. coli* bacterium is calculated (see Appendix I) a value of about 630 is found, a number much closer to the experimentally determined values.

In the light of these calculations it was decided to see if adsorbed colicin E2 was restricted to the girth (or other region) of the cell surface or if it was randomly dispersed. Fluorescently labelled colicin E2 was prepared for this purpose.
Methods

Preparation of purified colicin E2

Purified colicin E2 was required in milligram amounts for fluorescent dye conjugation, preparation and testing of anti-colicin E2 antisera. The purification procedure used was a modification of Herschman & Helinsky (1967).

(1) Growth and induction of cells colicinogenic for colicin E2

The colicinogenic strain carrying the Col E2-P9 factor was strain 906, a Salmonella typhimurium made colicinogenic for E2 by infection from Shigella sonnei P9. As mentioned in the general Introduction, colicin induction can be brought about by many different agents. Following Herschman and Helinsky (1967) induction with mitomycin C was chosen.

It was first necessary to establish the optimum growth, induction and harvesting conditions for the colicinogenic strain under study in order that high titres of colicin could be obtained. Fig. 2 shows the effect of mitomycin C upon cultures of strain 906 growing in supplemented minimal medium. Table 3 shows the cell density in Coulter Counter particles per ml at induction, the mitomycin C concentration used, the times of harvest and the colicin titres obtained. Similar results were found with strain 906 grown in NB medium. Since the maximum titres of colicin E2 achieved were as high as those
Fig. 2. Growth and Induction of Strain 906 Col E2

A 1 ml culture of strain 906 grown overnight in supplemented minimal medium was inoculated into 9 ml of fresh medium and incubated at 37°. Growth was measured using a Coulter Counter as described in the General Methods. At the times indicated by the arrows, cultures were treated with mitomycin C, final concentration 0.5 μg/ml flasks A, C and D or 1.0 μg/ml flask B. Samples were removed at various times and analysed for colicin E2 content as described in the General Methods. These results are recorded in Table 3.
HOURS INCUBATION AT 37°C.
## TABLE 3

Effect of Mitomycin C Concentration, Bacterial Cell Density at Induction, and Harvest Time on the Production of Colicin E2 by Strain 906

<table>
<thead>
<tr>
<th>Flask</th>
<th>Mitomycin C final conc. µg/ml</th>
<th>Cells/ml at induction</th>
<th>First harvest</th>
<th>Second harvest 16 hrs after induction</th>
<th>Colicin E2 titre u/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time hrs after induction</td>
<td>Colicin E2 titre u/ml</td>
<td>Colicin E2 titre u/ml</td>
</tr>
<tr>
<td>A</td>
<td>0.5</td>
<td>$1 \times 10^8$</td>
<td>4.6</td>
<td>$10^4$</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>B</td>
<td>1.0</td>
<td>$1 \times 10^8$</td>
<td>4.5</td>
<td>$10^4$</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
<td>$5 \times 10^8$</td>
<td>2.8</td>
<td>$8 \times 10^4$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>D</td>
<td>0.5</td>
<td>$1 \times 10^9$</td>
<td>2.0</td>
<td>$4 \times 10^4$</td>
<td>$2 \times 10^4$</td>
</tr>
</tbody>
</table>
previously reported (Herschman & Helinsky, 1967) cultures of strain 906 were routinely grown to $5 \times 10^8$ cells/ml at $37^\circ$, induced with mitomycin C at 0.5 $\mu$g/ml and harvested after overnight shaking at $37^\circ$.

(2) **Large scale purification of colicin E2**

(A) **Production of colicin E2.** Two six litre cultures of strain 906 (Col E2$^+$) were grown to $5 \times 10^8$ cells/ml in NB medium. Mitomycin C was added at this cell density to give 0.5 $\mu$g/final ml. Incubation was continued overnight (about 16 hr) and the cells harvested by centrifugation at 10,000 g for 10 min in 500 ml centrifuge bottles at $4^\circ$. The supernatant was kept, and the cells washed with two 100 ml lots of 1.0 M sodium chloride solution to remove any cell adsorbed colicin, followed by centrifugation at 10,000 g for 10 min at $4^\circ$. The washes were combined with the supernatant, and the volume and colicin activity determined.

(B) **Ammonium sulphate concentration.** Enzyme grade ammonium sulphate was added to 3 litre batches of the supernatant-wash mixture at the rate of 380 g of ammonium sulphate per litre of mixture. Solubilization of the salt was aided by magnetic stirring, all operations being carried out at $4^\circ$ in the cold room. The pH of the mixture was maintained at pH 7.2 by the periodic addition of 1.0 M sodium hydroxide solution. The preparation was then allowed
to stand for 20 min in the cold, and the precipitated protein collected by centrifugation at 10,000 g for 10 min. The resulting supernatant which contained 0.1% of the initial colicin E2 activity was discarded.

(C) Dialysis prior to sephadex treatment. The ammonium sulphate precipitate which contained about 50% of the initial colicin activity was resuspended in a minimum volume of $10^{-2}$ M sodium phosphate buffer pH 7.0 (henceforth abbreviated to buffer A in this section) and dialysed at $4^\circ$ overnight against three changes of 3 L of this buffer. The dialysate was measured for its volume and colicin activity and found to contain approximately 50% of the original E2 activity.

Depending on the scale of the preparation vis 6-12 L or up to 2 L, treatment with either (1) CM cellulose or (2) DEAE sephadex respectively, was next employed.

(D) (1a) Elution from CM-cellulose and second ammonium sulphate precipitation. For a 12 L original batch the ammonium sulphate dialysate was mixed with 20 g of CM-cellulose that had been thoroughly washed successively with 0.5 M sodium hydroxide, water, 0.5 M hydrochloric acid, water and finally equilibrated with buffer A, in a clean beaker. After 20 min gentle stirring at $4^\circ$, the mixture was filtered on a Buchner funnel and the filtrate mixed with 400 g
per litre of ammonium sulphate, the precipitate being collected as before. The supernatant was discarded, the pellet resuspended in buffer A and dialysed overnight against three changes of 3 L of the same buffer.

(b) Batch elution from hydroxylapatite. The second ammonium sulphate dialysate was stirred with 5 g of hydroxylapatite in 60 ml of 0.1 M sodium phosphate buffer pH 7.0, in a centrifuge bottle at 4°C. After 20 min to complete adsorption, the slurry was centrifuged at 10,000 g for 5 min at 4°C and the supernatant kept for assay. The pellet was suspended in 40 ml of 0.1 M sodium phosphate buffer pH 7.0, mixed and centrifuged at 10,000 g for 5 min at 4°C. This batch elution was repeated with two 40 ml lots of 0.2 M, and one lot of 0.3 M sodium phosphate buffer pH 7.0. Each elution sample was assayed, and those containing the highest colicin titres pooled, freeze dried and dialysed against the appropriate buffer prior to column chromatography on either hydroxylapatite or CM sephadex. Table 4 shows the recoveries of colicin E2 found at each step in the large scale procedure.

(2) Elution from DEAE sephadex. The first ammonium sulphate dialysate was mixed for 40 min with 2 g of DEAE sephadex per original litre of culture after equilibration of the sephadex with buffer A. The slurry was filtered on a Buchner funnel and
### TABLE 4

**A 12 Litre Scale Preparation of Colicin E2, Showing Colicin Recoveries**

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Volume ml</th>
<th>Colicin E2 Activity u/ml</th>
<th>Kept (+) Discarded (-)</th>
<th>Total Number of Colicin Units</th>
<th>Recovery as a % of Original Colicin E2 Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled cell supernatant and washings</td>
<td>11,550</td>
<td>$10^4$</td>
<td>+</td>
<td>$1.155 \times 10^8$</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant after first $(\text{NH}_4)_2\text{SO}_4$ precipitation</td>
<td>13,600</td>
<td>10</td>
<td>-</td>
<td>$1.36 \times 10^5$</td>
<td>0.1</td>
</tr>
<tr>
<td>Dialysate of first $(\text{NH}_4)_2\text{SO}_4$ precipitate</td>
<td>100</td>
<td>$4 \times 10^5$</td>
<td>+</td>
<td>$4 \times 10^7$</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>2 x 10^5</td>
<td>+</td>
<td>4.3 x 10^7</td>
<td>38.4</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----</td>
<td>----------</td>
<td>----</td>
<td>------------</td>
<td>------</td>
</tr>
<tr>
<td>CM cellulose filtrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant after second</td>
<td>320</td>
<td>10</td>
<td>-</td>
<td>3.2 x 10^3</td>
<td>40.1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysate of second</td>
<td>38.5</td>
<td>4 x 10^5</td>
<td>+</td>
<td>1.14 x 10^7</td>
<td>9.9</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite batch</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>washes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 M wash 1</td>
<td>90</td>
<td>10⁴</td>
<td>-</td>
<td>9 x 10⁵</td>
<td>0.08</td>
</tr>
<tr>
<td>0.1 M wash 2</td>
<td>40</td>
<td>10³</td>
<td>-</td>
<td>4 x 10⁴</td>
<td>0.04</td>
</tr>
<tr>
<td>0.2 M wash 1</td>
<td>38</td>
<td>2 x 10⁵</td>
<td>+</td>
<td>7.6 x 10⁶</td>
<td>6.6</td>
</tr>
<tr>
<td>0.2 M wash 2</td>
<td>50</td>
<td>10⁵</td>
<td>+</td>
<td>5 x 10⁵</td>
<td>4.3</td>
</tr>
<tr>
<td>0.3 M wash 1</td>
<td>47</td>
<td>10⁴</td>
<td>-</td>
<td>4.7 x 10⁵</td>
<td>0.4</td>
</tr>
<tr>
<td>Dialysate of</td>
<td>120</td>
<td>10⁵</td>
<td>+</td>
<td>1.2 x 10⁷</td>
<td>10.4</td>
</tr>
<tr>
<td>pooled 0.2 M washes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze* dried dialysate</td>
<td>4.6</td>
<td>8 x 10⁶</td>
<td>+</td>
<td>3.68 x 10⁷</td>
<td>31.7</td>
</tr>
</tbody>
</table>

*This material (28.3 mg protein) was chromatographed on hydroxylapatite and yielded 13.2 mg of purified colicin E2 having a total activity of 3.2 x 10⁷ units.
the filtrate measured for its colicin E2 activity. Colicin E2 does not adsorb to DEAE sephadex under these conditions but much inactive protein is adsorbed by the exchanger. The filtrate was freeze dried and dialysed overnight against 3 one litre changes of 5 x 10^{-2} M sodium phosphate buffer pH 6.0 prior to chromatography on CM sephadex.

(E) Column chromatography of colicin E2. Chromatography of colicin E2 was done either by pH gradient elution on CM sephadex or ionic concentration gradient on hydroxylapatite. The latter system was used for small scale work because only a small sample was required to determine the eluent molarity by refractive index, whereas the measurement of pH required fractions of several ml.

(1) Chromatography on CM sephadex. The colicin E2 preparation previously dialysed against 5 x 10^{-2} M sodium phosphate buffer pH 6.0 was loaded on to a column of CM sephadex previously equilibrated with this buffer. pH gradient elution was carried out by eluting with a mixture of the pH 6.0 buffer, and 5 x 10^{-2} M dipotassium hydrogen orthophosphate solution pH 9.0. Fig. 3 shows a protein elution profile using this system.

(2) Chromatography on hydroxylapatite. The colicin E2 preparation previously equilibrated in buffer A was loaded onto a column of hydroxylapatite previously equilibrated in buffer A. Elution by
Colicin E2 was partially purified from a 2 litre culture as described in the text. The freeze dried dialysate (approximately 6.5 mg in 1.8 ml) was loaded onto a 30 cm long, 1.5 cm dia. column of CM-sephadex previously equilibrated with $5 \times 10^{-2}$ sodium phosphate buffer pH 6.0. The column was eluted with a pH gradient formed by controlled mixing of the above buffer and $5 \times 10^{-2}$ dipotassium hydrogen orthophosphate pH 9.0. Fractions of 90 drops (= 5 ml) were collected and the O.D. at 260 m$\mu$ and 280 m$\mu$ determined. Colicin activity was determined by the spot dilution assay method as described in General Methods. The pH of certain fractions was determined using a Beckman-Pye pH meter. Fractions 35, 36 and 37 were pooled, dialysed against buffer A, freeze dried and kept until required at $-20^\circ$. 

Fig. 3. CM-sephadex chromatography of a medium scale preparation of colicin E2.
ionic concentration gradient was carried out by eluting with a controlled mixture of $10^{-2}$ M and 0.3 M sodium phosphate buffer pH 7.0. Fig. 4 shows a protein elution profile using this system.

(F) Recovery of colicin E2 activity. The data in Table 4 demonstrates the limitations of the spot assay method for colicin titre determinations. Since a two fold dilution series can give 100% error this may partly account for the reappearance of colicin activity at the final stage. It is possible also that purification removes some colicin inhibitor, or that the higher protein concentration after freeze drying protects against protein denaturation.

The final specific activity of purified colicin after column chromatography was between 1 and $3 \times 10^3$ u/$\mu$g of protein; this is in good agreement with the results of other workers ($1.6 \times 10^3$ u/$\mu$g protein (Herschman & Helinsky, 1967), $1.0 \times 10^3$ u/$\mu$g protein (Holland, 1968)) and therefore further purification and characterisation of colicin was not carried out.

Preparation of fluorescein labelled colicin E2.

Fluorescein (molecular weight 332) is a small molecule relative to colicin E2 (molecular weight 60,000). Thus although fluorescein is commonly conjugated to the appropriate antibody for use in fluorescent microscopy, it was thought that direct conjugation of this molecule to colicin E2 would not necessarily perturb the latter's
Fig. 4. Hydroxylapatite chromatography of a small scale radioactive* preparation of colicin E2

Colicin E2 was partially purified as described in the text and the freeze dried dialysate of volume 1 ml was put on a 10 cm long, 0.8 cm dia. hydroxylapatite column previously equilibrated in $10^{-2}$ M sodium phosphate buffer pH 7.0. The column was eluted with a molarity gradient formed by controlled mixing of $10^{-2}$ M and 0.3 M sodium phosphate buffers pH 7.0. Fractions of 35 drops (= 2.1 ml) were collected and their refractive index measured to determine the eluent buffer molarity from a standard curve. Colicin activity was measured by spot analysis. Radioactivity of the samples was determined by drying 0.1 ml aliquots onto millipore filters, suspending them in non-aqueous scintillation fluid and measuring the activity in a scintillation counter. Volume 53.5 to 61.0 ml was pooled, dialysed against buffer A, freeze dried and stored at -20°C.

*Radioactive colicin E2 was prepared for various adsorption studies, but its activity was lost too quickly for it to be of use. The conditions for the initial synthesis and early purification steps of radioactive colicin E2 are therefore not described.
in vivo activity.

(1) The conjugation method of Batty and Walker (1965). Purified colicin E2 (0.57 mg) was dissolved in 1.5 ml of buffer A, 1.0 ml of 0.5 M sodium carbonate/sodium bicarbonate buffer pH 9.0 was added and the mixture stirred in a beaker at 4°. Over a period of 15 min 0.15 ml of 0.5 mg/ml fluorescein isothiocyanate (FITC), dissolved in the pH 9 buffer was added dropwise from a Pasteur pipette. The mixture was stirred for a further 20 hr at 4°. The mixture was applied to a G-25 coarse-mesh sephadex column previously equilibrated and later eluted with buffer A made 1% w/v with sodium chloride. Fractions were collected manually, and the optical density at 495 μ (fluorescein's adsorption peak) and colicin E2 activity determined. The elution profile of this mixture is shown in Fig. 6.

Recovery. From the 0.57 mg of protein applied to the column, the pooled peak column fractions yielded 0.18 mg protein. The specific activity of the unconjugated protein was $7 \times 10^4$ u/mg protein, whilst that of the conjugate was $5.6 \times 10^3$ u/mg protein. This procedure led to more than 90% loss in biological activity of the colicin. The ratio OD 495 μ : OD 280 μ was measured and used as a degree of conjugation index (Brighton, Taylor & Wilkinson, 1967).
The conjugation method of Rinderknecht (1962). To 2.8 mg of purified colicin E2 in 3.2 ml of buffer A was added 6 ml of 0.5 M sodium carbonate/sodium bicarbonate buffer pH 9.0 at 25°. 0.45 mg of FITC as a 5% w/v dispersion of diatomaceous earth was added, and the mixture stirred for 25 min at 25°. The mixture was centrifuged at 25° for 10 min at 10,000 g to remove the diatomaceous earth. The supernatant dye-complex was concentrated by freeze drying, dissolved in 3 ml of buffer A plus 1% w/v sodium chloride, and dialysed against three one litre changes of this buffer at 4°. The dialysis step removed most of the unconjugated FITC, and the remainder was removed by chromatography on a G-25 sephadex column as described for the previous method.

Recovery. From the 2.8 mg of protein applied to the column, 0.6 mg was recovered in the pooled fractions. The specific activity of the unconjugated protein was $2 \times 10^6 \text{ u/mg protein}$ which dropped to $1.5 \times 10^3 \text{ u/mg protein}$. This particular preparation lost some 99% of its initial colicin E2 activity. The radio OD 495 $\text{m}_{\mu}$ : OD 280 $\text{m}_{\mu}$ was measured and used as a degree of conjugation index (Brighton et al., 1967).

Fluorescence Microscopy.

(1) Microscope and light source. Initially a Gillett and Sibert Conference Research Microscope with a quartz-iodide lamp was used.
No fluorescent bacteria could be detected with this microscope. It appears that few so-called UV fluorescent microscopes commercially available are capable of serious work with particles as small as bacteria (Professor C.E.D. Taylor, personal communication). Certainly a quartz-iodide lamp is not sufficiently powerful in the UV range to allow much excitation of fluorescein, for example. The microscope system developed by Professor Taylor and his associates (Lidwell, Taylor, Clark & Heimer, 1967) was however made available on one occasion. This consists of an optical bench, housing a very small mercury vapour arc lamp, a collector lens, exciting and barrier filters. Microscopic observation was with a Tiyoda microscope fitted with a Tiyoda UV wide filed, dark ground, substage condenser.

(2) **Accessories.** Good quality, thin 3 inch by 1 inch scratch-free microscope slides were selected and kept chromic acid cleaned. Ordinary square coverslips were employed. Instead of immersion oil for the 100x nosepiece and substage condenser, 50% v/v aqueous glycerol was used; specimens were mounted in this concentration of glycerol.

(4) **Fluorescent staining.** Several staining procedures were investigated with a view to ensuring good mixing of sensitive cells with a small volume of fluorescein-colicin E2 conjugate, and efficient washing of treated cells to remove unadsorbed conjugate. Small
centrifuge tubes were made by sealing 4 cm long, 2 mm i.d. glass tubes at one end. A few drops of washed, freshly grown bacteria were put into the tubes with the aid of a drawn out Pasteur pipette, followed by an equal volume of fluorescein-labelled colicin E2 (180 µg protein/ml). The tube contents were mixed and allowed to stand at 37°C for 30 min with occasional shaking. The tubes were centrifuged at 10,000 g for 5 min, the supernatant discarded and the cell pellet resuspended in buffer A. This was again centrifuged and finally resuspended at the original bacterial concentration in the buffer. One drop of treated bacteria was mixed with one drop of glycerol on a microscope slide, covered with a cover slip and examined as soon as possible.

The Conjugation of Ferritin to Anti E2 6.6s yglobulin

(1) Preparation of anti E2 6.6s yglobulin

(A) Preparation of colicin E2 suitable for immunization and the method of immunization. Purified colicin E2 (see Fig. 3) was re-chromatographed on a CM-sephadex column, peak fractions being pooled, freeze dried and dialysed against buffer A. One ml of this colicin preparation (2.8 mg/ml protein) was made up to 5 ml with 0.9% w/v sterile infusion saline containing 0.1% v/v chloroform to sterilize the colicin preparation. This method of sterilization was adopted since passage of colicin through filters can lead to
inactivation (Fredericq, 1957).

Injections of colicin E2 were made by Dr J.K.A. Beverley of Sheffield University. A white Australian rabbit was injected with 1.0 ml per injection of a mixture of 0.5 ml colicin preparation, 0.45 ml Bayol F and 0.05 ml of Arlacell A as adjuvant. Injections were made on alternate weekdays into nine different sites arranged as a 3 x 3 square on the rabbit's side. One bleeding was taken before colicin injection, and four subsequent to injection as outlined in Table 5.

(B) Tests of activity and specificity of anti-E2 serum. Sera from bleeds 1 and 2 (Table 5) were used for the initial tests.

(i) Precipitin ring test. Three drops of serum were carefully pipetted into small bore test tubes 2 cm long, 2 mm i.d. with the aid of a drawn out Pasteur pipette, and carefully overlayered with appropriate test solutions as shown in Fig. 5. In order to gain some quantitative measure of anti-E2 serum activity, dilutions of bleed 2 serum were made and layered on pure colicin E2 (1 mg/ml protein). Precipitin rings were visible after 30 min at 25° in 1, 1/10 and 1/100, but not 1/1000 fold dilutions of serum.

(ii) Anti colicin killing test. Pretreatment of colicin E2 with its antiserum would be expected to lead to a decrease in its killing activity (a) by antigen-antibody precipitation, (b) by perturbing
<table>
<thead>
<tr>
<th>Bleed number</th>
<th>Volume of serum ml</th>
<th>mg/ml protein in serum*</th>
<th>Days after final injection</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>NT</td>
<td>One week before first injection</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>66.2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>63.6</td>
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<td>4</td>
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<td>65.4</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>67.8</td>
<td>146</td>
</tr>
</tbody>
</table>

* Bovine serum albumin as protein standard.
**Fig. 5. The precipitin ring test.**

A few drops of each serum were carefully overlaid with various test sera and solutions. The tubes were incubated at 37° for up to 20 min before recording the presence or absence of a white precipitin ring between the two solutions (indicated by vertical lines in the diagram at the interface of the two solutions).

**Sera are:**
- Goat anti rabbit serum
- Rabbit control = Bleed 1
- Rabbit anti-E2 = Bleed 2

**Solutions are:**
- Saline 0.9% w/v
- Colicin E2 1 mg protein/ml.
SALINE    SALINE    SALINE
GOAT ANTI-RAB  RABBIT CONT.  RABBIT ANTI-E2

RABBIT CONTROL  RABBIT ANTI-E2  E2  E2
GOAT ANTI-RAB  GOAT ANTI-R  RABBIT CONT.  RABBIT ANTI-E2
normal colicin adsorption to sensitive cells through steric hindrance of immunoglobulin molecules attached to colicin molecules.

Serial dilutions of crude colicin E2 of known titre were made in NB. A suitable dilution of the serum to be treated was made, and aliquots of this mixed with aliquots of the colicin dilution series in small test tubes. Control sera and saline were mixed with the colicin series as controls. The mixture was incubated for 4 hr at 37° and spotted on to colicin sensitive lawns of bacteria to determine the final colicin titre. Undiluted serum from the uninjected rabbit (bleed 1) had no demonstrable effect upon colicin E2, but a 1/10 dilution of serum subsequent to injection (bleed 2) reduced the activity of colicin E2 from between 100 and 1000 u/ml to less than 1 u/ml.

(iii) **Ouchterlony double diffusion test.** To give some idea as to the number of antigen-antibody reactions occurring in the immune sera and purified 6.6s γ globulin, double diffusion tests in agar gels were carried out as described by Ouchterlony (1964). Tests were carried out either on microscope 3" x 1" slides (for small scale) or in 5.5 cm dia. glass petri dishes (for medium scale).

A flat layer of 1% Oxoid Ion Agar containing 0.1% w/v sodium azide and 1% sodium chloride was formed by pouring molten agar onto microscope slide or into petri dishes to give layers 1 mm and 5 mm
Immuno-double diffusion in agar gels: 6.6s Y globulin preparations.

Immuno-double diffusion in agar gel was carried out in 5.5 cm dia. glass petri dishes for 24 hr at 37° and stored overnight at 4° prior to photography. The central well contained undiluted goat and anti rabbit serum antiserum, the outer wells contained different 6.6s Y globulin preparations.

Key:

<table>
<thead>
<tr>
<th>Well No.</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4</td>
<td>purified 6.6s Y globulin 1.1 mg/ml</td>
</tr>
<tr>
<td>2,5</td>
<td>purified 6.6s Y globulin 0.55 mg/ml</td>
</tr>
<tr>
<td>3,6</td>
<td>suspected Y globulin dimers 1.0 mg/ml</td>
</tr>
</tbody>
</table>
thick respectively. A uniformly thick level layer was ensured by having the slides and petri dishes placed on plastic petri dishes floating on still water during layering and hardening. Six holes were symmetrically bored around a central well either with a set of seven mounted bores or with a cork borer.

For microscope slides the maximum bore centre-centre distance was 1.55 cm, bore holes being 3 mm dia., for petri dishes the maximum bore centre-centre distance was 2.5 cm, boreholes being 4 or 5 mm dia.

Antigen and anti sera were placed in the various wells, using a drawn out Pasteur pipette. Incubation was in a moist atmosphere (a vaccuum dessicator with a layer of water being used) for 4 to 24 hours at 37°. Photography of gels was usually after a further 24 hr at 4°.

(iv) Slide agglutination test. This test, which is explained and assessed in the Results and Discussion section, was developed as a quick, sensitive, specific and semi-quantitative assay for the presence of anti-E2 immune serum. Strain 906 (Col E2+) was grown in NB medium, washed and resuspended to 10^{10} cells/ml in buffer A and stored until required, at 4°. A drop of this suspension was mixed with a drop of the serum preparation to be tested. Another drop of cells was separately mixed with the serum's solvent on a
clean microscope slide. Observation of the mixture at 25° was continued up to 10 min, and the time taken for visible cell agglutination to occur noted, if it occurred in that period. Undiluted non-immune sera (bleed 1) produced no agglutination even though observation was continued for 30 min, however a 1/100 fold dilution of immune sera (bleed 2) caused agglutination in 6 min. Fig. 7 shows the dependence of the time of agglutination upon dilution of purified anti-E2 6.6s γ globulin (undiluted = 0.58 mg protein/ml).

(C) **Purification of anti-E2 6.6s γ globulin from anti-E2 serum**

Chromatography of immune sera on DEAE-cellulose pH 8.0. A modified version of the method described by Fahey (1962) and Baumstark, Laffin and Bardawil (1964) was used.

The amount of immune serum that was required to be chromatographed was previously dialysed against two 1 litre changes of 10^{-2} M sodium phosphate buffer pH 8.0 at 4°. The dialysate was applied to a DEAE-cellulose column previously washed successively in 0.5 M sodium hydroxide, water, 0.5 M hydrochloric acid, water and equilibrated with the pH 8.0 buffer. The serum was applied and allowed to adsorb into the column. Elution was with a gradient formed by the mixing of 10^{-2} M and 0.3 M pH 8.0 buffers. Fig. 8
shows the distribution of protein and anti E2 activity eluting down DEAE cellulose, the peak eluting with the $10^{-2}$ M buffer being the 6.6s γ globulin fraction (Fahey, 1962).

Better resolution of 6.6s γ globulin from the remaining serum proteins is obtained if elution is continued with $10^{-2}$ M buffer to remove 6.6s γ globulin prior to gradient elution (Baumstark et al., 1964). This procedure was adopted in subsequent fractionations.

Elution of 6.6s γ globulin at pH 7.0 from DEAE cellulose columns was also successfully utilized (Baumstark et al., 1964). Typically a yield of 7% of purified 6.6s γ globulin protein was obtained from the crude serum protein.

Prior to any conjugation studies the purified 5.6s γ globulin was re-chromatographed on DEAE cellulose as shown in Fig. 10.

(2) Ferritin conjugation to 6.6s γ globulin

(A) Preparation of bisdizotized benzidine. Bisdiazotized benzidine (BDB) was chosen as protein conjugating reagent for reasons outlined in the Results and Discussion section. BDB was made from benzidine (now no longer available commercially) as described by Gregory and Williams (1967). This preparation procedure was carried out in a fume cupboard, goggles and rubber gloves being worn. Benzidine and BDB are highly carcinogenic compounds.

Benzidine (1.8 g) was added to ice (20 g) and 6 ml of concentrated HCl was added with stirring. When the ice had just melted, a 5 ml
solution of sodium nitrate (1.4 g per 5 ml water) was added drop-wise with stirring at 0°. The reaction mixture was then filtered into an ice cold Buchner flask and made up to the appropriate volume with ice cold sodium potassium phosphate buffer pH 7.5, I = 0.1.

(B) **Conjugation procedure.** The optimum conditions for protein-protein conjugation with BDB described by Gregory and Williams (1967) were used throughout.

A 4% w/v solution of horse ferritin in sodium potassium buffer pH 7.5, I = 0.1 was mixed with an equal volume of 4% w/v solution of purified 6.6s γ globulin in this buffer. BDB was added to give a final molar total protein to BDB ration of 1:5. The reaction was continued for three hours at 0° with stirring. The mixture was then either dialysed against buffer A or treated with a 2% w/v solution of phenol in sodium borate buffer pH 9.3 I = 0.1 at 20°. The phenol was added at the rate of 100 moles phenol to 1 mole BDB. This phenol treatment was continued for 2 hr prior to dialysis against buffer A.

(C) **Conjugate mixture chromatography on sepharose 6B.** After numerous attempts to separate the constituents of the final conjugation mixture on several ion exchange and sephadex columns, Sepharose 6B was eventually chosen for fractionation purposes. Sepharose 6B separates
molecules on the basis of their molecular size in the high molecular weight range needed for the proteins in this study.

(i) Sepharose 6B column preparation. The dense suspension of Sepharose provided by Pharmacia contains 0.02% sodium azide to prevent microbial growth. The suspension was first diluted with an equal volume of distilled water, sucked dry on a Buchner funnel and repeatedly washed with water by resuspension and filtration. Equilibration with buffer A made 1% w/v sodium chloride was carried out by mixing 1 litre of original column material with several 1 litre changes of buffer with occasional stirring at 4° followed by Buchner filtration. The slurry was poured into a large glass column, a 660 ml column of gravity packed Sepharose being obtained. It was necessary to use such a large column in order to obtain good separation of the protein species within the 660 ml volume of eluting buffer.

(ii) Determination of void volume. It was necessary to determine the volume at which a molecular species entirely excluded from this gel, eluted. This enabled molecular weight calibration curves to be constructed and the column's usefulness determined. Any large molecular species which is entirely excluded from Sepharose 6B can be used to determine this volume. In this case the bacteriophage BF23 was used. Preparation, concentration and estimation of phage
particles is described in the General Methods section. A sample of phage BF23 was carefully added to the top of the 660 ml column of Sepharose 6B. After adsorption, elution was continued with buffer A made 1% w/v with sodium chloride. Fractions were automatically collected, and aliquots of these fractions were diluted and spotted onto sensitive lawns of bacteria as for the colicin spot assay method. After overnight incubation at 37° inhibition zones were examined, and aliquots of the fractions giving the largest zones were diluted and mixed with sensitive bacteria to allow plaque assay counts to be made. The volume of the fraction giving the highest phage titre was taken to be the void volume. This was found to be 205.5 ml for the 660 ml column (see Fig. 15).

(iii) Determination of the elution volumes for ferritin, ferritin dimers and 6.6s γ globulin. Prior to chromatographic separation of conjugation products, it was necessary to determine the elution volumes of the initial reactions. Firstly these volumes would determine the Sepharose 6B's suitability for separation and secondly they would be needed to predict the elution volumes of the 1:1 ferritin : globulin conjugate and other molecular species produced.

The molecular preparation whose elution volume was to be determined was dialysed against buffer A made 1% w/v with sodium chloride. A sample was applied to the 660 ml Sepharose 6B column
and eluted with this buffer. Fractions were monitored for protein content, and the volume of the peak fraction taken as the elution volume for that column.

The intense brown colour of ferritin solutions allowed its sensitive unambiguous assay in protein mixtures with the colourless γ globulin by determining the O.D. at 500 μ. Initially the o-phenanthroline assay (Granick & Michaelis, 1943) for ferritin iron was used but found to be less sensitive, cumbersome and unnecessary for the present study.

Results and Discussion

The use of fluorescein labelled colicin E2

Although fluorescein has a very small molecular weight (332 daltons) compared with that of colicin E2 (60,000 daltons) (Herschman and Helinsky, 1967) the killing activity of the fluorescein-colicin E2 conjugate appeared to be much reduced using either Batty and Walker's (1965) or Rinderknecht's (1962) method. It must be realised that these methods and other previous work on fluorescein labelling has been carried out with immune sera rather than with enzymes or colicins. It is possible therefore that fluorescein isothiocyanate preferentially reacts with active sites of molecules, such sites being more numerous and complex for immunoglobulins.
Fig. 6. Elution of fluorescein conjugated column E2 on a G-25 sephadex column.

The conjugate (0.57 mg protein), prepared by the method of Batty and Walker (1965) was applied to a 14 cm long 1.25 cm dia. column of G-25 sephadex previously equilibrated in buffer A made 1% w/v with sodium chloride, and eluted with this buffer. 10 drop (= 0.7 ml) fractions were collected manually. The presence of fluorescein was detected by measuring the O.D. of fractions at 495 mμ, that of colicin by the spot killing assay. Fractions containing more than 10 u/ml colicin E2 were pooled, dialysed against buffer A, freeze dried and stored at -20° until required.
compared with enzymes and colicins (Singer & Thorpe, 1968). Calculation of the average number of fluorescein molecules bound per colicin molecule from the O.D. 495 m\(\mu\) : O.D. 280 m\(\mu\) ratio (Nairn, 1964) gives a value of 2.8 for the conjugate made by Batty and Walker's method. The O.D. 495 m\(\mu\) : O.D. 280 m\(\mu\) for the conjugate made by Rinderknecht's (1962) method has a value of 1.64 which exceeded the recommended value of 0.5-1.0 for such conjugates (Brighton et al, 1967) and this conjugate was therefore not used.

From the Poisson distribution \(P = e^{-\lambda}\), where \(P\) = the probability of zero fluorescein molecules per colicin molecule, \(e = 2.1828\ldots\) and \(\lambda\) is the mean number of molecules of fluorescein per colicin, viz 2.8, a probability of 0.061 can be calculated. Thus 6.1% of colicin molecules would be expected to be unlabelled if fluorescein attachment was randomly distributed amongst the colicin molecules. Since the specific activity of this conjugate dropped to 8% of the non-conjugated colicin, this calculation is consistent with the view that all fluorescein labelled colicin molecules were inactivated. Nevertheless studies with Professor Taylor's fluorescent microscope allowed confirmation that adsorption, if not killing, of colicin molecules to sensitive bacteria did occur and this adsorption appeared to be specific.
Cells from strain IB11 and IB11/E (a BF23 phage resistant strain lacking the group E colicin receptor and derived directly from IB11) were treated and stained with fluorescein-E2 and mounted in glycerol as described in the text. In addition bacteria not treated with fluorescein-E2 were examined. All cells showed some faint blue autofluorescence, however only strain IB11 fluorescein-E2 treated cells showed yellow-green fluorescence characteristic of the fluorescein fluorochrome. Further, IB11 cells pre-treated with purified colicin E2 and post-treated with fluorescein-E2 showed no such fluorescence. These results are summarised in Table 6.

The distribution of the fluorescein upon the bacterial surface was consistent with a dispersive distribution of labelled colicin rather than a localized distribution. Unfortunately the low level of conjugation and the possibly reduced adsorption activity of the labelled colicin did not cause fluorescence sufficient to obtain good photographs. In the hope that a better preparation could be made by the method of Rinderknecht (1962) a second conjugate was made but not used because of its substantial loss in specific activity due to over conjugation.

Binding of fluorescein to colicin E2 was not therefore found to be possible without serious inactivation of the latter. The
**TABLE 6**

**Effect of fluorescein-E2 labelling of colicin sensitive and colicin resistant cells**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Pretreatment</th>
<th>Fluorescein-E2 treated</th>
<th>Cell fluorescent response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colicin E2 +</td>
<td>untreated -</td>
<td></td>
</tr>
<tr>
<td>IB11</td>
<td>-</td>
<td>+</td>
<td>fluorescein</td>
</tr>
<tr>
<td>IB11</td>
<td>+</td>
<td>+</td>
<td>none</td>
</tr>
<tr>
<td>IB11</td>
<td>-</td>
<td>-</td>
<td>none</td>
</tr>
<tr>
<td>IB11/E</td>
<td>-</td>
<td>+</td>
<td>none</td>
</tr>
<tr>
<td>IB11/E</td>
<td>-</td>
<td>-</td>
<td>none</td>
</tr>
</tbody>
</table>
alternative technique of fluorescein conjugation to anti-E2 γ globulin was considered, but was decided to be superfluous in view of the projected electron microscopic study with ferritin labelled γ globulin.

**Slide agglutination assay for anti-E2 immune serum**

The antigen-antibody agglutination reaction is considered to be a much more sensitive serological method than the precipitation reaction for demonstrating the specific antibody content of a serum (Coombs, Howard & Wild, 1952). Hence a convenient, sensitive, semiquantitative and specific assay system for anti-E2 serum was sought using agglutination as a basis. In any chromatographic work an assay system with the above characteristics is desirable so that protein denaturation, of dilute column fractions, is minimised. None of the other immunological tests described in the text fits all these criteria.

Sensitive bacteria cells with adsorbed colicin E2 might be expected to agglutinate with anti-E2 sera, however the amount of purified colicin required for such adsorption to a large preparation of cells would be prohibitive. It was argued that since strain 906 (col E2+) cells produce large amounts of colicin E2, sufficient colicin E2 may be surface located to allow cell agglutination to take place with anti-E2 sera. This was found to be the case with
immune, but not non-immune, sera induced rapid cell clumping. Neither sera produced cell agglutination when tested against non-colicinogenic *E. coli* K12 bacteria. This agglutination test carried out on microscope slides was very rapid (observation usually up to 10 min) as well as specific. A quantitative measure of the immune serum present was obtained by accurately timing the occurrence of agglutination after antigen-antibody mixing (see Fig. 7). Although direct observation of slide-agglutination was routinely made, a greater sensitivity was achieved upon microscope examination or by counting the appearance of cell clumps using a Coulter Counter.

**Heterogeneity of 6.6s γ globulin and horse ferritin.**

The gamma globulins. The antibody activity of immune sera is located in the γ globulin group of serum proteins. The three major classes of the γ globulins are termed IgG (6.6s γ globulin), IgA (9-11s B2 A) and IgM (18s macroglobulin) (Haurowitz, 1968). Chromatography of whole immune serum on DEAE cellulose pH 8.0 may be used for the separation of these major classes. Thus IgG protein is eluted with the starting buffer, the second protein peak contains IgA, the third IgM (Fahey, 1962) (see Fig. 9).

Depending upon the nature of the antigen and the age and nature of the immunized animal, the majority of the immune activity
Fig. 7. The dependence of the time of slide agglutination of strain 906 (Col E2*) cells upon anti-E2 γ globulin dilution

One loopful of strain 906 (Col E2*) cells at $10^{10}$ cells/ml was mixed with a loopful of different dilutions of purified 6.6s γ globulin. The undiluted preparation contained 0.58 mg protein/ml. The time of agglutination in 1/100 min was measured and the $\log_{10}$ of this time plotted against the dilution. Two determinations for each dilution were made, their averages being plotted.


![Graph showing the relationship between log₁₀ of time of agglutination and globulin dilution. The x-axis represents the log₁₀ of dilution, ranging from 1 to 8, and the y-axis shows the time of agglutination in minutes per 100. The graph demonstrates a linear increase in time of agglutination as the dilution increases.]
Fig. 8. DEAE-cellulose chromatography of immune serum (bleed 2) at pH 8.0

A 0.7 ml sample of bleed 2 serum (40.6 mg protein) was dialysed overnight against $10^{-2}$ M sodium phosphate buffer pH 8.0 and applied to a 14 cm long, 1.5 cm dia. column of washed DEAE cellulose previously equilibrated in the pH 8.0 buffer. Gradient elution was carried out with a controlled mixture of $10^{-2}$ M and 0.3 M sodium phosphate buffers at pH 8.0. 29 drops (= 2 ml) fractions were automatically collected on a LKB fraction collector. Fractions were measured for their O.D. 280 m\textmu, their anti-E2 activity by the slide agglutination test. Buffer molarity was calculated from a standard curve after determining the fraction's refractive index. Fractions 5, 6 and 7 containing 2.87 mg protein were pooled, dialysed against buffer A, freeze dried and stored until required at -20°.
Fig. 9. DEAE cellulose chromatography of immune serum

Bleed 5

A 10 ml (678 mg protein) sample of bleed 5 serum was layered on to a 25.4 cm long, 2.2 cm dia. DEAE-cellulose column previously equilibrated with $5 \times 10^{-3}$ M sodium phosphate buffer pH 8.0. Elution was with sodium phosphate buffer pH 8.0 in the following order:

1. $5 \times 10^{-3}$ M batch.
2. $10^{-2}$ to $10^{-1}$ M gradient.
3. $10^{-1}$ M to $2 \times 10^{-1}$ M gradient.
4. $2 \times 10^{-1}$ M batch.

Fractions of 40 drops (= 2.4 ml) were collected, and suitable dilutions measured at 280 mÅ. Anti-colicin E2 activity was measured by the slide agglutination test. Buffer molarity was determined from the fraction's refractive index.
Fig. 10. Re-chromatography of purified 6.6s γ globulin on DEAE cellulose pH 7.0

A 5 ml mixture of 6.6s γ globulin, purified from bleeds 2, 3 and 4 was dialysed overnight against 5 x 10^{-3} M potassium phosphate buffer pH 7.0. The dialysate was allowed to adsorb onto a 19.6 cm long, 1.25 cm dia. column of DEAE cellulose previously equilibrated in this buffer. 50 drop (= 3 ml) fractions were automatically collected and measured for anti E2 activity by slide agglutination. Suitable dilutions were made and their O.D. measured at 280 μ. Fractions 11, 12, 13 and 14 containing a total of 87.3 mg protein were pooled, dialysed, freeze dried and stored at -20°.
is seen to reside in the IgG fraction (see Fig. 8). However, with increasing immunological maturity and with increasing age, immune activity may be displaced to the IgA or IgM fractions (Fahey, 1962). Compare Fig. 8 and 9.

Electrophoretic heterogeneity of the γ globulins is due to several factors: (i) differing amounts of associated carbohydrate, (ii) extrinsic heterogeneity due to immune molecules being eluted by different parts of an antigen molecule, (iii) intrinsic heterogeneity due to a variety of immune molecules evoked by the same part of an antigen (Haurowitz, 1968). This heterogeneity precludes the use of electrophoresis for clear separation of a given γ globulin species from other molecules. Fortunately sufficient separation of 6.6s γ globulins from other γ globulins can be achieved on columns of DEAE cellulose. Heterogeneity of the IgG molecules is not reflected to any great extent in molecular size variation, and thus the use of molecular sieve columns was not precluded.

**Horse ferritin.** Ferritin as purified from horse spleen is found to consist of apoferritin, a protein of molecular weight 480,000 and an iron containing micelle of hydrated ferric oxide and some phosphate. The protein constitutes about 80% by weight of ferritin. The amount of iron present is variable, this variability accounting
for some of ferritin's electrophoretic, chromatographic and sedimentation heterogeneity. The other reason for ferritin's heterogeneity is its existence in dimer and higher oligomer forms (Granick, 1942; Granick & Michaelis, 1943; Williams & Harrison, 1968). Apoferritin has a clearly defined $S_{20}^0$ value of 17.6 but ferritin gives a broad diffuse boundary of average sedimentation coefficient 65s (Rothen, 1944) (see also Fig. 19). Unfortunately although the iron moiety can be readily removed from ferritin, re-addition of iron in various chemical forms has not been found possible (Granick, 1942). Since ferritin's use in the electron microscope depends on this mineral core, the heterogeneity of ferritin cannot be bypassed.

Ferritin conjugation to anti-E2 6.6s γ globulin

Choice of protein coupling agent. Singer and Schick (1961) have described the use of m-xylene diisocyanate (XC) and toluene 2,4-diisocyanate (TC) as reagents capable of conjugating proteins without substantial loss of biological activity. They favoured toluene 2,4-diisocyanate since its bifunctional nature allowed the para group to react with protein 1 in the first stage of the reaction, while the ortho group remained essentially intact until the second set of conditions were applied for its reaction to protein 2.
Although TC has been the reagent most often used in ferritin-protein conjugation studies (McLean & Singer, 1970; Rifkind, Hsu & Morgan, 1964; Nii, Morgan, Rose & Hsu, 1968; Lee, 1960), Williams and Gregory (1967) have criticized its use. They point out that the nature of the conjugation linking is part ionic, part covalent, resulting in some instability, also much reduction in biological activity occurs after such conjugation. Williams and Gregory (1967) have accordingly used bisdiazotized benzidine (BOB), whose structure is given in Fig. 11, as conjugant. In some comparative studies they found that the yield of bovine serium albumin dimers from monomers using BOB was six times that using TC under optimal conditions. Moreover BOB conjugates of goat anti-human γ globulin with ferritin retained at least 25% of the original antibody activity.

BOB was finally chosen for this study in consideration of the above findings and in view of the possibility of direct communication with Gregory and Williams at Sheffield University.
Fig. 11  The structural formulae of benzidine and its derivatives.

<table>
<thead>
<tr>
<th>Structural Formula</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H}_2\text{N} - \text{C}_6\text{H}_2\text{N} - \text{NH}_2 )</td>
<td>Benzidine</td>
</tr>
<tr>
<td>( \text{H}_2\text{N} - \text{C}_6\text{H}_2\text{N} + \text{Cl}^- )</td>
<td>Mono-diazotized Benzidine</td>
</tr>
<tr>
<td>( \text{Cl}^- + \text{N} + \text{N} - \text{C}_6\text{H}_2\text{N} + \text{Cl}^- )</td>
<td>Bis-diazotized Benzidine</td>
</tr>
</tbody>
</table>
Separation of conjugate, reactants and products.

From the unreacted mixture of ferritin, \( \gamma \) globulin and BDB, the following products can be expected: unreacted ferritin, unreacted \( \gamma \) globulin, ferritin dimers, and higher oligomers, \( \gamma \) globulin dimers and higher oligomers, 1:1 ferritin : \( \gamma \) globulin conjugate and other ratio conjugates. This large molecular variety may account for the complex pattern found when attempting to fractionate such a mixture on Sepharose 6B (see Fig. 12).

Sucrose density gradient centrifugation, although probably adequate to separate \( \gamma \) globulin from ferritin containing molecules, gives no further useful resolution due to ferritin's heterogeneous sedimentation behaviour (see Fig. 19).

Ion exchange materials, which often have superior separating properties, were initially tested for their usefulness in fractionating the conjugate mixture.

**Ion exchange columns.** Preliminary studies showed that pure ferritin could be eluted from CM-sephadex columns with \( 5 \times 10^{-3} \) M sodium phosphate buffer pH 8.0. It was also established during serum fractionation that 6.6s \( \gamma \) globulin was eluted from DEAE cellulose with low molarity buffers at pH 8.0. Hence it was assumed that both DEAE and CM cellulose or sephadex columns would be ideal for the separation of these molecules. Further study
Purified 6.6s γ globulin (23.7 mg) was mixed with ferritin (26.1 mg) and BDB (0.56 mg) in a final volume of 1 ml. Conjugation and dialysis were carried out as described in the text, no post-treatment with phenol being made. The dialysate, after centrifugation at 10,000 g for 5 min. to remove insoluble protein aggregates, was applied to a 650 ml 6B Sepharose column and eluted with buffer A made 1% w/v with sodium chloride. Initially 400 drop (= 24.6 ml) fractions were collected (fractions 1-8 inclusive), subsequently 75 drop (= 4.6 ml) fractions were collected (9-100) automatically. Each fraction was sampled and assayed by the Folin method for total protein (O.D. 750 mµ) and for ferritin by determining the O.D. at 500 mµ. Fractions 26-56 (pool 1), 62-66 (pool 2) and 67-80 (pool 3) were pooled since these elution volumes were those expected from column calibration studies to correspond to conjugate, γ globulin dimers and γ globulin monomers respectively. (All fractions had their anti-E2 activity tested by slide agglutination, but agglutination occurred after 3 min only with fractions 70, 71 and 72.)
showed however that 6.6s γ globulin chromatographed with CM-sephadex eluted with $5 \times 10^{-3}$ M sodium phosphate buffer pH 8.0. This lack of binding to either column probably arises from γ globulin's low intrinsic charge at physiological pH's (Fahey, 1962). When chromatography of ferritin was studied on DEAE cellulose the protein was adsorbed but could not be eluted even with 0.3 M sodium phosphate buffer pH 8.0. However the ferritin was eluted from DEAE-cellulose using $5 \times 10^{-2}$ M potassium phosphate buffer pH 7.0 (see Fig. 13). Quantitation of material eluted indicated that it contained only 68.5% of the initial amount of ferritin applied to the column. Furthermore the remaining ferritin, clearly visible at the top of the column, could not even be removed using the above buffer at 1.0 M.

To determine the reason for this result, the ferritin eluted at $5 \times 10^{-2}$ M phosphate buffer was collected, concentrated by freeze drying and reapplied to a column of DEAE cellulose. The same pattern of elution was obtained; this time 61.9% of the ferritin eluted at about $8 \times 10^{-2}$ M phosphate buffer, whilst the remaining ferritin was again visible at the top of the column and could not be removed at 1.0 M buffer.

The complicated behaviour of ferritin on DEAE cellulose precluded the use of this anion exchanger in conjugate isolation.
Fig. 13. DEAE-cellulose chromatography of horse ferritin

Ferritin solution (0.5 ml, 50 mg dry weight) was carefully layered on a 30 cm long, 1.3 dia. column of DEAE-cellulose previously equilibrated in 10^{-2} M potassium phosphate buffer pH 7.0. Molarity gradient elution was carried out by controlled mixing of 10^{-2} M and 1.0 M buffers, final elution was with 1.0 M buffer. Fractions of 67 drops (= 3.7 ml) were automatically collected, the O.D. of the fractions being measured at 500 m\textmu. Effluent buffer molarity was determined after measuring the fractions refractive index.
The two forms of ferritin that can undergo equilibration when in aqueous solution may possibly reflect monomer dimer (and higher forms) or ferritin iron complex + iron-depleted ferritin transitions (Granick, 1943; Rothen, 1944; Williams & Harrison, 1968; Fischbach et al, 1969).

**Molecular sieve columns.** In consequence of the difficulties encountered with ion exchange chromatography of ferritin, the use of molecular sieve columns was examined.

(i) **Sephadex G-200.** This sephadex preparation has the highest molecular weight range for globular proteins, with an estimated fractionation range between $5 \times 10^3$ and $8 \times 10^5$ daltons (Pharmacia Fine Chemicals Ltd.). As shown in Fig.14, however, although the protein peak shows a leading shoulder of ferritin this sephadex was quite unsatisfactory for the separation of proteins in the molecular range of Y globulin and ferritin dimers.

(ii) **Sepharose 6B.** This recently commercially available Sepharose preparation was tested for its usefulness in separating the molecular species expected in a conjugation mixture. Reference to Fig. 16 shows that the elution volume for ferritin dimers is close to that of the void volume (205.5 ml). If the manufacturer's data on Sepharose 6B is to be believed, then it appears that ferritin is again behaving atypically for a protein of its molecular weight.
Fig.14. Sephadex G-200 chromatography of a ferritin-γ globulin conjugation mixture

Ferritin (26.5 mg), purified 6.6s γ globulin (24 mg) and BDB (0.28 mg) were mixed in a final volume of one ml per 3 hr at 0°. The resulting conjugation mixture was treated with phenol as described in the text. The mixture (2 ml) was made 20% with 2 ml of 40% w/v sucrose, and carefully layered under 4 ml of buffer A which capped a 29.6 cm long, 2 cm dia. sephadex G-200 column. Elution and previous equilibration was with buffer A, made 1% w/v with sodium chloride. Fractions of 30 drops (≈ 1.73 ml) were automatically collected, aliquots being taken for total protein (Folin O.D. 750 μ) and ferritin (O.D. 500 μ).
Fig. 15. Sepharose 6B void volume determination

One ml of approximately $10^9$ BF23 pfu/ml was allowed to enter the top of a 660 ml column of 6B Sepharose. Elution was with buffer A made 1% w/v with sodium chloride. Initially 400 drop (= 24.6 ml) fractions were automatically collected, subsequently 50 drop (= 3.07 ml) fractions were collected. Aliquots of fractions and their dilutions were spotted onto lawns of BF23 sensitive bacteria. Those fractions indicating high bacteriophage content were taken, diluted and plaque assays made to determine the number of pfu/ml. The volume of the highest titre fraction (205.5 ml) being taken as the void volume for the 660 ml column.
Fig. 16. Sepharose 6B chromatography of ferritin and ferritin dimers

Ferritin (0.2 ml of 100 mg dry wt/ml) was adsorbed into the top of a 660 ml column of Sepharose 6B and eluted with buffer A made 1% w/v with sodium chloride. Initially 400 drop (= 24.6 ml) fractions, and subsequently 50 drop (= 3.07 ml) fractions were collected automatically. Aliquots of the fractions were measured for total protein content at 280 m\(\mu\) and by the Folin protein assay at 750 m\(\mu\). The elution volume for ferritin dimers was found to be 212 ml, the elution volume for ferritin monomers 398.4 ml.
Fig. 17. Sepharose 6B chromatography of purified 6.6s γ globulin

A 0.1 ml sample of purified 6.6s γ globulin (3 mg protein) was carefully layered and allowed to adsorb into a 660 ml column of Sepharose 6B, and eluted with buffer A made 1% w/v with sodium chloride. Fractions of 100 drops (≈ 6.14 ml) were collected and aliquots of these fractions measured for their total protein content by the Folin method. The elution volume for 6.6 s γ globulin was found to be 451 ml for this column.
Fig. 18. Dependence of elution volume upon the $\log_{10}$ molecular weight of the eluting protein using Sepharose 6B

The elution volumes were determined as described in the text.

0 = Elution volumes determined for the separate reactant molecules of ferritin dimer, ferritin monomer and 6.6s γ globulin.

1 = Peak volumes of the fractions taken to represent ferritin dimer, 1:1 ferritin-globulin conjugate, ferritin, globulin dimer and globulin.

Molecular weights assumed were those of Gregory and Williams (1967) viz.

Ferritin monomer 600,000 daltons

6.6s γ globulin 160,000 daltons
LOG<sub>10</sub> MOLECULAR WEIGHT

Effluent Volume

MLS

Globulin
Globulin Dimer
Ferritin
Ferritin Dimer

1:1 Conjugate
The Sepharose 6B material does allow separation of molecules in a predictable position (something which could be very difficult using ion exchange materials) and its degree of separation was considered to be superior to all other considered methods.

The preparation and assay of bisdiazotized benzidine.

Several initial conjugation attempts were abortive as judged by the absence, after Sepharose 6B fractionation, of any Y globulin-ferritin complexes. It was therefore decided to test the efficiency of the method for producing BOB. (For an explanation of this method see Appendix II). The outcome of this study was that benzidine can be converted to BOB with widely differing efficiencies, and since the method of Gregory and Williams (1967) assumed, and may have achieved, 100% conversion, it was thought that insufficient BOB had been added to some of the conjugation mixtures described herein. Subsequently all BOB conversions were measured for their efficiency by measuring the amount of sodium nitrate used up in the conversion, and the volume of BOB required calculated from this conversion efficiency.

Characterization of the ferritin-6.6s Y globulin conjugate.

Pooled fractions 1, 2 and 3 from Sepharose 6B fractionation of the conjugate shown in Fig. 12, corresponding to the expected positions of conjugate, Y globulin dimers and Y globulin were
dialysed against buffer A and concentrated by freeze drying. 

Aliquots of these pooled fractions, together with unconjugated 6.6s Y globulin, were tested for rabbit serum antibodies by agar double diffusion against goat anti-rabbit antiserum. The results of these different tests are seen in Plate 2. All fractions gave a precipitation line which appeared to join with the reference Y globulin. The precipitation line formed with pool 1 - the conjugate, was weak and the photograph of the gel is correspondingly poor. Although there are no precipitation spur lines characteristic of a different antigen, the precipitation is much closer to the conjugate well than in the other cases; this slower diffusion may reflect the antigen's greater size when conjugated to ferritin.

The supposed conjugate in pool 1 was also tested for anti-colicin E2 killing activity, as described in the Methods section. A 0.1 ml sample of conjugate (400 µg protein/ml), a 0.1 ml sample of purified 6.6s Y globulin (100 µg protein/ml) and a 0.1 ml sample of ferritin (1 mg protein/ml) were separately diluted with 0.9 ml of broth. Aliquots of the diluted mixtures were mixed with equal volumes of colicin E2 at different titres and subsequently tested for remaining activity. The Y globulin preparation inactivated 64 u/ml E2, the ferritin none, and the suspected conjugate 2-4 u/ml E2. (If this assay is directly proportional to Y globulin activity, then
Plate 2

Immuno-double diffusion in agar gels: pooled conjugation fractions from Sepharose 6B

Samples of the conjugation mixture separated on Sepharose 6B as described in Fig. 12 were tested by double diffusion in agar as described for Plate 1. The centre well contained undiluted goat anti-rabbit serum antiserum.

Key:

<table>
<thead>
<tr>
<th>Well No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>200 μg/ml conjugate (pool 1)</td>
</tr>
<tr>
<td>2</td>
<td>180 μg/ml γ globulin dimers (pool 2)</td>
</tr>
<tr>
<td>1</td>
<td>310 μg/ml γ globulin (pool 3)</td>
</tr>
<tr>
<td>4,5,6</td>
<td>100 μg/ml purified γ globulin</td>
</tr>
</tbody>
</table>
the conjugate has approximately 8% of the initial activity of its combined \( \gamma \) globulin.

The presence of ferritin and non-ferritin in the fractions pooled for pool 1 had been shown by the O.D. 500 \( \mu \) and O.D. 750 \( \mu \) Folin measurements of fractions shown in Fig. 12. That the O.D. 500 \( \mu \) was in fact indicative of the presence of ferritin and not just its iron core was tested by separate sucrose density gradient analysis of ferritin, and pool 1 conjugate. A mixture of ribosomes was included to allow some estimate of the sedimentation coefficient of ferritin and conjugate. The combined results are shown in Fig. 19 the 260 \( \mu \) traces being superimposed; \( s \) values of approximately 65 and 62.4 were found for ferritin and conjugate respectively.

**Ferritin Conjugation: Conclusions**

Protein conjugation studies have only been made with one conjugating species - BDB. The necessary use of ferritin as the electron dense stain for this type of electron microscopic study makes separation and characterization of a conjugate with 6.6s \( \gamma \) globulin difficult. BDB however has not been found in this study to be a very effective conjugating molecule for these two proteins. The only verified conjugate produced in this study was formed without the mixture being subsequently phenol treated.
Fig. 19. Continuous sucrose-density gradient profiles of ribosomes, ferritin and ferritin Y globulin conjugate

Samples (0.1 ml) of (a) ribosomes* (20 μg) comprising 30S, 50S, 70S and 100S particles, (b) horse ferritin (10 μg), (c) ferritin Y globulin conjugate** (10 μg) were separately layered onto a 4.8 ml, 5-20% w/v continuous sucrose density gradient as described in the General Methods. Gradients were centrifuged at 100,000 g for 2 hr at 4° and the tubes automatically analysed for adsorption at 260 mμ with an ISCO Density Gradient Analyser.

The three adsorption profiles are drawn to different vertical scales but the same horizontal scale, in order to demonstrate their relative positions.

Key: ............ ferritin-globulin
       --------- ferritin
          ------- ribosomes

*Kindly supplied by Dr B.W. Senior.

**From the preparation described in Fig. 12.
to remove unreacted BDB. Phenol treatment was advised by Gregory and Williams (1967) in order to minimize non specific staining by unsaturated diazo groups reacting with microscope specimens. Comparison of Fig. 12 with Fig. 16 shows that a large amount of ferritin dimers (and higher polymers) have been formed in this conjugation procedure. It is indeed unfortunate that ferritin has such a great affinity for self-conjugation mediated by BDB.

The more extensive and successful use by other workers in the field of ferritin labelling antibodies (Lee, 1960; Rifkind et al, 1964; Nü et al, 1968; McLean & Singer, 1970) of M toluene 2,4-diisocyanate (TC) would seem to validate the latter's further use. It is possible that the bifunctional groups of TC which are allowed to react with proteins in series as opposed to simultaneously with BDB would reduce the possibility of self-conjugation.

There appears to be no completely satisfactory chromatographic procedure for the separation of all the reactants and products resulting from the kind of protein-protein conjugation described here. The principal requirement for electron microscopic purposes is to remove unreacted Y globulin (and any of its oligomers) from the conjugation mixture, since the former's presence would compete with the minority and less active conjugate group for colicin interaction sites. This can probably best be achieved by high
speed centrifugation of the conjugate mixture on a more concentrated
(20%-60%) sucrose gradient. The conjugate's high s value (62s)
would effectively separate it from the lower oligomers of 6.6s Y
globulin. The problem of non-specific staining due to the presence of
ferritin and its oligomers in a conjugation mixture is probably best
overcome by their separation on Sepharose 6B.
SECTION II
ELECTRON MICROSCOPY OF PHAGE BF23 AND FERRITIN ANTI-E2 Y GLOBULIN ADSORBED TO BACTERIAL CELLS

Introduction

The observation that fluorescein labelled colicin E2 adsorbed to sensitive bacterial cells in a dispersed rather than a confined manner (see Section I) led to a search for the reason(s) why the adsorption of colicin E2 was so limited in amount and yet dispersely located. One explanation could be that the number of receptors is fixed by the metabolic or spatial capabilities of the cell, another that sufficient receptors do exist to allow a monomolecular covering of adsorbed colicin molecules but most of these receptors are masked by other cell surface constituents. A more interesting explanation could come from the recent observations by Bayer (1968a) arising from an electron microscopic study of bacteriophages adsorbed to E.coli B.

Bayer (1968a) found that by plasmolysing bacterial cells in 10% or 20% w/v sucrose solutions and using suitable stains, he could demonstrate the existence of 200 to 400 areas where the cytoplasmic membrane remained in contact with the cell wall. Moreover he demonstrated that the bacteriophages T, T3, T5 and T7 only appeared to attach to areas of the cell wall from which the cytoplasmic
connection arose. These connections were not artefacts of phage adsorption, as they existed to the same extent whether or not phage particles were added, nor were the phage particles preferentially desorbed from non-membrane attachment sites. Bayer (1968a) suggested that phage receptors were located at areas from which membrane attachment sites arose.

Since the bacteriophage BF23 receptor is closely involved with the colicin E group receptors (Fredericq, 1957; Hill & Holland, 1967) it was decided to study the adsorption of this bacteriophage, and colicin E2 to plasmolysed E.coli K12 cells with a view to (a) demonstrating the existence of these attachment sites in the K12 as opposed to B strains, and (b) determining the correlation, if any, between the adsorption sites of colicin E2 and the sites of membrane attachment to the cell wall.

If indeed the bacteriophage BF23 and E group of colicin receptors are located together, then an electron microscopic study of this phage's adsorption pattern would confirm or refute the findings on colicin receptors using fluorescein-labelled colicin E2.

**Methods**

*Preparation of high titre stocks of phage BF23 and T6.* The purification and preparation of bacteriophages is given in the Materials
and General Methods section. For very high titre stocks ($10^{10}$ to $10^{11}$ pfu/ml) 30 to 50 NB agar plates were seeded with purified phage stock to give confluent lysis of bacteria in soft agar overlayers. The phage was prepared for these soft agar overlayers as previously described, and the resulting chloroformed supernatant centrifuged at 27,000 g for 60 min to sediment the bacteriophage. The supernatant was discarded and the combined pellets carefully made up to a convenient volume in (ca 2 ml) $10^{-2}$ M sodium phosphate buffer pH 7.0 or NB medium as desired.

Quantitation of bacteriophage BF23 adsorption. A 14 ml culture of strain I811 was grown in NB medium at 37$^\circ$. Growth was followed by periodically taking 0.1 ml aliquots of the culture and determining the total cell counts with a Coulter Counter. Cells were harvested by centrifugation at 10,000 g for 5 min at 25$^\circ$ and resuspended in 30 ml fresh NB medium to give $1.5 \times 10^8$ cells/ml. One ml of these cells was mixed with one ml of an approximately 200 fold excess of BF23 bacteriophage. The mixture was kept at 37$^\circ$ with gentle shaking for up to 30 min (a period previously determined to be less than the replication and release time for this bacteriophage.) The mixture was diluted 10 times with fresh NB medium at 37$^\circ$ and centrifuged for 5 min at 10,000 g. The pellet was resuspended in 10 ml of fresh NB medium and centrifuged as before. The supernatants
were pooled, dilutions made to determine the unadsorbed bacterio-
phage titre as described in the Materials and General Methods
section. The input phage concentration was similarly determined,
and the bacterial viable count prior to phage mixing determined.

The ratio \( \frac{\text{input phage-unadsorbed phage}}{\text{input bacteria}} \)
was taken as a lower estimate of the number of phage BF23 phage
particles that could be adsorbed to a single bacterial cell.

**Electron Microscopy**

**Equipment.** Sections of bacteria embedded in araldite and suitably
polymerized, were cut on an L.K.B. Ultramicrotome using glass
knives made with an L.K.B. Glass Knife Maker. Sections placed on
carbon-coated formvar-covered grids were examined in a Siemens
Elmiskop 1A electron microscope. Manual operation of a shutter
allowed photographs to be taken on 6.5 cm x 9 cm photographic
plates (AGFA-Gevaert Scientia).

**Specimen preparation.** Individual specimen treatments are
indicated in the appropriate Plate legends, only general preparatory
procedures are described here.

1. **Whole bacterial cells and bacteriophages.** One drop of an
   aqueous suspension of bacteria \((10^8\) to \(10^9\) cells/ml) or bacteriophage
   \((10^{10}\) pfu/ml) was placed on a carbon counted formvar covered copper
grid (abbreviated to grid). The drop was removed with a clean filter paper after some 40 seconds duration. A drop of aqueous 0.4% w/v ammonium molybdate was placed on the grid and similarly removed after 1 min or less. Excess stain was removed by rinsing with a drop of water, which was removed with a filter paper. After a few minutes drying in the air, the grid was examined in the electron microscope.

Sections of bacteria and bacteriophage. Approximately $10^9$ pelleted bacteria, after appropriate experimental treatment with, for example, sucrose, colicin E2 or bacteriophage, were fixed by one of the following two methods, post stained with uranyl acetate/osmium tetroxide solution and dehydrated prior to araldite embedding. (a) Formaldehyde fixation followed by osmium tetroxide fixation. This method of fixation was taken from the method employed by Bayer (1968a) with minor modifications.

The experimentally treated cells were resuspended in 5 to 10 ml of 10% v/v formaldehyde in NB medium adjusted to pH 7.0 with sodium hydroxide solution. Fixation in formaldehyde was continued for 2 hr at 25°. The bacteria were then pelleted by centrifugation at 10,000 g for 5 min, washed in distilled water, again centrifuged and resuspended in 1 ml of 1% w/v osmium tetroxide solution made pH 7.0 with sodium hydroxide solution. After 1 hr at 25°, the cells were
again centrifuged and resuspended in 1 ml of 0.5% w/v uranyl acetate, 0.5% w/v osmium tetroxide solution in distilled water and kept overnight at 25°. (When the bacteria were first treated with 20% w/v sucrose, fixation was carried out as above except that all reagents were made 20% w/v with sucrose). Finally the cells were centrifuged again at 10,000 g for 5 min and washed with distilled water prior to the embedding treatments.

(b) Osmium tetroxide fixation. A modification of the method of Kellenberger, Ryter and Séchaud (1958) was used for osmium tetroxide fixation of bacteria and subsequent embedding in agar.

The experimentally treated cells were resuspended in 1 ml of 1% w/v osmium tetroxide in acetate-veronal buffer made pH 6.0 or pH 7.0 as required (Kellenberger et al., 1958). Treatment in this fixative was for 1 hr only since the suggested overnight fixation caused extensive blackening and aggregation of bacterial cells. The cells were pelleted by centrifugation at 10,000 g for 5 min and resuspended in 1 ml of 0.5% w/v uranyl acetate, 0.5% osmium tetroxide solution in acetate-veronal buffer and repelleted in glass centrifuge tubes. The pellet was then resuspended in 0.05 ml of distilled water and warmed to 50° for 2 min. An equal volume of 2% w/v molten agar at 50° was then added with rapid mixing at 50°. Drops of the molten agar mixture
were then allowed to solidify on clean plastic petri dish lids. Cubes (side 0.5 mm) were cut from the drop and dehydrated prior to araldite embedding.

**Dehydration and Araldite embedding.** The cells or agar blocks of cells were treated in series with the following solutions for 30 min at 25°, solutions being separated from cells at each stage by centrifugation and decantation: H₂O; H₂O; 50% v/v ethanol/H₂O; ethanol; ethanol; 50% v/v propylene oxide/ethanol; propylene oxide; 50% v/v araldite/propylene oxide. The cells were then transferred to araldite without accelerator for 24 hr at 37° followed by resuspension in araldite plus accelerator in pyramid capsules for 24 hr at 37°. The bacteria were pelleted by centrifugation of the capsules in a swing out rotor and the capsules kept at 60° for 2 or more days until hard. The resin blocks were removed from their capsules, trimmed and sectioned.

**Post staining of sections.** Fig. 20 shows a diagram of the waxed-slide method adopted for post staining with either a saturated solution of uranyl acetate in 50% v/v ethanol/water or Reynolds' (1963) lead hydroxide solution. The uranyl acetate staining was conducted in a moist atmosphere provided by a water wetted filter paper. Lead hydroxide staining was conducted in a moist, CO₂ depleted atmosphere provided by wetting a filter paper with...
POST-STAINING FOR ELECTRON MICROSCOPY

- waxed microscope slide
- copper grid
- Petri dish
- stain or wash droplet
- ultra-section
- water or potassium hydroxide saturated filter paper
saturated 1.0 M sodium hydroxide solution. Excess stain was removed from the grids by rinsing uranyl acetate stained sections in drops of 50% v/v ethanol, water and water, or rinsing lead hydroxide stained sections on drops of $2 \times 10^{-2}$ M sodium hydroxide solution and water. When both stains were used on a given specimen, the uranyl acetate preceded the lead hydroxide stain.

**Results and Discussion**

**Structure of BF23 bacteriophage and plasmolysed E.coli K12 cells**

Plate 3 shows the appearance of bacteriophage BF23 adsorbed to unsectioned cells of strain IB11, the shape of the phage head much resembles that for the phage T5 (Bayer, 1968a) and is to be compared with the shape of the T6 phage head shown in Plate 4. Plates 5 and 6 are higher magnifications of purified BF23 phage preparation, the hexagonal dried-down shape of the head measuring approximately 500 Å across, 750 Å long with a long tail $\approx 1800$ Å approximately 50 Å diameter.

Strain IB11 cells appear capable of adsorbing an average of at least 166 phage particles per bacterium when challenged with only a 200 fold ratio of phage : bacteria, this high minimal estimate indicates that the phage receptors are not located only in a small special area such as the girth of the cell. Sections of BF23 treated bacteria (see Plate 7) show that adsorption does
Whole cells of strain IB11 treated with bacteriophage BF23

Strain IB11 cells grown in NB medium were treated with a broth suspension of BF23 particles for 10 min at 37°. (10^9 cells + 10^11 pfu per final volume of 2 ml). A drop of the mixture was put onto a copper grid, removed and stained with 0.4% w/v ammonium molybdate solution as described in the Methods section.

Final magnification = 120 K
bar = 0.1 μ
PLATE 4

Whole cells of strain IB11 treated with bacteriophage T6

Cells were treated as described in Plate 3 except that an equivalent amount of bacteriophage T6 was used instead of BF23.

Final magnification = 120 K
bar = 0.1 μ
PLATE 5

Whole BF23 phage particles

A single drop of $10^{10}$ BF23 pfu/ml phage preparation was placed on a copper grid for 30 sec, removed with a filter paper and stained with a drop of 0.4% w/v ammonium molybdate solution for 1 min. The drop was removed with a filter paper, the grid washed with a drop of water, and dried with a filter paper prior to examination.

Final magnification = 240 K

bar = 0.1 μ
PLATE 6

Whole BF23 phage particles

This plate is a higher magnification of the phage particles prepared as for Plate 5.

Final magnification = 640 K

bar = 500 Å
Sections of BF23 treated cells of strain IB11

One ml of $10^9$ cells/ml of strain IB11 grown in NB medium at 37° to $5 \times 10^8$ cells/ml was mixed with an equal volume of $10^{11}$ BF23 phage pfu/ml NB for 10 min at 37°. Two ml of 20% v/v formaldehyde in NB pH 7.0 was then added, mixed and kept for 2 hr at 25°. The mixture was washed, fixed for 1 hr in 1% w/v osmium tetroxide solution, washed and stained overnight in 5% w/v uranyl acetate, 0.5% w/v osmium tetroxide solution. Sections were not post stained.

Final magnification = 65 K

bar = 0.1 μ
not appear to be restricted in any general areas along the cell surface, supporting the findings of the fluorescein labelled colicin E2.

Plates 8, 9 and 10 show the appearance of the cell wall and cytoplasmic boundary upon plasmolysis in sucrose for various lengths of time. A 30 second exposure to 20% w/v sucrose prior to fixation appears sufficient to demonstrate the effect of plasmolysis in some cells, although it could be argued that further plasmolysis is possible in the fixative medium (see Bayer, 1968b). Plate 11 demonstrates that the membrane attachment sites are also present in the K12 strain of E.coli, however it was found that post-staining with lead hydroxide was necessary to demonstrate these structures. Fig. 21 shows the relative sizes of a silver grain, ferritin and colicin at the same magnification for Plate 11. Sections of BF23-treated plasmolysed cells were not post-stained with lead hydroxide and the association of these particles with membrane connections was not established.

**Examination of colicin refractory mutants and ferritin anti-colicin E2-treated cells.**

Preliminary observations on E.coli K12 cells described above included an examination of the refractory mutants 56-3J, 56-3W and 56-3I. No obvious changes in cell wall or membrane structure were
Sections of sucrose treated strain IB11 cells

One ml of $10^9$ cells/ml of strain IB11 grown in NB medium at $37^\circ$ to $5 \times 10^8$ cells/ml was mixed with 1 ml of NB and 2 ml of 40% w/v sucrose in NB. After 0.5 min the bacteria were fixed by mixing with 4 ml of 20% v/v formaldehyde, 20% w/v sucrose in NB pH 7.0 for 2 hr at $25^\circ$. After washing in 20% w/v aqueous sucrose solution, bacteria were further fixed for 1 hr in 1% w/v osmium tetroxide in 20% w/v sucrose, followed by overnight staining in 0.5% w/v uranyl acetate, 0.5% w/v osmium tetroxide, 20% w/v sucrose solution. Sections were post-stained for 5 min in saturated alcoholic uranyl acetate solution.

Final magnification = 60 K

bar = 0.1 $\mu$
PLATE 9

Sections of sucrose treated strain IB11 cells

Cells were treated and stained as described in the legend for Plate 8 except that cells were treated for 2 min in 20 w/v sucrose prior to formaldehyde fixation. Sections were post-stained for 5 min in saturated alcoholic uranyl acetate solution.

Final magnification = 320 K
bar = 0.1 µ
Sections of sucrose treated strain IB11 cells

Cells were treated and stained as described in the legend for Plate 8 except that cells were treated for 5 min in 20% w/v sucrose prior to formaldehyde fixation. Sections were post-stained for 10 min in lead hydroxide solution.

Final magnification = 800 K

bar = 500 µ
PLATE 11

Membrane adhesion sites in plasmolyzed strain IB11 cells

Cells were treated and stained as described in the legend for Plate 8 except that cells were treated for 5 min in 20\% w/v sucrose prior to formaldehyde fixation. Sections were post-stained for 10 min in lead hydroxide solution.

Final magnification = 440 K

bar = 500 Å
Relative Sizes of a Silver Grain, a Ferritin Molecule and a Colicin Molecule. Magnification 440K.

- 0.1 μ dia. Silver Grain
- 122Å dia. Ferritin Molecule
- 50Å dia. Colicin E2 Molecule
apparent between these mutants and their parent 56-3. Even
the filament forming 56-3W strain grown on plates, although
showing abnormal lengths, did not show any other apparent
differences from the parent (see Plate 12). In addition, any
membrane changes brought about by colicin E2 treatment were not
discernable in the electron microscope.

Adsorption of the ferritin anti-E2 \( \gamma \) globulin preparation
to colicin E2 treated cells and to strain 906 cells colicinogenic
for colicin E2 and its demonstration in the electron microscope
was attempted in many different fixing and staining procedures with­
out success. Strain 906 cells were used for control purposes as
well as colicin E2 treated and untreated cells since it was known
that some of the colicin E2 was amenable to anti-E2 \( \gamma \) globulin
(the basis of the slide agglutination test in Section I). Further­
more, the use of formaldehyde as fixative for strain 906 cells was
known not to seriously interfere with their slide agglutination
capabilities.

Cells were either fixed for 10 min in 10% \( w/v \) formaldehyde
both after colicin adsorption prior to incubation with the
ferritin-label, or for 60 min after ferritin labelling (see Plates
13, 14, 15 and 16). Neither procedure eventually yielded any
ferritin labelled sections. The low amount of ferritin label
Sections of plate grown cells of strain 56-3W

Bacteria taken from an overnight growth of strain 56-3W on NB thymine agar plates at 37°C were treated for 1 hr in 10% v/v formaldehyde in NB pH 7.0. Bacteria were then washed and stained for 4 hr in 1% w/v osmium tetroxide solution, followed by overnight fixation and staining in 0.5% w/v uranyl acetate, 0.5% w/v osmium tetroxide solution. Sections were post-stained in saturated alcoholic uranyl acetate solution for 10 min.
A 5 ml culture of strain 56-3 was grown to $10^9$ cells/ml in NB thymine medium at $37^\circ$, diluted to 10 ml with fresh medium and divided into two 5 ml fractions. One portion was treated with 0.25 ml of $2 \times 10^4$ u/ml colicin E2 for 10 min at $37^\circ$, the other untreated. After the 10 min, 5 ml of ice cold NB thymine was added to both fractions which were then rapidly centrifuged at 10,000 g for 5 min at $4^\circ$. The cells were resuspended in 10 ml ice cold NB thymine and again centrifuged. The washed cells were then resuspended in 0.1 ml of warm NB thymine and the cell viabilities determined. Untreated cells had a colony forming ability of $4.45 \times 10^9$ cells/ml which was reduced to $8.2 \times 10^4$ cells/ml in the E2 treated cells. To 0.1 ml of cells, treated and untreated with colicin E2 was added 0.1 ml of ferritin anti E2 - γ globulin serum (400 μg/ml) for 30 min at $37^\circ$ with occasional shaking. Cells were washed and fixed by mixing with 5 ml of 10% v/v formaldehyde NB solution pH 7.0 for 1 hr. Cells were washed in 5 ml distilled water and resuspended in 1% osmium tetroxide for 90 min and treated as described in the Methods section according to Kellenberger et al., (1958). Sections were not post-stained.

Magnification = 120 K

bar = 0.1 μ
PLATE 14

Colicin E2, ferritin anti-E2 treated cells of strain 56-3

These cells are a higher magnification of cells prepared as described in Plate 13.

Magnification = 400 K

bar = 500 Å
PLATE 15

Ferritin anti-f2 treated cells of strain 56-3

These cells are a higher magnification of cells prepared as described for Plate 13.

Magnification = 120 K

bar = 0.1 μ
PLATE 16

Fenitin anti-E2 treated cells of strain 56-3

These cells are a higher magnification of cells prepared as described for Plate 13.

Magnification = 400 K
bar = 500 μ
available necessitated manipulation of small numbers of bacteria to ensure sufficient excess of label. The agar embedding procedure of Kellenberger et al (1958) was used to minimize loss of cells. However, numerous sectioning difficulties were encountered due to non-hardening of the araldite infused into the agar; these difficulties were eventually overcome by infusing with lower accelerator concentration in the araldite for longer periods of time at 37°.

The low yield and activity of the ferritin conjugate was probably the source of the failure in demonstrating adsorption. A new batch of colicin E2 was prepared for injection into rabbits but unfortunately Dr Beverley was unable to continue with a new immunization schedule.

Conclusions

The presence of membrane attachment areas and membrane extensions as described by Bayer (168a, b) for E.coli B have been demonstrated in the E.coli K12 strain. Although the association of these areas with the location of bacteriophage BF23 was not established, the disperse location of BF23 receptors was established. This supported the earlier finding using fluorescein labelled colicin E2 that the colicin E receptors were not localized in any confined cell surface area. Attempts were made to locate the adsorption sites of colicin.
E2 which could possibly be associated with the cytoplasmic connections. It was considered possible that colicin E2 might migrate through these channels to the cell cytoplasm or other parts of the cell membrane, or that these connections served as the sole sites of conduction of colicin-induced stimuli.

It was most unfortunate that the direct localization of colicin E2 molecules in or on the bacterial cell was not eventually made. The low yield and activity of the ferritin conjugate probably accounted for this lack of success rather than removal of conjugate in the fixing procedures. Ferritin-γ globulin labelling of the cells is one of the few highly versatile and specific electron microscopic stains available and it is to be hoped that the valuable information and experience gained in ferritin conjugation and electron microscopy will be of use in the localization of certain constituents of the cell envelope.
SECTION III

THE INTERACTION OF COLICIN E2 WITH THE BACTERIAL CELL SURFACE

Introduction

It was not the purpose of this investigation to study the biochemistry of colicin induced target degradation, or alteration; work along these lines is being carried out with colicin E2 in this laboratory by Holland and his co-workers and elsewhere by Reeves and also by Ringrose (personal communications). Rather, the mode of action of the colicin transmission system was the object of study. Since no evidence for a direct role of colicin in target degradation has yet been found, the weight of evidence for the indirect interaction of colicin was provisionally accepted and the therefore necessary colicin transmission system adopted as a working hypothesis for this study.

Under this hypothesis it would be expected that restricted mechanical disruption of the cell envelope might interfere with the transmission of the colicin's effect without having a gross effect on cell metabolism. Hence the effect of plasmolysis on the initial breakdown of DNA was studied by suspending cells in 20% w/v sucrose prior and subsequent to addition of colicin E2. Also, if part of the operation of the transmission system involved
the liberation of membrane associated degradative enzymes into
the cell cytoplasm, it was conceivable that release of the same
(or other) enzymes outwards into the cell wall and thence to the
cell's surrounding medium may also occur. Therefore known
surface-located enzymes were tested for their release into the
medium by colicin E2.

Involvement of surface-located enzymes or other systems was
also sought by subjecting cells to osmotic shock prior and
subsequent to addition of colicin E2. Qualitative and quantitative
differences in enzymes released by osmotic shock were made by using
high or low magnesium concentrations in the shock fluid (Nossal &
Heppel, 1966).

In addition to measuring the release of known surface-located
enzymes by colicin E2, the effect upon membrane envelopes was
tested by comparing SDS-acrylamide gel patterns of envelope
proteins, prepared from cells treated or untreated with colicin
E2.

To measure the effect of plasmolysis and osmotic shock upon
early colicin E2 action, the amount of DNA degradation was
monitored. This method was preferred to that of colicin induced
cell death, because the former but not the latter, was a more
immediate index of the efficiency of transmission of the colicin's
effect.
Methods

Assay of the surface-located enzymes 5'-nucleotidase and thymidine phosphorylase

5'-nucleotidase* is a zinc metallo-enzyme (Dvorak & Heppel, 1968) which can be assayed by measuring the amount of adenosine released from adenine monophosphate (AMP); the adenine being converted to inosine (I) by adenosine deaminase.

\[
\text{AMP} \xrightarrow{5'\text{-nucleotidase}} A + \text{Pi} \\
A \xrightarrow{\text{adenine deaminase}} I + \text{NH}_3
\]

The following reaction mixture (modification of Nossal & Heppel, 1966) was prepared, commencing the reaction by the addition of AMP.

- 0.025 ml to 0.5 ml bacterial enzyme extract
- 0.2 ml of 0.5 M sodium acetate/acetic acid buffer pH 6.0
- 0.05 ml of Co/Ca chloride salts 50 and 200 mM respectively
- 0.01 ml of 50 µg/ml adenine deaminase
- To 0.9 ml distilled water
- 0.1 ml of 0.1 mM AMP

*This enzyme was assayed by I.R. Beacham.
The reaction mixture was incubated at 37° and the amount of inosine formed measured by periodically determining the O.D. at 265 m\(\mu\).

Thymidine phosphorlase catalyses the reversible reaction between thymidine (dRT) and orthophosphate ion (Pi) to give deoxyribose phosphate and thymine (see Fig. 22). The released thymine was measured by taking aliquots of the reaction mixture at various times into 0.1 M sodium hydroxide solution which terminates the reaction and allows a quantitative measure of thymine.

\[
dRT + Pi \xrightarrow{\text{thymidine phosphorlase}} dR-1-P + T
\]

The following reaction mixture (Beacham, Eisenstark, Barth & Pritchard, 1968) was prepared, commencing the reaction by the addition of thymidine.

- 0.2 ml to 0.4 ml bacterial enzyme extract
- 0.1 ml of 0.1 M sodium phosphate buffer pH 6.7
- To 0.9 ml distilled water
- 0.1 ml of 0.05 M thymidine

The reaction mixture was incubated at 37°, 0.1 ml aliquots were removed and mixed with 0.9 ml of 0.1 M sodium hydroxide solution at various times. The optical densities of these mixtures at 300 m\(\mu\) were then determined as a measure of the
Fig. 22. Deoxynucleoside catabolism

Additional abbreviations:

- dRC = deoxycytidine
- dRT = thymidine
- dRU = deoxyuridine
- dRA = deoxyadenosine
- dRG = deoxyguanosine
- dR-1-P = deoxyribose-1-phosphate
- dR-5-P = deoxyribose-5-phosphate

DEOXYNUCLEOSIDE CATABOLISM

\[
\begin{align*}
\text{dRC} & \rightarrow \text{dT(U)} \\
\text{Pi} & \rightarrow \text{thymidine phosphorylase} \\
\text{T(U)} & \rightarrow \text{dR-1-P} \\
\text{dR-1-P} & \rightarrow \text{dR-5-P} \\
\text{dR-5-P} & \rightarrow \text{glyceraldehyde-3-P} + \text{acetaldehyde}
\end{align*}
\]
amount of thymine produced.

0smotic shocking of bacteria suspensions.

Preparation of cells. Typically, 300 ml of bacteria cells were grown in minimal medium plus 0.5% w/v glycerol, 0.1% w/v casamino acids (or required amino acids at 20 μg/ml) and thymine at 5 μg/ml. Occasionally glucose was added to 0.4% w/v.

Cells were radioactively labelled as indicated in the figures and grown from an initial inoculation of 5 x 10⁶ cells/ml to 5 x 10⁸ cells/ml at which density they were harvested by centrifugation at 10,000 g for 10 min.

Osmotic shock. The method of Nossal and Heppel (1966) was used throughout, although various volume modifications were made for individual experiments.

The harvested bacteria were resuspended in 10⁻² M Tris/HCl, 3.3 x 10⁻² M sodium chloride buffer pH 7.1 at 4°, centrifuged as before and resuspended in 100 ml of this buffer. The cells were again centrifuged and resuspended in a small volume (usually about 5 ml) of 3.3 x 10⁻² M Tris/HCl buffer pH 7.1 at 25°. This suspension was mixed with an equal volume of 40% w/v sucrose in 3.3 x 10⁻² M Tris/HCl buffer pH 7.1 with rapid shaking at 25° and 0.1 ml of sodium. EDTA solution added to give a final concentration of 10⁻⁴ M sodium EDTA. Stirring was continued for 10 min at 25°
and the cells centrifuged at 10,000 g for 10 min. The resulting supernatant was decanted and the pellet well drained prior to its shocking by careful dispersion in the ice-cold shock fluid. (Shock fluid was either distilled water, $5 \times 10^{-4}$ M or $2 \times 10^{-2}$ M magnesium chloride solution.) Gentle shaking of the suspension was continued for 10 min in an ice bath. The shocked cells were harvested by centrifugation at 10,000 g for 10 min; the supernatant (shockate) and pellet (shocked cells) were used immediately for subsequent study. Aseptic precautions and sterilized buffers were used throughout to allow cell viability determinations to be made at various stages in the shocking procedure.

**Isolation and testing of low thymine-requiring strains.**

Low thymine-requiring strain ($\geq 2 < 20 \mu g/ml$ thymine needed for growth) were desirable for this study since (1) incorporation of radioactive thymine into DNA is much facilitated and (2) the enzyme thymidine phosphorylase occurs in constitutive amounts in certain types of low requirers, its assay in osmotic shockates thereby being facilitated.

Thymine high-requiring strains ($\geq 20 \mu g/ml$ thymine needed for growth) were obtained using the folic acid analogue trimethoprim (2, 4-diamino-5-(3',4',5'-trimethoxy) benzyl pirimidine).
From these high requirers low-requiring strains were isolated following a second mutation. These mutants were either deoxyriboaldolase negative or deoxyribomutase negative; the former but not the latter are sensitive to thymidine and will not grow on minimal agar plates plus thymidine. The former class were more desirable because of the reason give in (2) above. (For an explanation of the complex regulatory pattern in deoxynucleoside catabolism see Beacham et al, 1968).

Trimethoprin selection for high thymine requiring strains. A culture of I811 was grown in minimal glucose medium plus 20 µg/ml methionine to 10⁸ cells/ml; 0.1 ml aliquots of this suspension were put into three sterile screw-capped bottles containing 10 ml of fresh medium. The bottles were supplemented as described below, no cells being added to the fourth bottle.

(1) thymine to 50 µg/ml, trimethoprin to 10 µg/ml
(2) trimethoprin to 10 µg/ml
(3) thymine to 50 µg/ml
(4) thymine to 50 µg/ml, trimethoprin to 10 µg/ml

After 2 or 3 days static incubation at 37° the bottles were examined for growth. Turbidity was found in bottles (1) and (3) only. An aliquot of cells from bottle (1) was taken into a bottle containing fresh medium plus thymine at 50 µg/ml and trimethoprin
at 10 μg/ml to give $10^6$ cells/ml. Incubation was continued for a further 2 or 3 days at 37° until turbid.

Aliquots of cells were plated on supplemented minimal agar plates with high thymine (50 μg/ml). Colonies arising were test-streaked on supplemented minimal agar plates with or without high thymine. Colonies only arising on the high thymine plates were purified by re-streaking on agar plates and kept as high thymine requiring mutants.

**Selection for low thymine requiring strains.** One of the high thymine requiring strains was grown in high thymine minimal medium and plated onto low thymine (2 μg/ml) supplemented minimal medium. Colonies arising were test-streaked on supplemented minimal medium with the following additions: high thymine (20 μg/ml), low thymine (2 μg/ml), no thymine and thymidine ($10^{-2}$ M). Strains that grew on low and high thymine plates only were designated *dra* mutants, those growing on all except no thymine plates were designated *drm* mutants. (See Fig. 22 and Table 1a)

**Measurement of DNA degradation**

The method of Holland (1968) was used throughout. Bacterial DNA was uniformly labelled by growing low thymine requiring cells in tritiated thymine medium for six or seven generations. Thymine labelled with tritium in the methyl group (14.2 C/mM) was usually
added to 5 μC/ml in the presence of 5 μg/ml unlabelled thymine.

After labelling, the cells were usually washed twice by centrifugation (10,000 g for 10 min) and resuspended in 10 ml fresh unlabelled medium plus 20 μg/ml unlabelled thymine at 25°.

Cells were experimentally treated as required, and the amount of DNA degraded measured by taking aliquots of cells into 5% w/v ice-cold TCA. After one hour at 0°, the samples were centrifuged at 10,000 g for 10 min at 0°. From the resulting supernatant 0.1 ml aliquots of the cold acid soluble material were mixed with 10 ml of aqueous scintillation fluid in glass vials. The latter were transferred to a liquid scintillation counter and their radioactivities determined.

Soluble counts were expressed as a percentage of total incorporated radioactivity determined by heating a sample cell suspension in 5% w/v TCA to 90° for 30 min and measuring the radioactivity of the supernatant fraction after centrifugation at 10,000 g for 10 min.

Analysis of Envelope Proteins

Cell growth and colicin treatments. A one litre culture of strain 56-3 and a 500 ml culture of strain 56-3/E were grown in NB plus 80 μg/ml thymine at 26°. Half of the 56-3 culture, when it had reached 5 x 10^8 cells/ml, was harvested by centrifugation at 10,000 g
for 10 min. To the remaining 500 ml was added 10 ml of unpurified colicin E2 to give a final concentration of $2 \times 10^4$ u/ml. Incubation at 26° was continued for 15 min (an aliquot of cells was taken for a viable estimation before and after colicin treatment) and cells harvested by centrifugation at 10,000 g for 10 min. The culture of 56-3/E was similarly harvested when it reached $5 \times 10^8$ cells/ml.

All harvested cells were washed with $10^{-2}$ M sodium phosphate buffer at pH 7.0 and envelopes prepared as described in Section IV. SDS-acrylamide gel electrophoresis of envelope proteins. Envelope samples were adjusted to 6 mg protein/ml and dissolved in 1% w/v SDS, 1% v/v mercaptoethanol in 0.1 M sodium phosphate buffer pH 7.0. Samples were dialysed overnight at 37° against 0.5% w/v SDS, 0.1% v/v mercaptoethanol in 0.05 M sodium phosphate buffer pH 7.0. Samples of envelope proteins were electrophoresed into 10% w/v acrylamide gels containing 0.5% w/v SDS in 0.05 M sodium phosphate buffer pH 7.0 as described in Fig.23. Gels were removed and stained overnight by shaking with aqueous 1% w/v naphthalene black, 10% v/v acetic acid, 50% v/v methanol at 25°. Gels were rinsed and electrophoretically destained in 7% w/v acetic acid and finally scanned in a Joyce-Loebl microdensitometer (see Section IV for details).
Incorporation of radioactive leucine into whole cells and TCA precipitable material.

Cultures of the low thymine requiring strain IB11a were grown in the appropriate medium and osmotically shocked if required as previously described. Shocked and unshocked cells were then resuspended in minimal medium supplemented with 0.5% w/v glycerol, 0.4% w/v glucose, 20 μg/ml methionine, 10 μg/ml thymine and 1 μg/ml unlabelled leucine. At appropriate times 0.1 ml of C$_4$, C$_5$ labelled H$^3$-leucine (22.2 C/mM) was added to each flask to give 1μC/final ml. 0.1 ml samples were then taken at various times, 0.5 ml of each sample was pipetted into 0.5 ml of ice-cold 10% w/v TCA and stored at 0°C for one hour. The remaining 0.5 ml was rapidly filtered onto 2.5 cm dia. Millipore filter discs 0.45μ dia. pore size, and rinsed with 5 ml of unlabelled growth medium at 37°C. TCA precipitated samples were collected onto similar filters and washed with 5% w/v ice-cold TCA. Both sets of filters were dried, suspended in non-aqueous scintillation fluid and their radioactivity determined.
Results and Discussion

Effect of colicin E2 on the release of surface located enzymes

The enzyme 5'-nucleotidase and thymidine phosphorylase are thought to be surface located on the basis of their release by osmotic shock from E.coli B cells (Nossal & Heppel, 1966). Electron microscopy of sites of 5'-nucleotidase activity has confirmed the surface location for this enzyme (Nisonson, Tannenbaum & Neu, 1969). It is not clear where the exact location of this enzyme is, but it is presumed that both the enzymes are located between the cell cytoplasmic membrane and the lipoprotein of the cell wall (Neu, 1967). Enzyme release was also studied in cells incubated under anaerobic conditions for it was thought that this treatment could also disturb the location of surface enzymes.

Tables 7 and 8 show the quantitative release of 5'-nucleotidase and thymidine phosphorylase upon treatment with colicin E2. It can be seen in the case of 5'-nucleotidase released from cells treated with colicin E2 for 40 min in Tris buffer, that a significant amount of enzyme activity was released compared with the enzyme activity that can be removed by osmotic shock. However the equally high levels of untreated and anaerobically incubated cells point to a general release of this enzyme and offers no evidence for specific release of colicin E2.
Legend to Table 7.

A 300 ml culture of strain C600 was grown at 37° in minimal medium plus 0.1% w/v casamino acids, 0.5% w/v glycerol, 1 µg/ml thiamine, and harvested at 6 x 10^8 cells/ml by centrifugation at 10,000 g for 10 min. Cells were thoroughly washed in 10^-2 M Tris/ HCl buffer pH 7.1 and resuspended in 15 ml of this buffer. Samples of these cells (2.5 ml) were treated as described below. After treatment, the cells were centrifuged at 10,000 g for 10 min and the supernatant measured for 5' nucleotidase activity.

Treatments
(1) Cells were osmotically shocked with distilled water.
(2) Cells were treated in 10^-2 M Tris/HCl buffer pH 7.1 (resuspension buffer) for 40 min with unpurified colicin E2 at 40 u/final ml at 37°. (This reduced the colony forming ability of the cells some 10^3 fold from 1.2 x 10^10 to 1.5 x 10^4 cells per ml).
(3) Cells in resuspension buffer were flushed 5 times with argon gas, followed by evacuation in resuspension buffer in a Thunberg tube. The cell suspension was then incubated at 37° for 40 min in an atmosphere of argon.
(4) Cells were shaken at 37° for 40 min in the resuspension buffer.
TABLE 7

The release of 5' nucleotidase from treated cell suspensions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage Enzyme activity released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotically shocked cells</td>
<td>100</td>
</tr>
<tr>
<td>Colicin E2 treated cells</td>
<td>9.86</td>
</tr>
<tr>
<td>Anaerobically incubated cells</td>
<td>7.41</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>8.38</td>
</tr>
</tbody>
</table>
Legend to Table 8.

A 300 ml culture of strain C600 was grown at 37° in minimal medium plus 0.1% w/v amino acids, 0.5% w/v glycerol and 1 μg/ml thiamine, and harvested at 5 x 10^9 cells/ml by centrifugation at 10,000 g for 10 min. Cells were washed and resuspended in 12 ml of fresh medium. Samples of these cells (2.0 ml) were treated as described below. After treatment the cells were centrifuged at 10,000 g for 10 min and the supernatants measured for thymidine phosphorylase activity.

Treatments.

(1) Cells were osmotically shocked with distilled water.

(2) Cells in growth medium were treated for 2 min with purified colicin E2 at 50 u/final ml at 37°. (Colony forming ability decreased 20 fold from 7.5 x 10^9 to 3.5 x 10^8 cells/ml).

(3) Cells in growth medium were treated with 50 u/final ml of colicin E2 for 40 min as described above. (Colony forming ability decreased 100 fold from 7.5 x 10^9 to 6.4 x 10^7 cells/ml).

(4) Cells in growth medium were incubated in argon for 2 min at 37° (see Table 7).

(5) Cells in growth medium were incubated in argon for 40 min at 37° (see Table 7).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage Enzyme activity released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotically shocked cells</td>
<td>100</td>
</tr>
<tr>
<td>Cells treated with colicin E2 for 2 min</td>
<td>1.92</td>
</tr>
<tr>
<td>Cells treated with colicin E2 for 40 min</td>
<td>10.6</td>
</tr>
<tr>
<td>Cells anaerobically incubated for 2 min</td>
<td>1.63</td>
</tr>
<tr>
<td>Cells anaerobically incubated for 40 min</td>
<td>13.78</td>
</tr>
<tr>
<td>Untreated cells</td>
<td>7.66</td>
</tr>
</tbody>
</table>
The release of thymidine phosphorylase from colicin-treated and untreated cells was studied over short and long time periods after colicin addition, in growth medium rather than in the Tris medium used for 5'-nucleotidase release, in order to avoid any 'Tris' effects (Neu, Ashman & Price, 1967). Table 8 shows that enzyme release over the 40 min period is comparable to that for 5'-nucleotidase. There appears to be no significant difference between the control and E2 treated cells, but it is possible that the anaerobically incubated cells differ significantly. That the 10% enzyme release for cells treated with colicin E2 for 40 min is not significant, is supported by the observation that cells treated with E2 for only 2 min release only 2% of the enzyme. Since the transmission of the colicin E2 stimulus is largely completed in this short period (Ringrose 1970b) any significant and specific E2 induced release of surface enzyme would be expected to be almost complete in this time period.

It appears therefore that colicin E2 does not induce a general non-specific alteration in the surface located enzymes 5'-nucleotidase and thymidine phosphorylase. This finding is consistent with the observation that colicins have no overall permeability effect upon sensitive cells as already mentioned in the general Introduction. Other surface located enzymes or
enzyme systems not necessarily associated with the transmission system may well be dislocated or released upon colicin treatment, but this alteration does not appear to affect the release of known surface enzymes.

Effect of colicin E2 on the composition of envelope proteins.

The ability of SDS-acrylamide gel electrophoresis to discern quantitative differences in cell envelope proteins down to 3% of the total envelope protein (Samson & Holland, 1970) (See Section IV) was made use of to study the effect of colicin E2 upon the composition of cell envelope proteins. Fig. 23 compares the Joyce-Loebl microdensitometer traces of envelope proteins of strain 56-3 treated and untreated with colicin E2 and 56-3/E, a derivative of 56-3 made receptor-negative for the E group of colicins. Despite the high level of colicin used for a short time (the conditions most likely to show a specific induced protein change if it occurred), no significant differences in profiles were found.

The conclusion that no gross compositional alterations in envelope proteins occur after colicin treatment is again consistent with the highly specific effects on the cell that colicins are known to elicit.

Effect of plasmolysis upon colicin E2 induced DNA degradation

The plasmolysis of E.coli cells by their immersion in 20% w/v sucrose solution, can be demonstrated by electron microscopic
Envelope proteins were prepared as described in the Methods section. Samples (0.05 ml) were mixed with an equal volume of 40% w/v sucrose, 0.001% bromophenol blue, 0.5% w/v SDS in 0.05 M sodium phosphate buffer pH 7.0 and electrophoresed into 10% acrylamide gels for 6 hr 20 min at 3 mA per gel. Gels were stained in aqueous 1% w/v napthalene black, 10% w/v acetic acid, 50% v/v methanol and destained electrophoretically in 7% v/v acetic acid. (The dotted lines indicate areas of opacity characteristic of gels stained in 50% v/v methanol.)

Trace 1 = Strain 56-3
Trace 2 = Strain 56-3 treated with $2 \times 10^4$ u/ml colicin E2 for 15 min at 26°C. (Colony forming ability decreased $10^5$ fold from $6.4 \times 10^8$ cells/ml to $5.3 \times 10^3$ cells/ml.)
Trace 3 = Strain 56-3/E.
examination in some cells 30 seconds after treatment (see Plate 8). This plasmolysed state eventually reverses for most cells approximately 15 min after sucrose treatment (Bayer, 1968b). The effect of plasmolysis, in particular the partial separation of the cell wall from the cytoplasmic membrane, can be judged from some of the electron micrographs in Section II. Other workers (Beppu & Arima, 1967) have studied the effect of sucrose on colicin treated bacteria over long time periods. It was, however, considered desirable to study the primary effects of sucrose, that is the mechanical separation of the cell wall from the cytoplasmic membrane - rather than possible secondary effects of plasmolysis. The effects of sucrose upon the kinetics of colicin E2 induced DNA breakdown were therefore examined in the initial 15 to 20 minute period prior to reversal of plasmolysis.

Fig. 24 shows the effect of colicin E2 upon DNA degradation with and without sucrose treatment. DNA degradation of non-sucrose treated cells can be demonstrated approximately 5 min after colicin E2 addition at 10 u/ml or 20 u/ml. The time of addition of an equal volume of 40% w/v sucrose medium to the cell suspension is shown by the arrow in Fig. 24, colicin E2 being added at time zero. The effect of sucrose appeared to be to delay the time of initiation of DNA degradation and also to
Fig. 24. The effect of plasmolysis by sucrose upon the initial rate of colicin E2 induced DNA breakdown.

A 24 ml culture of strain 206 labelled with 5 μCi/ml, 2.5 μg/ml H$^3$-thymine in NB medium was grown and harvested at 10$^8$ cells/ml by centrifugation at 10,000 g for 10 min. Cells were washed and resuspended in 24 ml of fresh NB medium plus 25 μg/ml unlabelled thymine. The washed cells were distributed amongst seven flasks A to G and treated at various times with an equal volume of NB medium plus 25 μg/ml thymine with or without 40% w/v sucrose.

Colicin E2 was added to flasks A, C, D, E and F to give 10 μ/ml, 20 μ E2/ml being added to flask B. The control flask G (not shown) had no colicin added. Results are expressed as a percentage of the total radioactivity available after subtracting the control's released radioactivity.

Times of addition of sucrose are shown by the arrows. Colicin E2 was added at 0 min, equivalent volumes of NB being added to flasks A, B and G at -12 min. Aliquots (0.5 ml were taken at various times into 0.5 ml of 10% TCA to determine the cold acid soluble counts as described in the Methods section. (Final concentrations: cells 5 x 10$^7$/ml, colicin E2 10 or 20 μ/ml.)
reduce the subsequent initial rate.

Induction of DNA degradation was most effectively delayed by treatment with sucrose for 2 min prior to addition of colicin E2. Sucrose added 8 min prior to colicin E2 addition was less effective. Sucrose addition subsequent to colicin treatment was also less effective than sucrose addition prior to treatment. Again the 2 minutes subsequent to colicin treatment with sucrose was more effective than the 8 minute subsequent treatment with sucrose.

Cell Viability measurements by colony forming ability were also made 30 min after the addition of colicin E2 to gain some idea of the efficiency of colicin adsorption. The viability of non-plasmolysed cells, 'A' in Fig. 24, treated with 10 u/ml colicin E2 was reduced $5 \times 10^3$ fold, and that of cells 'B' with 20 u/ml E2 was reduced $5 \times 10^4$ fold. Survival of all plasmolysed cells with 10 u/ml colicin E2 was comparable, being reduced $10^3$ fold. This viability data was consistent with the possibility that sucrose does have a slight effect upon colicin E2 adsorption kinetics (see Table 9). However the effect of sucrose addition subsequent to colicin treatment cannot be explained merely by a decrease in adsorption efficiency, as adsorption is almost complete in this period (Reeves, 1965).
### TABLE 9

Flask treatments and cell viabilities for Fig. 24

<table>
<thead>
<tr>
<th>Flask</th>
<th>Final colicin E2 titre u/ml</th>
<th>Time of addition of sucrose relative to colicin E2 addition (min)</th>
<th>Cell viability 30 min after colicin addition colony forming units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>-</td>
<td>5 x 10³</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>-</td>
<td>6 x 10³</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>-8</td>
<td>2 x 10⁴</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>+8</td>
<td>2.3 x 10⁴</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>-2</td>
<td>2.1 x 10⁴</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>+2</td>
<td>1.2 x 10⁴</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>-</td>
<td>2.8 x 10⁷</td>
</tr>
</tbody>
</table>
Since sucrose treatment only delayed the initiation of DNA degradation for maximally 10 minutes, and was most effective during the period of maximum plasmolysis, it seems reasonable to conclude that mechanical distortion of the cell surface during plasmolysis can interfere with colicin E2 action.

**Effect of osmotic shock upon colicin E2 induced DNA degradation.**

(A) **Effect of colicin E2 concentration on DNA degradation.** Prior to the isolation of low thymine requiring strains of the more extensively studied strain IB11, preliminary experiments on other low thymine requiring strains were made. Fig. 25 shows the effect of different concentrations of colicin E2 upon DNA degradation in strain CR-34. Increasing E2 concentration appeared to increase the rate of DNA degradation, and to reduce the time of initiation of DNA degradation. From the known specific activity of purified colicin E2, and the known number of cells used, the concentrations of E2 used can also be expressed in terms of molecules of colicin E2 available per bacterial cell. For the concentration of bacteria used in Fig. 25, this is approximately 200 for 1 u/ml. (All degradation experiments were carried out in growth medium at 37°C. The unusually high TCA soluble counts present before colicin E2 addition found in Fig. 25 were due to insufficient prior washing with unlabelled thymine medium.)
Fig. 25. Effect of colicin E2 concentration on induced DNA breakdown

A 7 ml culture of strain CR-34 was grown at 37°C in NB medium plus 20 µC/ml H3 thymine, 5 µg/ml unlabelled thymine and harvested at 2.2 x 10^8 cells/ml by centrifugation at 10,000 g for 10 min. Cells were washed with ice-cold NB plus 100 µg/ml unlabelled thymine. Cells were resuspended in 30 ml of NB + 100 µg/ml thymine, 5 ml aliquots were distributed to 5 flasks and incubated at 37°C. At 5 min (indicated by an arrow on the figure) 0.1 ml of various concentrations of colicin E2 were added to give a 0.1 to 100 u/final ml; control cells were not treated with colicin E2. Aliquots (0.5 ml) were taken into 0.5 ml of 10% w/v TCA to determine the acid soluble radioactivity as described in the Methods section. (Final cell concentration 5 x 10^7/ml.)

Key:

○ = No colicin E2
● = 0.1 u/ml colicin E2
▲ = 1 u/ml colicin E2
▲ = 10 u/ml colicin E2
□ = 100 u/ml colicin E2
Effect of colicin E2 on DNA degradation in osmotically shocked cells. The effect of osmotic shock upon the initiation of DNA degradation in E2 treated cells was next examined. Cultures of strain CR-34 grown in supplemented minimal medium were washed, either shocked or not shocked in $2 \times 10^{-2} \text{M} \text{MgCl}_2$ solution, resuspended in growth medium and treated or untreated with colicin E2. The results of this treatment are shown in Fig.26. (The efficiency of osmotic shock was determined in all experiments by determining the release of thymidine phosphorylase. Typical release patterns are given in Table 10 which shows that 75% of TdR phosphorylase activity of a sonicated cell extract was released by distilled water shock. Only 5.6% of TdR phosphorylase activity being released in high magnesium shock.) The reduced amount of DNA degradation in the shocked cells could not be increased by adding back shockate to such cells. The decreased amount of TCA soluble material prior to colicin E2 treatment in shocked cells was probably due to release of the soluble thymine pool upon osmotic shock. Cell viability was again monitored, but the drastic effect of osmotic shock alone upon the cell viability of this and other strains of *E.coli* K12 reduced the viability to about 1% of unshocked cells. Nossal and Heppel (1966) however, working
Fig. 26. Effect of osmotic shock in high magnesium upon colicin E2 induced DNA degradation

A 50 ml culture of strain CR-34 was grown at 37° in supplemented minimal medium plus 0.5% w/v glycerol, 0.1% w/v casamino acids, 20 μC/ml H3-thymine, 5 μg/ml unlabelled thymine and harvested at 5 x 10^8 cells/ml by centrifugation at 10,000 g for 10 min. Cells were washed and resuspended in 100 ml of 3.3 x 10^-2 M NaCl in 10^-2 M Tris/HCl buffer pH 7.1 prior to washing and resuspension in 5 ml of 3.3 x 10^-2 M Tris/HCl pH 7.1. Two ml of these cells were stored at 0°, the remainder were treated for osmotic shock in 2 x 10^-2 M MgCl2 solution. Aliquots of shocked or unshocked cells (approximately 2.5 x 10^8 cells) were resuspended in 10 ml of fresh supplemented minimal medium plus 10 μg/ml unlabelled thymine. Colicin E2 was added to three of the six flasks to give a final concentration of 10^3 μ/ml at 8 min (indicated by the arrow in the figure). Two ml of shockate or 2 x 10^-2 M MgCl2 was added to certain flasks as described below. Samples were periodically removed to determine the acid soluble radioactivity as described in the Methods section. (Final concentrations: cells 2.5 x 10^7/ml, colicin E2 10^3 μ/ml.)

Key: ○ = Unshocked cells + MgCl2
● = Unshocked cells + E2 + MgCl2
△ = Shocked cells + MgCl2
▲ = Shocked cells + E2 + MgCl2
□ = Shocked cells + shockate
■ = Shocked cells + E2 + shockate
<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Percentage total protein</th>
<th>Percentage TdR phosphorylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicate of unshocked cells</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sonicate of H₂O shocked cells</td>
<td>68.2</td>
<td>21.4</td>
</tr>
<tr>
<td>Sonicate of 2 x 10⁻² M MgCl₂ shocked cells</td>
<td>87.5</td>
<td>88.9</td>
</tr>
<tr>
<td>H₂O shockate</td>
<td>28.4</td>
<td>74.6</td>
</tr>
<tr>
<td>2 x 10⁻² M MgCl₂ shockate</td>
<td>7.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Sucrose pre-H₂O shock</td>
<td>3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Sucrose pre-MgCl₂ shock</td>
<td>5.4</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*Cells were prepared according to Fig.27, and shocked as described in the Methods section.*
with E.coli B obtained 80-90% viability of cells after osmotic shock. Several attempts were made to achieve these high viabilities by using other E.coli K12 strains without success. The poor viability found, detracted from the effect upon colicin E2-induced DNA degradation because of the possibility of generalized cellular damage. An index of cell integrity other than viability was therefore sought.

(C) Protein synthesis in osmotically shocked cells. Fig. 27 shows the effect of osmotic shock in high \((2 \times 10^{-2} \text{ M MgCl}_2)\) and low \((5 \times 10^{-4} \text{ M MgCl}_2)\) magnesium shock medium upon the uptake of \(^3\text{H}-\text{leucine}\) into strain IB11a whole cells and acid precipitable material. The results indicated that leucine was not incorporated by osmotically shocked cells. Since there was not uptake, no measure of protein synthesis by measuring TCA precipitable material was possible. Recent experiments have now shown that several, but not all, permeases are removed from cells upon osmotic shock among them the permease for leucine (Anraku, 1968). Alternative measurement of \(\beta\)-galactosidase synthesis from endogenous amino acids indicated no net protein synthesis possibly because the strain used required methionine whose uptake was also perturbed in osmotically shocked cells.

A measure of cell integrity that did not require the uptake of compounds into osmotically shocked cells was therefore utilized,
Fig. 27. Effect of osmotic shock on amino acid incorporation into TCA precipitable material and whole cells

A 50 ml culture of strain IB11a cells was grown at 37° in minimal medium supplemented with 0.5% w/v glycerol, 0.5% w/v casamino acids, 20 μg/ml methionine, 1 μg/ml leucine and 2.5 μg/ml thymine. Cells were harvested at 5 x 10^8 cells/ml by centrifugation at 10,000 g for 10 min. Cells were washed and resuspended in 10 ml of 3 x 10^-2 M sodium chloride in 10^-2 M Tris/HCl buffer pH 7.0. One third of these cells was kept in the above buffer at 4° whilst the remainder were osmotically shocked with either distilled water or 2 x 10^-2 M MgCl₂. All cell fractions were harvested and resuspended in the supplemented minimal medium without casamino acids. At 0 min H³-leucine was added to give 1 μC/final ml of cells. Aliquots of cells (0.5 ml) were taken into 0.5 ml ice cold 10% w/v TCA for TCA precipitable radioactivity, or filtered onto Millipore filters for whole cell radioactivity incorporation, as described in the Methods section. Results are expressed as a percentage of the total H³-leucine available. Aliquots of shockates and sonicates of cells were taken to determine the amount of thymidine phosphorylase released as indicated in Table 9.

Key:
- ▲ = Whole H₂O shocked cells
- ▲ = TCA precipitate H₂O shocked cells
- ■ = Whole 2 x 10^-2 M MgCl₂ shocked cells
- □ = TCA precipitate 2 x 10^-2 M MgCl₂ shocked cells
and the effect of mitomycin C upon DNA degradation was chosen for simplicity. In all subsequent experiments on osmotic shock, cells were monitored for the degradation induced by both colicin E2 and mitomycin C. Fig. 28 shows the effect of different concentrations of mitomycin C upon DNA degradation in unshocked cells.

**Effect of osmotic shock on the continuation of DNA degradation induced by mitomycin C and colicin E2.**

(A) **High magnesium shock.** Nossal and Heppel (1966) showed that quantitative and qualitative differences in enzyme release could occur depending upon the nature of the osmotic shock fluid. In particular, it was found that 90% of the RNA-inhibited DNA endonuclease of *E. coli* B was released by high magnesium osmotic shock (2 x 10^{-2} M MgCl_{2}), but only 11% was released by low magnesium shock (5 x 10^{-4} M MgCl_{2}). It was conceivable that this DNA degradation enzyme could be involved in colicin E2 induced DNA breakdown.

Fig. 29 shows the effect of high magnesium osmotic shock upon bacteria previously treated with colicin E2. It is seen that osmotic shock does not significantly effect the course of DNA degradation by either colicin E2 or mitomycin C when compared with unshocked cells. The initial high rate of colicin E2 induced DNA degradation prior to osmotic shock did appear to be reduced.
**Fig. 28. Mitomycin C induced DNA degradation**

A 10 ml culture of strain IB11a was grown at 37° in glucose minimal medium plus 0.5% w/v glycerol, 0.5% w/v casamino acids, 10 μC/ml H³-thymine plus 2.5 μg/ml unlabelled thymine and harvested at 5 x 10⁸ cells/ml by centrifugation at 10,000 g for 10 min. Cells were washed and resuspended in 10 ml fresh medium plus 10 μg/ml unlabelled thymine. Various concentrations of mitomycin C were added at 5 min (at the position of the arrow in the figure) and the amount of cold TCA soluble radioactivity determined as described in the Methods section. (Final cell concentration 5 x 10⁸/ml.)

**Key:**

- ○ = No mitomycin C added
- ● = 1 μg/final ml mitomycin C added
- △ = 10 μg/final ml mitomycin C added
- ▲ = 50 μg/final ml mitomycin C added
- □ = 100 μg/final ml mitomycin C added
Fig. 29. *Effect of high magnesium osmotic shock upon the continuation of DNA degradation induced by mitomycin C and colicin E2*.  

A 12.5 ml culture of strain IB11a cells was grown at 37° in glucose minimal medium plus 0.5% w/v glycerol, 0.5% w/v casamino acids, 10 μg/ml H<sup>3</sup>-thymine plus 2.5 μg/ml unlabelled thymine and harvested at 5 x 10<sup>8</sup> cells/ml. Cells were washed and resuspended in 10 ml of fresh medium plus 10 μg/ml unlabelled thymine. Three ml of this suspension were put into each of three flasks and treated with either 1 ml of medium, 1 ml of 500 μg/ml colicin E2 or 1 ml of 400 μg/ml mitomycin C at 37°. DNA degradation was measured as previously described in the Methods section. Cells were harvested by centrifugation at 10,000 g for 10 min at 0° and either not osmotically shocked (kept at 4°) or shocked in 2 x 10<sup>-2</sup> M MgCl<sub>2</sub> solution. Cells were finally resuspended in fresh supplemented minimal medium plus 10 μg/ml thymine and further DNA degradation measured. (Final concentrations: cells 4.7 x 10<sup>8</sup>/ml, Colicin E2 125 μg/ml.).

Colicin E2 or mitomycin C was added at 5' (arrow).

The time for shocking took 1 hr 35 min.

Key: ○ = untreated, unshocked cells
• = untreated, shocked cells
□ = mitomycin C treated, unshocked cells
■ = mitomycin C treated, shocked cells
△ = colicin E2 treated, unshocked cells
▲ = colicin E2 treated, shocked cells
subsequent to treatment, but this effect was independent of osmotic shock. This can in part be explained by loss of the already solublized DNA during cell resuspension, and possibly also by a decreased efficiency of DNA degradation during the period taken for osmotic shock (90 min.). This effect was also found for the data in Fig. 30.

Comparison of the above results with the data from high magnesium shock prior to colicin E2 addition shown in Fig. 26 implies that high magnesium shock influences some early event in colicin E2 action, but not the degradation process once initiated. Alternatively, it could be suggested that colicin E2 protects cells from certain effects of osmotic shock to some extent as it does sphaeroplast membranes (Nose, Ono & Mizuno, 1970), however no significant changes in TdR phosphorylase release were noticed.

(B) Low magnesium shock. The effect of low magnesium osmotic shock upon the continuation of colicin E2 and mitomycin C is shown in Fig. 30. In contrast to high magnesium shock, the continuation of DNA degradation after both treatments is very much reduced by low magnesium shock. This effect is not therefore specific to colicin E2 action unless the enzymes involved in both types of degradation are the same.

If endonuclease I is similarly released from E.coli K12 upon
Fig. 30. Effect of low magnesium osmotic shock upon the continuation of DNA degradation induced by mitomycin C and colicin E2

The conditions of the experiment were as described for Fig. 29 except that cells were shocked in $5 \times 10^{-4}$ M MgCl$_2$ solution and the colicin E2 concentration was 62 u/final ml, half that used in Fig. 29.

Colicin E2 or mitomycin C was added at 5' (arrow).

The time taken for osmotic shock was 1 hr 45 min.

Key:

○ = untreated, unshocked cells
● = untreated, shocked cells
□ = mitomycin treated, unshocked cells
■ = mitomycin treated, shocked cells
△ = colicin E2 treated, unshocked cells
▲ = colicin E2 treated, shocked cells
high or low magnesium shock as found for \textit{E.coli} B (Nossal & Heppel, 1966) then it could be concluded that this endonuclease was not necessary for continued DNA degradation in either case. The effect of high magnesium shock prior to the addition of colicin E2 could be interpreted as a role for endonuclease in early degradation of DNA. However it is also possible that shocking prior to colicin E2 addition may affect the adsorption of colicin.

\textbf{Conclusions}

\textbf{Colicin induced enzyme release.}

The effects of colicin E2 upon the nonspecific release of surface located enzymes and of overall protein changes have been studied. No significant release of the two surface located enzymes 5' nucleotidase or thymidine phosphorylase above that released by untreated cells was observed. The implication that colicins affect cell envelopes in highly specific ways, not initially leading to diverse effects on the membrane, is consistent with the necessarily specific transmission systems that must exist for each of the different colicins. The lack of any gross ($\geq 3\%$) quantitative change in envelope proteins evoked by large doses of colicin E2 upon sensitive cells corroborates the former, more sensitive, observations.
Plasmolysis.

Effects have been found on colicin E2 induced DNA degradation brought about both by protoplasting cells in 20% w/v sucrose and by osmotic shock. Although sucrose may in part affect the rate and extent of adsorption of colicin E2 to sensitive cells, for example by viscosity, its effect subsequent to the addition of colicin E2 indicates an additional effect. The partial separation of the cytoplasmic membrane from the cell wall that occurs upon plasmolysis would be expected to seriously interfere with the early fate of the adsorbed colicins. Depending upon whether the colicin remains on the cell wall, or whether it migrates to the cell membrane during the normal course of colicin action (Bhattacharyya, Wendt, Whitney & Silver, 1970). The plasmolysing effect of sucrose upon the initiation of DNA degradation would be expected to be more or less severe respectively.

Assuming the colicin remained upon the cell wall throughout normal (and abnormal) colicin action, then plasmolysis just prior to colicin adsorption would prevent transmission of the colicin induced stimulus across the cell membrane, the efficiency of this prevention depending on the extent of plasmolysis. Initiation of further DNA degradation would have to await some protoplast reversion. Protoplasting of cells subsequent to addition of colicin would prevent
any further initiation of DNA degradation occurring. If, however, colicins themselves migrated to the cell membrane, plasmolysis would only slightly prolong this migration if the findings of Bhattacharyya et al are accepted.

The effects of sucrose treatment upon colicin E2 induced DNA breakdown can be interpreted by either colicin transmission system depending on the assumed efficiency of plasmolysis. Significant delays in the appearance of degraded DNA seems to favour the cell wall location for active colicin molecules. A similar conclusion was reached in the recent study by Beppu and Arima (1967) on protoplasts formed and held in 0.8 M sucrose, $10^{-2}$ M MgSO$_4$. They achieved greater protection from E2 induced DNA degradation due, probably, to the prolonged stabilization of protoplasts by magnesium.

**Osmotic shock.**

The severe effect of osmotic shock on cell viability detracted from the observed effects of osmotic shock upon colicin E2 induced DNA degradation. Osmotically shocked cells do appear to be temporarily metabolically inactive for up to 60 min after shock (Nossal & Heppel, 1966). In view of the known energy requirement for colicin E2 induced DNA degradation (Holland & Holland, 1970) the effect of osmotic shock upon colicin E2 and mitomycin C induced DNA degradation may merely be due to an effect upon energy metabolism.
Despite the inability to demonstrate quantitative differences of the effect of osmotic shock upon colicin E2 as compared with mitomycin C induced DNA degradation, it must be pointed out that the degradative enzymes involved must, in part at least, be different. Thus UV$^\text{R}$ \textit{E. coli} mutants are blocked in mitomycin C but not in colicin E2 induced degradation (see Holland & Holland, 1970).

It is not clear whether the differences in colicin induced DNA degradation observed in high and low magnesium shocked cells reflect a difference in the class of osmotically released substances or just an overall difference in cell metabolic integrity. However, the non-involvement of the RNA-inhibited DNA endonuclease in the continuation of colicin E2 and mitomycin C DNA degradation is suggested by these studies, and is consistent with the observation that \textit{E. coli} strains lacking this endonuclease are still capable of colicin E2 induced DNA degradation (Holland, personal communication).
SECTION IV

FRACTIONATION OF THE BACTERIAL CELL ENVELOPE

Introduction

The two membranes of the *E. coli* cell envelope consist of lipoprotein. The lipid composition of the envelope, as mentioned in the general Introduction, comprises by weight of total lipid approximately 75% phosphatidylethanolamine, 18% phosphatidyl glycerol and 5-10% cardiolipin, minority groups including phosphatidic acid and phosphatidyl serine (Ames, 1968). Protein composition studies of *E. coli* cell membranes has only recently been attempted but several groups have shown that a large variety of proteins exists in the cell envelope as a whole (Schnaitman, 1969; 1970a,b; Jones & Kennedy, 1969; Inouye & Guthrie, 1969). The work of Rothfield and Pearlman-Kothencz (1969) and Schnaitman (1970a,b) indicates however that the protein moiety of the cell wall lipoprotein layer consists primarily of one type of protein. In contrast studies on partially purified *E. coli* cytoplasmic (or inner) membranes (Schnaitman, 1970a,b) and the cytoplasmic membranes of *Mycoplasma* (Rottem & Razin, 1967) indicate that these membranes do contain a large variety of protein species.

Proteins are characteristically the main agents of cell function and specificity and the apparent simplicity of the lipid
moiety relative to the protein moiety of bacterial cell membranes
is consistent with the view that functional specificity of membranes
is mainly determined by proteins. In support of this, it may be
noted that one fatty acid may be replaced by another fatty acid in
auxotrophic mutants of *E. coli* without apparent deleterious effects
to the cell (Wilson, Rose & Fox, 1970; Razin, Tourtellotte, McElhaney

In view of the probable importance of proteins in envelope
function, the protein composition of *E. coli* cell envelopes from
various colicin E2 refractory mutants was analysed by sodium
dodecylsulphate (SDS) acrylamide gel electrophoresis, using a
modification of a recently published method (Jones & Kennedy, 1969).
In addition, a method of analysing lipoproteins was investigated
following a suggestion by Daniels (personal communication).

Colicin E2 refractory mutants (CetC) which may show associated
properties, for example, sodium deoxycholate sensitivity and UV
sensitivity, were initially studied as mutants likely to have membrane
alterations (see for example Nagel de Zwaig & Luria, 1967).
Methods

Analysis of total envelope proteins by sodium dodecyl sulphate (SDS) acrylamide gel electrophoresis.

Preparation of envelope fraction(s)

(1) The method of Jones and Kennedy (1969). Bacterial cell envelopes were prepared by a modification of the above method. Typically 500 ml of bacterial cells were grown in supplemented glucose minimal medium or NB medium at 26° or 37°. Cells were harvested at 5 x 10^8 cells/ml by centrifugation at 10,000 g for 10 min at 4°, washed with two batches of 200 ml of 10^-2 M sodium phosphate buffer pH 7.0 (phosphate buffer A) resuspended in 12 ml of this buffer and sonicated for three 2 min periods at 0° with 30 sec cooling breaks. The sonicate was centrifuged at 10,000 g for 10 min at 4° to remove cell debris and the decanted supernatant made up to 15 ml with phosphate buffer A. Samples were taken at various stages to monitor protein contents of fractions.

The 15 ml sonicate supernatant was centrifuged at 100,000 g at 4° for 30 min in three 5 ml cellulose nitrate tubes in an SW50L Spinco centrifuge rotor. The supernatants were discarded and the brown cytochrome-containing pellet resuspended in 0.1 ml of phosphate buffer A with the aid of a glass rod. Pellet resuspensions were pooled and made up to 3ml with phosphate buffer A and dispersed.
with the aid of brief (0.2 min) sonication at 25°. The suspension was made up to 5 ml and centrifuged again at 100,000 g for 30 min at 4° to separate the faster sedimenting envelope fraction from the slower sedimenting ribosomes and cytoplasmic proteins. Three cycles of resuspension and centrifugation were found to be sufficient to give consistent envelope protein profiles on SDS acrylamide gels.

The final brown pellet was usually resuspended with the aid of a glass rod and brief sonication, in 1% w/v SDS, 1% v/v mercaptoethanol in phosphate buffer A at 25° to give a 6 mg protein/ml solution. The envelope preparation was then usually dialysed overnight at 37° against two, one litre changes of 0.1% w/v SDS, 0.1% v/v mercaptoethanol in phosphate buffer A prior to SDS acrylamide gel electrophoresis.

(2) The method of Schnaitman (1970a,b). Crude cell envelopes were prepared essentially as described above except that cells were disrupted in a French Press, digested with RNase and DNase and only once centrifuged at 100,000 g for 30 min at 4°. The supernatant was discarded and the pellet resuspended with the aid of a glass rod in 10^{-2} M HEPES/sodium hydroxide buffer pH 7.4 to 10-30 mg protein/ml.

Fractionation of this crude envelope protein into cell wall and cytoplasmic membrane enriched fractions was achieved by
discontinuous sucrose density gradient centrifugation. The envelope preparation was carefully layered onto a pre-formed 28 ml discontinuous sucrose density-gradient made up in $10^{-2}$ M HEPES/NaOH buffer pH 7.4 at $4^\circ$. (See Fig. 38A). The preparation was centrifuged at 25,000 rpm at $4^\circ$ for 16 hr in an SW25.1 Spinco rotor. Multi-drop fractions were collected manually from a hole made in the bottom of the cellulose nitrate centrifuge tube. Peak fraction positions were located by determining the O.D. at 280 m\(\mu\). Alternatively, fractions were individually dialysed overnight against 4 changes of 4 litres of $10^{-2}$ M Tris/HCl buffer pH 7.0 at $25^\circ$, and the protein content determined by the Folin method.

Peak fractions were subsequently solubilized in 1% w/v SDS prior to SDS-acrylamide gel electrophoresis.

**Sodium dodecyl sulphate acrylamide gel electrophoresis.**

**Composition and preparation of gels.** SDS acrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (1969). Gels were usually 10% w/v acrylamide, occasionally 7.5% w/v. The SDS concentration of the gels was usually 0.1% w/v, occasionally 0.5% w/v. Compositions of stock solutions and amounts used for a 10% w/v acrylamide, 0.1% w/v SDS gel are given below for 30 ml of mixture.

1. 10 ml of 30% w/v acrylamide, 0.8% w/v methylenebisacrylamide
2. 15 ml of 0.2% w/v SDS in 0.2 M sodium phosphate buffer pH 7.0
3. 3.98 ml of distilled water
(4) 1 ml of 15 mg/ml ammonium persulphate solution
(5) 0.02 ml of N,N,N',N' tetramethylethylenediamine (TEMED)

Solutions (1) and (5) were kept at 4° in amber-glass bottles. Solutions (1), (2) and (3) were filtered through Whatman No. 1 filter paper and stored in dust-free screw capped bottles. Solution (4) was made freshly just prior to use. To minimize contamination by dust and fibre particles, all solutions were transferred with chromic acid cleaned pipettes that were kept dust free. (Filtration and other dust-free precautions were necessary to minimize irregularities in the subsequent optical scanning of the gels.)

Solutions (1), (2) and (3) were transferred to a clean Buchner flask containing a few glass beads, gently swirled and deaerated with the aid of a mechanical vacuum pump. Solutions (4) and (5) were then added with gentle swirling and the resulting mixture transferred into vertically held 9 cm long 6mm id. thin-bore glass tubes suitably sealed with a rubber cap at one end. The acrylamide mixture was very carefully overlaid with approximately 0.1 ml of 0.1% w/v SDS solution and allowed to polymerize at 25° (20 to 30 min). Suitably formed gels were placed in the electrophoresis unit (see Materials and General Methods section) ready for sample application.
Sample application and electrophoresis. Envelope protein samples (0.02 to 0.05 ml of 6 mg protein per ml solution) were mixed with an equal volume of 20% w/v sucrose solution with or without tracking dye (0.001% w/v bromphenol blue). These gels were immersed at both ends in 0.1% w/v SDS in sodium phosphate buffer A and electrophoresed towards the anode at 3 mA per gel for several hours followed by 6 mA per gel as required.

The gels were freed from their retaining tubes by gently driving a warm (approximately 35°C) soapy solution between the glass and the gel using a syringe. The freed gels were briefly rinsed in distilled water, their lengths and the position of the tracking dye recorded prior to fixing and staining.

Fixing and protein staining. Several published and unpublished fixing and staining methods for the dye coomassie brilliant blue were tested and found to be unsatisfactory (for example Schnaitman, 1969; Jones & Kennedy, 1969; Weber & Osborn, 1969). Eventually the method of Laemmli (1970) was used for coomassie brilliant blue fixation and staining. In view of the initial difficulties of staining with coomassie brilliant blue, most gels were stained with the less sensitive dye napthalene black.

Napthalene black fixation and staining was carried out by shaking each gel overnight at 25°C with 25 ml of 1% w/v napthalene
black (with or without 50% v/v methanol) in 10% v/v acetic acid.
Gels were destained electrophoretically in 7% v/v acetic acid after
preliminary rinse in 7% v/v acetic acid solution.

Gels were stained with coomassie brilliant blue by shaking
each gel overnight at 37° with 50 ml of 50% w/v TCA solution,
followed by staining in 50 ml per gel of 0.1% w/v coomassie
brilliant blue in fresh 50% w/v TCA at 37° for 60 min. Gels were
destained by shaking with several changes of 150 ml per gel of 7%
v/v acetic acid solution.

Gel scanning. Stained acrylamide gels were placed in a gel holder
sealed onto a Joyce-Loebl microdensitometer optical glass plate. The
gels were completely immersed in a polymerized acrylamide solution
(forming by allowing the mixture of 6 g of acrylamide, 0.1 ml TEMED,
0.1 g ammonium persulphate, to polymerize at 25° for 10 min in 100
ml aqueous solution followed by dilution to 200 ml with water).
This viscous liquid effectively damped any equipment vibrations which
would otherwise cause the recording pen to 'chatter'. All gels were
scanned with the b269 optical wedge which gave a vertical scale of
0.043 O.D. units/cm. The horizontal scale was set at a magnification
of 5. No filter was used when scanning naphthalene black stained
gels; a red filter was used for scanning coomassie brilliant blue
stained gels.
The gels were routinely scanned along their entire lengths in two parallel non-overlapping positions in order to identify any minor fluctuations due to dust particle contamination of the gel. The base line for a given scan was set by zeroing the trace on the end of the gel that the tracking dye had not reached. Quantitation of napthalene black stained protein peaks was carried out by counting graph squares under the traced peaks (Fambrough, Fujimura & Bonner, 1968).

Selection of UV<sup>R</sup> revertants of CetC mutants.

**Irradiation and replica plating.** Bacterial cultures of the UV sensitive (CetC) colicin E2 refractory mutants 56-3J and 56-3W were grown in NB thymine medium at 37° to 5 x 10<sup>8</sup> cells/ml. Aliquots of these suspension were diluted and spread in triplicate onto NB thymine agar plates to give approximately 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup> and 10<sup>2</sup> cells per plate.

Plates were then UV irradiated at 6 ergs/mm<sup>2</sup>/sec at 254 mμ for 10 secs (see Materials and General Methods section). Plates were then incubated at 26° for two days in the dark to minimize photoreactivation. Colonies arising were then replica-plated onto fresh NB thymine agar plates in triplicate and either irradiated with 600 or 900 erg/mm<sup>2</sup> with UV light or unirradiated and incubated at 25° in the dark for two days. Colonies arising were purified.
and tested for their UV, colicin E2 and colicin E3 sensitivities (see Materials and General Methods section). This selection procedure was a modification of that described by Ganesan and Smith (1968).

**Ultraviolet sensitivity screening.** Colonies or strains to be tested for their response to UV light were purified by re-streaking and picking from single colonies on agar plates, and grown to $5 \times 10^8$ cells per ml in NB thymine medium at 37°. Parallel streaks of these suspensions were made in duplicate on dried NB thymine agar plates with the aid of a sterilized mounted wire loop. Different portions of these streaks were then sequentially irradiated with UV light in dark surroundings at 6 erg/mm$^2$/sec for 0, 10, 30, 60 and 100 seconds using a thick cardboard mask. Plates were then incubated in the dark at 37° or 26°.

**Cadmium N-lauroyl sarcosinate elution of radioactively labelled proteins and lipids (See page 225).**

**Radioactive labelling of lipid and protein.** Bacterial proteins were labelled by growth in the presence of C$^{14}$ universally labelled lysine (209 mC/mM) supplemented with 1 μg/ml unlabelled lysine. Cold TCA insoluble material was determined and taken to represent amino acids incorporated into protein. Cell lipid was radioactively labelled by growth in the presence of H$^3$ C2 glycerol (500 mC/mM).
Cold TCA insoluble material was determined and taken to represent glycerol incorporated into lipids (Daniels, 1969).

Preparation of the cell extract-cadmium N-lauroyl sarcosinate complex. Bacterial strains were grown from an initial inoculum of $5 \times 10^6$ cells per ml to $1 \text{ to } 2 \times 10^8$ cells per ml in $\text{C}^{14}$ lysine, $\text{H}^3$ glycerol labelled, supplemented glucose minimal medium at $37^0$. Cells were harvested and disrupted by either (a) sonication or (b) lysozyme-EDTA treatment.

(a) **Sonication.** Harvested cells were washed in 10 ml of $10^{-2}$ M Tris/HCl buffer pH 7.4 (Tris buffer A), centrifuged and resuspended in 2 ml of this buffer. Sonication of this suspension was carried out for 2 min at $0^0$ in 3 ml glass vials. The sonicate was centrifuged at 10,000 g for 5 min to remove cell debris. The clarified supernatant was made up to 4.4 ml with Tris buffer A.

(b) **Lysozyme-EDTA disruption.** Harvested cells were washed in 10 ml of $3.3 \times 10^{-2}$ M Tris/HCl buffer pH 8.1 and resuspended in 2.8 ml of this buffer plus 0.1 ml of 4 mg/ml EDTA and 0.1 ml of 10 mg/ml lysozyme solutions. Incubation at $37^0$ was continued for 10 to 20 min and the cells further disrupted by washing with two 10 ml batches of Tris buffer A followed by centrifugation at 14,000 g for 20 min. The crude envelope pellet was resuspended in Tris buffer A to a final volume of 4.4 ml.
The sonic extract of lysozyme-EDTA disrupted cells (4.4 ml) were then gently shaken at 25° for 60 min in a 25 ml flask containing 0.4 ml of 0.1 M CdCl₂ and 0.2 ml of 5% w/v sodium N-lauroyl sarcosinate (SNLS).

**Cadmium N-lauroyl sarcosinate elution.** The equilibrated cadmium N-lauroyl sarcosinate (CNLS) - cell extract precipitate was filtered at 25° onto a Whatman No. 541, 2.5 cm dia. filter paper held in the apparatus shown in Fig. 31 (A) or (B). (Results presented later were obtained using apparatus (B)). The filtrate was recycled through the filter four times, the fifth filtrate being collected in 5% w/v cold TCA solution. The precipitate was subsequently eluted at 25° with 5 ml batches of increasing concentrations of KCl or sodium deoxycholate (DOC) solutions in Tris buffer A.

All filtrates were collected in 8 ml glass test tubes containing 1 ml of 30% w/v TCA solution. The resulting 6 ml of 5% w/v TCA solutions were mixed and kept at 0° for 2 hours prior to filtration and 5% w/v cold TCA washing onto Whatman GB/f 2.5 cm dia. glass fibre discs. The washed discs were dried and their radioactivity determined as described in the General Methods and Appendix III.

**Protein analysis by SDS-acrylamide gel electrophoresis of fractions eluted from cadmium N-lauroyl sarcosinate.**

In order to obtain sufficient protein in fractions eluted from CNLS-cell extract complexes for acrylamide gel electrophoretic
Fig. 31. Apparatus used for elution of cadmium N-lauroyl sarcosinate.

(A) plunger

plastic 10 ml syringe

precipitate

filter paper

filter holder

collection tube

(B) filter funnel

vacuum release

To vacuum

Buchner filter funnel

collection tube

precipitate

filter papers
analysis, a large scaling up of the procedure was necessary. Direct scaling-up was impractical because of the large volumes involved and various adjustments of the cell extract:CNLS:Tris buffer A ratios were therefore made.

Bacterial strains were grown in 500 ml of supplemented glucose minimal medium and harvested by centrifugation at 10,000 g for 10 min at 0° when the culture reached 5 x 10^8 cells/ml. The cells were washed with two 200 ml batches of Tris buffer A followed by centrifugation at 10,000 g for 10 min at 0°. Cells were resuspended in 12 ml of this buffer and sonicated at 0° three times for 2 min with 30 sec. breaks. Cell debris was removed by centrifugation at 10,000 g for 10 min.

Part of this clarified sonicate (50 mg protein) was mixed for 60 min at 25° with 10 ml of 0.1 M CdCl₂ and 5 ml of 5% w/v SNLS in Tris buffer A of final volume 71 ml. The CNLS-sonicate precipitate was then eluted with KCl and DOC solutions as previously described, but using a large Buchner funnel and Whatman No. 1 filter paper.

The protein content of each filtrate was first determined by the Folin method, filtrates were then dialysed overnight at 4° against 10^-3 M Tris/HCl buffer pH 7.4 three times against 31 of buffer to remove effluent KCl and DOC. The dialysates were concentrated by freeze-drying and dialysed as before. These dialysates were then adjusted to 2 mg protein/ml with 0.1% w/v SDS in dialysate
buffer and analysed on 0.1% w/v SDS, 10% w/v acrylamide gels as previously described.

Results and Conclusions

Colicin E2 refractory mutants.

Several workers have recently described the isolation and physiological properties of mutants of *E. coli* K12 that are resistant to the action of certain E group colicins but nevertheless still adsorb colicin. These mutants have been termed refractory by Hill and Holland (1967) and tolerant by Nomura and Witten (1967) and Nagel de Zwaig and Luria (1967). The term refractory will be employed here. Some of these mutants have been mapped by previous workers and their positions on the *E. coli* linkage map are shown in Fig. 1.

The mutants used in this study, originally designated as RefII mutants (Refractory to colicin E2) have now been re-named Cet mutants (Colicin E two) and this terminology will be used here (Holland, Threlfall, Holland, Darby & Samson, 1970). Cet mutants are refractory to colicin E2 only, being still sensitive to the closely related colicin E3 (Hill & Holland, 1967) which is thought to compete with E2 for the same colicin receptor (Maeda & Nomura, 1966). Cet mutants are also temperature conditional to a greater
or lesser extent, colicin E2 refractivity being maximal between 25° and 30° and minimal between 37° and 40°. In a small minority of mutants, refractivity under certain conditions is however almost identical at 30° and 40° (Holland, et al, 1970).

Hill and Holland's (1967) study showed that certain laboratory strains of _E. coli_ K12 did not give rise to colicin E2 refractive mutants. Threlfall and Holland (1970) subsequently demonstrated that a second mutation (CetA) was required to allow the expression of colicin E2 refractivity. The Cet phenotype may or may not be accompanied by a variety of additional co-transducible characters such as UV light sensitivity, recombination deficiency, abortive growth of bacteriophage λ and filament formation (Holland, et al, 1970; Threlfall & Holland, 1970). The genetic locus giving rise to E2 refractivity alone is arbitrarily termed _cetB_ and that giving rise to E2 refractivity plus other characteristics, _cetC_. The relative map positions of these closely linked _cet_ loci to those of known chromosomal markers was determined by Threlfall and Holland (1970) and is shown in Fig. 32.

The associated surface alterations of some refractory mutants including Cet mutants (Nagel de Zwaig & Luria, 1967; Holland et al, 1970) implied some underlying envelope structural changes. Studies were therefore initiated into the bacterial envelope structure of certain colicin E2 refractory mutants.
Fig. 32 P1 Transductional analysis of Cet mutants.

hsp = host specificity
serB = serine B
thr = threonine
leu = leucine

Numbers refer to the percent linkage between genetic markers.
SDS acrylamide gel electrophoresis.

(A) General properties of envelope protein profiles. The method of envelope preparation by Jones and Kennedy (1969) followed in this study, would be expected to yield preparations containing only those proteins firmly attached to the bacterial surface layers. Thus if Cet mutants contained altered proteins only weakly associated with the cell membrane no differences in protein profiles would be expected. The properties and derivations of Cet mutants used in this SDS acrylamide gel study are shown in Table 11.

In preliminary control studies of envelope proteins in SDS gels, no significant differences were obtained between NB or minimal medium grown cells. This finding was consistent with the expectation that major protein constituents of the envelope do not grossly vary under different growth conditions. Moreover it was found that the envelope protein profiles from bacteria grown at either 25° or 37° were also identical. In most subsequent studies, therefore cells were grown in NB thymine medium at 26° and cell envelopes prepared and thoroughly washed as described in the Methods section. A typical set of washed envelope protein preparations separated on SDS acrylamide gels, is shown in Figs. 33, 34, 35 and 36. Amounts of protein removed by successive washing during the preparation of envelopes are shown in Table 12.
* Numbers 4, 3, 2, 1 and 0 signify decreasing gradations of the quantity of growth relative to the unirradiated control ( = 4).
# TABLE 11

UV, colicin E2 and colicin E3 sensitivity of strains used in SDS acrylamide gel studies of envelopes

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Immediate parental derivation</th>
<th>Post incubation at 26°</th>
<th>Post incubation at 37°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colicin response</td>
<td>UV response* (erg/mm²)</td>
<td>Colicin response</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>E3</td>
<td>0</td>
</tr>
<tr>
<td>56-3</td>
<td>-</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>56-3/E</td>
<td>56-3</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>56-3/w</td>
<td>56-3</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/I</td>
<td>56-3</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/J</td>
<td>56-3</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/w/I</td>
<td>56-3/W</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/w/2</td>
<td>56-3/W</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/W/E</td>
<td>56-3/W</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>56-3/J/1</td>
<td>56-3/J</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/J/2</td>
<td>56-3/J</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>56-3/J/3</td>
<td>56-3/J</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>56/33/E</td>
<td>56-33</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>56-33/E</td>
<td>56-33</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>
Fig. 33. Envelope proteins of strain 56-3, 56-3I and 56-3W

Envelope proteins were prepared from 500 ml NB thymine cultures of 56-3, 56-3I and 56-3W strains grown at 26°.
Proteins were dissolved in 2% w/v SDS, 1% v/v mercaptoethanol in 0.1 M sodium phosphate buffer pH 7.0 to give a 5 mg protein/ml solution, and dialysed overnight at 37° against 0.5% w/v SDS, 0.1% v/v mercaptoethanol in 0.05 M sodium phosphate buffer pH 7.0. Aliquots (0.05 ml) of the dialysate envelope proteins were mixed with an equal volume of 40% w/v sucrose. Electrophoresis was continued for 5 hr at 3 mA/gel and 1 hr at 6 mA/gel at room temperature. Gels were fixed and stained in 50% v/v methanol, 10% v/v acetic acid, 1% napthalene black overnight at 25°. Gels were destained electrophoretically after rinsing in 7% v/v acetic acid. Mobilities were expressed as a fraction of the mobility of the tracking dye in an envelope preparation run with 0.001% w/v bromophenol blue.

(Dotted lines signify the opacity of certain peaks when stained in 50% methanol.)

Key: 1 = 56-3
2 = 56-3I
3 = 56-3W
Fig. 34. Envelope proteins of strains 56-33, 56-332 and 56-333

Envelopes were prepared and analysed as described for Fig. 33.

Key:  1 = 56-33  
      2 = 56-332  
      3 = 56-333
Fig. 35. Envelope proteins of 56-3, 56-3I and 56-3W

Envelope proteins were prepared according to the method of Jones and Kennedy (1969) as described in the text and Fig. 33.

0.02 ml samples of envelope protein (5 mg/ml) in 0.1% SDS, 0.01 M sodium phosphate buffer pH 7.0, 0.1% v/v mercaptoethanol were mixed with an equal volume of 20% w/v sucrose, 0.001% bromophenol blue and electrophoresed in 0.1% w/v SDS, 0.1 M sodium phosphate buffer pH 7.0, 10% w/v acrylamide gel for 3 hr 10 min at 3 mA/gel and 4 hr 47 min at 6 mA/gel. Reservoir buffers were 0.1% SDS, 0.04 M sodium phosphate buffer pH 7.0.

Gels were fixed and stained with 0.1% w/v coomassie brilliant blue in 50% w/v TCA as described in the Methods section.

Key: 1 = 56-3
     2 = 56-3I
     3 = 56-3W
**Fig. 36. Envelope proteins of strains 56-3J, 56-3J2, and 56-3J3**

Envelope proteins were prepared and analysed as described in Fig. 35.

Key:  
1 = 56-3J  
2 = 56-3J2  
3 = 56-3J3
PLATE 17

SDS acrylamide gel electrophoresis of envelope proteins

stained with coomassie brilliant blue

Legend as for Fig. 35.

Key:  
1 = 56-3  
2 = 56-3J  
3 = 56-3W  
4 = 56-3I  
5 = 56-3J2  
6 = 56-3J3
<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>SUP 1</th>
<th>SUP 2</th>
<th>SUP 3</th>
<th>PELL 3</th>
<th>SUP 4</th>
<th>PELL 3-SUP 4 as a % total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>56-3</td>
<td>85.1</td>
<td>4.13</td>
<td>1.34</td>
<td>12.85</td>
<td>0.63</td>
<td>11.8</td>
</tr>
<tr>
<td>56-3J</td>
<td>101.2</td>
<td>6.6</td>
<td>1.56</td>
<td>13.55</td>
<td>0.75</td>
<td>10.4</td>
</tr>
<tr>
<td>56-3I</td>
<td>112.9</td>
<td>5.0</td>
<td>1.08</td>
<td>11.25</td>
<td>0.57</td>
<td>8.08</td>
</tr>
<tr>
<td>56-3W</td>
<td>142.9</td>
<td>6.15</td>
<td>1.39</td>
<td>10.6</td>
<td>0.61</td>
<td>6.18</td>
</tr>
<tr>
<td>56-332</td>
<td>142.9</td>
<td>6.85</td>
<td>1.54</td>
<td>15.15</td>
<td>0.75</td>
<td>8.67</td>
</tr>
<tr>
<td>56-333</td>
<td>125.3</td>
<td>7.6</td>
<td>1.7</td>
<td>14.1</td>
<td>0.76</td>
<td>8.99</td>
</tr>
</tbody>
</table>

a. Cells were prepared according to the legend for Fig. 33.

b. Protein determined by the Folin method is expressed in mg equivalents of Bovine serum albumin.

c. SUP 1, 2, 3 and 4 are supernatants after centrifugation at 100,000 g for 30 min at 4°.

SUP 1 = supernatant from clarified cell sonicate.

SUP 2-4 = supernatant after 1st - 3rd envelope pellet wash.

PELL 3 = third pellet of envelopes prior to 4th wash.
Examination of Figs. 33 - 36 shows that although quantitative differences occur between the naphthalene black and coomassie blue stained gels, some 10 or 11 major peaks can be discerned, a to k. It must be pointed out that this SDS acrylamide gel system only separates the polypeptide subunits of proteins according to their molecular weights (Weber & Osborn, 1970) and therefore these peaks represent the predominance of certain polypeptide molecular weight classes, not necessarily individual proteins. Under the experimental conditions used, the lipid fraction of envelope membranes is supposed to dissociate from the proteins (see Fig. 37) and migrate with the tracking dye (Rothfield & Pearlman-Kothencz, 1969; Salton & Schmitt, 1967). Schnaitman, however, removed lipid from cell envelopes with dimethyl formamide/HCl prior to SDS acrylamide gel electrophoresis of envelope proteins and claimed much greater resolution in SDS gels (Schnaitman, 1969; 1970a, b). The effect of removing lipid prior to analysis was not investigated in this study.

(B) Envelope protein analysis of CetA, CetB and CetC mutants. Only minor differences in overall gel patterns in repeated analysis of the same wild-type or mutant strain were observed, however one striking and reproducible difference in peak a was evident when wild-type and mutant envelopes were compared (Figs. 33 - 36).
Fig. 37. G200 Sephadex chromatography of cell envelopes dissociated by SDS

Twenty five ml of strain IB11 was grown at 37° in supplemented glucose minimal medium plus 0.5% w/v casamino acids and 2 μg/ml H^2 C2 glycerol (500 mC/mM). Cells were harvested at 3 x 10^8 cells/ml, washed in 12 ml of 8 x 10^{-3} M Tris/HCl pH 7.4 and resuspended in 3 ml of this buffer. Cells were sonicated at 0° twice for 2 min with a 30 sec break. Cell debris was removed from the sonicate by centrifugation at 10,000 g for 5 min. The clarified supernatant was made up to 5 ml in the buffer and centrifuged at 4° at 100,000 g for 30 min. The supernatant was discarded and the crude envelope pellet resuspended in 5 ml of 1% SDS in 8 x 10^{-3} M Tris/HCl, 4 x 10^{-3} M sodium acetate, 4 x 10^{-3} M EDTA buffer pH 7.5. The envelope fraction was allowed to dissolve in this buffer at 20° for 20 min, any undissolved debris removed by further centrifugation at 100,000 g for 30 min at 20°.

One ml of the envelope supernatant (7.6 x 10^4 CPM/ml) previously dialysed overnight at 25° against 500 ml of 10^{-4} M EDTA, 10^{-3} M mercaptoethanol, 0.5% w/v SDS in sodium phosphate buffer pH 7.4, was carefully layered onto an 18 ml column of G200 Sephadex previously equilibrated in this buffer. Twenty drop (≈ 0.32 ml) fractions were manually collected and monitored for their protein (Folin) and lipid (H^3 glycerol) content as described in the Materials and General Methods section.
Whereas the wild type parent 56-3 and the revertant 56-3J of 
cetC mutant 56-3J both have low levels, the cetC mutants 56-3J, 
56-3W and cetB mutant 56-3I have high levels of peak e to the 
extent of a two-fold difference (see Table 13). It should be 
emphasized, as indicated above, that the increased levels of peak 
e in the mutants, were obtained whether the cells were grown at 
26° or 37°, although it has been shown previously that at the 
higher temperature strains 56-3I and 56-3W are largely sensitive 

That the Cet mutants were still sensitive to colicin E3 
suggested that peak e was not concerned with any alteration in 
receptor function. Confirmation of this was gained by analysing 
the SDS gel profiles of receptor negative derivatives of all the 
strains used; no change in the peak e level was observed in going 
from receptor plus to receptor minus.

(C) Envelope protein analysis of CetC revertants. Both a CetB 
mutant (56-3I) and two CetC mutants (56-3J and 56-3W) showed 
similar increases in peak e, and it was decided to see if colicin 
E2 sensitive revertants would have the envelope protein peak e 
reduced to its low parental value.

As the CetC mutants were UV sensitive, particularly at 26°, 
it was decided to select for E2 sensitive revertants by selection
TABLE 13

Quantitation of peak e\!* in strains 56-3, 56-3J, 56-3W, 56-3J, 56-3J2 and 56-3J3*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sum of mm squares in peaks c + d</th>
<th>Sum of mm(^2) in peak e</th>
<th>e as a % of c + d</th>
<th>e as a % of all peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>56-3</td>
<td>1947</td>
<td>815</td>
<td>41.86</td>
<td>3.12</td>
</tr>
<tr>
<td>56-3J</td>
<td>1725</td>
<td>1906</td>
<td>110.49</td>
<td>6.14</td>
</tr>
<tr>
<td>56-3I</td>
<td>1576</td>
<td>1428</td>
<td>90.61</td>
<td>-</td>
</tr>
<tr>
<td>56-3W</td>
<td>2859</td>
<td>1986</td>
<td>69.5</td>
<td>-</td>
</tr>
<tr>
<td>56-3J2</td>
<td>2160</td>
<td>2177</td>
<td>100.79</td>
<td>-</td>
</tr>
<tr>
<td>56-3J3</td>
<td>1885</td>
<td>853</td>
<td>45.25</td>
<td>-</td>
</tr>
</tbody>
</table>

*Measured directly from the Joyce-Loebl microdensitometer scan by counting squares under the respective peaks. Gels were those described in Figs. 33 and 34.
to UV resistance. In order to isolate such strains the method of Ganesan and Smith (1968) was followed (see Methods section). Although many colonies were eventually obtained which resisted the 600 or 900 erg/mm² UV irradiation on NB agar plates, subsequent testing of purified colonies showed in the main no significant increases in UV resistance. Eventually two UV⁰ derivatives of 56-3W (viz 56-3W₁ and 2) and of 56-3J (viz 56-3J₁ and 2) were obtained. Surprisingly these strains were still refractory to colicin E2 although in accordance with expectations the filament forming ability of 56-3W was lost from these UV⁰ revertants. When the membrane profiles of these mutants were examined (see Figs. 34 and 36 for example) no changes in the levels of peak e were found from the CetC mutant levels.

Despite numerous attempts to obtain UV resistant, E2 sensitive revertants of CetC mutants, none was found, however one UV sensitive, E2 sensitive revertant of 56-3J (viz 56-3J₃) was obtained following UV irradiation and screening. On analysis of envelope proteins from 56-3J₃, it was found that the amount of peak e was reduced to approximately the same level as that of the cet⁺ parental level (see Table 13 and compare profiles in Figs. 33 and 34 or Figs. 35 and 36). The implications of these unexpected findings are discussed later.
(D) **Location of the altered polypeptide 'e' in the bacterial cell envelope.** Good separation of the cell wall from the cytoplasmic membrane of *E. coli* cell envelopes is very difficult to achieve. A few claims at significant separation have recently been made (for example Miura & Mizushima, 1968) but none has yet been confirmed. A recent method to be published by Schnaitman (1970a,b and personal communication) which separates French press disrupted cell envelope preparations by sucrose density gradient analysis, was used in this study (see Methods section).

Various Cet mutants and their parent 56-3 were subjected to this separatory procedure. Fig. 38B shows a typical sucrose density gradient envelope profile after centrifugation. Successful separation into two bands was invariably found corresponding to cell wall enriched and cytoplasmic membrane enriched fractions claimed by Schnaitman. However upon SDS acrylamide gel analysis of the two sets of fractions, no qualitative or quantitative difference between gel patterns could be discerned.

In contrast using this technique, Schnaitman showed that the two fractions did contain different amounts of the whole envelope proteins, in particular, he identified a peak (probably peak e of this study) as being the main constituent of the cell wall fraction and only a minor constituent of the cytoplasmic membrane. Reasons
Fig. 38. *Discontinuous sucrose gradient centrifugation.*

(A) Volumes and molarities of sucrose solution in HEPES/NaOH buffer pH 7.4 used, and band positions of cytoplasmic membrane and cell wall fractions according to Schnaitman (1970a,b) as described in the Methods section.

(B) Optical Density 280 μ profile of a discontinuous density gradient fractionation of 56-3 envelopes resuspended and analyzed according to Schnaitman (1970a,b) and as described in the text.
0.5 mL SAMPLE
10 ML 0.77 M
10 ML 1.44 M
8 ML 2.02 M

CENTRIFUGE AT 4°, 25,000 g FOR 16 hrs.

CYT. MEM.
CELL WALL

(A)

OPTICAL DENSITY 280 Mm

(B)
for this disparity in results is unclear but alternative separatory methods are currently being investigated and the Schnaitman method re-tested.

**Cadmium N-lauroyl sarcosinate elution.**

Earhart, Tremblay, Daniels and Schaechter (1968) and Trembley, Daniels and Schaechter (1969) have described the use of magnesium N-lauroyl sarcosinate as a precipitated detergent capable of combining with membrane-associated bacterial cell fractions. This combination is thought to arise from interactions of the membrane lipid with the detergent complex, pure non-membrane proteins (for example lysozyme in Table 14) do not associate with the detergent complex. Following a suggestion by Daniels (personal communication) the cadmium rather than the magnesium salt of the detergent was used because of its improved temperature independent characteristics. Daniels also suggested that this complex might be useful in investigating bacterial membrane structure. Membranes with their associated proteins would be allowed to interact with the soap complex, unadsorbed material being washed off with low ionic strength buffers. Different fractions of lipid protein complexes could then be sequentially eluted with increasing concentrations of, for example, KCl and sodium deoxycholate.

The fractionation procedure requires the intimate mixing of bacterial cell extracts containing protein and lipid (previously
Legend to Table 14

Ten mg of lysozyme was mixed with 4.4 ml of 10^{-2} M Tris/HCl buffer pH 7.4 (Tris buffer A), 0.4 ml of 0.1 M CaCl_2 and 0.2 ml of 5% w/v SNLS at 25^0 for 60 min. The mixture was filtered and eluted with 5 ml of increasing concentrations of KCl and DOC in Tris buffer A. Eluted proteins were measured by the Folin method, and compared with standard calibration curves of lysozyme in the respective eluents. Protein eluted is expressed as a percentage of the total protein recovered from the elution as this had a slightly higher value than that calculated to have been applied.
<table>
<thead>
<tr>
<th>Fraction number and composition</th>
<th>Percentage protein eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 4th wash</td>
<td>83.5</td>
</tr>
<tr>
<td>2 $10^{-2}$ Tris</td>
<td>9.7</td>
</tr>
<tr>
<td>3 &quot;</td>
<td>0.8</td>
</tr>
<tr>
<td>4 &quot;</td>
<td>0.46</td>
</tr>
<tr>
<td>5 &quot;</td>
<td>0.42</td>
</tr>
<tr>
<td>6 0.05 M KCl</td>
<td>1.01</td>
</tr>
<tr>
<td>7 &quot;</td>
<td>0.59</td>
</tr>
<tr>
<td>8 &quot;</td>
<td>0.59</td>
</tr>
<tr>
<td>9 0.1 M KCl</td>
<td>0.38</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>0.13</td>
</tr>
<tr>
<td>11 &quot;</td>
<td>0.55</td>
</tr>
<tr>
<td>12 0.2 M KCl</td>
<td>0.25</td>
</tr>
<tr>
<td>13 &quot;</td>
<td>0.21</td>
</tr>
<tr>
<td>14 0.4 M KCl</td>
<td>0.34</td>
</tr>
<tr>
<td>15 &quot;</td>
<td>0.34</td>
</tr>
<tr>
<td>16 0.8 M KCl</td>
<td>0.25</td>
</tr>
<tr>
<td>17 &quot;</td>
<td>0.17</td>
</tr>
<tr>
<td>18 0.05% DOC</td>
<td>0.17</td>
</tr>
<tr>
<td>19 0.2% DOC</td>
<td>0.17</td>
</tr>
<tr>
<td>20 0.4% DOC</td>
<td>0.17</td>
</tr>
</tbody>
</table>
radioactively labelled) with cadmium N-lauroyl sarcosinate (CNLS). The latter was first formed by mixing sodium N-lauroyl sarcosinate (SNLS) with excess cadmium chloride solution. Subsequently the precipitated membrane complex was fractionated by sequential washing with increasing concentrations of KCl solutions followed by increasing concentrations of sodium deoxycholate. In contrast to SDS acrylamide gel electrophoretic analysis of washed envelopes, elution of CNLS complexed to lysozyme-EDTA disrupted cells, or sonic extracts of cells, should not be limited to analysing only tightly bound envelope proteins.

(A) **Radioactive protein-lipid profiles.** A preliminary elution profile of 56-3 and 56-3W lysozyme-EDTA disrupted cells complexed to CNLS is shown in Fig. 39. It was generally found that little lipid was eluted in the earlier fractions, more being eluted in the later DOC fractions. Conversely more protein was eluted in early fractions than later fractions. The amounts of lipid and protein retained on the filter after DOC elution varied however from preparation to preparation. This variation was presumed to arise from fluctuations in the extent and efficiency of lysozyme-EDTA cell breakage.

The more reliable cell breakage method of sonication was later employed although this involved the analysis of a whole cell extract.
Fig. 39. Cadmium N-lauroyl sarcosinate elution profiles of strains 56-3 and 56-3W lysozyme-EDTA disrupted cells

Strains 56-3 and 56-3W were separately grown at 37° in 6 ml of supplemented glucose minimal medium plus 20 μg/ml thymine and 1 μg/ml unlabelled lysine. H³ C₂ glycerol was added to 10 μC/ml and C¹⁴ U lysine to 0.5 μC/ml. Cells were harvested at 2 x 10⁸ cells/ml, washed and resuspended in 2.8 ml of 3.3 x 10⁻³ M Tris/HCl pH 8.0 and lysozyme-EDTA treated according to the Methods section. Disrupted cells were washed and resuspended in 10⁻² M Tris/HCl pH 7.4 and used for cadmium N-lauroyl sarcosinate elution as described in the Methods section.

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluent</th>
<th>Fraction No.</th>
<th>Eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4th wash</td>
<td>36-40</td>
<td>0.4 M KCl</td>
</tr>
<tr>
<td>2-5</td>
<td>10⁻² M Tris</td>
<td>41-45</td>
<td>0.6 M KCl</td>
</tr>
<tr>
<td>6-10</td>
<td>0.05 M KCl</td>
<td>46-50</td>
<td>0.8 M KCl</td>
</tr>
<tr>
<td>11-15</td>
<td>0.1 M KCl</td>
<td>51-55</td>
<td>0.05% w/v DOC</td>
</tr>
<tr>
<td>16-20</td>
<td>0.15 M KCl</td>
<td>56-60</td>
<td>0.2% w/v DOC</td>
</tr>
<tr>
<td>21-25</td>
<td>0.2 M KCl</td>
<td>61-65</td>
<td>0.4% w/v DOC</td>
</tr>
<tr>
<td>26-30</td>
<td>0.25 M KCl</td>
<td>FILTER</td>
<td></td>
</tr>
<tr>
<td>31-35</td>
<td>0.3 M KCl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: 1 = 56-3   2 = 56-3W

(Radioactive determinations were calculated as described in Appendix III and expressed as a percentage of the total radioactivity put onto the eluting filter.)
rather than a crude envelope fraction as obtained with lysozyme
-EDTA breakage. The reproducibility of CNLS elution profiles using
sonic extracts of 56-3 and 56-3W is shown in Fig. 40. Again it was
generally found that more protein was eluted in the earlier KCl
fractions than the later DOC fractions and *vice versa* with the
lipids. More protein appeared to be eluted from earlier fractions
than with lysozyme-EDTA disrupted cells, a property expected of the
cytoplasmic proteins of the cell. Reproducibility between elution
profiles of the same extract was fair, but the detail afforded by
the 15 eluent solutions appeared insufficient to demonstrate
definite differences between the strains. The unfortunately high
(approximately 40%) retention of protein on the eluted filter may
have masked any real difference(s) between the two strains.

(B) Qualitative analysis of different protein fractions eluted
from CNLS complexes. The observation that different fractions
eluted from CNLS-cell extract complexes contained different amounts
of lipid and protein, and lipid:protein ratios, was taken by
Daniels (personal communication) to indicate that different classes
of lipoproteins were being eluted with the different eluents.
Confirmation of this supposition was sought by analysing eluted
proteins in SDS acrylamide gels using sonic extracts of strains
56-3J and 56-3J3. Owing to the relatively large amounts of protein
Strains of 56-3 and 56-3W were separately grown at 37° in 6 ml of supplemented minimal glucose medium plus 20 μg/ml thymine, 1 μg/ml unlabelled lysine. H3 C2 glycerol was added to 10 μC/ml, C14 U lysine to 0.33 μC/ml. Cells were harvested at 2 x 10^8 cells /ml, washed in 10^-2 M Tris pH 7.4, resuspended in 2 ml of this buffer and sonicated for 2 min at 0°. After removal of cell debris by centrifugation at 9,000 g for 5 min the supernatant was made up to 6 ml with the Tris buffer. Three ml was kept overnight at 4° for the second determinations (Runs II), the remaining 3 ml was immediately used for cadmium N-lauroyl sarcosinate elution Rune I, as described in the Methods section.

Key:  
A = 56-3 Run I  
B = 56-3 Run II  
C = 56-3W Run I  
D = 56-3W Run II

(Radioactive determinations were calculated as described in Appendix III and expressed as a percentage of the total available radioactivity put onto the eluting filter.)
material required for acrylamide gel analysis rather than radioactive eluent analysis, the amount of cell extract, CNLS and suspension buffer had to be considerably increased and altered in relative amounts. The gel profiles of strain 56-33-CNLS complex eluted with KCl and DOC are shown in Fig. 41 and 42, and those for 56-333 in Fig. 43 and 44.

It can be seen from the fractionation of a given strain that there is a great variation in the protein content of different fractions eluted by increasing concentrations of KCl and DOC. Nevertheless the same eluent gives a very similar gel profile for the two strains. Although it is possible that some proteins are eluted in more than one fraction, it is clear that elution of CNLS-cell extract complexes can give fractions with reproducibly different protein compositions. The identification of any differences in protein peaks corresponding to peak e were sought but not found. Again the high amount of protein retention upon the filter paper may contain much envelope protein which has not therefore been analysed (see Table 15).

Discussion

Cadmium N-lauroyl sarcosinate as a useful tool in bacterial membrane studies.

A preliminary study of the properties of CNLS in binding bacterial cell extracts have been described. Although a purified non membrane

271
<table>
<thead>
<tr>
<th>Eluent composition</th>
<th>Effluent volume ml</th>
<th>56-3J protein in effluent fractions μg/ml</th>
<th>56-3J3 protein in effluent fractions μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNLS-membrane filtrate</td>
<td>71</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>Tris buffer A</td>
<td>15</td>
<td>37</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>17</td>
<td>46</td>
</tr>
<tr>
<td>0.05 M KCl</td>
<td>15</td>
<td>178</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>138</td>
<td>80</td>
</tr>
<tr>
<td>0.2 M KCl</td>
<td>15</td>
<td>252</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>130</td>
<td>153</td>
</tr>
<tr>
<td>0.8 M KCl</td>
<td>15</td>
<td>212</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>100</td>
<td>207</td>
</tr>
<tr>
<td>0.1% w/v DOC</td>
<td>15</td>
<td>197</td>
<td>562</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>260</td>
<td>LOST</td>
</tr>
<tr>
<td>0.4% w/v DOC</td>
<td>14</td>
<td>372</td>
<td>190</td>
</tr>
<tr>
<td>Percentage recovery of input protein</td>
<td></td>
<td>64.5</td>
<td>53.0</td>
</tr>
</tbody>
</table>
A sonic extract of 56-33 cells grown in 500 ml of supplemented glucose minimal medium, was prepared as described in the text. Part of the sonicate (48.4 mg protein) was mixed with 10 ml 0.1 M CdCl₂ and 5 ml 5% w/v SNLS in a total volume of 71 ml in Tris buffer A for 60 min at 25°C. Elution, protein concentration and subsequent acrylamide gel analysis was carried out as described in the Methods section.

Key:  
1 unfraccionated sonicate extract  
2 CNLS-sonicate filtrate  
3 pooled tris buffer A effluents  
4 pooled 0.05 M KCl effluents
Fig. 42. SDS-acrylamide gel electrophoresis of 56-33 proteins eluted from cadmium N-lauroyl sarcosinate

Cell extracts were prepared and analysed as for Fig. 41.

Key: 1 pooled 0.2 M KCl effluents
     2 pooled 0.8 M KCl effluents
     3 pooled 0.1% DOC effluents
     4 0.4% DOC effluent
A sonic extract of 56-333 cells grown in 500 ml of supplemented glucose minimal medium, was prepared as described in the text. Part of the sonicate (68.25 mg protein) was mixed with 10 ml 0.1 M CdCl₂ and 5 ml 5% w/v SNLS in a total volume of 71 ml in Tris buffer A for 60 min at 25°. Elution, protein concentration and subsequent acrylamide gel analysis was carried out as described in the Methods section.

Key:
1. unfractionated sonicate
2. pooled Tris buffer A effluents
3. pooled 0.05 M KCl effluents
Fig. 44. SDS-acrylamide gel electrophoresis of 56-333 protein eluted from cadmium N-lauroyl sarcosinate

Cell extracts were prepared and analysed as for Fig. 43.

Key:
1  pooled 0.2 M KCl effluents
2  pooled 0.8 M KCl effluents
3  0.1% DOC effluent
4  0.4% DOC effluent
protein lysozyme (see Table 14) and non-membrane bound DNA (Earhart et al, 1968) do not appear to bind to CNLS or MNLS respectively, it is conceivable that non-membrane bound molecules could subsequently interact with the membrane-detergent complexes. Studies with purified envelopes should obviate this objection. To study proteins comprising, and tightly bound to, bacterial envelopes, use of purified envelopes would clearly be of advantage to reduce the amount of eluent protein to be analysed.

One disadvantage of the system is the large percentage of adsorbed protein and lipid that cannot be eluted from the CNLS complex on the filter paper. It has not yet been demonstrated whether this tightly bound fraction preferentially contains the envelope proteins or not. If substances can be found that do effectively elute all CNLS bound protein and lipid, an important use can be made of this system to study envelope structure. Lipo-protein complexes rather than simply proteins could be selectively eluted and subsequently studied separately. This procedure would allow reproducible separation of envelopes into many fractions which could then be categorized by their lipid and protein constituents. If the lipo-protein association of these eluted fractions represents an in vivo association, this technique could prove to be a powerful tool in the elucidation of membrane lipoproteins.
Envelope protein changes in CetB and CetC mutants.

(A) Properties of CetB and CetC mutants. The finding of an altered envelope protein associated with colicin refractivity is the first direct demonstration that some system (other than the receptor) exists in the bacterial cell envelope that can be specifically altered to block colicin action, a finding predicted by the Nomura (1963) model of colicin action.

Previous studies had shown that (1) E2 refractivity is usually temperature conditional, (2) that although cetB and cetC loci are closely linked, the pleiotropy of the latter implies two functionally distinct cistrons, and (3) that the complex pleiotropy of each CetC mutant can be ascribed to a single mutational event (Threlfall, 1969; Threlfall & Holland, 1970). On the basis of these earlier findings, the results of envelope protein analysis of CetB and CetC mutants and their revertants cannot therefore be explained by a simple hypothesis. The construction of more elaborate hypotheses is required, two of which are considered below.

(B) Nature of envelope protein changes. Although the 100% increase in the amount of peak e has invariably been obtained with both CetB and CetC mutants, it is not clear whether this similar increase arises in the same way. Moreover, if the altered polypeptide(s) only constitute for example 10% of the non-mutant levels of peak e
then increases in this polypeptide(s) level is not twofold, but
eleven-fold. Also it is not yet clear whether the exaggeration
of peak \( e \) is due to increased amounts of normal peak \( e \) constituent(s)
being incorporated into envelopes, or the incorporation of non-
envelope protein into envelopes. Finally it may be that increases
in peak \( e \) do not reflect quantitative differences in protein levels
but rather increased dye affinity. The similar increase found with
both protein dyes napthalene black and coomassie brilliant blue
makes this explanation unlikely, but if correct, equally deserving
of an explanation.

(C) **Hypotheses to explain the properties of CetB and CetC mutants.**

Bearing in mind all the above considerations, two types of
hypotheses are presented which are designed to explain the properties
of CetB and CetC mutants in a reasonable fashion. For simplicity
(but not inconsistent with alternative assumptions) it will be assumed
that the increase in peak \( e \) is due to a 100% increase in a normal
envelope polypeptide constituting parental and mutant peaks \( e \) in
both CetB and CetC cases. Further it will be assumed that the
pleiotropic effects of various CetC mutants are derived from a single
mutational event. The fact that \texttt{cetB} and \texttt{cetC} genes are very closely
linked will also be taken into account but they will be assumed to be
in different cistrons in the initial hypotheses formulations. In
In both hypotheses (Figs. 45 and 46) part of the membrane transmission system for colicin E2 is represented by T, the modification leading to UV sensitivity, filament formation and detergent sensitivity represented by M, and finally the peak polypeptide by e. All envelope components, T, M and e function normally in the square configurations, and function abnormally in the parallelogram configurations. In addition both models require only a unit dose of e or e' to disturb M, but a two unit dose to disturb T. The temperature conditional nature of CetB and some CetC mutants is designed into the hypothesis by supposing that e or e' can change from the parallelogram form at 30° to the square form at 40°, for example.

(1) Polypeptide 'e' modification hypothesis. This type of hypothesis (see Fig. 45) supposes that the control of the amount of incorporation of polypeptide e into the bacterial cell envelope is affected in CetB and CetC mutants. In addition, in CetC mutants, this polypeptide is also altered qualitatively to e'.

Selection of UV$^R$ E2$^R$ revertants of a cetC mutant would involve the modification of M to M' which is not affected by e' whereas selection of UV$^S$ E2$^S$ revertants from the cetC mutant returns the level of e' to the parental e level (see Fig. 45).
### Poly peptide 'e' modification hypothesis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype @ 30°C</th>
<th>revertant phenotype @ 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A⁻B⁺C⁺</td>
<td>UV⁺ E₂⁺</td>
<td>UV⁺ E₂⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A⁻B⁻C⁺</td>
<td>UV⁺ E₂⁺</td>
<td>UV⁺ E₂⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A⁻B⁻C⁻</td>
<td>UV⁻ E₂⁺</td>
<td>UV⁻ E₂⁺</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A⁻B⁻C⁻</td>
<td>UV⁻ E₂⁻</td>
<td>UV⁻ E₂⁻</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
To explain this arrangement in genetic terms assuming only point mutations, it may be suggested that a mutation in the cetB gene alters a regulatory process for polypeptide e incorporation. Mutations in the cetC locus could give rise to an altered polypeptide e' which, due to its decreased functional efficiency in the membrane is incorporated in greater amounts by some regulatory process.

(2) Polypeptide e' location variation hypothesis. This type of hypothesis (see Fig. 46) supposes that polypeptide e is normally apposed to the T system but displaced from the M system in the cell membrane. Mutations in the regulatory cetB locus would give rise to increased amounts of e in the membrane but at its normal location. This increased level would be capable of disturbing T's function. Mutations in the cetC locus would allow increased amounts of polypeptide e but at a different location, such that it could affect both M and T's function.

Selection of UV^R E2^R revertants of cetC mutants would again involve alteration of M to M' not affected by e. In contrast selection of UV^S E2^S revertants of cetC mutants could lead to reduction of the level of e to normal, but remaining in the altered location.
Fig. 46  Polypeptide 'e' location-variation hypothesis.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype @ 30°</th>
<th>revertant phenotype @ 30°</th>
</tr>
</thead>
<tbody>
<tr>
<td>A⁻B⁻C⁺</td>
<td>UV⁺ E₂⁻</td>
<td>UV⁺ E₂⁺</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>M'</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>e</td>
</tr>
<tr>
<td>A⁻B⁻C⁺</td>
<td>UV⁺ E₂⁻</td>
<td>UV⁺ E₂⁺</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>M'</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>e</td>
</tr>
<tr>
<td>A⁻B⁺C⁻</td>
<td>UV⁺ E₂⁻</td>
<td>UV⁺ E₂⁺</td>
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<td></td>
<td>M</td>
<td>M'</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>e</td>
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<td>A⁻B⁻C⁻</td>
<td>UV⁺ E₂⁻</td>
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<td></td>
<td>M</td>
<td>M'</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>e</td>
</tr>
</tbody>
</table>
Again explaining this arrangement in genetical terms assuming only point mutations, it may be suggested that the $\text{cetB}$ gene regulates quantitatively the incorporation of polypeptide $e$ whereas the $\text{cetC}$ gene product determines the location of $e$ in the envelope. Mutations in the $\text{cetB}$ gene would then allow the insertion of excess polypeptide $e$. The altered location of polypeptide $e$ by mutations in the $\text{cetC}$ gene may make the functioning of $e$ less efficient, more $e$ being incorporated in an attempt to redress this deficiency, alternatively the altered location would be less amenable to incorporation regulation.

(D) Tests of the hypotheses. It must be remembered that these two hypotheses are simple examples of their class, many modifications could be introduced to explain hitherto unestablished data, or alternative classes of hypothesis could be proposed. Although the genetic interpretation of the two hypotheses assumed that $\text{cetB}$ and $\text{cetC}$ loci were in different cistrons, these hypotheses are also tenable, assuming $\text{cetB}$ and $\text{cetC}$ loci are in one cistron. Thus the product $e$ would in part at least be responsible for its own regulation of incorporation and location in the cell envelope.

Hypothesis 1 requires that the increased polypeptide $e'$ in CetC strains is different to the polypeptide $e$ in CetB or parental strains. Such a difference may be demonstrable in conventional acrylamide gel systems that separate proteins on a charge basis,
not solely on molecular weight as in SDS acrylamide gel electro-
phoresis. Both models predict that $UV^R E_2^R$ revertants have an
altered M component, this could be tested by the genetic mapping
of these revertants.

Finally, identification of T or M (in acrylamide gels?) may
be possible by studying mutants affected in the T and M systems.
Work along these lines, in particular separatory methods for
envelope proteins according to a charge basis is now being carried
out in the eventual hope of characterizing all the envelope proteins
involved in colicin transmission.
DISCUSSION

Concepts of Membrane Structure.

Natural membranes which can be isolated in a form not contaminated with other cell constituents are known to comprise of essentially protein and lipid. Details of the composition of these constituents varies considerably depending on the nature of the membrane.

The problem of how these two molecular species, lipid and protein, are arranged in the membrane has called forth an abundance of models attempting to explain the structural and biological properties of membranes. A selection of the principal models would include those of Danielli and Davson (1935), Robertson (1960), Green and Perdue (1966), Weier and Benson (1967) and Vanderkooi and Green (1970). Experimental support for these models is discussed at length in the relevant articles. The continued viability of such diverse models confirms that insufficient data exists for an exclusive choice amongst them.

The original membrane model of Danielli and Davson (1935), proposed that the membrane consisted of a bimolecular leaflet of phospholipid molecules whose polar heads interacted with a monolayer of globular proteins on each side of the leaflet. The area between the hydrophobic tails of the phospholipid molecules was
thought to comprise of some lipid material, the amount of which varied amongst different membranes. A more recent appraisal and presentation of this concept was put forward by Robertson (1960) as the 'Unit Membrane' hypothesis. This model proposed that membrane lipid was in the form of a bimolecular leaflet with no inner lipid material, the polar phospholipid heads being embedded in the polar side chains of a sandwiching monolayer of proteins in an unfolded conformation. In both these types of models the bonding between protein and lipid was primarily electrostatic, and the protein and lipid were in continuous, and essentially distinct, phases. (A criticism of the observations leading to the presentation and support of these models has been made by Korn (1966).)

The demonstration of subunits in the membranes of mitochondria and chloroplasts led Green and Perdue (1966) and Weiher and Benson (1967) respectively, to propose that membranes were basically subunit assemblies rather than continuous sheets. By analogy with the self-assembly of Tobacco Mosaic Virus (Caspar, 1963), bacteriophage heads (Polglazov, Mesyazhinov & Kosourov, 1967), bacterial flagella (Asakura, Eguchi & Iino, 1966) and ribosomes (Traub & Nomura, 1968), the subunit hypothesis was teleologically satisfying when the problem of ordered membrane growth was considered. The recent demonstration of reaggregation of Mycoplasma laidlawii membrane fragments (Engelman
& Morrowitz, 1968a,b) suggests that membranes, in addition to the above mentioned complex structures, may indeed be capable of self-assembly without pre-existing membranes as templates.

The feasibility of forming bimolecular phospholipid membranes that have electrical resistance, microscopic appearance, refractive index, surface tension and other physical properties similar to natural membranes, has been adequately demonstrated by Thompson and his co-workers (Thompson, 1964). There appears to be no doubt that bimolecular phospholipid membranes do have remarkably similar properties to natural membranes. The bimolecular leaflet is the energetically most favourable form of pure lipid membrane (Danielli, 1966), and sufficient lipid exists in the human erythrocyte to cover this cell with such a membrane (Engelman, 1969). However, the recent observation by Jost and Jones (1970) that gas vacuole membranes of *Microcystis aeruginosa* are comprised solely of protein molecules, demonstrates that lipids are not essential for the composition of natural membranes. These 'membranes' however are of limited size (cylinders of diameter 710 Å, length 3,600 Å) and appear to have only a mechanical,storage function.

It is possible that an important role of lipid in conventional, more extensive membranes is to give mechanical support and plasticity. A recent model proposed by Vanderkooi and Green (1970) envisages
membranes as double layers of protein molecules with single lipid bilayers filling the pores between them, bonding between lipids and proteins being hydrophobic in nature.

Until more in vivo studies into the structure of membranes are made using such techniques as optical rotatory dispersion (Lenard & Singer, 1966), circular dichroism (Glaser, Simpkins, Singer, Sheetz & Chan, 1970), nuclear magnetic resonance (Chapman, Kamat, De Gier & Penkett, 1968), electron spin resonance (Tourtellotte, Branton & Keith, 1970), X-ray diffraction and electron microscopy (Finean, Coleman, Knutton, Limbrick & Thompson, 1968), it would be wise to maintain a broad concept of membrane structure, embracing pertinent facts and flexible enough to allow individual variations.

Colicins and Genetic Analysis as Useful Tools in Discerning the Structure of the E.coli Cell Membrane.

It would appear that the physical techniques outlined previously to probe membranes are by their nature averaging, and at best can only give an overall picture of membrane structure. An understanding of the workings of highly function-specific membranes containing only one type of repeating unit, is more likely to be achieved with these techniques than workings of vastly complex membranes such as exist in unicellular organisms.

The original purpose of this investigation was to try and
understand the transmission of the colicin stimulus in terms of cell membrane (or cell envelope) structure. The findings of this study have transposed this into demonstrating how colicins can be used to study membrane structure. The use of colicins in conjunction with genetic analysis will now be outlined as a basis for studying the composition, interactions and assembly of proteins in membranes.

Since colicins appear to affect their biological targets indirectly via the cell membrane, some mutants with severely altered membranes (temperature conditional-lethals) might be expected to be refractory to one or more colicins because of impairment of the transmission system. This defect giving rise to refractivity at the lethal temperature or perhaps at some sub-lethal temperature. Thus colicin refractivity could be an initial screening method for distinguishing potential membrane, temperature conditional-lethal mutants, from other temperature conditional mutants. Subsequent acrylamide gel electrophoresis analysis of purified membrane or envelope proteins, according to a charge rather than a molecular weight basis, would allow these mutants to be assigned non membrane-protein mutants, or membrane-protein mutants. The latter class would comprise membrane proteins altered in the amount of incorporation into the membrane, in electrophoretic mobility or altered in both parameters. (It must be remembered that electrophoretic mobility changes of proteins detected by SDS
gel electrophoresis would only be expected in proteins with grossly altered molecular weights, due perhaps to ochre and amber mutations. Such vastly altered proteins would however be unlikely to be incorporated into membranes.)

When a large number of known membrane protein mutants have been collected and characterized, genetic mapping and complementation analysis can be made to further assign functional groups. Intragenic complementation would be an important pointer to multi-subunit protein assemblies in the membrane. Further, it might be expected that a certain type of intergenic complementation might exist whose basis would arise from interactions between non-identical subunits analogous to the tryptophan synthetase in microorganisms. Such an analysis of interacting proteins in membranes would be a very powerful tool in assessing the importance and extent of cooperative effects between proteins in membranes as proposed to exist (Changeux & Thierry, 1967; Wyman, 1969).

Combining the techniques of radioactive labelling with acrylamide gel electrophoretic analysis of membrane proteins, studies into the biogenesis and mode of protein incorporation into membranes can be initiated. Membrane protein systems specifically involved in for example chromosome replication or cell division might be expected to be incorporated at particular times in the growth division cycle.
Radioactive pulse labelling of division synchronized cells and subsequent gel analysis may indicate which membrane proteins are preferentially incorporated at certain times. The feasibility of separating newly synthesized membranes from old membranes using density labelled lipids (Fox, personal communication) would allow an investigation into the association between lipid and protein incorporation into membranes.

The problem of the necessarily elaborate workings of cell membranes is now becoming experimentally amenable. As with other basic advances in biochemistry, the study and solution of how the *E. coli* cell membrane works will possibly be the rerunner of membrane analysis and a representative example of membrane organization in general.
APPENDIX I

Calculation of the number of colicin molecules that could be close-packed on a typical E.coli cell surface

(1) The number of closely packed squares side 50 Å that would cover a closed cylinder 3 µ long 1 µ diameter.

Total outer surface area of a closed cylinder

\[ \text{Area of } \frac{50}{10^8} \text{ side square} = \frac{50 \times 50}{10^8} \text{ sq } \mu \]

\[ = 2.5 \times 10^{-5} \text{ sq } \mu \]

Thus number of squares per cylindrical area

\[ = \frac{11 \times 10^5}{2.5} \]

\[ = 4.4 \times 10^5 \]

(2) The number of spheres diameter 50 Å that can be packed as a monolayer round a circle diameter one micron.

Perimeter of a circle

\[ = 2 \pi r \]

\[ = 3.14 \mu \]

Diameter of the sphere

\[ = \frac{50}{10^4} \mu = 5 \times 10^{-3} \mu \]
Thus the number of sphere diameters per circle perimeter

\[ = \frac{3.14 \times 10^3}{5} \]

\[ = 6.28 \times 10^2 \]
APPENDIX II

Benzidine: Its efficiency of bis-diazotization

The reaction mixture used to make bis-diazotized benzidine (BDB) was allowed to react, and the product collected and dried to a powder in a vacuum dessicator over several changes of silica gel at 4°C. Pastes of benzidine and the reaction mixture were then made with a Nujol (a paraffin carrier used in infra-red studies) and applied between two rock salt discs. The paste was scanned in an infra red spectrometer.

Part of the trace around the -NH₂ group stretch is shown for benzidine and suspected BDB in Fig. 47. The -NH₂ group which peaks around 3,700 cm⁻¹ is present in both compounds, but a reduction of the peak heights is found with the suspected BDB. The common peak N corresponds to that of the Nujol carrier.

These spectra suggested that BDB formation was not complete (for BDB should have no -NH₂ groups) and it was decided to see if the mixture formed was benzidine plus BDB, or benzidine plus mono-diazotized benzidine plus BDB. A volumetric analysis of the reaction mixture filtrate was therefore carried out to distinguish between these possibilities. The diazotization reaction had to be carried out with conc. sulphuric acid instead of conc. hydrochloric acid because the large amount of Cl⁻ ions would interfere with the
Fig. 47 Infrared spectra of Benzidine and Bisdiazotized Benzidine.

Percent Transmittance

Wavenumber (cm⁻¹)

Benzidine

Bisdiazotized Benzidine

N
subsequent volumetric analysis.

**Volumetric analysis.** A sample of the diazotization mixture filtrate was dried at 40° over silica gel to determine its weight. Another filtrate sample was treated with potassium iodide which liberated the nitrogen from any diazo groups formed. Titration of the potassium iodide remaining with silver nitrate solution therefore gave a measure of the original diazo groups formed.

**Reactions involved in the analysis**

- **Monodiazotized benzidine, reaction with excess KI**
  \[ \text{Ar}_2\text{NH}_2\text{N}_2\text{Cl} + 3\text{KI} = \text{N}_2 + \text{Ar}_2\text{NH}_2\text{I} + \text{KCl} + 2\text{KI} \]

- **Bisdiazotized benzidine, reaction with excess KI**
  \[ \text{Ar}_2(\text{N}_2\text{Cl})_2 + 3\text{KI} = 2\text{N}_2 + 2\text{Ar}_2\text{I}_2 + 2\text{KCl} + \text{KI} \]

- **Titration with AgNO₃ using rose bengale as adsorption indicator**
  \[ \text{KI} + \text{AgNO}_3 = \text{AgI} + \text{KNO}_3 \]

(here Ar = aromatic ring)

From the weight of compound formed and the number of diazo groups, it was concluded that although diazotization was inefficient for benzidine (10-50% conversion), the filtrate consisted of bisdiazotized benzidine rather than monodiazotized benzidine.

Monitoring of the efficiency of subsequent diazotizations was carried out by measuring the amount of sodium nitrite used up in its
formation and adjusting the amount of BDB added to the conjugation mixture. (When excess NaNO₂ is added it releases iodine from starch KI paper turning the paper blue-black.)
APPENDIX III

Estimation of $^3$H and $^{14}$C isotopes in double labelling experiments.

Nature of the problem. In the cadmium N-lauroyl sarcosinate (CNLS) elution studies, variable mixtures of the two isotopically labelled compounds $^{14}$C lysine and $^3$H glycerol are obtained from which the individual isotope contributions to the total radioactivity must be computed. This calculation is relatively simple when no biased quenching of the isotopes exists. When non-biased quenching exists it is desirable to have the isotopes' unquenched radioactivities computed.

Unquenched samples. If the radioactive count contribution of the two isotopes are arranged by judicious channel settings such that the maximum overlap of the $^3$H radioactivity into the $^{14}$C channel setting is 0.1%, the unquenched mixtures of $^3$H and $^{14}$C can have their individual contributions computed.

For example:

<table>
<thead>
<tr>
<th>Channel setting</th>
<th>CPM $^3$H only</th>
<th>CPM $^{14}$C only</th>
<th>CPM $^3$H &amp; $^{14}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>B</td>
<td>0.1</td>
<td>100</td>
<td>100.1</td>
</tr>
</tbody>
</table>

From the mixed isotope counts and the known isotope overlaps from the unmixed isotopes, calculation of individual isotope contributions can be made.
Contribution to channel B is negligibly affected by H³, therefore C¹⁴ contribution to the mixture is 100 CPM. Now from the experimentally determined constant for unmixed C¹⁴ Channel A ÷ Channel B (20 ÷ 100) the mixed C¹⁴'s contribution to Channel A is 100 x 20 ÷ 100 = 20 CPM which is subtracted from 120 CPM to give 100 as the H³ isotope contribution.

*Samples quenched with deoxycholate.* Cadmium N-lauroyl sarcosinate fractions that have been eluted with sodium deoxycholate form dense white precipitates when made 5% w/v with trichloroacetic acid, this precipitate is the source of quenching dealt with here.

Following the rationale for unquenched samples, a three channel scintillation counter can be used to monitor the degree of quenching with the third channel.

<table>
<thead>
<tr>
<th>Channel setting*</th>
<th>Isotope(s)</th>
<th>Algebraic notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C¹⁴</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>C¹⁴</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>C¹⁴</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>H³</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>H³</td>
<td>E</td>
</tr>
<tr>
<td>3</td>
<td>H³</td>
<td>F</td>
</tr>
<tr>
<td>1</td>
<td>C¹⁴ &amp; H³</td>
<td>G</td>
</tr>
<tr>
<td>2</td>
<td>C¹⁴ &amp; H³</td>
<td>H</td>
</tr>
<tr>
<td>3</td>
<td>C¹⁴ &amp; H³</td>
<td>I</td>
</tr>
</tbody>
</table>
Channel 1 is the $^{14}$C optimum channel (10% gain, 250 to 1000 gate). Channel 2 is the $^{14}$C suboptimal channel (5% gain, 250 to 1000 gate). Channel 3 is the $^3$H optimum channel (80% gain, 50 to 1000 gate).

This third channel (setting 2) can be assigned to receive a smaller contribution from $^3$H than setting 1, so that even under extreme quenching conditions less than 0.1% of the $^3$H counts enter this channel. This channel was also chosen to give a reasonable count for $^{14}$C (approximately 50% of channel setting 1 for unquenched samples). The channel ratio for settings 1 and 2 is a constant for any unquenched mixtures of $^{14}$C and $^3$H, but varies for quenched samples. This altered ratio (arising solely from the $^{14}$C contribution) was used as an internal index of DOC quenching. The unquenched contributions of both isotopes in any quenched mixture can therefore be computed using a set of experimentally determined standard curves for the pure isotopes quenched with different amounts of DOC. (See Figs. 48 and 49). The resulting calculation is best presented algebraically.

For unquenched samples:-

\[
^{14}\text{C counts} = G \\
^3\text{H counts} = I - G\left(\frac{C}{A}\right)
\]

For quenched samples:-

\[
^{14}\text{C unquenched counts} = G \times \frac{100}{\% \text{ efficiency for } ^{14}\text{C} \text{ at the } ^{3}\text{H}/G \text{ value}}
\]
Various known mixtures of quenched and unquenched $^{14}C$ and $^3H$ samples were prepared and measured. The calculated individual contributions agreed well with the predicted values, a finding taken to indicate the validity of the method for measurement and calculation.
Fig. 48. Standard calibration curve for the $C/A$ value at different $H/G$ values.

A 10 ml culture of strain IB11 was grown in M9 glucose supplemented minimal medium plus 1 $\mu$g/ml lysine. The cultures were divided into two portions; to one was added $^{14}C$ lysine (209 mC/mM) to 0.5 $\mu$C/ml, and to the other added $^3H$ glycerol (500 mC/mM) to 2 $\mu$C/ml. After growth in radioactive medium for 4 generations, cells were harvested at $5 \times 10^8$ cells/ml, washed in unlabelled medium and resuspended in 5% w/v cold TCA solution. Aliquots of these suspensions were mixed with equal volumes of either 5% w/v TCA, or various KCl concentrations in 5% w/v TCA, or various concentrations of sodium deoxycholate in 5% w/v TCA. The resulting $^{14}C$ or $^3H$ mixtures were filtered onto 2.5 cm dia. glass fibre discs and washed five times each with 5 ml ice-cold 5% w/v TCA solution, dried and their radioactivities determined (General Methods).

The $C/A$ value is for pure $^{14}C$ quenched with the DOC-TCA precipitate. (KCl gives no quenching due to its removal and non-precipitation with TCA.) The $H/G$ value was also determined for pure $^{14}C$ as the $B/A$ value.
Fig. 49. Standard calibration curve for the counting efficiency of $^3$H glycerol and $^{14}$C lysine at different H/G values.

Radioactive samples were prepared as described in Fig. 48. The counts produced at different levels of DOC quenching as measured by the $H/G$ ($= B/A$) value, are expressed as percentages of the unquenched counts for pure $^{14}$C and pure $^3$H.
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ENVELOPE PROTEIN CHANGES IN MUTANTS OF ESCHERICHIA COLI REFRACTORY TO COLICIN E2

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1. Introduction

Colicins are antibacterial proteins which induce specific changes in membrane-associated functions of sensitive bacteria. The fixation of colicin E2 is followed by a complex series of surface changes apparently culminating in the formation of a specific conformational state of the membrane which promotes rapid degradation of the bacterial chromosome [1].

Previous studies have indicated that colicin action may be blocked at several post-adsorption steps [2–4] and that at least some of these steps involve specific proteins [5]. In this study we have attempted to confirm that mutants which still adsorb colicin E2 but remain insensitive to its presence, produce altered envelope proteins. The mutants examined were of two types: CetB mutants are specifically refractory to colicin E2 at low temperatures (25°) but are largely sensitive at high temperature (37°) [6]; CetC mutants are also refractory to colicin E2 at 25° but show varying degrees of refractivity at 37°. CetB and CetC loci are closely linked [7] but CetC mutants, as distinct from CetB mutants, as distinct from CetB mutants, are also UV-sensitive [8, 9]. Genetic studies have also shown that mutations to CetC refractivity are pleiotropic [7] and that in addition to UV-sensitivity, these strains also show detergent sensitivity, recombination deficiency, and in some cases filament formation or abortive growth of bacteriophage λ [9].

In this report we show that envelope preparations of both types of Cet mutant, when analysed on sodium dodecyl sulphate (SDS) acrylamide gels, have an almost identical large increase in one polypeptide peak when compared to the wild type parent. This enlarged peak is not observed in revertants of CetC mutants which have a wild type response to colicin E2. In contrast, UV-resistant revertants which are still refractory to E2 still produce enhanced amounts of the polypeptide.

2. Materials and methods

Litre cultures of each bacterial strain were grown in nutrient broth plus 80 μg/ml thymine at 25°, harvested in the exponential phase at 5 × 10^8 cells/ml and washed with 10 mM sodium phosphate buffer pH 7.1. Cell envelopes were prepared by sonication of whole cells followed by low and high speed centrifugation [10]. The pellets obtained by centrifugation at 100,000 g for 30 min were rigorously washed by resuspension in wash buffer with the aid of brief (0.2 min) sonication followed by centrifugation at 100,000 g for 30 min. This procedure was then repeated twice more. Typically, the final pellet contained approximately 9% by weight of the sonicate protein and less than 0.03% of the sonicate RNA. The washed pellet was resuspended at 5 mg protein/ml in 10 mM sodium phosphate buffer pH 7.1, containing 1% w/v SDS and 1% v/v 2-mercaptoethanol and dialysed 14 hr at 37° against the same buffer containing 0.2% w/v SDS, 0.1% v/v 2-mercaptoethanol. Acrylamide gels were prepared as described by Weber and Osborn [11] and had the final composition: 10% w/v acrylamide, 0.26% w/v N,N'-methylenebisacrylamide 0.1% w/v SDS, 0.06% v/v N,N,N',N'-tetramethylenediamine, 0.05 w/v ammonium persulphate, 100 mM sodium phosphate buffer pH 7.0.

Envelope preparations (0.02 ml, 100 μg protein) were mixed with an equal volume of 20% w/v sucrose containing 0.001% w/v bromphenol blue as anti-con-
vectant and tracking dye respectively, and applied to 6 mm × 90 mm gels. Both buffer reservoirs contained 0.1% w/v SDS in 40 mM sodium phosphate buffer pH 7.0. Samples were electrophoresed at 3 mA per gel for 3 hr followed by 6 mA per gel for 5 hr. Gels were fixed in 50% w/v aqueous trichloroacetic acid (TCA) overnight at 37°, stained with 0.1% w/v coomassie brilliant blue in fresh 50% w/v TCA for one hr at 37°, and destained by several changes of fresh 7% v/v aqueous acetic acid. Gels were scanned on a Joyce-Loebl microdensitometer using a red filter.

3. Results and discussion

The CetB, CetC and the revertant strains used in this study are shown in table 1. Envelope fractions of bacterial strains were prepared as described above and microdensitometer tracings of typical gel profiles are shown in figs. 1 and 2. The appearance of the gels is shown in fig. 3. Eleven major polypeptide peaks (or groups) can be discerned which occur in all six strains. On the basis of repeated gel analysis of these strains, however, we conclude that peak b at least is probably complex. Furthermore, the variation in this region observed in different runs is in part due to its proximity to the top of the gel where adverse heating effects are maximal, and protein concentrations are initially highest. Examination of the gel profiles clearly demonstrates that both CetB (ASH101) and CetC mutants (ASH114 and ASH111) show an increased peak e compared with the wild type ASH1. The enlarged peak e was in all cases obtained whether the Cet mutants were originally grown at 25° or 37°, and this feature is not therefore conditional upon the growth temperature of the cells. Mutants resistant to colicin E2 may also be obtained which have lost the capacity to adsorb E2 (and related colicins). These receptor-negative strains do not show pleiotropic effects and are genetically quite distinct from Cet mutants [2]. Analysis of envelope preparations of these mutants has also been carried out, but no deviations from the wild type pattern have been revealed.

Table 1

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Immediate parental derivation</th>
<th>Cet status</th>
<th>Response to colicin E2*</th>
<th>Response to UV*</th>
<th>Area ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASH1</td>
<td>--</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>41.86</td>
</tr>
<tr>
<td>ASH101</td>
<td>ASH1</td>
<td>CetB</td>
<td>S</td>
<td>R</td>
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</tr>
<tr>
<td>ASH111</td>
<td>ASH1</td>
<td>CetB</td>
<td>S</td>
<td>R</td>
<td>69.5</td>
</tr>
<tr>
<td>ASH114</td>
<td>ASH1</td>
<td>CetC</td>
<td>S</td>
<td>R</td>
<td>110.49</td>
</tr>
<tr>
<td>ASH706</td>
<td>ASH114</td>
<td>--</td>
<td>S</td>
<td>R</td>
<td>100.79</td>
</tr>
<tr>
<td>ASH711</td>
<td>ASH114</td>
<td>--</td>
<td>S</td>
<td>S</td>
<td>45.25</td>
</tr>
</tbody>
</table>

* R = resistant; S = sensitive to colicin E2 and UV [8].
** Area e expressed as a percentage of the sum of areas c and d measured on naphthalene black stained gels.
Confirmation of the specificity of the observed changes in gel profiles was obtained when revertants of ASH114 were examined. As shown in fig. 2, envelope preparations of ASH711, a colicin E2-sensitive revertant of CetC mutant ASH114, shows peak e to be reduced to the original parental level. It is important to note that this revertant strain is however still UV-sensitive. Moreover, all UV-resistant revertants of ASH111 and ASH114 (e.g. ASH706) which are still refractory to E2 retain the enlarged peak e characteristic of their mutant parents. The enlargement of peak e therefore appears to be specific to changes in E2 refractivity of CetC mutants. Since previous genetic analysis of CetC mutants has nevertheless indicated that both UV-sensitivity and E2 refractivity arise through a single point mutation, the precise relationship between these two characters remains obscure.

Analysis of the gel profiles shown in figs. 1, 2 and 3 also provides some information with regard to the properties of the altered polypeptide(s) in mutant strains. The mean relative dye mobility of peak e is 33.7% which according to the data of Weber [11] indicates a molecular weight of about $4.3 \times 10^4$ daltons. Similar results have also been obtained using napthalene black as protein stain and this has facilitated a quantitative estimate of the protein content of peak e [12]. Thus in wild type strains peak e constitutes about 3% of the total envelope protein and increases to about 6% in most mutant strains.

The results described above do not yet establish the precise location within the cell surface of the altered polypeptide(s) present in the mutants. Although the properties of these strains strongly suggest that they have altered cytoplasmic membranes, modification of other protein containing layers in the cell surface has not been excluded. Further studies will now attempt to clarify this question and also how increased amounts...
of polypeptide e in the cell envelope can arise. The appearance of an enlarged peak e may for example result from (1) the binding of a protein normally present in the cytoplasm to the altered cell surface; (2) incorporation of increased amounts of defective envelope protein into the cell surface; (3) increased incorporation of a normal envelope protein into the cell surface in compensation for loss of the mutant protein; (4) changes in a regulatory gene controlling the synthesis and/or incorporation of a specific surface protein. Alternatively, although this seems unlikely, the increase in peak e may only be apparent and result from increased binding of the two dyes to an altered polypeptide. Purification of polypeptide e and further genetic analysis of Cet mutants and their revertants may now be necessary in order to establish the nature of the altered cell surface protein e, its precise relationship to colicin E2 and its normal functional role in the cell.

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References

Mutants of Escherichia coli with Altered Surface Properties which are Refractory to Colicin E2, Sensitive to Ultraviolet Light and which can also Show Recombination Deficiency, Abortive Growth of Bacteriophage λ and Filament Formation

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SUMMARY

Mutants refractory to colicin E2, previous phenotypic symbol RefII, have now been divided into two major phenotypic groups. CetC mutants in distinction to CetB mutants show increased sensitivity to u.v. and to a detergent (DOC). CetC mutants may also show other properties, including abortive growth of bacteriophage λ and defective division. CetB mutants are refractory to E2 at 30° but largely sensitive at 37°. CetC mutants are also refractory at 30° but may be either sensitive or completely refractory at 37°. In contrast to Rec− mutants, CetC strains are not sensitive to gamma rays but two mutants show enhanced DNA breakdown after u.v. irradiation. CetC mutants seem defective in a specific membrane component which renders them insensitive to E2 and alteration of which can affect several aspects of DNA metabolism.

INTRODUCTION

One approach to the study of the functional organization of the bacterial membrane appears to be through the use of certain protein antibiotics, colicins. The general properties of colicins (see Review, Nomura, 1967) suggest that their primary site of action is the cytoplasmic membrane although they do not appear to induce detectable permeability changes in sensitive cells (Nomura, 1964). It is assumed, therefore, that colicins induce subtle changes in the physical properties of the cell membrane, which in tum induce the intracellular changes specific to each colicin. To establish the nature and normal functional role of colicin-specific sites in the cell surface, efforts have therefore been made to isolate mutants, resistant to colicin, with altered membranes. The isolation of mutants possibly of this type, which still adsorb colicin normally but remain refractory to its effect, has been described previously (Hill & Holland, 1967). The mutants included a class which were specifically refractory to colicin E2 at 30° but largely sensitive at higher temperatures. Further studies confirmed that E2 refractivity in one mutant, ASH 101, was temperature-dependent and that the mutant growing at 40° immediately became resistant to E2 after a shift of temperature to 30° (Holland, 1968). At 30° inhibition of cell division and induction of rapid degradation of DNA, characteristic effects of E2 on sensitive cells, were not observed in this mutant. Studies of other mutants refractory to E2 have shown that a small proportion were also
u.v.-sensitive, although this property was not apparently temperature-dependent (Holland, 1967).

In all previous communications E2-refractory strains were designated as Ref11 mutants. In accordance with the recommendations of Clark, Demerec, Adelberg & Hartman (1966), E2-refractory mutants are now identified by the symbol Cet (Colicin E-two). For convenience, mutants of normal u.v. sensitivity are designated CetB, whilst u.v.-sensitive, E2-refractory mutants are designated CetC. In this paper additional CetC mutants are described and the basis of their u.v. sensitivity further examined. The temperature dependence of E2-refractivity in these mutants has been found to be more complex than that of CetB mutants and they have acquired, in addition to u.v. sensitivity, several other mutant characteristics including altered cell surface properties. The properties of these mutants suggest that colicin E2 may normally interact with a part of the cell membrane which is involved in the regulation of cell division and in some aspects of DNA metabolism.

METHODS

The general properties of strains used in this study are shown in Table 1. Mutant strains ASH 101, 110, 111, 112 (Holland, 1967, 1968) and ASH 102, 113 (Holland & Threlfall, 1969) have been studied previously. Strains ASH 114, 115 and 116 are described for the first time. E2-refractory strains ASH 101, 111 and ASH 114 were derived from parental strain ASH 1 (HfrB11, thi- met- thy- leu- str- λ-) whilst ASH 102, 112, 113, 115 and 116 were derived from the parental strain ASH 10 (F- thi- met- thy- lac- str- λ+). The E2-refractory strain ASH 110 was derived from HfrH (thi- thy- lac- str+ λ-). The isolation and initial characterization of the mutants has also been described previously (Holland & Threlfall, 1969).

Table 1. Summary of phenotypic properties of CetB and CetC mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Response to E2</th>
<th>Response to u.v.</th>
<th>Response to X-ray</th>
<th>DOC</th>
<th>λ</th>
<th>RecA formation</th>
<th>Filament formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental ASH 1</td>
<td>Hfr</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>/</td>
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<tr>
<td>ASH 10</td>
<td>F-</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>CetC ASH 110</td>
<td>Hfr</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>/</td>
<td>S</td>
<td>S</td>
<td>/</td>
</tr>
<tr>
<td>ASH 111</td>
<td>Hfr</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>Rec-</td>
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<tr>
<td>ASH 114</td>
<td>Hfr</td>
<td>R</td>
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<td>S</td>
<td>R</td>
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<td>S</td>
<td>Rec-</td>
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<tr>
<td>ASH 112</td>
<td>F-</td>
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<td>R</td>
<td>S</td>
<td>R</td>
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<td>R</td>
<td>Rec-</td>
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<tr>
<td>ASH 113</td>
<td>F-</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>Rec-</td>
</tr>
<tr>
<td>ASH 115</td>
<td>F-</td>
<td>R</td>
<td>R</td>
<td>S</td>
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<td>+</td>
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<tr>
<td>ASH 116</td>
<td>F-</td>
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<td>S</td>
<td>/</td>
<td>S</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>CetB ASH 101</td>
<td>Hfr</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>/</td>
<td>R</td>
<td>S</td>
<td>/</td>
</tr>
<tr>
<td>ASH 102</td>
<td>F-</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>/</td>
<td>R</td>
<td>S</td>
<td>+</td>
</tr>
</tbody>
</table>

R denotes resistance, or refractivity; S denotes sensitivity. The response to colicin E2 was determined in liquid-grown cultures as in Fig. 1. With phage λ, three strains, ASH 114, 112 and 116, showed increased but not complete resistance to the phage (see text); * denotes strains not tested for this character.

The RecA strain KMBL 239 (rec 34) was obtained from Professor A. Rörsch, and the RecB mutant, JC 4457, was kindly provided by Dr A. J. Clark. Culture methods and production and assay of colicin E2 (P9) obtained from Salmonella typhimurium LT2 906 were described previously (Hill & Holland, 1967).
Irradiation procedures. Ultraviolet irradiation. The source of u.v. was a low-pressure mercury lamp (Hanovia Ltd) used without filter and with an incident dose rate of 6.5 ergs/mm² at 46 cm. For quantitative determination of the u.v. sensitivity of mutant strains, the procedure described previously was used (Holland, 1967). Exponential cultures of various strains grown in tryptone broth (TB) were harvested, washed and resuspended in 0.07 M-phosphate buffer at 10⁸ bacteria/ml. Appropriate dilutions of bacteria were then plated on tryptone (1 % Oxoid tryptone, 0.5 % w/v NaCl) agar plates, irradiated and incubated in the dark for 14 h. at 37° before counting.

Gamma irradiation. Exponential cultures (5 × 10⁸ bacteria/ml.) of mutants in TB media were harvested, washed and resuspended in 0.07 M-phosphate buffer at 10⁸ bacteria/ml.; 2 ml. aliquots in 14 in. x ₃ in. glass vials were then irradiated in a Gammacell 200 (Atomic Energy of Canada, Ltd) output 0.13 megarads/h. Irradiated samples were diluted, plated on TB plates, and survival determined after 14 h. at 37°.

Determination of u.v.-induced DNA breakdown. Exponentially growing cultures of strains in Difco nutrient broth (NB) were labelled with H³ thymine (16 µCi/µg. thymine/ml.) in the presence of 100 µg./ml. deoxyuridine, for five generations and harvested when the density reached 3 × 10⁸ bacteria/ml. Labelled cells were washed twice and finally resuspended (2 × 10⁷ bacteria/ml.) in 0.07 M-phosphate buffer pH 7.2 plus 25 µg./ml. cold thymine; 8 ml. samples were irradiated in a thin layer (2 mm.) and then diluted with an equal volume of NB and reincubated in dim light at 37°. Samples (0.5 ml.) were removed at intervals, mixed with 0.5 ml. 10 % cold trichloroacetic acid and cold and hot acid soluble counts analysed as described by Howard-Flanders & Theriot (1966). Full details of labelling procedures and methods of the determination of E2-induced DNA breakdown have been described previously (Holland, 1968).

Growth of bacteriophage λ. Production and assay of λ and λgv (Jacob & Wollman, 1953) have been described previously (Holland, 1967). One-step growth experiments were carried out by the standard technique based upon that described by Ellis & Delbrück (1939) using Escherichia coli C600 as indicator. The medium used in one-step growth experiments consisted of an M9 basal medium (Anderson, 1946) with 1.4 % (w/v) maltose as carbon source and supplemented with 1 % (w/v) Casamino acids (Difco), 40 µg./ml. thymine and 10 µg/ml. vitamin B1. For the assay of intracellular phage, 0.5 ml. aliquots of infected cultures were removed, mixed with an equal volume of 10 % (v/v) chloroform in phage buffer (0.02 M-tris pH 7.2, 0.01 M-MgSO₄, 0.005 % gelatin) and blended vigorously on a Vortex mixer. Finally, the suspension was incubated 10 min. at 37° to complete lysis, and, after cooling, 0.1 ml. samples were assayed for free phage.

RESULTS

Response of Cet mutants to colicin E2

In the initial characterization of E2-refractory mutants (Hill & Holland, 1967), refractivity to colicin was determined in cross-streak tests on nutrient broth agar. The majority of the mutants, although pregrown at 37°, were refractory to E2 at 25° but completely sensitive at 37°. In one mutant, ASH 112, some growth in the presence of E2 was observed at 37° and so sensitivity in this strain was not complete at high temperature. Similar results were again obtained in cross-streak tests with all the mutants examined in this study. When sensitivity to colicin was determined in liquid culture, although all strains grown and tested at 30° were still refractory to E2, the response at 37° varied
widely in different mutants. As shown in Fig. 1, some strains, including \textit{ASH} 112, were completely refractory to E2 whilst others, e.g. \textit{ASH} 111 were quite sensitive. Strains \textit{ASH} 101 and \textit{ASH} 102, which appeared to be typical of the majority of Cet mutants, showed, as described previously (Holland, 1968), an intermediate sensitivity at 37°. The induction of DNA breakdown by colicin E2 at 37° was also examined in all the mutants. As found previously and as shown in Fig. 2, the extent of E2-induced DNA breakdown at 37° closely parallels the E2-sensitivity of each strain at this temperature.

\textbf{Fig. 1} \hspace{1cm} \textbf{Fig. 2}

![Graphs showing sensitivity and DNA breakdown](image)

\textit{Fig. 1.} Sensitivity of CetC mutants to colicin E2 at 37°. Exponentially growing cultures in NB medium of various CetC mutants, two CetB mutants (\textit{ASH} 101 and 102) and a wild-type strain, \textit{ASH} 10, were adjusted to \(10^8\) bacteria/ml. After treatment with different concentrations of colicin E2 for 40 min. at 37°, the surviving fractions were determined by plating on NB agar plates.

\textit{Fig. 2.} Colicin-induced DNA breakdown in CetC mutants at 37°. Bacteria were labelled by growth in NB media plus H\(^3\) thymine as described in Methods, harvested, washed and resuspended in NB at \(2 \times 10^7\) bacteria/ml. E2 was added and the cells reincubated with shaking at 37°. Samples (1-0 ml.) were removed at intervals, mixed with 4 ml. 10 % ice-cold TCA and the content of acid-soluble and acid-precipitable radioactivity determined as described previously (Holland, 1968). The colicin concentrations used were 1000 units/ml. for \textit{ASH} 110, 112; 400 units/ml. for \textit{ASH} 101, 113 and 111; 100 units/ml. for \textit{ASH} 10.

\textit{Ultraviolet-sensitive Cet mutants.} When further properties of Cet mutants were examined they were found to be divisible into two major groups. CetB mutants, e.g. \textit{ASH} 101 and \textit{ASH} 102, apparently differ from the wild-type only in their refractivity to colicin E2. CetC mutants, e.g. \textit{ASH} 112, in contrast have acquired a varied range of additional properties (Table 1). However, CetC mutants, which contribute about 15 %
Surface mutants of E. coli

of all Cet mutants, i.e. seven out of about 50 tested, have one property in common: they are all u.v.-sensitive. The u.v. sensitivity of several CetC mutants at 37° is shown in Fig. 3. The mutants again show a wide range of response with those mutants showing maximum refractivity to E2 at 37° (ASH 112 and ASH 114) being the least sensitive to u.v. The u.v.-sensitivity of ASH 111 varied somewhat in different experiments (see also Holland, 1967) but was usually similar to that shown in Fig. 3.

It was previously reported (Holland, 1967) that CetC mutants ASH 110 and ASH 113, like Rec- mutants, are still capable of host-cell reactivation (i.e. they are Hcr+). However, unlike Rec- mutants, CetC mutants are not particularly sensitive to gamma-irradiation (Fig. 4).

Ultraviolet-induced DNA breakdown in CetC mutants. Howard-Flanders & Theriot (1966) and Willetts & Clark (1969) have shown that whilst at least some RecB and RecC mutants show a ‘cautious’ (less than wild-type) DNA breakdown, RecA mutants show ‘reckless’ DNA breakdown after u.v. irradiation. The CetC mutant ASH 111 was previously found to show enhanced DNA breakdown after u.v. irradiation (Holland, 1967) and several other CetC mutants have now been tested for this property. The u.v.-induced DNA breakdown patterns obtained (Fig. 5) demonstrate that none of the mutants fall clearly into the two categories originally defined by Howard-Flanders & Theriot (1966). Although ASH 114 and ASH 111 and possibly ASH 113 showed increased
breakdown over the parental type after large doses of u.v., they could still be distinguished from typical RecA mutants which characteristically show extensive spontaneous DNA breakdown, have large intracellular nucleotide pools and rapidly degrade their DNA after small doses (50 ergs) of u.v. (Howard-Flanders & Theriot, 1966). With other CetC mutants DNA breakdown after irradiation was similar to the parental strain ASH 10 or the CetB mutant ASH 101.

*Detergent sensitivity of CetC mutants.* De Zwaig and Luria (1967) reported that some mutants refractory to both colicins E2 and E3 showed increased sensitivity, under certain cultural conditions, to sodium deoxycholate (DOC) and to EDTA. They suggested that this and other properties of the mutants indicated that refractivity to colicin was associated with a change in properties of some component of the cytoplasmic membrane. When grown in nutrient broth, all CetC mutants showed greatly increased sensitivity to DOC whilst CetB mutants were largely unaffected (Fig. 6). For these tests the bacteria were first grown at 37° and then tested for DOC-sensitivity at either 25° or 37°. The CetC strains were almost equally sensitive at both temperatures as with their response to u.v. As in other tests the mutants showed a wide range of response to the treatment with, to some extent, the mutants most sensitive to u.v. being least sensitive to the detergent. Some CetB mutants also showed slight sensitivity to DOC (e.g. ASH 102) but the effect was much less than that observed with CetC strains.

Other u.v.-sensitive mutants of *Escherichia coli* K 12 including RecA, RecB and Uvr− strains and an Hsp− (host specificity) mutant were also tested for DOC sensi-
Surface mutants of *E. coli*

...activity and found to be resistant. Increased DOC-sensitivity therefore appears to be a specific change associated with the CetC phenotype.

**Effect of temperature shift on E2-induced DNA breakdown in CetC mutant ASH 113**

Mutants refractory to colicin E2 should conceivably arise through a change in the activity of the nuclease normally activated by the presence of the colicin. Furthermore, since particular strains, e.g. ASH 111 and ASH 113, are very sensitive to E2 at high temperature but refractory at low temperature, it was of interest to determine whether such mutants produced a cold-sensitive nuclease. A culture of ASH 113 was first pre-

[labelled with H³ thymine at 37° and colicin E2 was added to several different samples. At various times treated cultures were shifted to 30° and the effect on DNA breakdown determined at intervals. As shown in Fig. 7b, temperature shift, even at time zero, did not prevent E2-induced DNA breakdown. The observed reduction in the rate of breakdown is compatible with the reduced activity of a normal enzyme operating at 30° rather than at 37° and in no way indicates instability of a nuclease at low temperature. Moreover, cultures shifted to 30° for at least 20 min. before addition of E2 still showed considerable DNA breakdown upon subsequent addition of E2 (Fig. 7a). Similar results were obtained with the CetC mutant ASH 111. Neither of these strains therefore produce an E2-specific nuclease which is unstable at low temperature. The results do not, however, exclude the possibility that the mutants produce an altered...
nuclease in the form of an aggregate, functioning at both 37° and 30°, which can be assembled at 37° but not at 30°.

The results shown in Fig. 7a also indicate a further possible difference between CetB and CetC mutants. Previous studies with CetB mutant ASH 101 (Holland, 1968) showed that this strain produced an altered cellular constituent which, after temperature shift from high to low temperature, immediately underwent a change which rendered the cells insensitive to the bacteriocidal effects of E2. In the present study the

![Fig. 7](image1)

![Fig. 8](image2)

**Fig. 7**. Effect of temperature shift on E2-induced DNA breakdown in CetC mutant ASH 113. Strain ASH 113 was labelled with H³ thymine during growth at 37° as described in Fig. 2 and Methods. The labelled bacteria were washed, resuspended at $2 \times 10^8$ bacteria/ml. in NB medium and kept at 37° for 10 min. before use.

(a) At time zero E2 (50 units/ml.) was added to each of two cultures, one was incubated at 37° and the other immediately shifted to 30°. E2 was also added to other cultures after 5, 10 and 20 min. incubation at 30° respectively. Release of H³ thymine as cold acid-soluble material was determined, as for Fig. 2, following temperature shift and the addition of colicin. In a control culture (not shown) growing at 30° throughout, addition of colicin E2 produced no increase in the release of soluble H³ thymine over a 2 h. period.

(b) Colicin E2 (50 units/ml.) was added to several cultures of ASH 113 and one culture shifted to 30° at each of the times indicated by the curves. One culture was maintained at 37° throughout.

**Fig. 8**. Abortive growth of bacteriophage λ in some CetC mutants. The number of infectious centres obtained in one-step growth experiments is plotted against time for each phage-bacterial system. (a) Growth of λφv on the lysogenic strains ASH 102 (CetB) and ASH 112 (CetC). (b) Growth of λ on non-lysogenic strains ASH 101 (CetB) and ASH 114 (CetC).

bacteriocidal effects of E2 under conditions of temperature shift were not measured. Nevertheless, the results shown in Fig. 7a indicate that in this CetC mutant extensive growth at low temperature is required before E2-refractivity is expressed. The cold-sensitive lesion leading to E2 refractivity in CetB mutants and in at least some CetC mutants may therefore be different.
Increased resistance to bacteriophage λ amongst CetC mutants. Since the replication of λ and ΦX174 DNA has been reported to take place at specific membrane sites (Knippers & Sinsheimer, 1968; Salivar & Sinsheimer, 1969), the sensitivity of various Cet mutants to phage λ was also determined. So far, out of 30 strains tested, three CetC mutants (Table 1) displayed increased resistance of some kind of λ or its virulent derivative λgv. This was reflected in the formation of minute plaques and reduced plating efficiencies. All three strains (ASH 112, 114 and 116) nevertheless appeared to adsorb λ with an efficiency equal to that of wild-type cet+ strains. When one-step growth experiments were carried out with ASH 112 and ASH 114, infectious centres were formed at normal frequencies indicating that both adsorption and injection of λ DNA were normal under these conditions. As shown in Fig. 8b, however, the average burst-size for λ or λgv in ASH 114 was only 5. This compares with burst sizes of 30 to 35 in the CetB mutant ASH 101 or in Cet+ parental strains. Growth of λgv in ASH 112 was found to be even more restricted with a maximum burst size of 1 to 2 phage particles per bacterium compared to 25 to 30 in the CetB control ASH 102. Liberation of mature phage was also greatly delayed in the ASH 112 host, but the low burst was not apparently due to lysis inhibition since premature disruption of infected cells with chloroform did not increase the yield. The reason for the restriction of λ multiplication in these CetC mutants is as yet unknown, but further experiments will be carried out to determine which particular step in the growth cycle is blocked.

Other properties of CetC mutants. At least two out of the seven CetC mutants so far studied show a further mutant phenotype, filament formation. These strains, ASH 110 and ASH 111, which both plate λ normally, produce, especially on solid nutrient agar media, large numbers of filaments up to 30 μm in length, unlike all the other CetC mutants so far studied. With one exception (ASH 116) CetC mutants do not form mucoid colonies on minimal or nutrient agar. In these respects they are clearly distinct from u.v.-sensitive mutants of the Lon-type (Howard-Flanders, Simson & Theriot, 1964) in which u.v. sensitivity has been attributed to defective control of cellular polysaccharide synthesis (Walker & Pardee, 1967). CetC mutants may be further distinguished from Lon-type mutants by their recombination deficiency which is described in the accompanying paper (Threlfall & Holland, 1970).

Table 1 summarizes the main properties of CetC mutants described so far, but a further important characteristic of the mutants should be noted. CetC strains, in contrast to CetB mutants, grow more slowly than wild-type strains in both nutrient and minimal media, and in some cases give reduced (40 to 50%) colony counts when plated on agar. These characteristics are probably a reflexion of surface defects which may be clearly seen by phase contrast microscopy, particularly with stationary phase cultures. Many free spheroplasts, organisms showing extrusion of almost free spheroplasts and even branched organisms are present. These characteristics have not been observed in CetB, Uvr−, RecA, RecB and RecC mutants examined by us.

DISCUSSION

Adsorption of colicin E2 is normally followed by rapid degradation of DNA and the inhibition of division. Cet mutants, refractory to E2, have now been isolated from several Escherichia coli K12 strains. These strains still adsorb E2 but remain completely refractory to its effect at 30° although the majority are largely sensitive at 37°. All Cet
mutants so far studied show normal sensitivity to colicin E1, bacteriophage BF23 and to colicin E3, a colicin which appears to utilize the same initial receptor as E2 (Maeda & Nomura, 1966; Hill & Holland, 1967). The mutants also appear unchanged in their sensitivity to colicin K and to bacteriophage TI (Naysmith, 1967). Colicin E2 refractivity appears therefore to be associated with a highly specific surface or intracellular change rendering interaction with this colicin ineffective.

Mutants refractory to E2 can be divided into two phenotypic groups: those, CetB, which have acquired E2-refractivity only, and those, CetC, which are also u.v.- and DOC-sensitive. Most CetC mutants also appear to be recombination-deficient in some way (Holland & Threlfall, 1969; Threlfall & Holland, 1970). In addition, two CetC mutants show filament formation under normal growth conditions whilst three other mutants support only poor growth of bacteriophage λ. Despite these wide variations in phenotype, genetic studies have shown that \textit{cetB} and \textit{cetC} loci are very closely linked. Furthermore, all the additional characteristics associated with CetC refractivity appear to be the pleiotropic effects of a single gene closely linked to, if not identical with, the \textit{cetC} locus, and not the result of several independent mutations (Threlfall & Holland, 1970). Despite the close linkage of \textit{cetB} and \textit{cetC} loci, the radical differences in the properties of these mutants indicate that at least two distinct cistrons may be involved, and complementation studies are being carried out to test this.

Since colicins appear to exert their effects at the level of the cytoplasmic membrane, some mutants refractory to their action might be expected to have altered membranes. Both the increased detergent sensitivity of the CetC mutants and the microscopic evidence of surface abnormality strongly suggest that the mutants do have altered surfaces. Moreover, preliminary experiments have shown that membrane preparations from strains \textit{ash} III and \textit{ash} 114 also have altered protein patterns on acrylamide gels when compared to wild-types (Samson & Holland, unpublished results).

The complex pleiotropy of CetC mutants may be best explained on the basis of either of the following two models. The first model assumes that the \textit{cet}+ gene constitutes part of an operon concerned with DNA metabolism and cell surface formation. Mutations in this gene might then arise having strong polar or pseudopolar effects upon adjacent genes (cf. Fink, 1966; Giles, Case, Partridge & Ahmed, 1967; Zipser, 1969). The second model supposes that the \textit{cet}+ gene determines the synthesis of a component of the cell membrane, malformation of which can directly or indirectly affect u.v. sensitivity, recombinant formation, cell division and, in some cases, growth of λ. Different mutations in the \textit{cetC} gene would then give rise to the pleiotropic effects observed. Further genetic analysis of CetC mutants is now being carried out to test the validity of the first model. To test the second model, a qualitative study of the proteins present in membrane preparations from CetB and CetC mutants and their revertants is in progress. Further investigation of the molecular basis of the u.v. sensitivity of CetC mutants may also aid the understanding of CetC pleiotropy. Results obtained so far indicate that the u.v. sensitivity of these mutants differs from that of all Uvr, Rec and Lon mutants so far described. It is still not clear, however, whether CetC mutants are defective in repair of DNA or in some aspect of cell division which prevents normal recovery from u.v. irradiation.

The mode of action of colicin E2 and the properties of Cet mutants indicate that E2 normally interacts with a unique membrane site. This could be the part of the membrane which binds the DNA and which has been implicated in the cell division
Surface mutants of E. coli

process (Jacob, Brenner & Cuzin, 1963; Ryter, 1968). Comparison of the properties of this membrane fraction which can perhaps be isolated with its attendant DNA (see Tremblay, Daniels & Schaechter, 1969), with those of the remainder of the cell membrane in wild-type and in Cet mutants, should provide a crucial test of our hypothesis.

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SUMMARY

The Mode of Action of Colicin E2 with Regard to the Structure of the Escherichia coli Cell Envelope

The importance of cell membranes in maintaining the structural integrity of living organisms has been known for some time. A very complex and important role for membranes as the spatial and temporal organizers of the cell's numerous metabolic functions is presently being unfurled. Although overall membrane structure may appear to be similar amongst the vast range of living organisms, the basic framework is necessarily adorned with specialized coordinated systems relevant to each cell's functions.

The mechanism of colicin E2 interaction with the cell surface of Escherichia coli is examined and discussed. Colicin action upon the bacterial cell's biochemical processes is both indirect and highly specific, and is mediated by some transmission system in the bacterial cell membrane. Fluorescein labelled colicin E2 and ferritin labelled anti-E2 Y globulin have been prepared and used in an attempt to locate the positions of colicin E2 molecules in or on the cell surface of sensitive bacteria.

Generalized disturbance of cell envelope integrity subsequent to colicin action has been sought but not found. The highly specific nature of colicin action upon the membrane is supported. Treatments
designed to interfere with bacterial cell envelope integrity do perturb the action of colicin E2.

Membrane fractionation procedures suitable for studying bacterial membrane structure are presently becoming available. An additional membrane fractionation procedure is presented, based upon the interaction of cadmium N-lauroyl sarcosinate with bacterial cell extracts. Sodium dodecyl sulphate acrylamide gel electrophoretic separation of *E. coli* envelope proteins from mutants refractory to the action of colicin E2 has shown that these membrane-containing structures have an altered protein component compared to the non-refractory parental strain.

Hypotheses explaining a certain type of colicin E2 refractivity are presented. Future studies involving the use of colicins and genetic analysis as effective tools in probing the complexities of the *E. coli* cell membrane structure are outlined.