MODE OF ACTION OF COLICIN E_2-P9

by

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PREFACE

This dissertation describes studies into the mode of action of the protein antibiotic colicin E_2-P9 with reference to its *in vivo* effects on DNA metabolism in *Escherichia coli*, its *in vitro* activity on DNA and the effects of other antibacterial agents on colicin E_2-P9 induced DNA degradation. This work was carried out during the period January 1968 to May 1970 in the Sub-department of Chemical Microbiology, Department of Biochemistry, University of Cambridge.

Some of the results described in Chapter 5 have been published in the following paper:

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It is a pleasure to record my appreciation and thanks to Dr. David Kerridge for his advice and encouragement. I am also indebted to my colleagues in the Sub-department of Chemical Microbiology for valuable discussions and to my wife for encouragement under trying circumstances. In addition my thanks are due to the Medical Research Council for a Scholarship for Training in Research Methods.

I certify that this dissertation describes my own unaided work and is not substantially the same as one which has been submitted to any other University.

P S Pingrose

August, 1970

"Natural philosophy ought to be esteemed the great mother of the sciences. Even among those who have attended to it, it has scarcely ever possessed a disengaged and wholeman, but that it has been made merely a passage and bridge to something else. Let no man look for much progress in the sciences - especially in the practical part of them - unless natural philosophy be carried on and applied to particular sciences, and particular sciences be carried back again to natural philosophy. For want of this, astronomy, optics, music, a number of mechanical arts, medicine altogether lack profoundness, and merely glide along the surface of things."

Francis Bacon, (1620) Novum Organum.

CONTENTS

ABBREVIATIONS

The following abbreviations have been used;

AC	Acriflavin
ACT D	Actinomycin D
ATP	Adenosine triphosphate
BBOT	2,5-bis-(5′-t-butylbenzoxazolyl-2′)-thiophene
CAP	Chloramphenicol
Col.factor	Colicinogenic factor
DAP	α:ϵ diaminopimelic acid
DAU	Daunomycin
DNase	Deoxyribonuclease
DNP	2:4 dinitrophenol
E_2	Colicin E_2-P9
E_3	Colicin E_3-CA38
EB	Ethidium bromide
EDTA	Ethylenediamine tetracetic acid
GTP	guanosine triphosphate
MGIC	Minimum growth inhibitory concentration
MITO	Mitomycin C
ng	nanogram (g x 10^{-9})
NOV	Novobiocin
ORD	Optical rotatory dispersion
PEA	β Phenethyl alcohol
PHL	Phleomycin
Poly U	Polyuridylic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid

Tris	Tris(hydroxy-methyl)-amino-methane
UV	Ultraviolet

Other abbreviations conform to the usage in the Biochemical Journal incorporating the Systeme Internationale recommended symbols.

GENERAL INTRODUCTION

Colicins are protein antibiotics which initiate specific lethal processes in sensitive strains of intestinal bacteria and are synthesised by other strains of such bacteria that harbour the corresponding genetic determinant or colicinogenic factor.

The antagonistic effect of certain strains of Escherichia coli toward other strains was first reported by Gratia in 1925 when he showed that E. coli V excreted a diffusible antibiotic substance that was particularly lethal for E. coli Ø. Fredericq (1948) later showed that the phenomenon of reciprocal antibiotic action was not restricted to E. coli but could be found in many other genera of the Enterobacteriaceae such as Salmonella and Shigella. It also became obvious that more than one antibiotic substance or colicin was involved and that an active strain could produce more than one colicin type. In addition bacterial strains producing one colicin to which they were necessarily immune were inhibited by strains producing other colicins (Fredericq, 1957).

By 1953 sufficient numbers of bacterial species in addition to the coliform bacteria had been reported as showing this antagonistic effect within their own genera, that Jacob et al. introduced the more general term "bacteriocin" which was defined as a highly specific antibacterial protein produced by certain strains of bacteria and active against some other strains of the same or related species. Hamon (1964) listed 27 bacteriocinogenic families including strains of Bacillus megaterium, Pseudomonas aeruginosa, Vibrio cholerae and Pasteurella pestis and found that bacteriocinogeny was as prevalant in nature as lysogeny.

Many bacteriocins have been poorly characterised and this introduction will be largely restricted to a discussion of colicins, in particular colicin E_2. Bacteriocins in general are more extensively covered in reviews by Nomura (1967) and Holland (1967.b). Colicins have been shown to interfere with macromolecular metabolism in bacteria by initiating chromosomal degradation (colicin E_2), cessation of protein synthesis (colicin E_3) or inhibition of energy production (colicins K and E_1), (Nomura, 1963). Each colicin has its own characteristic effect but it is questionable whether these observable effects are the direct cause of cell death or the result of a primary lethal process associated with cell membrane, since the killing action of colicins is a single hit process (Jacob et al., 1952; and see text) yet the observed biochemical effects are frequently dependent in nature and extent on the number of colicin molecules per bacterium (Nomura, 1967; Luria, 1970). Owing to the reversibility of their action by trypsin, colicin molecules are believed to remain near the cell surface (Nomura and Nakamura, 1962; Reynolds and Reeves, 1963) but the exact location of their site of operation will be discussed later.

(a) Classification

Colicin nomenclature involves the use of an alphabetical letter to indicate receptor specificity, followed by a number to indicate the specificity of immunity of the producing strain. Colicins are now classified into 23 different classes (Hamon and Peron, 1963) according to the scheme of Fredericq (1949) who originally found 17 classes. All colicins in group E use the same receptor sites in sensitive strains and it was believed that these sites were similar to those used by phage (Fredericq, 1949 and 1957),

so that phage BF_{23} used the colicin E site and phage T_6 used the colicin K site (Nomura, 1967). However these findings and the whole concept of colicin receptors has recently been questioned by Šmarda and Taubeneck (1968) and their observations will be discussed in section (d).

Group E is subdivided by virtue of the immunity of a colicinogenic strain to the lethal action of a homologous colicin. Although colicinogenic strains possess the potentiality of synthesising colicin coded for by the particular colicinogenic or Col. factor very few do so spontaneously and induction is necessary as in lysogenic strains. Therefore a bacterium carrying the Col. factor for colicin E_2 is immune to extracellular E_2 but is sensitive to colicins E_1 and E_3 (see Table 1i).

Colicins are usually further identified by giving the coding of the original producing strain preceeded by the classification number. Thus colicin E_2 is usually described as E_2-P9 or E_2-CA42 according to the origin of the Col. factor. The receptor specificity classification of Fredericq, (1949) relies to a large extent on the isolation of resistant mutants that have lost the specific receptor sites for the group of colicins studied. The situation is however far more complex since there are mutants which, despite being resistant to the lethal action of a particular colicin, are still able to adsorb the antibiotic molecule, i.e. the tolerant mutants of Nomura and Witten (1967) and the refractory mutants of Hill and Holland (1967). In addition it is reported (Hamon and Peron, 1966; Hill and Holland, 1967) that mutants have been isolated which had lost the adsorption capacity for colicins E_2 and E_3 but not E_1. It therefore looks as if E_1 has a separate receptor site to E_2 and E_3. In contrast another colicin which was given the letter P because of its different heat stability to the E group was found to have a similar mode of action to colicin E_2 and may

probably use the E receptor site (Hamon and Peron, 1965). Colicin P may well have been misclassified since in addition its heat stability was found to be dependent on purity (Nomura, 1967).

Bacteriocins from B. megaterium or Megacins are divided into groups A, B and C according to their general properties and mode of production owing to the difficulty of isolating resistant mutants (Holland, 1967.b).

(b) Chemical Nature

Bacteriocins in general vary widely in their chemical composition; the E group colicins (Herschman and Helinski, 1967.a) and megacin A (Holland, 1961) appear to be pure proteins whilst others resemble incomplete bacteriophage, e.g. colicin 15 (Endo et al., 1965), certain pyocins from Ps. aeruginosa (Nomura, 1967) and two peculiar bacteriocins from Listeria monocytogenes (Bradley and Dewar, 1966) and Enterobacter cloacae (De Graaf et al., 1968). In contrast a few bacteriocins are very similar to the somatic antigen or part of the gram -ve cell wall of the parent colicinogenic strain, i.e. colicins K, A and V (Nomura, 1967; Hinsdill and Goebel, 1964). Goebel and Barry (1958) first showed that colicin K activity was associated with a lipopolysaccharide protein complex and that phenol extraction produced a protein that was 10-100 times more active. Hinsdill and Goebel (1966) later showed that this protein moiety was not characteristic of the bacterial species carrying the K Col. factor but that the lipopolysaccharide component resembled part of the cell envelope of the producing strain.

The molecular weights of most protein bacteriocins are in the range of 30,000 to 100,000 and colicins in particular are characterised by their partial resistance to heat, sensitivity to proteolytic enzymes and diffusibility through agar. Colicins differ amongst themselves

in their antigenic properties and electrophoretic mobilities (Fredericq, 1957; Nomura, 1967).

Herschman and Helinski (1967.a) demonstrated that colicins E_2-P9 and E_3-CA38 are entirely protein with molecular weights close to 60,000. In addition colicin E_2 is reported to have two electrophoretically distinguishable but interconvertable forms at pH 8.6 but not pH 4.0. This finding is not the result of aggregation and is believed to involve a change in secondary or tertiary protein structure causing a reduction of one net positive charge in going from the higher to the lower pH isoelectric form (Herschman and Helinski, 1967.a). Amino acid analysis has revealed that both colicins have relatively low amounts of hydrophobic amino acids and only one cysteine residue per protein molecule consistent with the properties of certain other extracellular proteins (Pollock and Richmond, 1962). Immunochemically colicins E_2 and E_3 have a region that is common to both and may be associated with receptor specificity, also a characteristic region which may be associated with immunity and target specificity.

In contrast Reeves (1963) had shown earlier that colicin E_2-CA42 or F contained 5-16% carbohydrate and perhaps some lipid material (Hinsdill and Goebel, 1964). Its mode of action and trypsin reversibility appear to be identical however to colicin E_2-P9 described above.

(c) Production of colicins

Colicinogenic or Col. factors are extrachromosomal supercoiled duplex DNA molecules (Roth and Helinski, 1967) present in colicinogenic strains, responsible on induction for the synthesis of colicin protein and can under certain conditions infect noncolicinogenic strains of Enterobacteriaceae (Stocker et al., 1963). They contain 10^4-10^5 nucleotide pairs and resemble resistance transfer

factors, fertility factors and prophage to varying degrees (DeWitt and Helinski, 1965; Ozeki, 1965).

In 1953 Fredericq established that the ability to synthesise colicin could be transferred by cell contact to non-colicinogenic strains. However Nagel de Zwaig et al., (1962) showed that the frequency of transfer depended upon the nature of the particular Col. factor and that if an Hfr noncolicinogenic strain were crossed with a F^- colicinogenic strain then the noncolicinogenic property of the Hfr strain was not transmitted to the recombinants. It was concluded from these and other experiments by Stocker et al., (1963) that Col. factors could be divided into at least four distinct classes (see reviewed by Nomura, 1967) with properties ranging from those of a temperate phage (classes III and IV, i.e. colicin groups E, K and V_1) to those of the fertility factor (class I, i.e. colicin groups B, V_2 and V_3).

The molecular properties of the Col. factors substantiate this genetic classification since it has been shown that plasmid Col. factors of the E group have small molecular weights of 5×10^6 and there are several copies per chromosome unlike the episomal-like Col. factors of the B and $V_{(2 \text{ and } 3)}$ groups which have molecular weights approaching 10^8 and more closely resemble the fertility factor which has only one copy per chromosome (Bazaral and Helinski, 1968). For a discussion of episomes and plasmids see Hayes (1968).

The size of the E group Col. factors (approx. 10^4 nucleotide pairs) necessarily infers a certain degree of informational repetition assuming that the colicin molecule (approx. 500 amino acids) is the only product. The phenomenon of repeated sequences is however consistent with the observations of Corneo et al., (1970) on human placental DNA and may reflect the genetic provision of efficient production of a particular protein necessary for the self

preservation of a bacterial strain (however see Ikari *et al.*, 1969) or differentiation of an animal cell. The Col. factor may also code for a protein responsible for the immunity phenomena.

Synthesis of colicin is a lethal process for the colicinogenic strain but unlike lysogenic phage there is no immediate cell lysis (Ozeki *et al.*, 1959). Spontaneous colicin production occurs only at low frequency. In 1952 Jacob *et. al.*, first showed that synthesis of colicin E_1-ML could be induced by UV irradiation, and later Iijima (1962) found that low concentrations of mitomycin C induced class III Col. factors (group E colicins). Class I Col. factors are not induced by mitomycin (Herschman and Helinski, 1967.*b*). Other methods previously used for inducing lysogenic phage such as thymine deprivation and high temperature treatment of temperature sensitive mutants also proved successful for induction of certain colicins (see Nomura, 1967). More recently Borunova *et al.*, (1968) have reported the inducibility of colicin E_1 by triphenylmethane dyes, the effect being enhanced by illumination.

Helinski and Herschman (1967) found that recombination deficient (rec^-) mutants of class III colicinogenic strains did not produce colicin spontaneously nor could be induced to do so. A similar phenomenon was observed with lamda phage. However indirect induction was possible by mating a UV irradiated rec^+ F^+ strain with a $F^-rec^-col^+$ strain. The enzymes determined by the rec gene are therefore probably involved in the formation of an inducer from UV damaged DNA. The rec gene is not necessary for the expression of immunity or the ability of bacteria to acquire, maintain and transfer class III Col. factors. Expression of class I Col. factors is unaffected by the rec^- mutation.

After induction of class III colicinogenic bacteria with mitomycin C there is unregulated DNA synthesis with the production of 30-100 Col. factors per bacterium (DeWitt and Helinski, 1965). However this multiplication of Col. factors does not appear to be necessary for colicin production since Kohiyama and Nomura (1965) observed that normal induction occurs in a temperature inducible col^+ E_2-P9 mutant strain under conditions of inhibited DNA synthesis. Colicin activity is first detected 20-30 min after induction (Herschman and Helinski, 1967.b) during which time there was no inhibition of normal cellular protein synthesis. Chloramphenicol prevents colicin synthesis, but if present during the 20-30 min lag period and then removed, colicin synthesis is immediately initiated (Herschman and Helinski, 1967.b).

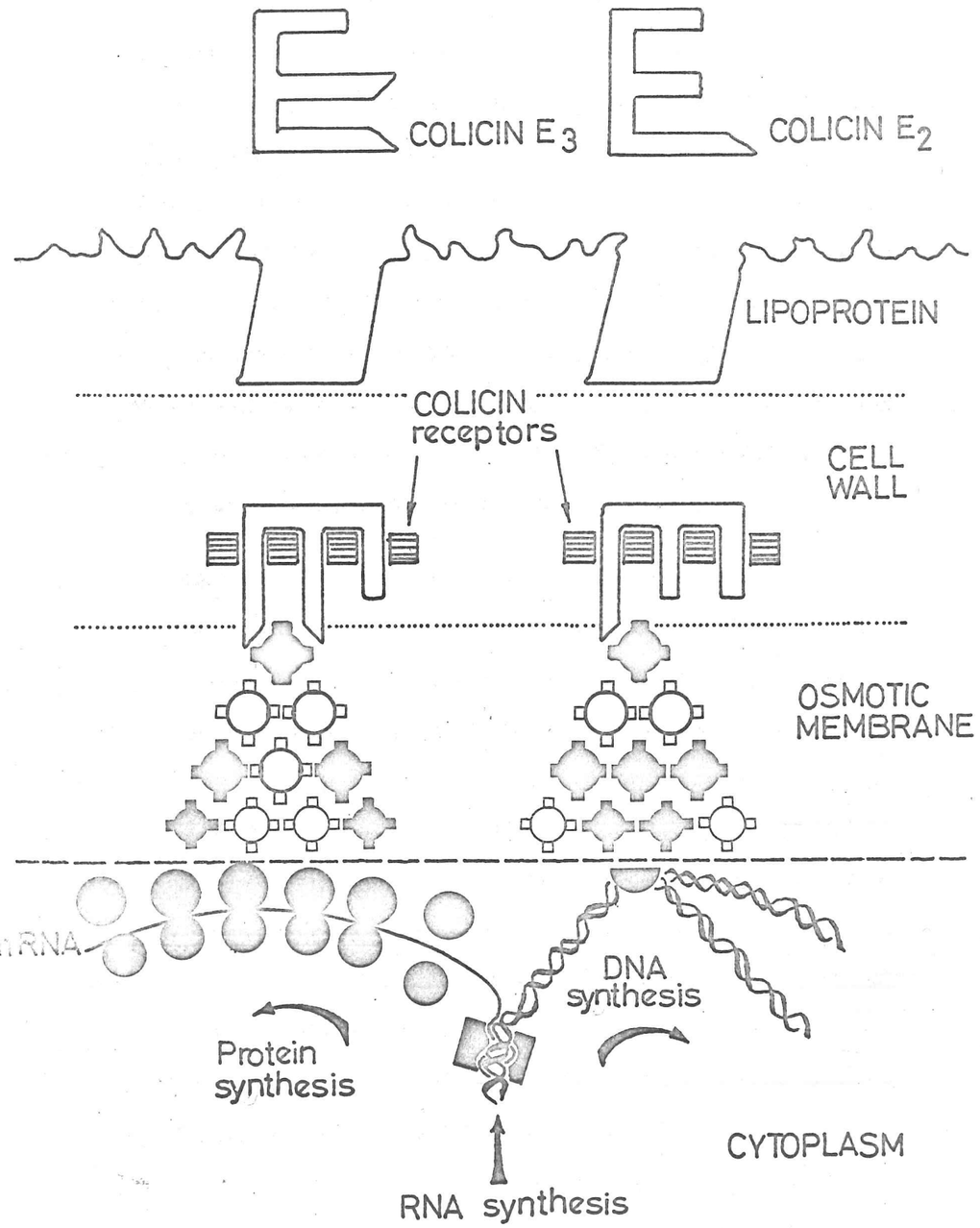
(d) Mechanism of action of colicins

Jacob et al., (1952) originally demonstrated colicin adsorption to sensitive bacterial strains by the loss of colicin activity from the culture medium. Maeda and Nomura (1966) have shown more directly, using radioactively labelled colicins E_2 and E_3, that adsorption occurs on sensitive cells, tolerant cells and even the immune parent colicinogenic cells but not on receptor deficient mutants. It was also confirmed that colicins E_2 and E_3 bind at the same site since they exhibit an inverse binding ratio to each other.

As mentioned previously the killing action of colicins is believed to be a single hit process and not to involve a multiple co-operative effect due to several colicin molecules (Hedges, 1966; Shannon and Hedges, 1967). However the 37% lethal dose (see Ch. 4) has been found to correspond to a colicin molecule per cell ratio of approx. 100 (Maeda and Nomura, 1966). This means that for a single

lethal event to occur at least 100 colicin molecules must be adsorbed by the bacterium. This apparent paradox may be resolved by assuming that (i) the colicin molecules are heterogeneous (ii) the receptor sites are heterogeneous, or (iii) there is an inefficient transmission process occuring when a colicin molecule binds to its receptor site with a chance of 1:100 of initiating the lethal process (Holland, 1967.b). Evidence is increasingly supporting (ii) whereby colicin molecules may bind to non-lethal receptor sites in addition to the effective (lethal) receptors (Shannon and Hedges, 1967).

The location of the colicin receptor site was originally hypothesised as near the cell surface owing to the ability of trypsin to digest colicin from treated cells and hence prevent the lethal action if added within a certain time after adsorption of colicin to cells (Nomura and Nakamura, 1962; Reynolds and Reeves, 1963). The work of Maeda and Nomura (1966) with subcellular fractionation after treatment of cells with labelled colicin supported this and because of the proposed similarity between colicin and phage adsorption (Fredericq, 1957) the receptor site was thought to be in the mucopeptide region between the inner and outer membranes of the gram-ve bacterial cell envelope. Nomura (1963 and 1964) proposed his transmission theory whereby the colicin molecule is specifically and irreversibly bound at the cell wall receptor site and from this position initiates a lethal sequence of events which are transmitted through the murein and cytoplasmic membrane layers to the biochemical target which is on the inner surface of the membrane. The effect of colicin on the "biochemical target "gives rise to the observable biochemical changes (i.e. DNA degradation for colicin E_2) but these may not necessarily correspond to colicin action on the "killing target" which gives rise to the lethal event (Nomura, 1964). The killing target may however sometimes be the same as the biochemical target or could be a membrane component necessary for a vital cellular control process such as replication.



COLICIN effect "transmitted" via membrane "control circuits"

(adapted from Bayer and Anderson, 1965; and Holland, 1967.a)

The transmission theory was supported by the subsequent isolation of numerous tolerant (Nomura and Witten, 1967) and refractory (Hill and Holland, 1967) mutants which were proposed to result from a malfunction in one of the "steps" between colicin adsorption and final action on the target. Temperature sensitive tolerant mutants were used to demonstrate the involvement of vital protein in the transmission system (Nomura and Witten, 1967) and Nagel de Zwaig and Luria (1967) have shown that certain tolerant mutants are hypersensitive to EDTA and detergent indicating some alteration in the structural components of the bacterial cell envelope.

Hill and Holland (1967) modified Nomura's theory by proposing a dual role for the cell surface "receptor", the first for binding of the protein and the second for correct orientation of the bound molecule relative to the cytoplasmic membrane. In addition Holland (1967.a) postulated that the transmission system interrupts "regulatory circuits" associated with the cytoplasmic membrane (Jacob and Monod, 1962) which normally control and co-ordinate replication and repair of DNA, protein synthesis and energy production (see Fig. opposite). In support of this theory there is substantial evidence that the 3 main biochemical targets for colicin attack are associated with the cytoplasmic membrane (see Chs. 5 and 6), i.e. chromosomal DNA (Jacob et al., 1963) ribosomes (Schlessinger, 1963; Abrams et al., 1964) and oxidative phosphorylation (Lascelles, 1965).

More recently colicin action has been more closely associated with membranes. Changeux and Thiery (1967) proposed an allosteric conformational change of hypothetical membrane subunits to explain the amplification of the single hit lethal event to the sometimes numerous biochemical targets. This theory was further extended by Hunter et al., (1968) to explain the mechanism of action of scrapie "virus". The

Changeux and Thiery theory envisaged a more direct colicin/membrane interaction and this was confirmed by Šmarda and Taubeneck (1968) who showed that L-forms of colicin sensitive bacteria, that is cells without a mucopeptide and outer membrane layer (protoplasts), had an increased colicin susceptibility yet were resistant to phage. Colicin and phage receptor sites differ therefore in that one is on the membrane and the other is in the cell wall. Šmarda and Taubeneck proposed that colicin adsorption may occur at either the outer cell wall or cytoplasmic membrane but it is only the direct and probably reversible interaction of the colicin molecule with the cytoplasmic membrane that results in the effect on the killing target. They dismiss a possible fixation of colicin in the cell wall and transmission of the lethal stimulus to the killing and biochemical targets as proposed by Nomura (1964). In addition it was found that trypsin recovery of colicin treated L-forms was greatly reduced. It would therefore appear that colicin can bind non-lethally and irreversibly to outer cell wall receptors but it is the direct specific interaction with membrane that is lethal to the cell. This may explain the 100 colicin molecules per single hit paradox and is consistent with the lethal and non lethal binding results of Shannon and Hedges (1967) and the results of Bhattacharyya et al., (1970) who found that proline accumulation in isolated membrane vesicles from colicin resistant and tolerant mutants was respectively inhibited and unaffected by colicin. Colicin killing action on whole cells therefore probably involves colicin penetration of the outer lipoprotein and lipopolysaccharide layers in the cell wall via channels (Bayer and Anderson, 1965), which could narrow in resistant mutants, and subsequent direct colicin interaction with the plasmamembrane. Two stage colicin adsorption has also been detected by Maeda and Nomura (1966) and Reynolds and Reeves (1969) but the

overall mechanism of colicin action, adsorption and the nature of tolerant mutations are far from clear at present. The above results on adsorption may explain the great disparity on the number of colicin receptor sites per bacterium ranging from 100 to 2-3000, (Reeves, 1965; Mayr-Harting and Shimeld, 1965; Maeda and Nomura, 1966).

In order that a biochemical effect is satisfactorily defined at a particular colicin concentration it is necessary to use the multiplicity or number of colicin killing units per bacterium (Maeda and Nomura 1966). One killing unit is defined as the minimum number of colicin molecules needed to kill a single bacterium and has been found to approximate to 100 (Maeda and Nomura, 1966). The degradation of DNA induced by colicin E_2 and inhibition of macromolecular synthesis by colicins E_2 and E_3 are strongly influenced by the multiplicity in contrast their killing effect which is single hit (Luria, 1970). Colicins E_1 and K however have a reasonable correlation between their killing effect and interference with membrane associated energy production (Luria, 1970).

The effects of colicin which result in cell death are specific and not the result of general cell damage. It was shown (Nomura, 1963) that colicin K did not cause any leakage of phosphate or Beta galactosidase from sensitive cells at multiplicities up to 50 killing units per cell. In addition phage infection and multiplication was not significantly affected at low colicin E_2 multiplicities. This demonstrates that despite the colicin induced host chromosomal DNA degradation, most of the normal cellular synthetic processes remain intact. The trypsin recovery results with colicin K (Nomura and Nakamura, 1962) also indicate no permanent cell damage. Colicin E_2 differs however because of the irreversible degradation of the chromosome and trypsin recovery only works efficiently in the presence of metabolic inhibitors that prevent the DNA breakdown.

Colicin E_2 at low multiplicities specifically induces degradation of the chromosomal DNA. Phage DNA is relatively insensitive to colicin induced nuclease attack (Nomura, 1963). Other biochemical effects for example (i) stimulation of genetic recombination (Nomura, 1967, (ii) induction of lambda phage (Endo et al., 1963), (iii) slow inhibition of DNA synthesis in contrast with the rapid inhibition by UV irradiation (Clark et al., 1966) and (iv) inhibition of cell division (Holland, 1968) are probably direct consequences of colicin E_2 induced DNA degradation. In contrast to the work of Šmarda and Taubeneck (1968) with L-forms, Nomura and Maeda (1965) and Šmarda (1965) found that although E. coli spheroplasts adsorbed colicin E_2 the sensitivity was reduced. This was probably due to the partial protection of bacteria from colicin attack by high osmotic pressure reported by Beppu and Arima (1967).

At higher multiplicities of colicin E_2 protein and RNA syntheses are inhibited (Nomura, 1967) and ribosomal RNA degraded (Nose et al., 1966; Nose and Mizuno, 1968). There is no activation of phospholipase activity by colicin E_2 in contrast to colicins K, H, Q, A, E_1 and E_3 (Cavard et al., 1968) and no change in lipid composition of the membrane (Nose et al., 1970). At excessively high multiplicities Persiel (1965) found that respiration is inhibited.

Colicin E_2 closely resembles colicin E_3 in chemical composition, molecular weight and adsorption properties (Herschman and Helinski, 1967.a) however their biochemical effects are very different since colicin E_3 specifically alters a protein in the 23S core of the 30S ribosomal subunit and thereby inhibits protein synthesis (Konisky and Nomura, 1967). There is no DNA or RNA degradation. Other colicins have not been investigated in as much detail except Ia and Ib which appear to act similarly to colicins E_1 and K (Levisohn et al., 1968). Colicin P will induce lambda phage but there is no detectable DNA degradation (Hamon and Peron, 1965).

Preliminary investigations by Nomura (1963 and 1964) have shown that colicin E_2 is not a nuclease and does not initiate the synthesis of a nuclease. In contrast megacin C also induces DNA degradation but appears to need protein synthesis for this to occur (Holland, 1967.b). Genetic evidence (Threlfall and Holland, 1968) suggests that colicin E_2 may use a nuclease system similar to that involved in repair of UV damaged DNA and recombination. Colicin E_2 was therefore an ideal candidate for investigating control of nucleic acid metabolism in E. coli and the mechanism of action of colicins in general.

(e) The Nature of the Problem

Many questions arise from the biochemical effects of colicin E_2 and are listed below;

(1) How does colicin E_2 specifically initiate nuclease attack by supposedly acting on the outer surface of the plasma membrane?

(2) Since there is no new nuclease synthesised, are the nucleases which are normally present in the cell activated by colicin E_2 or decontrolled by colicin interference with the "membrane circuits" which normally only allow restricted nuclease activity under certain circumstances in replication, repair, recombination etc?

(3) What nucleases are involved in this attack on chromosomal DNA and why is phage DNA relatively insensitive?

(4) If the nucleases are not decontrolled or activated, is the physical state of the DNA altered in any way such as local distortion, denaturation or a change in the association of DNA with membrane at the replication site?

In addition to these questions it is possible to propose conflicting theories of colicin action. All present theories assume non-penetration of the inner bacterial membrane. It could be that only one colicin molecule in a hundred gets through, interacts directly with its biochemical target and relies on the other colicin molecules bound to the outer surface of the membrane for some initial effect. This would still be consistent with the reversibility of colicin action by treatment of colicin incubated bacteria with trypsin and the location of radioactively labelled colicin in the cell envelope (Maeda and Nomura, 1966). Also since colicin E_2 has only one primary biochemical target (the bacterial chromosome) there is no apparent problem in explaining the "amplification effect" of colicin action. However direct interaction of colicin E_3 with its multiple biochemical targets (the ribosomes) is difficult to envisage using this penetration theory.

Colicin could also be regarded as a two component system, in which a large carrier molecule attaches itself to the surface of the bacterial cytoplasmic membrane enabling a small lethal component to penetrate into the cell, or as a defective membrane protein which is incorporated into the cytoplasmic membrane disrupting the associated cellular control mechanisms. Although these theories are becoming less likely, they were still worthy of investigation. Nomura's initial experiments on <u>in vitro</u> action of colicin were very preliminary and further investigation was needed to establish whether colicin E_2 directly interacts with DNA in any way.

This thesis describes attempts to answer some of these questions on the biochemical effects of colicin E_2 and investigations designed to eliminate some of the possible conflicting theories of colicin action. In the last few months reports have shown that colicin E_2 does not activate any known nucleases <u>in vivo</u> but does initiate attack of DNA using an endonuclease (Obinata and Mizuno, 1970)

also that there are detectable changes in the bacterial membrane which are irreversible and occur after 5 min incubation of sensitive bacteria with colicin E_2 (Nose *et al.*, 1970).

GENERAL METHODS

(a) BACTERIAL STRAINS

The bacterial strain routinely used for production of colicin E_2 was *Escherichia coli* K12 TR2 which received colicinogenic factor E_2 from *Shigella* P9 and was donated by Professor P. Fredericq. The sensitive strain routinely used was *E. coli* K12 ROW Str^r Met^- Stock No. CL 142 which was donated by Professor G. Meynell.

For the purposes of cross-characterisation of colicinogenic strains the following bacteria were used (see Ch. 1);

Stock No.	Description
(i) Colicinogenic strains	
M388	col^+E_1 *E. coli* ML
CL125	col^+E_2 *E. coli* CA42 (equivalent to col F)
CL124	col^+E_3 *E. coli* CA38
CL131	col^+K *E. coli* 235
CL223	col^+Ia-CA53 in strain CL142
CL232	col^+Ib-P9 in strain CL142
(ii) Sensitive strains	
CL145	*E. coli* ROW/E resistant to E group of colicins
CL147	*E. coli* ROW/I resistant to I group of colicins
CL18	*E. coli* strain ∅ (Gratia 1925)

All were donated by Professor G. Meynell. *E. coli* CL125 produced colicin E_2-CA42 (Reeves 1963; Reynolds and Reeves 1963; Nomura 1967) which differed in chemical composition from colicin E_2-P9 routinely used in the following work (Hershman and Helinski 1967).

E. coli K12 1000 and its endonuclease I^- mutant strain 1100 were donated by Professor H. Hoffmann-Berling and were used in Ch. 2. *E. coli* K12 uvr^+ AB1157 and *E. coli* K12 uvr^- AB1885 were donated by Professor P. Howard-Flanders and were also used in Ch. 2.

E. coli B, phage T4 and *Micrococcus lysodiekticus* were from the laboratory culture collection.

(b) MEDIA

(i) The synthetic medium M9 described by Anderson (1946) was used for growth of all *E. coli* K12 strains and contained 50 mM Na_2HPO_4, 20 mM NH_4Cl, 20 mM KH_2PO_4, 20 mM glucose, 0.1 mM $MgSO_4$, 10 µM $FeCl_3$ and 3g casamino acids (Difco) per 1. The standard indicator strain *E. coli* ROW was also grown in the presence of 200 ug streptomycin per ml. and where indicated 1 mM methionine instead of casamino acids.

(ii) For production of T4 phage using *E. coli* B as host the following medium was used (adapted from Thomas and Abelson, 1966); 100 mM Tris-HCl pH 7.4, 10 mM NaCl 1 mM $MgSO_4$, 0.64 mM KH_2PO_4, 0.2 mM $CaCl_2$, 5 µM $FeCl_3$, 20 mM glucose, 1 mM tryptophan and 2g casaminoacids (Difco) per 1.

(iii) Growth medium for *M.lysodiekticus* was 5% (w/v) Difco peptone, 0.1% (w/v) yeast extract and 0.5% (w/v) NaCl adjusted to pH 7.5 with NaOH.

(c) GROWTH OF BACTERIA

Stock cultures of *E. coli* K12 were maintained by subculturing on Dorset Egg slopes each month and stored at room temperature. Cultures used less frequently were lyophilised and used when needed. The 2 strains used routinely (TR2 and CL142) were maintained by daily subculturing into M9 liquid medium. All bacteria were grown under aerobic conditions at 37° and culture density was estimated by absorbance at 600 nm, measured in a Unicam SP-500 spectrophotometer.

The approximate relationship was established for *E. coli* K12 OD_{600} of 2.0 = 1 mg dry weight cells per ml = cell density 10^9 bacteria per ml.

MATERIALS

(i) Enzymes

Creatine phosphokinase (180 units/mg)	Boehringer
Deoxyribonuclease I (RNase free; 2200 units/mg)	
Lysozyme (egg white)	Worthington Armour Pharmaceuticals
Pronase (grade B)	Calbiochem
Ribonuclease A (3560 units/mg)	Worthington
Ribonuclease T_1 (grade III)	Sigma
RNA polymerase (2000 units/mg)	Biopolymers (gift)
Trypsin (pancreatic)	B.D.H.
Yeast Invertase	Dr. D. Kidby (gift)

(ii) Proteins

Bovine serum albumin (fraction V)	Armour Pharmaceuticals
Ovalbumin	Sigma

(iii) Antimicrobial agents

Acriflavin	Dr. M.J. Waring (gift)
Actinomycin D	Mercke & Co. (gift)
Chloramphenicol	Parke, Davis & Co.
Daunomycin	Dr. M.J. Waring (gift)
Ethiduim Bromide	"
Mitomycin C	Sigma
Nisin	Dr. L. Ingram (gift)
Nogalomycin	Dr. M.J. Waring (gift)
Novobiocin	Upjohn
2-Phenethyl alcohol	Koch-Light
Phleomycin (Lot No. 64 L 1238)	Bristol Laboratories
Polymixin	Pfizer
Streptomycin sulphate	Glaxo

(iv) Radiochemicals

(^{3}H) diaminopimelic acid	Radiochemical Centre Amersham
(^{14}C) guanosine triphosphate	"
(^{14}C) phenylalanine	"
(methyl-^{3}H) thymine and thymidine	"
(2-^{14}C) thymidine	"
(5-^{3}H) uridine	"
(^{14}C) valine	"

(v) Other chemicals

BBOT	Ciba
5-Bromouracil	Sigma
Caesium Chloride	B.D.H. Fisons Harshaw
Caesium Sulphate	B.D.H.
Calf thymus DNA	Sigma
Creatine phosphate	Boehringer
Hydroxylapatite (Biogel HTP)	Bio Rad (Calbiochem)
Mercaptoethanol	Kodak
Nucleotide triphosphates	Sigma
Polyuridylic acid (2.2 μmol /mg)	Boehringer
Sarkosyl NL (35%)	Geigy (gift)
Sephadex G_{200}	Pharmacia
Carboxymethyl (CM-C50)	"
Diethylaminoethyl (DEAE-A50)	"

All other chemicals were AnalaR grade.

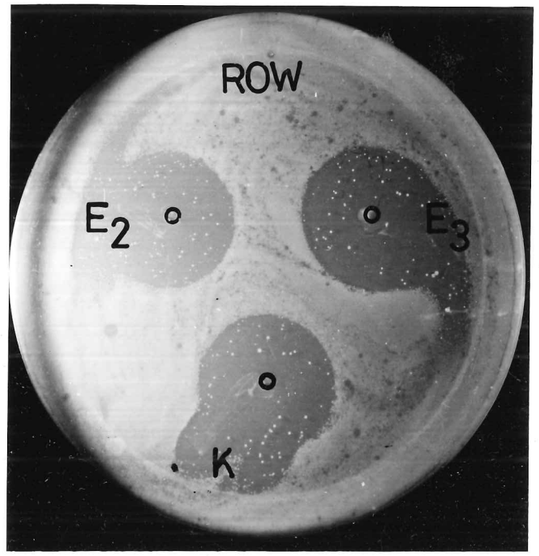
EXPERIMENTAL RESULTS AND DISCUSSION

The experimental section is divided into 6 chapters; each chapter has its own methods and results sections and chapters 3-6 have separate discussion sections. Owing to the diversity of experimental procedures and techniques used a certain amount of discussion is also included in the results sections. Specific introductions are given at the beginning of chapters 3-6 in order to give a more detailed account of previously published work relevant to the particular chapter and the reasoning behind the experimental approach. The results from all the chapters are correlated in CONCLUSIONS.

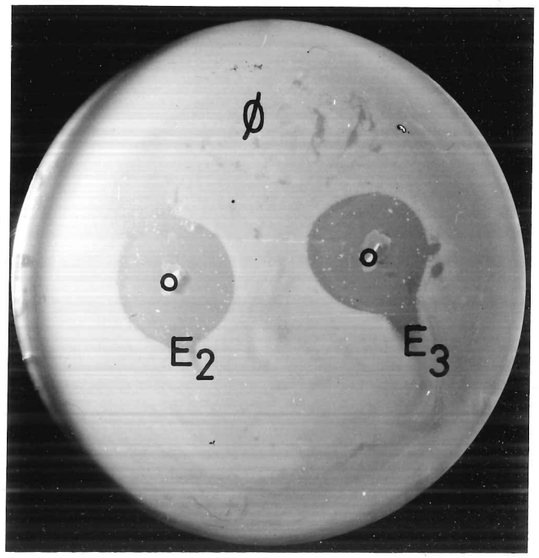
PLATE 1 i

Characterisation of col$^+$ strains by the zone method.

Colicinogenic strains were stabbed on to nutrient agar plates and incubated for 48h . The plates were then chloroformed and the indicator strain added in liquid agar using the double layer technique. The photographs were taken after a further 24h incub.



(a) Col$^+$strains TR2(E2), CL124(E3), CL131(K)
Indicator strain CL142 (ROW)



(b) Col$^+$ strains TR2(E2) & CL124(E3); Indicator strain CL18(Ø)

CHAPTER 1

Purification and Characterisation of colicin E_2-P9

In order to eliminate artefacts due to impure colicin preparations whilst investigating the biochemical effects of colicin E_2 *in vivo* and more especially *in vitro*, it was necessary to purify and characterise the E_2 protein.

METHODS 1

(a) Cross characterisation of colicinogenic strains

The methods used for detecting and characterising the biological activity of colicins are essentially the same as those described by Fredericq (1957).

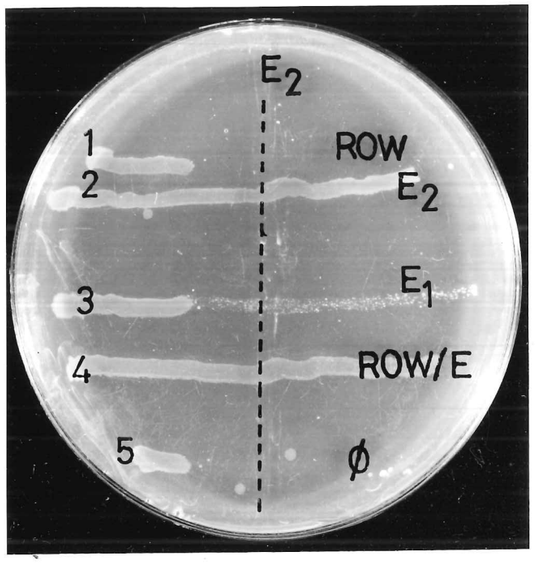
(i) Zone method

Colicinogenic strains were stabbed on to 2% (w/v) nutrient agar (Difco) plates (in glass petri dishes) and incubated for 48h at 37°. The plates were then inverted over chloroform to kill the bacteria. After removal of the chloroform, the plates were overlayered with 4 ml liquid agar (0.7% (w/v) and at 47°) containing an indicator strain in late exponential phase at approx. 10^7 bacteria per ml. Incubation was continued for a further 24h at 37°. Inhibition zones surrounding the original colicinogenic colonies were observed on the resulting confluent growth (Plates 1i(a) and (b)). The sizes of the zones caused by the diffusion of different colicins are shown in Table 1i using various indicator strains to characterise particular colicins. Colicins E_2, E_3 and K produced the largest zones, with colicins Ia and Ib sometimes being hardly detectable. Colicin E_2-P9 was characterised as an E group colicin by the resistance of indicator strain ROW/E and as E_2 by the resistance of *E. coli* col$^+$ E_2-CA42.

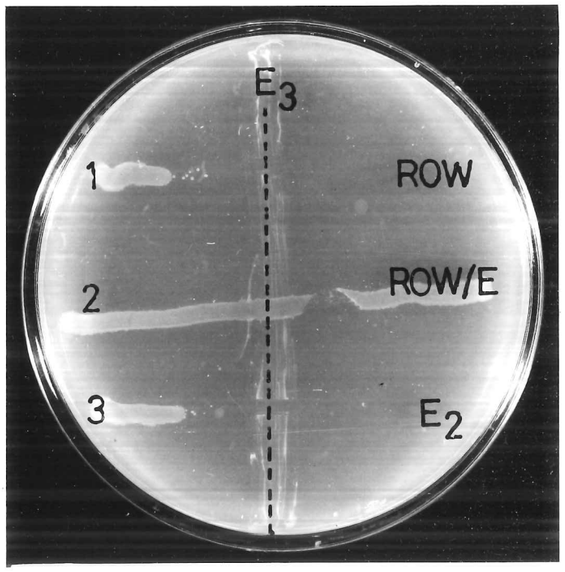
PLATE 1 ii

Characterisation of col$^+$ strains by the streak method.

A colicinogenic strain was streaked across a nutrient agar plate and incubated for 48h . The growth was scraped off and the plate chloroformed. The indicator strains were streaked across and photographs taken after a further 24h incubation.



(a) Col$^+$strain TR2(E2); Indicator strains CL142(ROW), TR2(E2), M388(E1), CL145(ROW/E) and CL18(ø).



(b) Col$^+$strain CL124(E3); Indicator strains CL142(ROW), CL145(ROW/E) and TR2(E2).

The other colicinogenic strains were all sensitive to colicin E_2 and each colicin had its own particular resistance pattern with respect to the indicator strains (Table 1i).

(ii) Streak method

A colicinogenic strain was streaked across a nutrient agar plate and incubated for 48h at 37°. The growth was scraped off and the plate sterilized with chloroform vapour for 30 min. The indicator strains were streaked across the original streak and incubation continued for a further 24h. When the indicator strain was sensitive to the particular colicin, inhibition of growth occurred where the indicator streak crossed over the colicin diffusion band (Plate 1ii). This method was only applicable to the very active colicinogenic strains (E_1, E_2, E_3 and K) and not colicins Ia and Ib (see Table 1i).

(b) Method of colicin bioassay (Nomura, 1964)

(i) A chloroform treated colicinogenic cell supernatant fluid or colicin preparation was serially diluted in sterile 10 mM phosphate buffer pH 7.0 containing 1 mg bovine serum albumin per ml (to prevent denaturation of the colicin). The colicin dilutions were spotted (by use of a loop) onto a nutrient agar plate which had been previously freshly overlayered with 4 ml liquid agar containing 200 µg streptomycin per ml and the indicator strain E. coli ROW in late exponential phase at 10^7 bacteria per ml. The plates were incubated for 16h at 37°. The colicin titre was taken as the reciprocal of the highest dilution giving minimal inhibition of growth of the indicator strain. The zones of growth inhibition resulting from the plating of serial dilutions of a preparation containing 10 µg purified colicin E_2 per ml are shown in Plate 1iii. The numbers indicate the negative logarithms of the serial dilutions, hence the colicin preparation had a specific activity of approx. 10^6 units per mg protein.

TABLE 1i

Cross characterisation of colicinogenic strains

COLICINOGENIC STRAINS	M388 E1	TR2 E2	CL124 E3	CL131 K	CL223 Ia	CL232 Ib
INDICATOR STRAINS						
CL142 (ROW)	++	+++	+++	+++	+	+
CL145 (ROW/E)	-	-	-	+	+	+
CL147 (ROW/I)	++	+++	+++	+++	-	-
M388 (COL.E1)	-	+++	+++	+++	+	+
TR2 (COL. E_2-P9)	+	-	+++	++	-	-
CL125 (COL.E_2-CA42)	+	-	+++	++	-	-
CL124 (COL.E3)	-	+	-	++		
CL131 (COL.K)	+	++	++	-		
CL223 (COL.Ia)	++	+++	+++	+++	-	+
CL232 (COL.Ib)	++	+++	+++	+++	+	-

SIZE OF INHIBITION ZONE

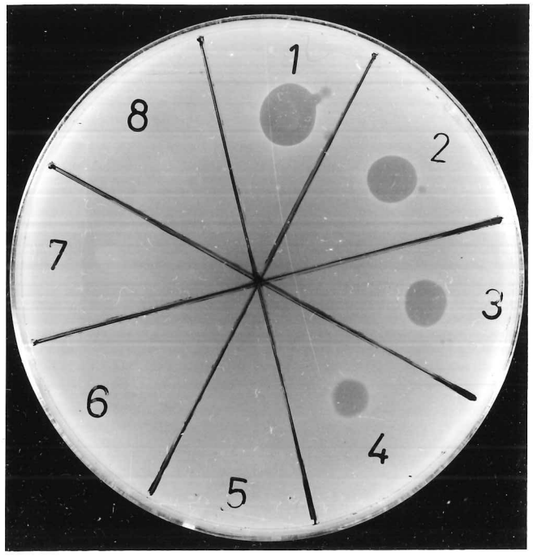
(+) Less than 5 mm.

(++) Between 5 and 10 mm.

(+++) Greater than 20 mm.

PLATE 1 iii

Bioassay of colicin E2



A solution containing 10µg colicin E2 per ml was serially diluted in 10mM phosphate buffer pH 7.0 containing 1mg bovine serum albumin per ml. The diluted samples were spotted on to a nutrient agar plate previously seeded with E.coli ROW (using the double layer technique) by means of a loop. The numbers on the plate are the negative logarithms of the dilutions. The photograph was taken after 16h incubation at 37° .

The activity of the crude culture supernatant of an E group colicinogenic strain 4h after induction was usually between 10^4 and 10^5 units per ml (see Table 1ii), colicin E_3 tending to be more active than colicin E_2. Some of the E group colicinogenic strains used (TR2 and CL124) have been reported to harbour the I Col. factor (Nomura, 1967), but this was not induced by mitomycin (Iijima, 1962) as demonstrated by the absence of inhibition zones when using strain ROW/E as indicator and undiluted induced culture supernatant from strains TR2 and CL124. Further tests also showed the absence of significant levels of other antibacterial agents and phage.

A more convenient method for assaying column or gradient fractions for colicin activity was to spot each fraction on to a previously seeded agar plate as described above and simply to measure the diameter of the growth inhibition zone after 16h incubation using a Nikon stereoscopic microscope (Model SM5) with mm squared graph paper below the agar plate (see Fig. 1iii).

(ii) A more accurate but tedious method of assay involved incubation of washed cells of the indicator strain with various colicin concentrations and estimation of the percentage of bacteria killed. The amount of colicin giving 37% survival corresponded to a multiplicity of one killing unit of colicin per sensitive cell used. The specific activity of pure colicin E_2 was 10^{13}-10^{14} killing units per mg protein.

The indicator strain was grown to a cell density of 3×10^8 bacteria per ml, harvested, washed and resuspended in M9 medium at a density of $1\text{-}3 \times 10^8$ bacteria per ml with various dilutions of colicin. The treated and control bacteria were incubated for 20 min at 37° and the number of survivors estimated by plating out suitable dilutions in sterile saline (0.85% (w/v) NaCl) on to nutrient agar. The survival curve shown in Fig. 2ii was obtained using this method and its implications will be discussed later.

(c) Quantitative determination of macromolecules

Protein was estimated using the method of Lowry et al., (1951) and bovine serum albumin as standard. DNA was estimated using the diphenylamine method of Burton (1956) and calf thymus DNA as standard. RNA was estimated using the orcinol method of Meijbaum (1939) and ribose as standard. Carbohydrate was estimated using the anthrone method of Trevelyan and Harrison (1952) and glucose as standard. Nucleic acid phosphate and free phosphate were determined by the method of Fiske and Subbarow (1925).

(d) Ultracentrifugal Analysis

Sedimentation velocity analysis was performed in a Beckman-Spinco model E ultracentrifuge equipped with schlieren optics, at 20° using one standard cell and one 1° wedge window cell to displace the schlieren image of one cell. The schlieren images were photographically recorded at intervals during centrifugation and the distance moved by the peak determined from the photographic plate using a Nikon profile projector (model 6C).

(e) Polyacrylamide gel electrophoresis

Electrophoresis was carried out using the horizontal block method (Ornstein, 1964) at pH 8.6 with a 7% (w/v) gel owing to the large molecular weights of colicins E_2 and E_3. The gel was prepared by mixing 200 ml 7% (w/v) cyanogum BDH 41 (95% acrylamide and 5% ethylene bisacrylamide) in 0.1 M tris-citrate buffer pH 8.6 with 1 ml 2-dimethyl amino ethyl cyanide and 1 ml freshly prepared 10% (w/v) ammonium persulphate. The mixture was poured into a perspex former (volume 60 ml) and 30 min was allowed for polymerisation. A discontinuous buffer system was used with 0.1 M borate buffer pH 8.6 in the electrode tanks and tris-citrate buffer in the gel. Electrophoresis of 20 µl samples was carried out at 4° for 4h using a current density of 2.8 mA/cm^2.

When it was necessary to carry out electrophoresis in the presence of 8 M urea, the gel was prepared in the normal way and then allowed to equilibrate overnight with a solution of 8 M urea in tris-citrate buffer since polymerisation in the presence of urea inhibits cross link formation.

After electrophoresis the proteins were stained with a 1% (w/v) solution of amido-black in a water:methanol:glycerol:acetic acid (50:50:20:1) mixture for 10-15 min and excess stain removed by washing with 1% (v/v) acetic acid.

For disc electrophoresis potassium ferricyanide (5 mM) was used to retard gel polymerisation and the 6% gel mixture (Herschman and Helinski, 1967.a) was deaerated before addition of the initiator ammonium persulphate. A 0.1 ml protein sample was introduced at the top of the gel rod (cathode end) in a 10% (w/v) solution of sucrose containing a bromophenol blue marker. Electrophoresis was for 2-3h at 20° using a potential difference of 200V.

(f) UV spectroscopy

The UV spectra of column fractions were determined using the Cary 15 automatic recording spectrophotometer at 20° using 1 cm quartz cells that were "multipotted" against water blanks.

(g) Gel filtration

This method was used in the determination of the molecular weight of colicin E_2. 4g G200 sephadex (Pharmacia) was swollen in 50 mM tris-HCl buffer pH 7.5 containing 100 mM KCl (Andrews, 1964 and 1965) for 3 days with continuous stirring. On each successive day the gel suspension was boiled for 1h. Fines were removed by decantation five times with the loss of about one third of the original dry

weight of sephadex. The gel suspension was slowly dripped into a 2 x 40 cm chromatography column filled with buffer and the sephadex particles allowed to sediment on top of a layer of G25 sephadex followed by ballotini beads over the glass sinter at the column base. These secondary layers were necessary to prevent column blockage by the small G200 sephadex particles. When the sephadex column was approx. 30 cm high, a glass fibre filter disc was floated onto the top of the bed to avoid disturbance of the sephadex particles during addition of the sample. The packing procedure was performed under conditions of minimum pressure at 4° and the flow rate was 10 ml/h during particle sedimentation. The column was used after allowing it to settle for 48h. The maximum flow rate was 30 ml/h but this was restricted to 10 ml/h by means of a peristaltic pump (LKB Instruments Ltd.,) in order to decrease the band width of eluted samples. UV absorbing material was eluted through the column and 1 ml fractions collected after passing through a UVicord (LKB Instruments Ltd.,) recording spectrometer (operating at 254 nm). The eluant volume of a particular sample was taken as the volume of buffer eluted from the column between addition of the sample and the appearance of the peak maximum obtained on the UV tracing or by bioassay in the case of colicin. The column void volume (V_o) was found to be 31 ml using blue dextran 2000 and the total column volume (V_t) was 89 ml using the peptide antibiotic polymixin.

(h) Isolation of colicinogenic factor E_2

The procedure was adapted from the method due to Bazaral and Helinski (1968).

Exponentially growing cells of *E. coli* col^+ E_2 (strain TR2) were harvested, washed and resuspended in 20 ml of fresh M9 medium containing 10 µCi (methyl-^{3}H) thymine (specific activity 1 Ci/mmol)

per ml and 200 µg deoxyadenosine per ml, at a cell density of 2×10^8 bacteria per ml. The culture was incubated at 37° under aerobic conditions for 2-3 generation times. The cells were harvested by centrifugation, washed with NET buffer * at 4° and resuspended in 1.5 ml NET buffer containing 25% (w/v) sucrose and 1 mg lysozyme per ml. The cell resuspension was incubated for a further 10 min at 37° followed by addition of 0.5 ml 2% (w/v) sarkosyl detergent to complete cell lysis. A further 1 ml NET buffer was added and the viscous lysate sheared by repeatedly drawing through a 1 ml pipette followed by short vortex mixing.

2 ml sheared lysate was mixed with 13.1g CsCl, 7.6 ml water and 4.0 ml fresh ethidium bromide solution (1 mg/ml in 0.1 M phosphate buffer pH 7.0). Two 8 ml aliquots were transferred to two 13 ml polyallomer centrifuge tubes and overlayered with sufficient liquid paraffin to fill the tubes. Samples were centrifuged in a Ti-50 fixed angle rotor at 20° for 44h at 44,000 rev/min using a Beckman Spinco model L2 ultracentrifuge. After centrifugation 0.15 ml fractions were collected by carefully inserting a long canular needle down through the liquid paraffin layer to the tube bottom and pumping out the tube contents using an LKB peristaltic pump operating at 80 ml/h. Fraction collection by piercing the tube was found extremely difficult with polyallomer tubes also CsCl solutions were found to flow out too fast to count the drops. This last method was therefore not attempted.

(i) Optical Rotatory Dispersion

ORD measurements were made using a Bendix-Ericsson Polarmatic 62 Recording Spectropolarimeter. The conditions are described in Methods 3.

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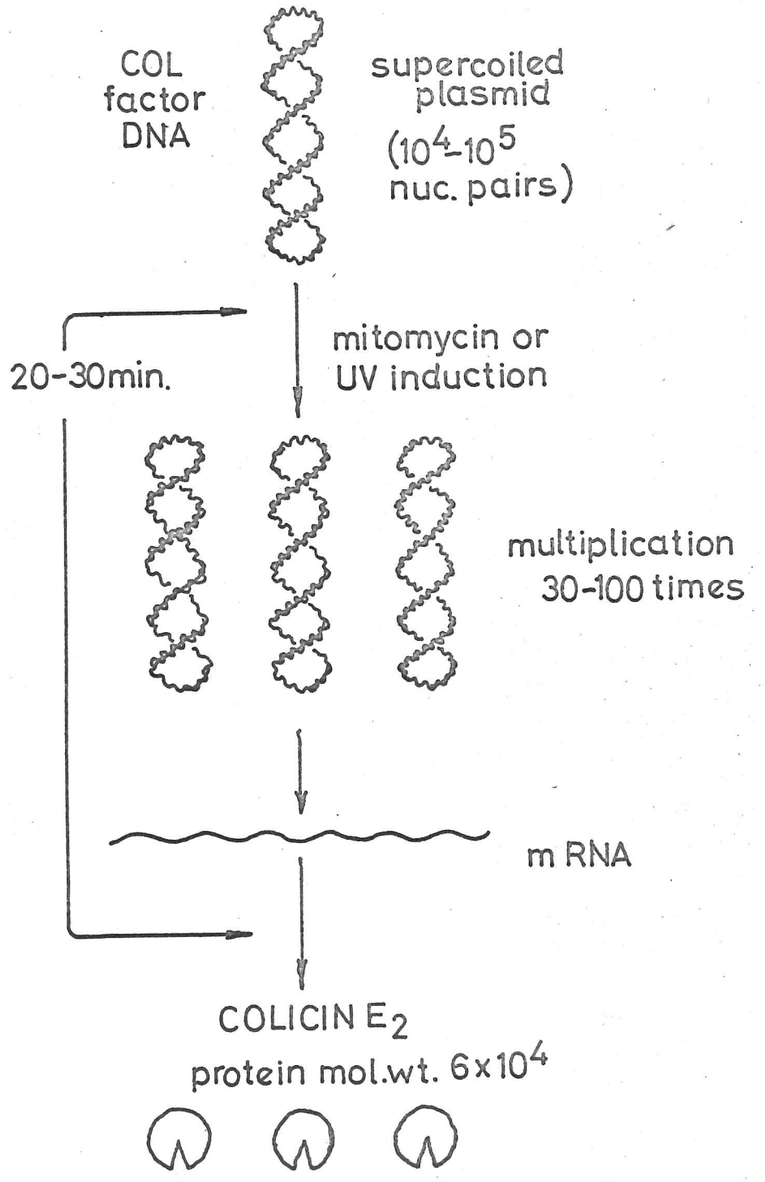
* NET buffer (Smith and Hanawalt, 1967) contains 10 mM tris-HCl pH 8, 10 mM EDTA and 100 mM NaCl.

FIG. 1 i



Induction of colicinogenic E.coli

RESULTS 1

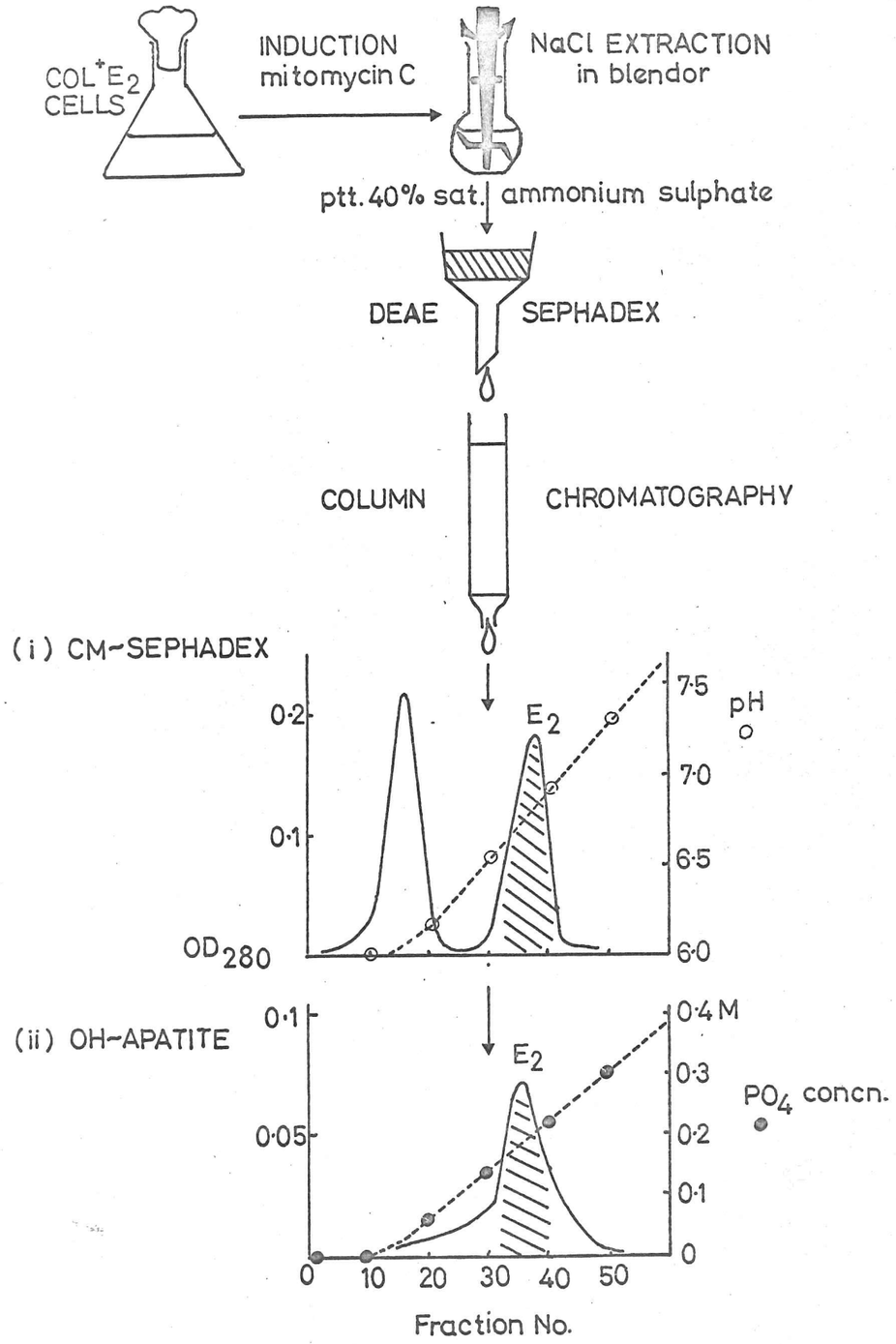
(a) Induction and purification of colicin E_2-P9

Production of colicin E_2, like prophage, can be induced with certain agents active on DNA metabolism (see General Introduction and Fig. 1i). The methods used for the purification of colicin E_2 fall into three distinct catagories. The first, used by Fredericq (1957) and Holland (1968), relies on the release of colicins from the colicinogenic bacteria into the medium after approx. 4-6h incubation under aerobic conditions at 37°. The dying cells are removed by centrifugation and colicin purified from the supernatant fluid. This method suffers from the disadvantages that the volumes involved for large scale production are inconvenient for ammonium sulphate precipitation and that after 4-6h incubation of colicinogenic cells after induction, cell lysis frequently occurs thus contaminating the supernatant protein.

The second method was developed by Herschman and Helinski (1967.a) and was used in an adapted form in this work. This involves incubation of the induced colicinogenic bacteria for approx. 2h under anaerobic conditions and salt extraction of the colicin from the outer surface of the cell wall after harvesting. Herschman and Helinski (1967.a) found that under anaerobic conditions colicin E_2 synthesis was unaffected but bacterial growth was inhibited. Therefore the initial specific activity of colicin is not reduced by excessive continued synthesis of other cell proteins. Also by using short post-induction incubation times, most of the colicin activity (90%) remains attached to the cell surface and can be conveniently extracted for ammonium sulphate precipitation by using molar salt solutions after harvesting the cells by centrifugation. In the studies described below (see Table 1ii) approx. 70-80% colicin activity remained associated with the cells after 2h incubation.

FIG. 1 ii

PURIFICATION OF COLICIN E_2



The third method was developed largely for use on very small scale colicin production (Herschman and Helinski, 1967.b) for example in the preparation of radioactively labelled colicins E_2 and E_3 (Maeda and Nomura, 1966). This simply involves EDTA-lysozyme-SDS lysis of the induced cells after 2-3h incubation and hydroxylapatite chromatography of the lysate.

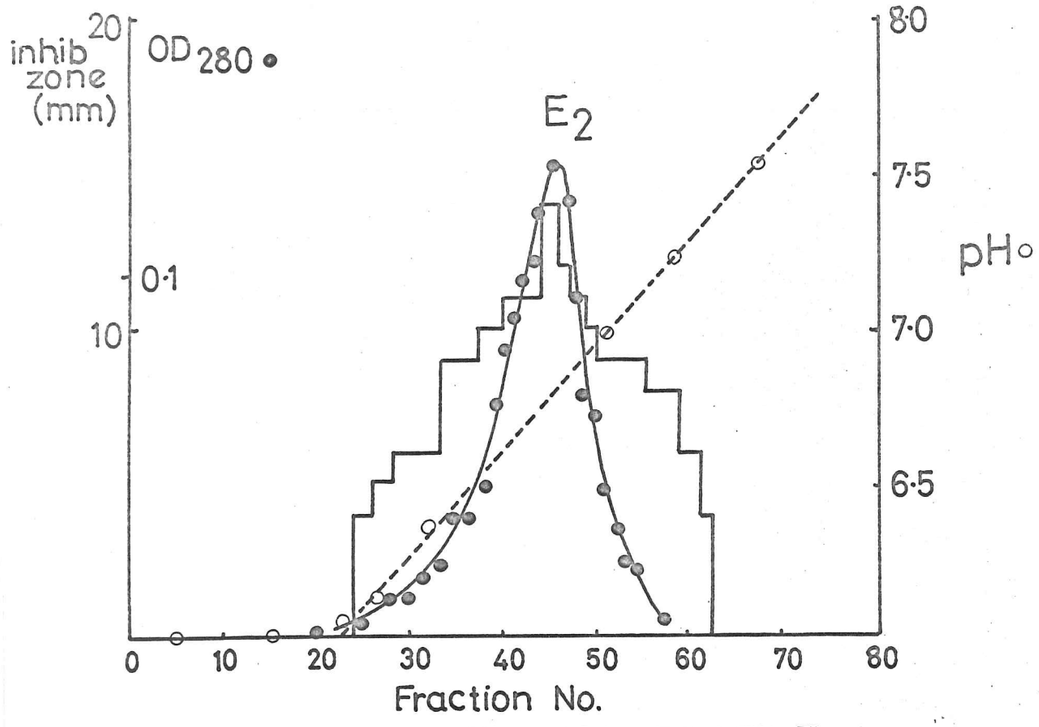
The extraction and purification of colicin E_2 used in this work involved the second extraction method described above and a combination of the purification methods of Holland (1968) and Herschman and Helinski (1967.a), see Fig 1ii. All operations except the post-induction incubation were performed at 0-4° and are described below.

Mitomycin C (final concentration 0.4 µg/ml) was added to an exponentially growing culture of the colicinogenic strain TR2 ($col^+ E_2$) when the cell density was 4×10^8 bacteria per ml and the incubation continued for a further 2h at 37°. The bacteria were harvested by centrifugation and the colicin was extracted by resuspending the cells at a density of $5 \times 10^{10} - 10^{11}$ bacteria per ml in 10 mM phosphate buffer pH 7.0 containing 1 M NaCl and blending at low speed for 15-20 min in a MSE microblendor (using the 80 ml flask attachment). This procedure was repeated twice. The saline washings were combined and precipitated routinely with ammonium sulphate at 40% saturation.

The yields and distribution of activities on ammonium sulphate fractionation are shown in Table lii. 60-80% of the total activity appeared in the first two fractions.

The precipitate was resuspended in 10 mM phosphate buffer pH 7.0 and dialysed overnight against the phosphate buffer. The protein solution was then centrifuged at 48,000g for 15 min to remove a fine precipitate that formed during dialysis. This was followed by mixing with pre-equilibrated DEAE sephadex at 4° for 30 min in standard phosphate buffer so as to remove the more acidic proteins and nucleic acids (see OD280/260 ratio in Table lii). Colicin was not adsorbed under these conditions and appeared in the filtrate after filtering the DEAE sephadex suspension through a filterpad in a Buchner funnel. The DEAE sephadex was prepared by first allowing it to swell in distilled water and then washing with 0.5 N NaOH on a Buchner funnel until free of chloride. Excess NaOH was removed by rinsing with distilled water and the DEAE sephadex allowed to equilibrate in excess standard phosphate buffer until pH 7.0 was attained. The equilibration procedure took 3-4 days using 1-2g DEAE sephadex (approx. amount for 5l original culture). Inadequate preparation of the DEAE sephadex did not remove contaminating 260 nm absorbing material and sometimes reduced the specific activity of the colicin.

After dialysis and centrifugation, the DEAE sephadex filtrate was reduced in volume using Carbowax 20M (approx. 2h). This had advantages over vacuum distillation in that it was performed at 0° and caused less protein denaturation. The concentrated preparation (approx. 10 ml) was further dialysised against 0.05 M phosphate buffer pH 6.0 and then applied to a CM-sephadex column (2 x 10 - 15 cm), equilibrated with the same buffer. The column was eluted with an increasing pH gradient formed by mixing equal volumes (2 x 100 - 150 ml) of 0.05 M phosphate buffer pH 6.0 and 0.05 M K_2HPO_4 (pH 8.8).



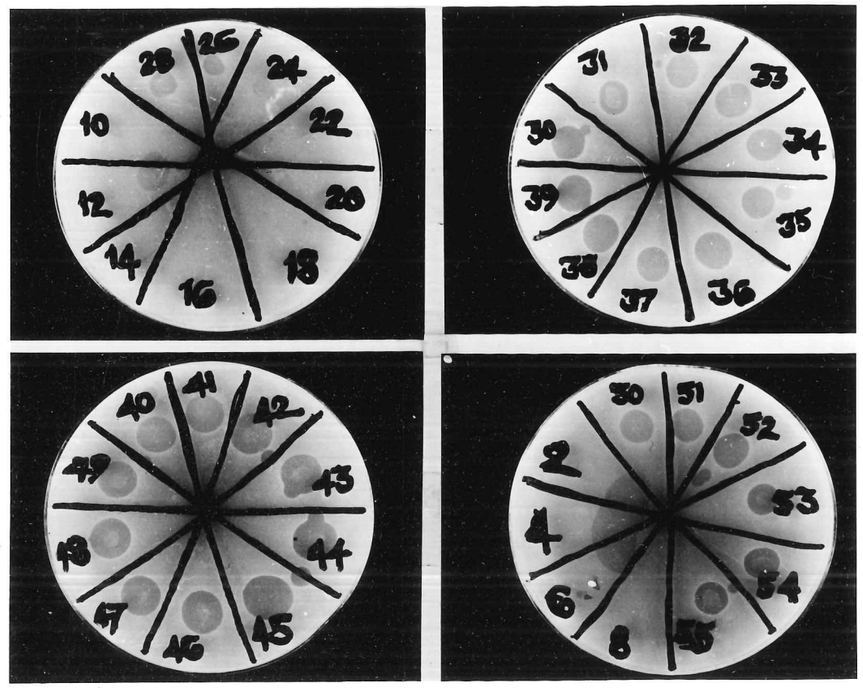
CM SEPHADEX ELUTION PROFILE

FIG. 1 iii

After treatment of the dialysed saline cell extract from strain TR2 with ammonium sulphate and DEAE sephadex, the protein solution was concentrated with carbowax and applied to a CM sephadex column. The proteins were eluted by an increasing pH gradient formed by mixing 0.05M phosphate buffer pH 6.0 and 0.05M K_2HPO_4. The column fractions were tested for growth inhibitory activity (see PLATE 1 iv) and the inhibitory zone size plotted. The OD_{280} of the fractions is also plotted.

PLATE 1 iv

Bioassay of CM Sephadex column fractions



The fraction numbers correspond to those in FIG. 1 iii and the inhibition zone diameters are plotted in FIG. 1 iii.

TABLE liii

Purification table for colicin E_2-P9

	Volume (mls)	Total Activity (units) x 10^{-7}	Protein (mg)	Specific Activity (units/mg) x 10^{-5}	Yield %	OD_{280}/OD_{260}
Induced Culture Supn.	5000	4.0	230	1.7	-	-
Cells [a]		11.8	1550	0.76	100	
Saline Extract	45		102			
	35		61			
	30		18			
total	110	6.6	181	3.7	56	0.63
Ammonium sulphate ptt.						
0-20	40	1.2	18	6.7		
20-40	44	4.4	72	6.1		
40-60	40	0.4	23	1.7		
60-80	25	0.01	5	0.2		
Dialysis and centrifugation [b]	115	4.6	71	6.5	39	0.87
DEAE Sephadex	105	4.2	52	8.0	36	1.36
CM Sephadex	60	3.0	24	12.5 [c]	25	1.45
HO-Apatite	65	0.9	13	7.0	8	1.46

a. Values derived from cell lysate aliquot.

b. Ammonium sulphate fractions 0-20 and 20-40% satn. were combined, dialysed and centrifuged.

c. 16.5 fold purification with respect to cell lysate.

Colicin activity was detected as described in Methods 1 and Fig. liii and was eluted at a pH value of 6.80 (see Herschman and Helinski, 1967.a).

CM sephadex (approx. 1 g) was prepared similarly to DEAE sephadex except that after swelling in distilled water the ion exchanger was treated with 0.5 N HCl and equilibrated for 3 days with changes of 0.05 M phosphate buffer pH 6.0.

The results of the purification procedure are shown in Table liii. The protein associated with the colicin activity peak on CM sephadex was characterised by the methods described in the following section. A 16.5 fold purification was achieved with respect to the total cell protein and the yield was 25%. The specific activity of colicin preparations was of the order of 10^6 units per mg protein.

Further purification and characterisation was attempted using hydroxylapatite columns (Maeda and Nomura, 1966; Holland, 1968). Commercially prepared hydroxylapatite was mixed as a slurry with successive changes of 10 mM phosphate buffer for 2 days. The slurry was allowed to gently sediment in a chromatographic column filled with phosphate buffer. The column (2 x 8 cm) was then eluted with 2 column volumes of phosphate buffer and the colicin sample applied immediately. Hydroxylapatite columns were slow running if allowed to stand for more than 1 day before use. Removal of fines and column packing under low pressure was essential. Cellite(diatomaceous earth) was mixed with hydroxylapatite initially to increase the eluant flow but considerable colicin inactivation resulted. Protein was eluted from the column using a linear gradient of 0.01 M to

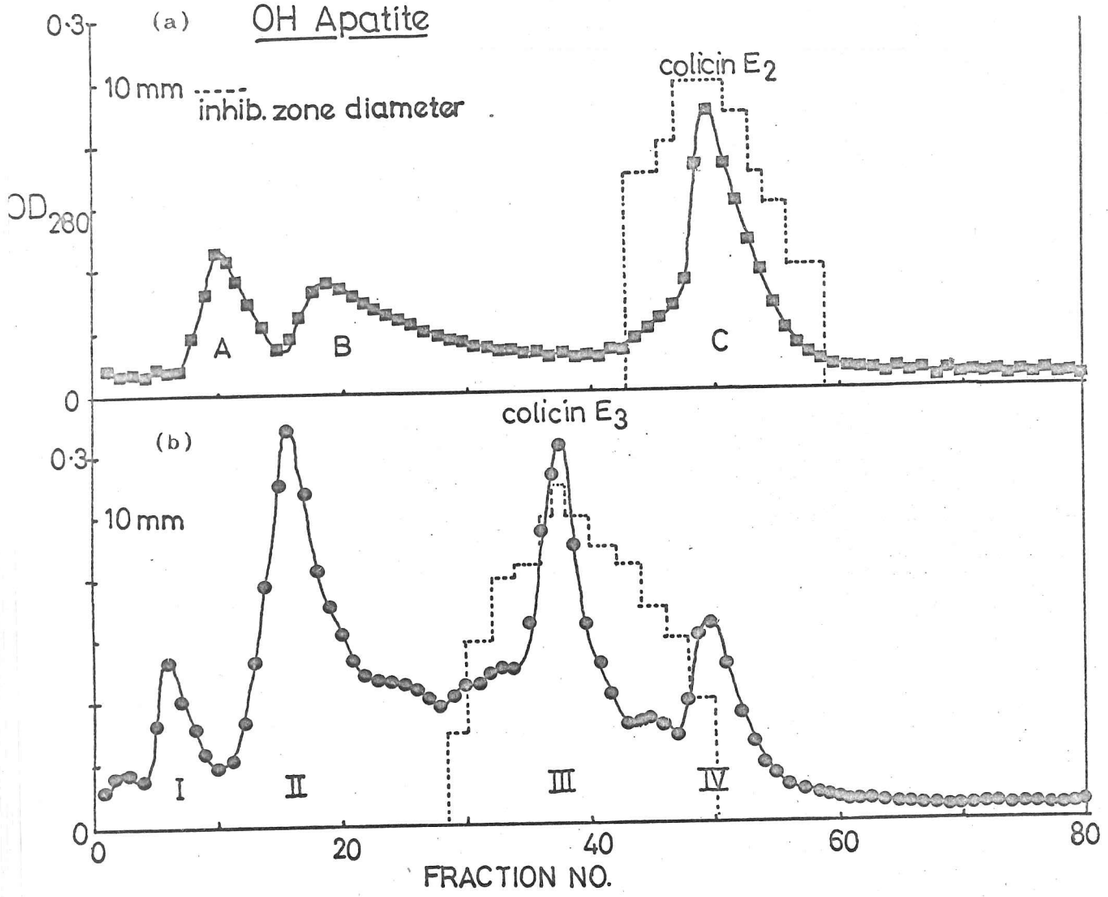


FIG. 1 iv

Hydroxylapatite elution profile of colicin E2 and E3

The ammonium sulphate precipitates of saline extracts from strains TR2 and CL124 were dialysed, concentrated and applied to hydroxylapatite columns. Proteins were eluted by an increasing phophate gradient formed by mixing 10mM phosphate buffer pH 7.0 with 400mM phosphate buffer pH 7.0 . The growth inhibitory activity and OD_{280} of the fractions were determined.

PEAK	FRACTION NOS.
A	7 - 14
B	17 - 25
C	44 - 56
I	4 - 9
II	12 - 20
III	34 - 42
IV	47 - 54

0.40 M phosphate buffer pH 7.0. Colicin activity was eluted at 0.18 M phosphate (see Holland, 1968) as detected by the method of Fiske and Subbarow (1925), see Fig. 1ii. No further purification was achieved and the specific activity usually decreased from that obtained after CM sephadex ion exchange chromatography (Table 1ii). This step was therefore left out.

Initial studies showed that if after ammonium sulphate precipitation and dialysis the colicin preparation was applied directly to an hydroxylapatite column (Fig. 1iv(a)) the colicin activity peak (c) ran in the same position as some 260 nm absorbing material, (the colicin peak fraction had an OD $^{280}/_{260}$ ratio of 0.8 - 1.0). The method of Holland (1968) was similar to this initial method except that DEAE cellulose was used prior to hydroxylapatite.

(b) Induction and Purification; colicin E_3-CA38

Colicin E_3 was purified for later comparison with colicin E_2 (see Chs. 2 and 3). In contrast to colicin E_2 production, Herschman and Helinski (1967.a) found that colicin E_3 synthesis was inhibited by anaerobic conditions and the cells started to lyse 1-2h after mitomycin induction. The procedure for colicin E_2 production was therefore modified for colicin E_3 by incubating the mitomycin induced cells (strain CL 124) for only 1 h under aerobic conditions. The cells were extracted with M NaCl in 10 mM phosphate buffer pH 7.0 as described for colicin E_2 and the dialysed ammonium sulphate fraction (0-40%) applied directly to a freshly prepared hydroxylapatite column. The elution procedure was the same as above and the elution profile is shown in Fig. 1iv(b). Colicin E_3 activity was eluted earlier than E_2 and corresponded to peak III (Fig. 1iv(b)). Peak IV however had a low OD $^{280}/_{260}$ ratio (0.5 - 0.7) and was in the same

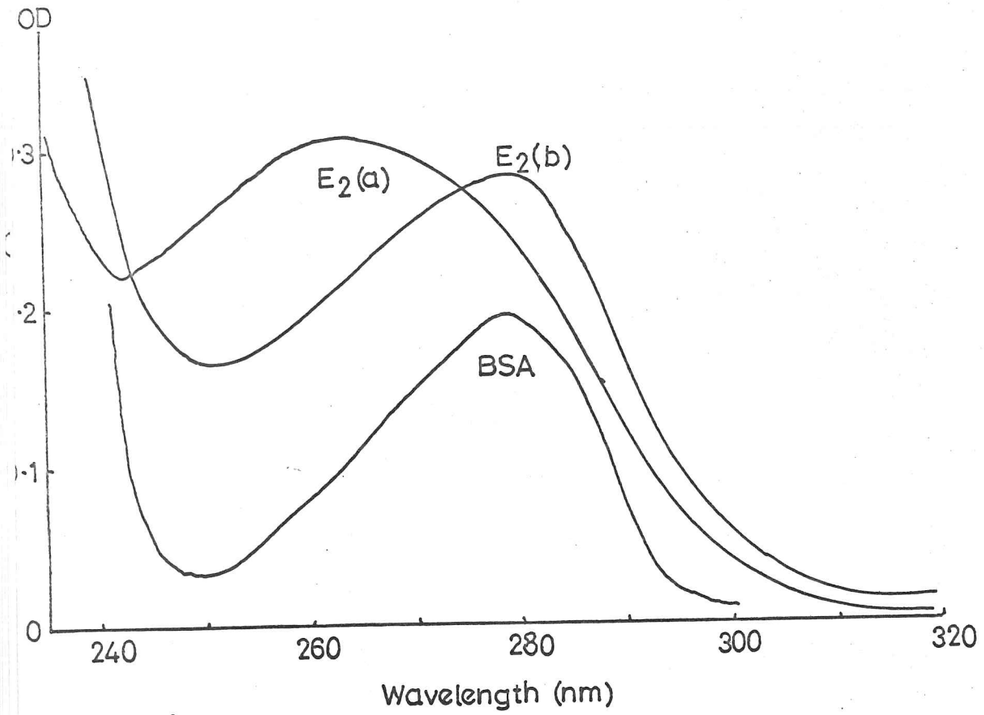


FIG. 1 v

Absorption spectra of colicin E2 preparations

Preparation E_2(a) is fraction C (FIG. 1 iv) after hydroxylapatite chromatography.

Preparation E_2(b) is the colicin activity peak (fractions 38-50, FIG. 1 iii) after CM sephadex chromatography.

BSA is bovine serum albumin.

TABLE 1 iii

Chemical analysis of colicin E_2-P9 preparations

	Prepn. a	Prepn. b
Protein	300 µg/ml	350 µg/ml
DNA	20 "	< 2 "
RNA	< 2 "	< 2 "
Carbohydrate	< 4 "	< 4 "

Macromolecules were determined as described in Methods 1. Prepn. a was fraction C after hydroxylapatite chromatography (Fig. 1 iv) and prepn. b was the colicin activity peak (fractions 38-54, Fig. 1 iii) after DEAE and CM sephadex chromatography.

position as the colicin E_2 peak with respect to the phosphate gradient, (Fig. 1ii). This was probably the contaminating material obtained in colicin E_2 prepared by the hydroxylapatite procedure. The large amount of other protein shown in Fig. 1iv was probably due to the susceptibility of col^+ E_3 cells to lysis after induction. The yield of colicin E_3 (peak III) from 21 original culture was 11 mg (protein).

(c) Characterisation of colicin E_2-P9

The final colicin preparation after CM sephadex chromatography was shown to be pure by sedimentation analysis and polyacrylamide gel electrophoresis, and characterised by determination of its molecular weight, sedimentation value and α helical content.

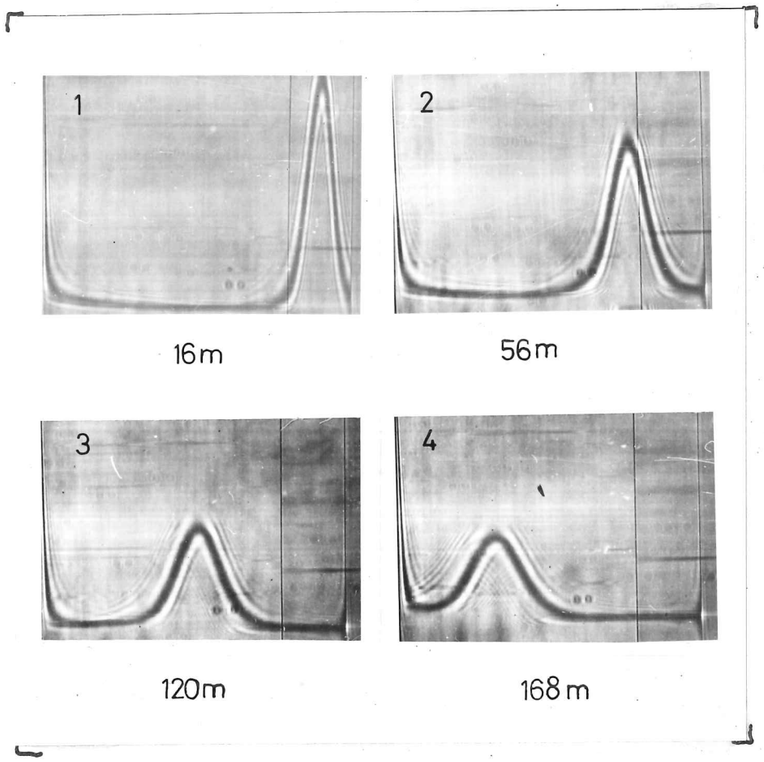
(i) Chemical analysis

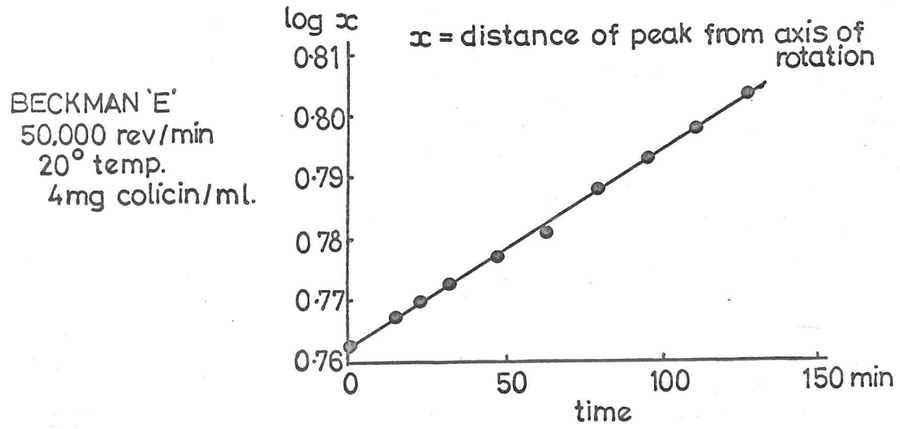
Colicin E_2-P9 preparations after CM sephadex chromatography were analysed for the presence of nucleic acid and carbohydrate because certain methods of purification (hydroxylapatite) produced varying amounts of 260 nm absorbing material associated with the colicin E_2-P9 fractions (see Fig. 1v) and it has been reported that carbohydrate is a component of colicin F (E_2-CA42), (Reeves, 1963). CM sephadex purified colicin E_2-P9 was shown to contain less than 1% nucleic acid and carbohydrate (Table 1iii).

(ii) Ultracentrifugal Analysis

A purified colicin preparation (4 mg protein per ml) was centrifuged in 10 mM phosphate buffer pH 7.0 and the schlieren pictures are shown in Fig. 1 vi. No contaminating peaks were observed and the colicin peak was symmetrical. The sedimentation coefficient was determined at concentrations of 2, 4 and 6 mg protein/ml and was found to be equal to 4.2 ± 0.2. There was no observable effect due to

FIG. 1vi Velocity Sedimentation of COLICIN E_2





$$S_{20w} = \frac{2.303 \log x_1/x_2}{\omega^2 (t_2 - t_1)} = 4.2$$

Sedimentation analysis of colicin E2 was carried out in a Spinco model E analytical ultracentrifuge. The sedimentation coefficient is given by the above equation, ω is the angular velocity.

concentration (see Herschman and Helinski, 1967.a). In order to calculate the sedimentation coefficient of a colicin preparation, the distances moved by the schlieren peak from the axis of rotation were determined at intervals. The log. of these distances (cm) was plotted against time (Fig. 1 vi) and the slope of the line determined. The sedimentation coefficient (S_{20w}) was given by dividing this slope by the square of angular velocity of the rotor and multiplying by 2.303 (Fig. 1 vi). The value obtained compares well with that found by Herschman and Helinski (1967.a), i.e. S°_{20w} = 4.0 ± 0.1 for colicin E_2.

(iii) Polyacrylamine gel electrophoresis

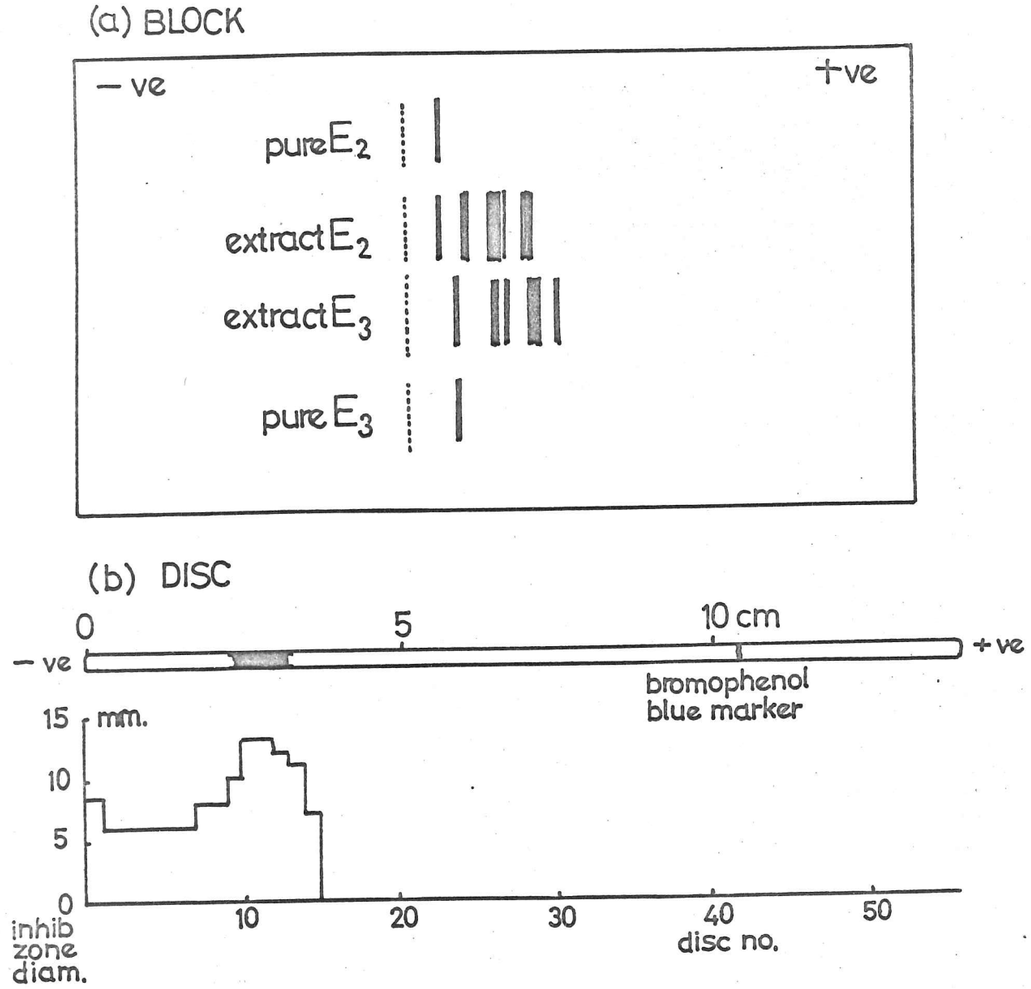
As a further test for purity, purified colicin E_2 and fraction III of colicin E_3 (Fig. 1 iv(b)) were electrophoresed with dialysed saline extracts of the original cultures (Fig. 1 vii(a)). Colicin E_3 was more electronegative than colicin E_2. This result is consistent with the isoelectric point determinations of Herschman and Helinski (1967.a). No contaminating proteins were observed even when 100 µg colicin E_2 was electrophoresed.

Attempts were made to demonstrate possible sub-unit structure of colicin E_2 by electrophoresis of the protein in the presence of 8 M urea. Pretreatment of colicin E_2 with dithiothreitol (10 mM) and 8 M urea was also tried to detect possible S-S bridged subunits as found in the gamma globulins. There was no detectable shift in band position of the treated samples from the control except for a slightly increased diffuseness and the investigation was not continued.

In addition colicin E_2 preparations were subjected to disc gel electrophoresis at pH 8.6 in an attempt to observe the apparent "isocolicin" phenomenon of colicin E_2 described by Herschman and

FIG. 1 vii

POLYACRYLAMIDE GEL ELECTROPHORESIS



(a) Samples of crude and purified preparations of colicins E2 and E3 were electrophoresed for 4h in a discontinuous buffer system of tris-citrate and borate at pH 8.6 .

(b) A pure preparation of colicin E2 was electrophoresed for 3h in the same buffer system as (a). The gel was cut in two, one half was stained for protein and the other half cut into discs and colicin activity determined.

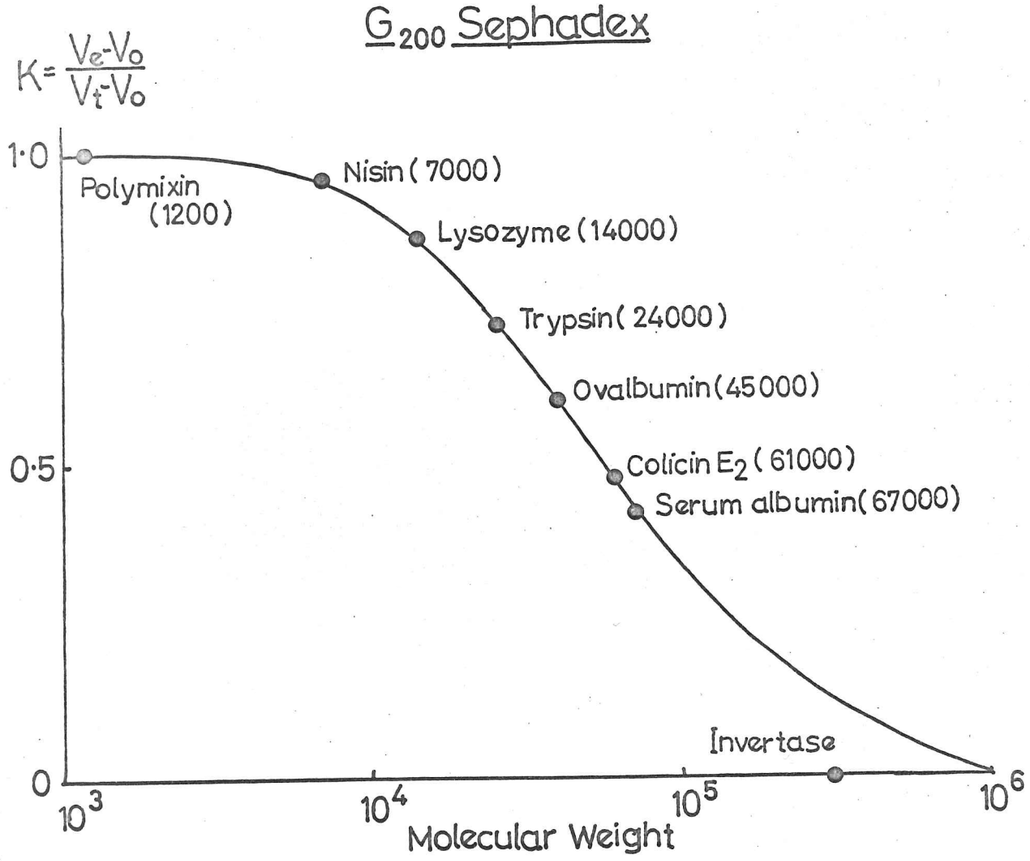
Helinski (1967.a). After electrophoresis colicin activity was located by cutting the gel rod into two along its length and placing 3 mm thick discs cut serially from the half-rod on agar plates previously seeded with E. coli ROW. The inhibition zones were measured after incubation of the plates at 37° for 16h. The other half of the rod was stained and the only protein band coincided with the peak of colicin activity (Fig. 1 vii(b)). There was however no detectable bifurcation of the protein band or activity peak.

(iv) Gel filtration

The molecular weight of colicin E_2 was determined using the method of Andrews (1964 and 1965). A G_{200} sephadex column was prepared (see Methods) and calibrated with proteins of known molecular weights (Fig. 1 viii). The elution volumes and K values were determined as described in Methods and Fig 1 viii and the K values plotted against the log. of the molecular weight. For small protein molecules of less than 10^4 molecular weight K was equal to unity, i.e. the elution volume was equal to the total column volume. The relationship of K to the log. of molecular weight was approximately linear for proteins of molecular weight from 2×10^4 to 10^5. The theoretical molecular weight of pure protein at which K was zero was 10^6 and for pure polysaccharide 2×10^5. Yeast invertase, which was believed to have an approximate molecular weight of 3×10^5 (Lampen, 1968), had an elution volume equal to the void volume (K = 0). This was therefore consistent with the observation that this enzyme contained approximately 50% carbohydrate.

Preparations of colicin E_2 and colicin E_3 gave elution volumes of 58 and 59 ml respectively. The variation between duplicate determinations of all standards and colicins was no more than ± 1 ml with respect to the elution volumes. The molecular weights of the two colicins were determined from Fig. 1 viii to be 61,000 ± 4,000

FIG. 1 viii



Determination of protein molecular weight by Gel Filtration

Proteins of known molecular weight were eluted through a G200 sephadex column and their elution volumes Ve determined. The constant K was calculated using the values of 31ml for the void volume Vo and 89ml for the total volume Vt, and plotted against the log protein molecular weight.

and 58,000 ± 4,000 respectively. This method however assumes that the proteins are 100% pure and globular. The consistency of these values with those obtained by Hershman and Helinski (1967.a) using the Yphantis short column technique would suggest that this assumption was justified.

On a few isolated occasions with different colicin E_2 preparations an additional activity peak was observed corresponding to a protein with a molecular weight of approx. 180,000. This peak was small and diffuse compared with the main 60,000 molecular weight peak and was not consistently reproducible. It could perhaps be interpreted as colicin E_2 trimer formation at high concentrations, however polyacrylamide gel electrophoresis did not detect a similar phenomenon.

(v) Optical rotatory dispersion

The helical content of colicin E_2 was determined from its ORD spectrum (Ch. 3 and Fig. 3 vi). Two methods were used (Fasman, 1963); the first and most accurate method involved measurement of the "b_o" value by use of the Moffitt equation:

$$(m')_\lambda = \frac{a_o \lambda_o^2}{\lambda^2 - \lambda_o^2} + \frac{b_o \lambda_o^4}{(\lambda^2 - \lambda_o^2)^2}$$

(Moffitt and Yang, 1956)

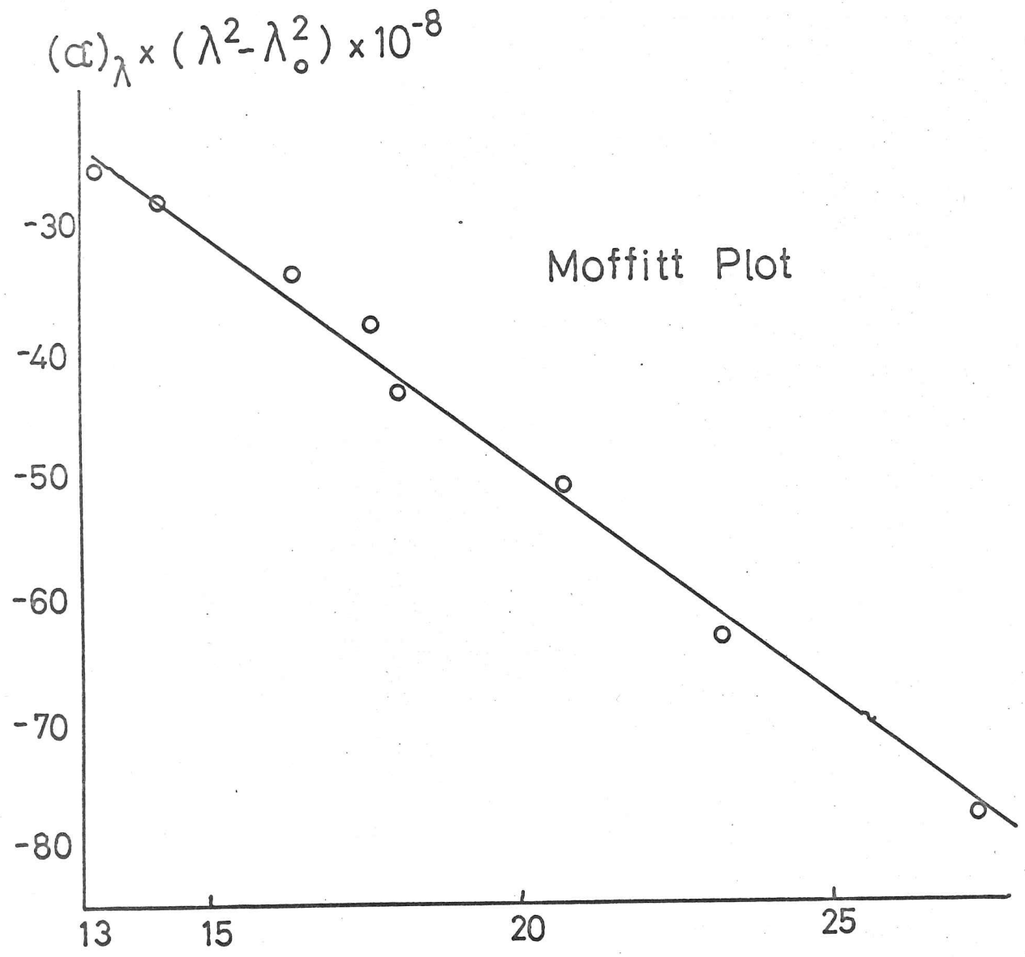
a_o, b_o and λ_o are constants. $(m')_\lambda$ is the reduced mean residue rotation at wavelength λ. b_o and λ_o are functions of the helical backbone and a_o is a function of environment.

$$(m')_\lambda = \frac{3}{(n^2+2)} \cdot \frac{MRW}{100} \cdot (\alpha)_\lambda$$

MRW is the mean residue weight (= 115 for most proteins)

n is the refractive index of the solvent at wavelength λ

FIG. 1 ix



$$b_o = \frac{\text{slope}}{K.\lambda_o^4} = -108$$

ie. Colicin E_2 helical content
=17%

Determination of colicin E2 helical content

The Moffitt plot was obtained by measuring the degree of specific rotation $(\alpha)_\lambda$ caused by colicin E2 at certain wavelengths λ from the ORD spectrum (FIG. 3 vi) . K and λo are constants (see text). b_o is -630 for a protein of 100% helical content.

$(\alpha)_\lambda$ is the specific rotation and is derived from the observed rotation (Fig. 3 ii) see Ch. 3.

Therefore if $(\alpha)(\lambda^2-\lambda_o^2)$ is plotted against the reciprocal of $(\lambda^2-\lambda_o^2)$ a straight line should be produced with a slope equal to: $b_o / K.\lambda_o^4$

Where $K = \frac{100}{MRW} \cdot \left(\frac{n^2+2}{3}\right)$

λ_o was taken to be equal to 216 nm (Fasman, 1963) and the straight line plot is shown in Fig. 1 ix. The slope was found to be equal to -3.77 and hence b_o = -108.

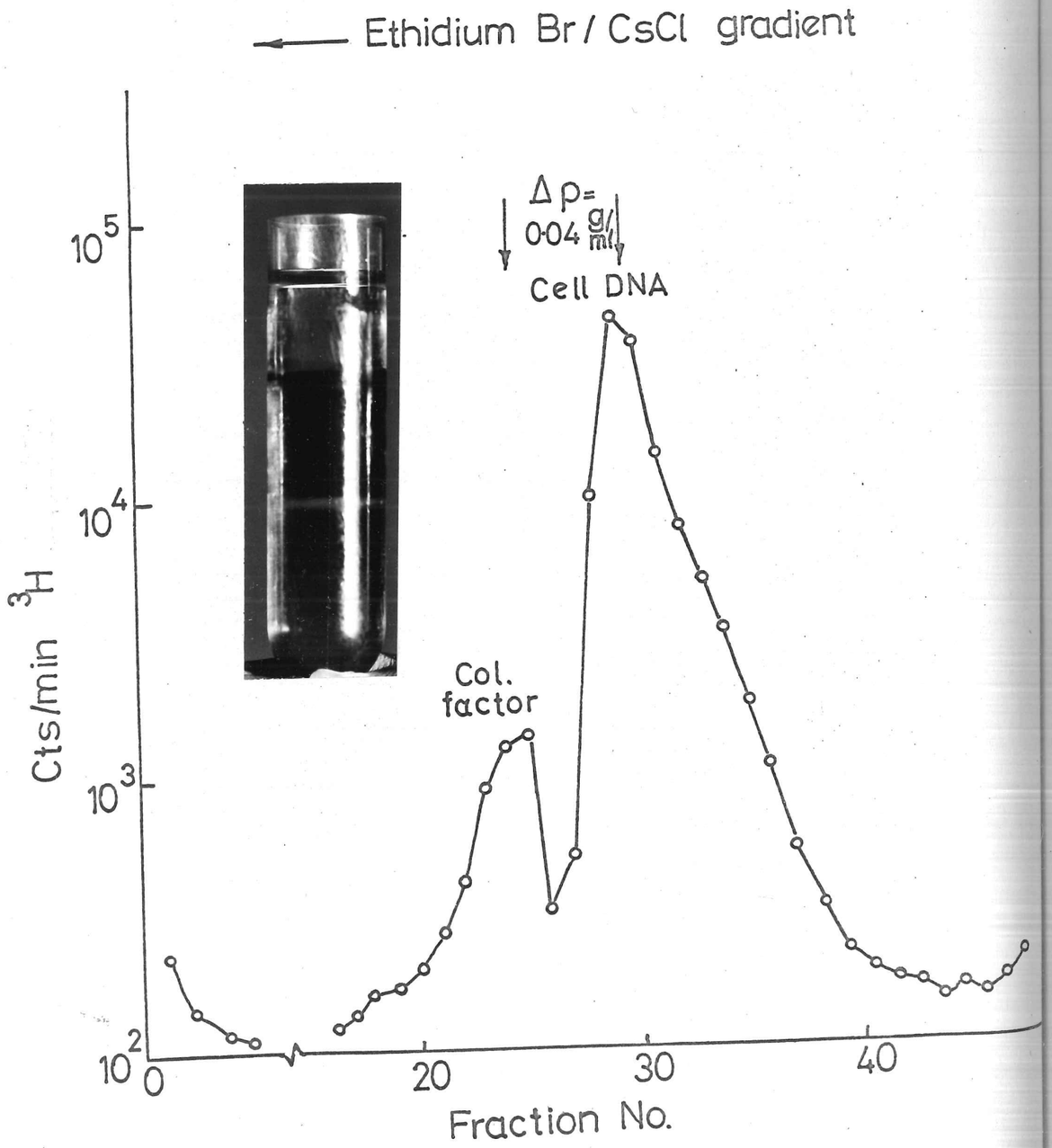
b_o was assumed to be zero for random chains and -630 for completely helical chains of L-amino acids using poly-L-glutamic acid (pH 4.2) as standard (Fasman, 1963). Therefore the observed b_o value for colicin E_2 would indicate a helical content of 17.2%. This may also be interpreted as the excess right handed helix over left handed helix in the protein.

The second simpler method involves calculation of the reduced mean residue rotation $(m')_\lambda$ at 233 nm from the specific rotation $(\alpha)_{233}$ (see Ch. 3). This method relies on the observations that $(m')_{233}$ for a 100% helical polypeptide is $-12{,}700^\circ$ and for a random coil is -1800°, (Fasman, 1963). A value of -3565° for colicin E_2 indicates a helical content of 16.2%.

(d) Isolation and characterisation of Col. factor E_2

The Col. factor has been shown to be a closed circular duplex DNA molecule (Roth and Helinski, 1967) and its isolation by the ethidium bromide -CsCl technique of Radloff et al., (1967) provided a convenient method for characterising the strain of E. coli used for

FIG. 1 x



Isolation of Col. factor E2 DNA

(^{3}H) thymine labelled cells of E.coli col $E2^{+}$ (TR2) were lysed and centrifuged to equilibrium in CsCl containing 250µg ethidium bromide perml. Aliquots of the tube fractions were cold-acid precipitated and the radioactivity determined.

colicin E_2 production and for obtaining a DNA standard for subsequent sedimentation studies.

A culture of E. coli TR2 labelled with (^{3}H)-thymine was lysed and subjected to isopycnic centrifugation in CsCl containing the intercalating drug ethidium bromide. At high concentrations this drug binds preferentially to non-circular DNA duplexes and hence the bouyant density of chromosomal DNA decreases by approx. 0.04 g/ml (see Radloff et al., 1967) relative to circular duplex DNA. The radioactivity profile after equilibrium sedimentation in ethidium bromide -CsCl is shown in Fig 1 x. There was a distinct satellite peak on the heavy side of the main chromosomal peak containing 2-3% of the total radioactivity. A UV photograph of a centrifuge tube and its contents after centrifugation to equilibrium is also shown in Fig. 1 x. The main DNA band fluoresced brightly because of the bound ethidium bromide. The Col. factor band was too small to be detected.

The pooled satellite DNA fractions were dialysed against NET buffer and analysed by sucrose gradient centrifugation (Fig. 5 i) and CsCl isopycnic centrifugation (Fig. 5 xiii). The sedimentation value obtained was 25S and this value is approximately equal to that observed by Bazaral and Helinski (1968). The bouyant density was the same as that of E. coli chromosomal DNA.

CHAPTER 2

In vivo activity of colicin E_2-P9

After colicin E_2-P9 had been purified and characterised physicochemically, its biological activity was investigated by studying its effect on cell viability, nucleic acid stability and macromolecular synthesis in sensitive strains of E. coli K12 and some of their nuclease deficient mutants.

METHODS 2

(a) Labelling of cell nucleic acid

An overnight culture of E. coli ROW was diluted one hundred fold with prewarmed M9 medium and incubated aerobically until the cell density was 3×10^8 bacteria per ml. These exponentially growing cells were diluted tenfold with M9 medium containing 200 µg deoxyadenosine per ml to facilitate thymine uptake, (Boyce and Setlow, 1962) and 5-10 µCi (methyl-^{3}H) thymine per ml (specific activity 1 Ci/mmol) to label cellular DNA.

RNA was labelled by diluting the cells in M9 medium containing 5 µCi (5-^{3}H) uridine per ml (specific activity 5 Ci/mmol) and 10 µg unlabelled uracil per ml.

Incubation was continued for a further 2-2½h until the cell density was again 3×10^8 bacteria per ml. The labelled bacteria were then diluted tenfold with prewarmed M9 medium containing 100 µg unlabelled thymine or uracil per ml and colicin E_2 at the required concentration. Purified colicin preparations were diluted in the presence of 1 mg bovine serum albumin per ml to prevent denaturation. The final dilution was carried out immediately before addition of the labelled bacteria and the final concentration of albumin was 10 µg/ml in the incubation medium.

TABLE 2 i

Characterisation of cold-acid insoluble labelled material

Treatment	% (^{3}H) counts insoluble after treatment	
	(methyl-^{3}H) thymine label	(5-^{3}H) uridine label
TMK buffer 30 min, 37°	93	96
TMK buffer + DNase (10 µg/ml) 15 min, 37°	2	97
TMK buffer + RNase (10 µg/ml) 15 min, 37°	91	4
TMK buffer + trypsin (10 µg/ml) 15 min, 37°	95	96
N NaOH overnight 37°	90	1
5% (w/v) TCA 30 min, 90°	1	2

At intervals after addition of colicin E_2 to the labelled bacteria, 0.5 or 1 ml aliquots were mixed with an equal volume of ice cold 10% (w/v) TCA. After 30 min at 4°, the precipitates were collected on 25 mm diameter Oxoid membrane filters (or glass fibre filters) washed twice with 2.5 ml cold 5% (w/v) TCA and twice with 5 ml 1% (v/v) acetic acid. The filters were dried at 80-100° for 30 min and the radioactivity measured in a Packard Tri-Carb scintillation spectrometer using a toluene base scintillation fluid containing 4g BBOT per l.

(b) Identification of labelled material (Cundliffe, 1967.a)

The nature of the label positions in (methyl-^{3}H) thymine and (5-^{3}H) uridine ideally necessitated incorporation of label into specific macromolecules, so that the former would only label DNA and the latter RNA.

Cells were incubated in the presence of (methyl-^{3}H) thymine and (5-^{3}H) uridine. Aliquots were cold-acid precipitated and collected on Oxoid membrane filters. After washing and drying the filters, the radioactivity was determined. Samples were selected to give approx. 100,000 cts/min. The filters were then removed from the scintillation fluid, gently rinsed in toluene and dried in a stream of air. The residual scintillation fluid contained less than 0.5% of the radioactivity on the filter. The dried filters were immersed in 5 ml TMK * buffer, N NaOH or 5% (w/v) TCA and treated as described in Table 2 i. After each treatment TCA was added to a final concentration of 5% (w/v) and the mixture chilled to 4°. The filter was removed and the radioactivity determined. The remaining liquid was filtered through a second Oxoid filter and the radioactivity determined. The total radioactivity after treatment was expressed as a percentage of the initial radioactivity.

---

* TMK buffer contained 10 mM tris-HCl pH 7.9, 10 mM magnesium acetate and 100 mM KCl.

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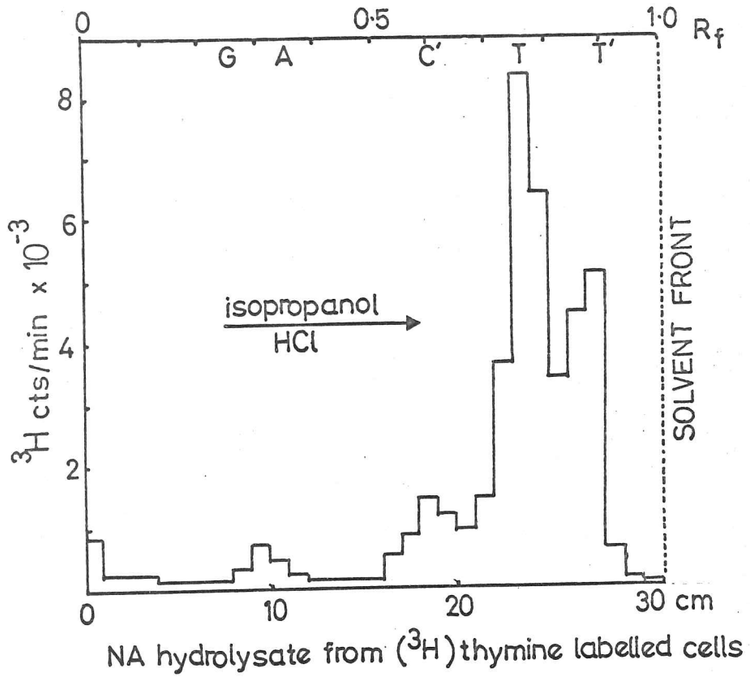
PAPER CHROMATOGRAPHY



NA hydrolysate from (^3H) thymine labelled cells

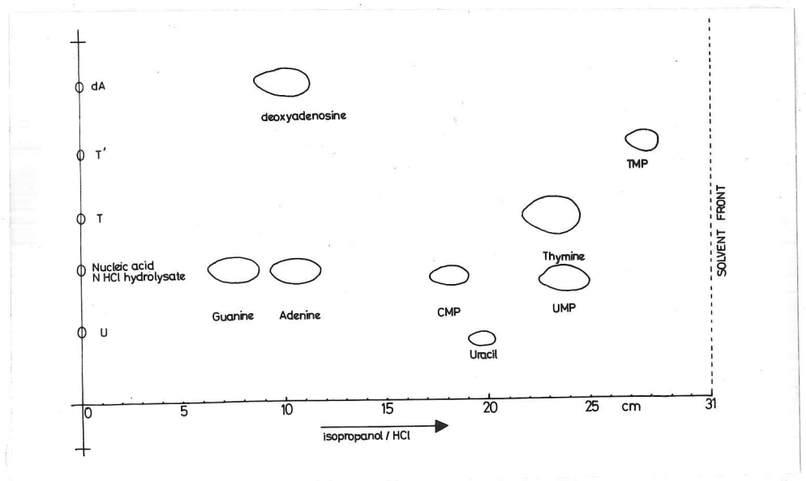


FIG. 2 i

Paper chromatography of a nucleic acid hydrolysate from (^3H) thymine labelled cells.

The cold-acid insoluble material from labelled cells was extracted with hot TCA and the extracts hydrolysed with N HCl . The hydrolysate was resolved by paper chromatography using isopropanol-HCl . The location of the UV absorbing bases and nucleotides is shown in the photograph and the distribution of radioactivity in the figure.

The bases are abbreviated by their initials (X) and the nucleoside monophosphates as X' .

The (^3H) thymine labelled material was sensitive to DNase and hot TCA but not to RNase, trypsin or NaOH suggesting that it was DNA, whereas (^3H) uridine labelled material was sensitive to RNase, NaOH and hot TCA but not to DNase or trypsin suggesting that it was RNA.

Other (^3H) labelled filters were treated with 2 ml 5% w/v TCA at 90° for 30 min after washing with ether: 75% ethanol (1:1) to remove lipid material (less 1% total radioactivity). The hot TCA extracts were shaken with equal volumes of ether three times to remove the TCA and hydrolysed with N HCl in a boiling water bath for 60 min. The hydrolysate was evaporated to dryness. The residue was dissolved in 100 µl or less of H_2O and applied to Whatman No. 1 chromatography paper. The hydrolysate was run overnight using a descending 65% (v/v) isopropanol: 2 N HCl system, (Fig. 2 i). The chromatogram was dried at 45° and photographed using ultra-violet light. The paper was then cut into 3 x 1 cm strips and the radio-activity measured in the liquid scintillation counter.

The positions of the radioactive peaks, UV absorbing spots and predicted Rf values are shown in Fig. 2 i. The acid hydrolysate from (^3H) thymine labelled cells gave two main radioactive peaks corresponding to the positions of thymine or thymidine and TMP (methyl-^3H) thymine ran in the same position as the major radio-active peak with an Rf value of 0.77. Acid hydrolysates from (^3H) uridine labelled cells also gave two main radioactive corresponding to uridine and UMP. The two nucleic acid precursors were shown to be chromatographically pure with (^3H) uridine running in the same position as CMP. Recovery of radioactivity was routinely 80-90%.

It was concluded from these experiments that under the experimental conditions used the radioactive precursors could be used for specifically labelling DNA and RNA.

(c) Incorporation studies

DNA, RNA, protein and mucopeptide synthesis were followed by radioactive incorporation studies using (methyl-^{3}H) thymine, (5-^{3}H) uridine, (^{14}C) valine and (^{3}H) α, ε-diaminopimelic acid.

Exponentially growing cells of E. coli ROW were washed and resuspended in M9 medium (without casaminoacids) containing 100 µg methionine per ml at a cell density of 10^{8} bacteria per ml. The bacteria were incubated for 10 min at 37° followed by addition of radioactive precursors to separate tubes. The final concentrations were; 1 µCi (^{3}H) thymine (1 Ci/mmol) per ml (plus 200 µg deoxyadenosine per ml); 1 µCi (^{3}H) uridine (5 Ci/mmol) per ml (plus 10 µg unlabelled uracil per ml); 0.1 µCi (^{14}C) valine (6.9 mCi/mmol) per ml and 1 µCi (^{3}H) diaminopimelic acid (76 mCi/mmol) per ml (plus 100 µg unlabelled lysine per ml to prevent (^{3}H) incorporation into protein as lysine).

Colicin E_2 was added as required, 1 ml aliquots were taken at intervals, mixed with equal volumes of 10% (w/v) cold TCA and left at 4° for 30 min (for thymine, uridine and DAP incorporation) or heated to 90° for 30 min (for valine incorporation). The precipitates were collected on glass fibre filters, washed and the radioactivity determined.

Mitomycin C and actinomycin D were used as a further check on the incorporation of labelled thymine and uridine into DNA and RNA respectively. Mitomycin C was used at a final concentration of 5 µg/ml (Fig. 2 vi). Actinomycin D was only effective on E. coli if the cells

were pretreated with EDTA according to the method of Leive (1965). Exponentially growing cells were washed with 10 mM tris-HCl buffer pH 8, resuspended in tris buffer at a cell density of 10^9 bacteria per ml and treated with 1 mM EDTA for 2 min at 20°. The treated cells were then diluted tenfold with prewarmed M9 medium and incubated for 10 min at 37°. (^3H) uridine was added to the culture as described above together with 100 µg actinomycin D (Fig. 2 vii). The EDTA treated cells (in the absence of actinomycin D) incorporated (^3H) uridine at a comparable rate to untreated cells.

(d) Total cell analysis

Exponentially growing cells of E. coli were washed and resuspended in M9 medium at cell densities of 2.5×10^8 or 10^9 bacteria per ml together with colicin E_2 as required. Incubation was continued at 37° for 2 h. The cells were harvested by centrifugation and resuspended in 0.2 N perchloric acid at 4° for 30 min. The precipitate was removed by centrifugation and the procedure repeated. The cold-acid insoluble material was resuspended in 0.5 N perchloric acid and extracted three times at 70° for 20 min. The pooled hot-acid extract was analysed for DNA and RNA (Methods 1). The hot-acid insoluble material was dissolved in N NaOH and analysed for protein (Methods 1).

(e) Characterisation of E. coli K12 mutant strains

(i) UV sensitivity

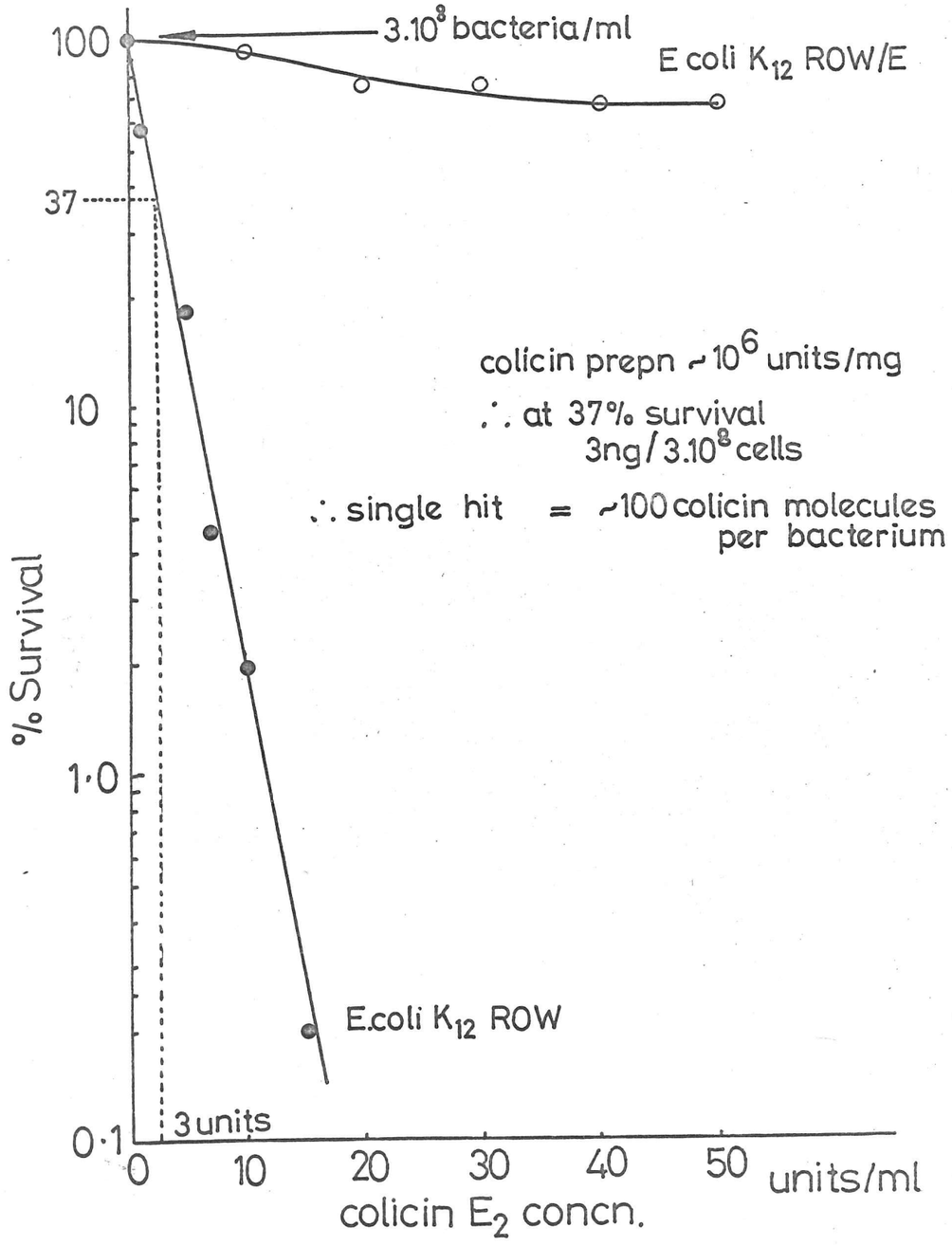
E. coli K12 strain AB1157 and its UV sensitive mutant AB1885 were tested for UV irradiation sensitivity by plotting a survival curve. Exponentially growing cells (2×10^8 bacteria per ml) were harvested, washed and resuspended in sterile saline at a cell density of 2×10^7 bacteria per ml. 10 ml suspensions were pipetted into petri dish lids irradiated with UV light from a 30 watt low pressure mercury vapour

lamp (Hanovia B13/A 1836) at an incident dose rate of 12.5 ergs/mm^2/sec. Suitable serial dilutions in sterile saline were plated on nutrient agar and the number of survivors determined after incubation at 37° for 16h. Results are shown in Fig. 2 ix(a).

(ii) Endonuclease I activity

E.coli K12 strain 1000 and its endonuclease I deficient mutant (strain 1100) (Dürwald and Hoffmann-Berling, 1968) were tested for nuclease activity using an adapted version of the method due to Korn and Weissbach (1963) and Obinata and Mizuno (1968). Exponentially growing cells were harvested by centrifugation, washed and resuspended at 5 x 10^9 bacteria per ml in 10 mM tris-HCl pH 8.0 containing 5 mM $MgCl_2$. The bacteria were then broken by sonic oscillation using a 0.5 inch probe (Soniprobe Type 1130/1A Dawe Instruments Ltd.) at 4 amps for 1-2 min. The broken cell extract was diluted tenfold with tris-Mg^{++} buffer pH 8.0. The assay system contained 2.5 µg (^3H)-thymine labelled DNA (approx. 10,000 cts/min, see Ch. 3) and 0.05 ml diluted crude cell extract in a total volume of 0.25 ml tris-Mg^{++} buffer pH 8.0. The system was incubated for 30 min at 37° and the reaction stopped by adding 1 ml ice cold 5% (w/v) TCA. The cold-acid insoluble radioactivity was determined and the decrease taken as a measure of other nuclease activity. In order to determine specifically endonuclease I activity 0.05 ml diluted crude cell extract was incubated with 5 µg heated ribonuclease in 0.2 ml tris-Mg^{++} buffer pH 8.0 for 15 min at 37° prior to addition of (^3H) DNA; this treatment removed tRNA from the extract and therefore activated endonuclease I (see Lehman et al., 1962). Endonuclease I activity is given by subtracting the DNA degradation obtained without RNase from DNA degradation obtained with RNase.

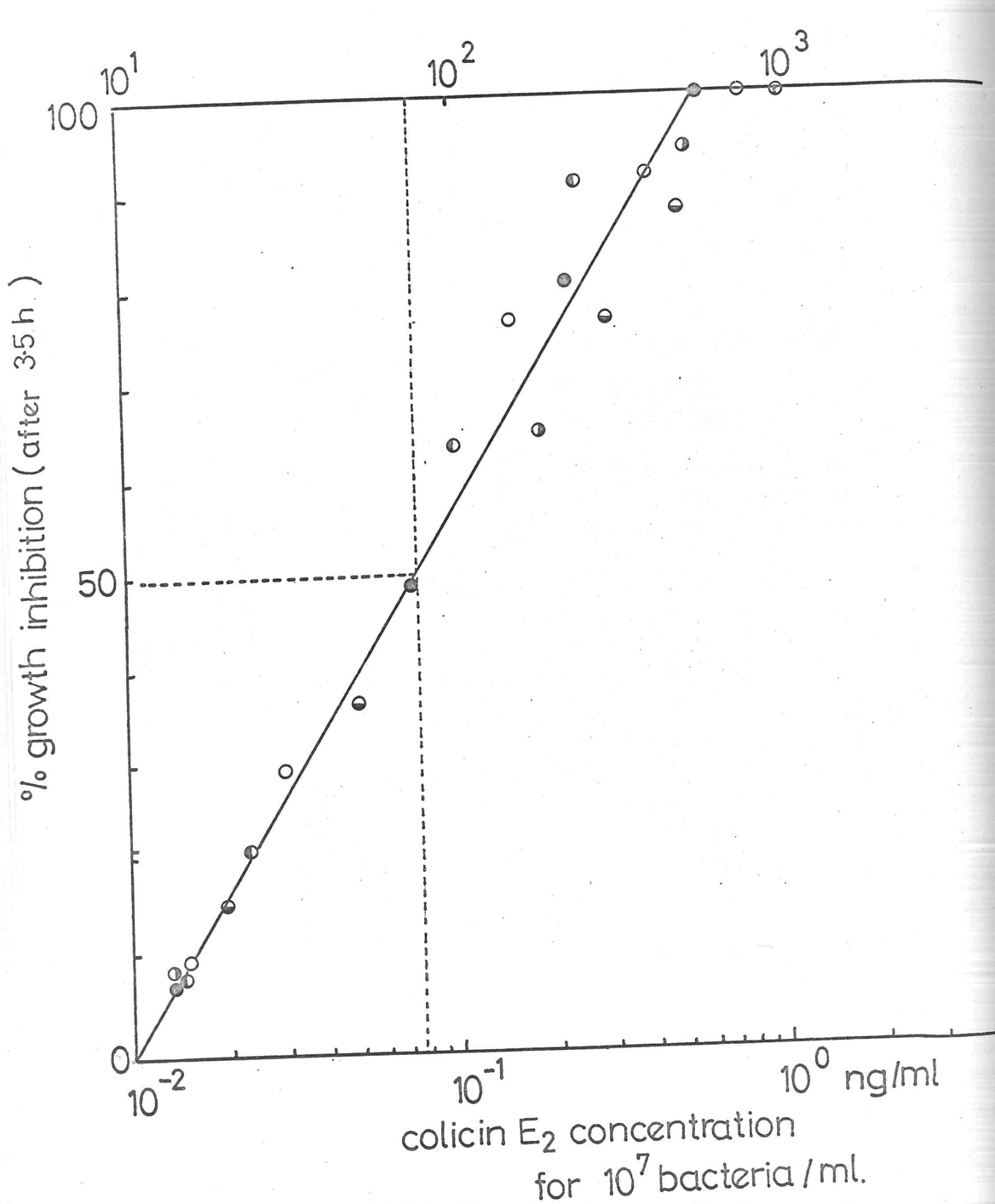
FIG. 2 ii



Survival curves for E.coli ROW and ROW/E

Exponentially growing cells of E.coli were incubated with the appropriate concentrations of colicin E2 for 20 min, serially diluted and the number of cell survivors determined. 1 unit refers to the minimum amount of colicin required to produce a growth inhibition zone of E.coli ROW on agar.

FIG. 2 iii

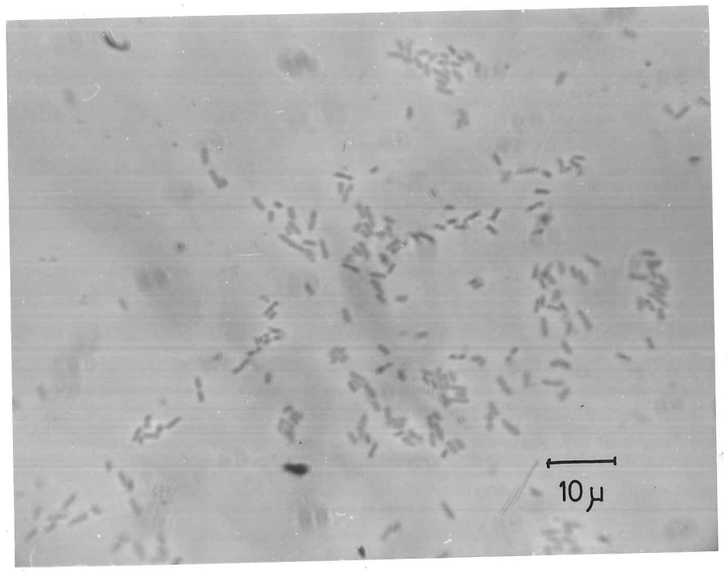


Growth sensitivity of E.coli ROW to colicin E2

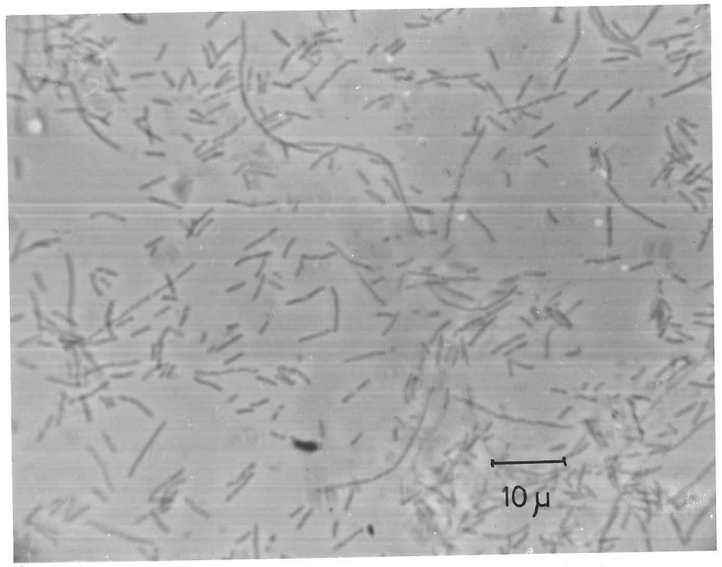
Exponentially growing cells of E.coli ROW were incubated with the appropriate concn. of colicin E2, (10^7 bacteria per ml) and the OD_{600} after 3.5 h determined . The different symbols indicate different E2 preparations.

PLATE 2 i

Filament formation on incubation of cells with colicin E2



(a) Control E.coli ROW



(b) E.coli ROW after 2h incubation with colicin E2 (250 molecules per bacterium).

RESULTS 2

(a) Effect of colicin E_2 on cell viability and growth

Colicin activity is sometimes expressed as arbitrary units per ml where the units are derived from the highest dilution of a colicin preparation able to produce an inhibition of bacterial growth on nutrient agar (see Methods 1). This is unsatisfactory from a biochemical point of view and a more meaningful figure would be the number of colicin molecules required to kill one bacterium (i.e. one killing unit). The number of killing units (KU's) in a colicin preparation may be determined from a survival curve (Methods 1 and Fig. 2 ii) where the colicin arbitrary units are plotted against the log. of the percentage surviving bacteria after incubation with colicin. The survival curve was shown to be exponential consistent with the single hit theory proposed by Jacob et al., (1952) and therefore the mean lethal dose or the amount of colicin statistically needed to initiate one lethal event per bacterium according to target theory (see Lea, 1946) was at 37% survival rate. This corresponded to approx. 100 colicin molecules per bacterium (cf. Maeda and Nomura, 1966) which means that the colicin preparation has a specific activity of approx. 10^{14} KU's/mg. At multiplicities approaching 1 killing unit of colicin E_2 per bacterium, filamentous cell forms were observed after 1-2h incubation (Plates 2 i(a) and (b)). Similar results have been observed with colicin E_2 by Holland (1968) and with mitomycin C by Reich et al., (1961). At higher multiplicities however this phenomenon was less noticeable probably due to the more rapid inhibition of macromolecular synthesis (see text).

The effect of colicin E_2 on the growth of a bacterial culture as determined by measuring the OD_{600} is shown in Fig. 2 iii. A typical drug-response curve was obtained from which the minimum growth inhibitory concentration (MGIC) was determined and found to correspond to approx.

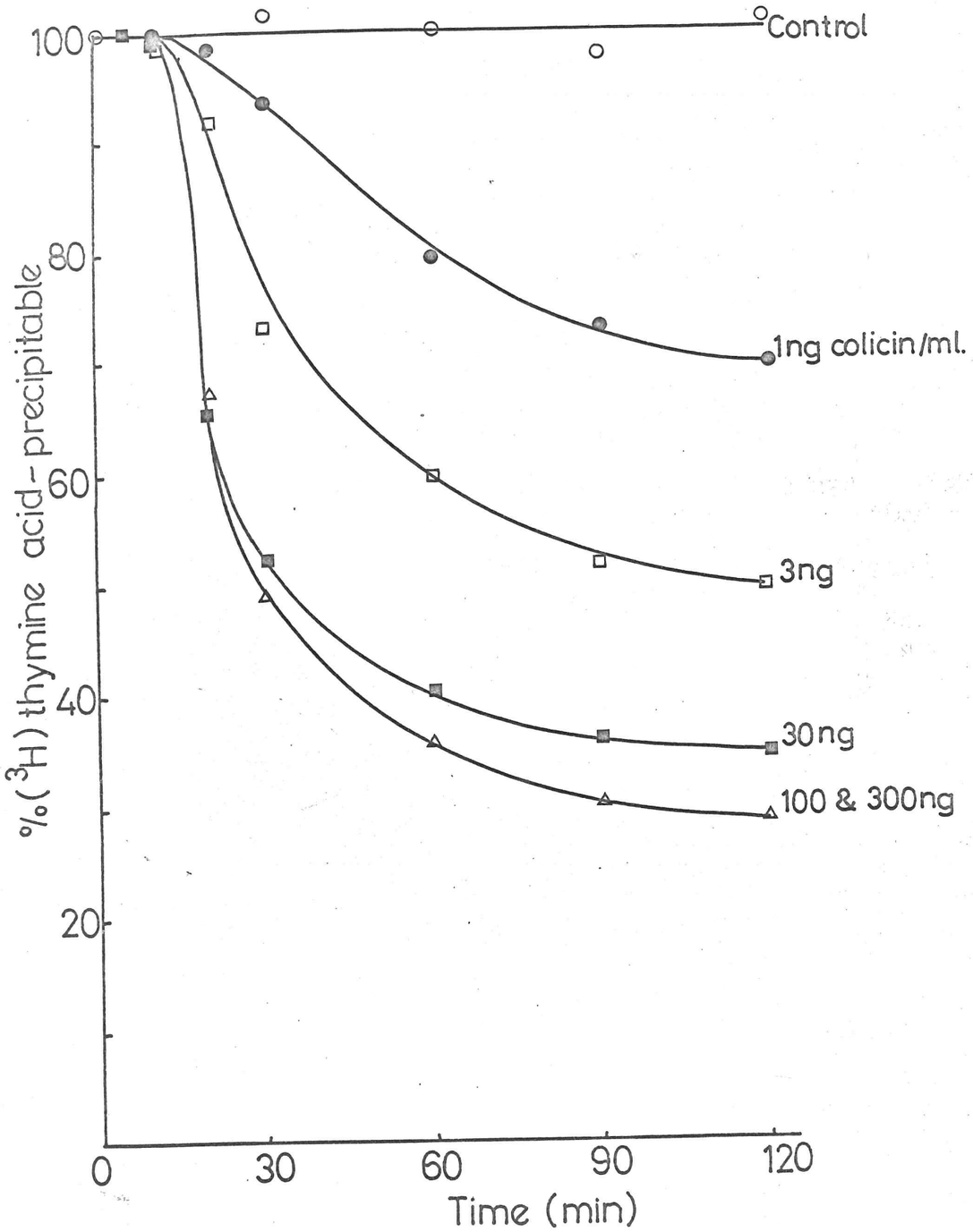


FIG. 2 iv Colicin E2 induced DNA degradation

Exponentially growing (^{3}H) thymine labelled cells of E.coli ROW were washed and incubated with colicin E2 at a cell density of 3.10^7 bacteria per ml. At intervals, aliquots were taken and the cold-acid precipitable radioactivity determined. The results are expressed as a percentage of the radioactivity at zero time.

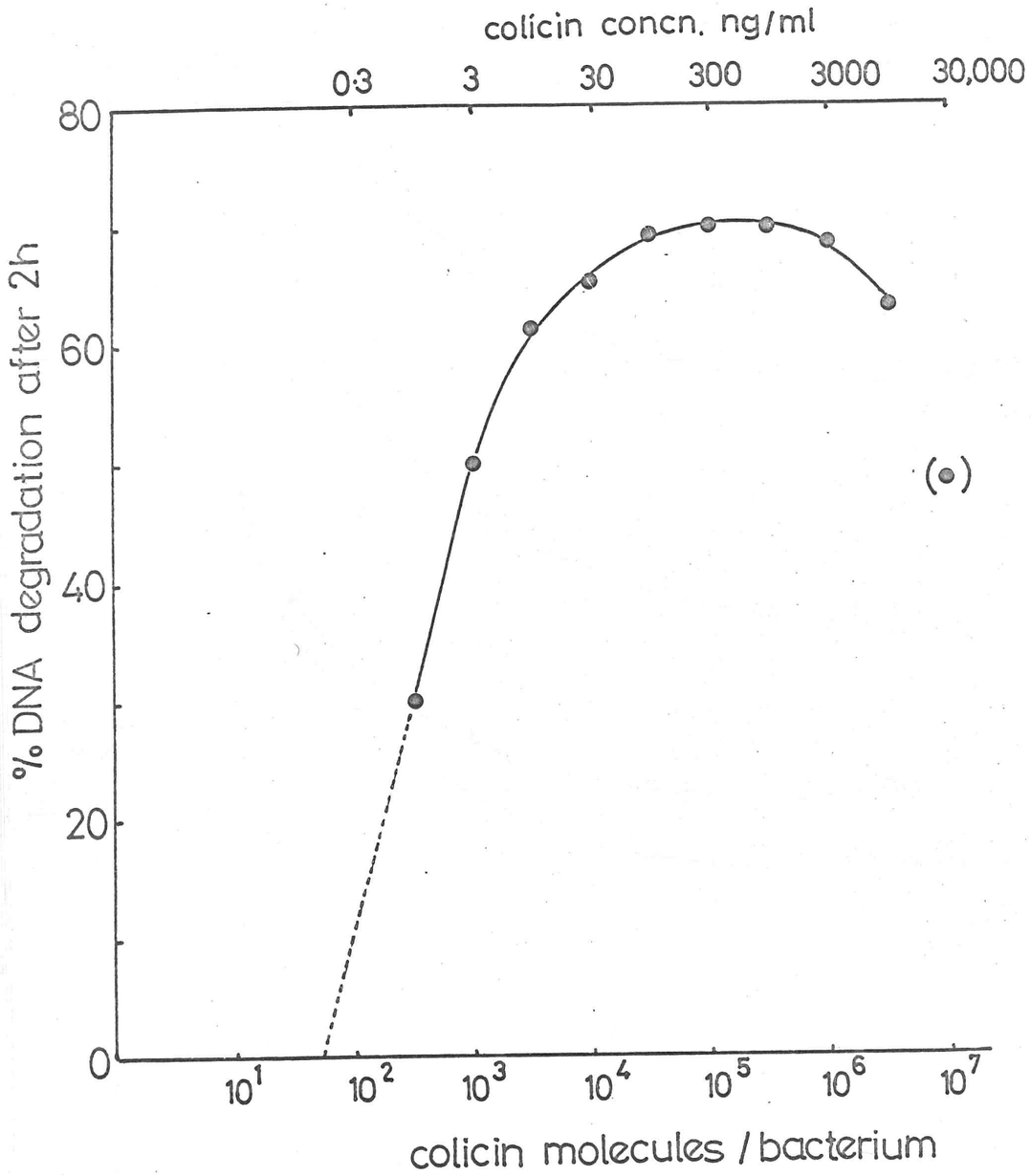


FIG. 2 v Effect of colicin E2 concentration on induced DNA degradation

The percentage DNA degradation after 2h incubation of cells with colicin E2 was derived from FIG. 2 iv and plotted against the ratio of colicin molecules per bacterium or the concentration of colicin at a cell density of 3.10^7 bacteria per ml.

75 colicin E_2 molecules per bacterium. Measurements of this kind were frequently used as a quick guide to the activity of a particular colicin sample.

(b) Effect of colicin E_2-P9 on nucleic acid stability in vivo

(i) DNA

E. coli ROW labelled with (methyl-^{3}H) thymine was incubated with various concentrations of colicin E_2. After a lag period of 10-15 min the cold-acid precipitable radioactivity decreased with time (Fig. 2 iv). The maximum decrease was approx. 70% after 2h incubation. At colicin E_2 concentrations greater than 3 ng/ml, most of the DNA degradation occurred between 15 and 50 min incubation and was largely over by 2h. The value for the residual cold-acid insoluble radioactivity was reasonably constant (± 3%) for a particular colicin concentration and was dependent on the concentration of colicin E_2 added initially from 10^0 to 10^2 ng/ml (Fig. 2 v). Above 10^4 ng colicin E_2 per ml a relative decrease in DNA degradation was consistently observed but the amount of degradation varied by ± 10%. Large variations were also observed at colicin concentrations less than 1 ng/ml (i.e. multiplicities near to unity).

The inhibition of colicin E_2 induced DNA degradation at high colicin concentrations has not been reported previously and may result from high secondary colicin binding (Maeda and Nomura, 1967; Shannon and Hedges, 1967) interfering with the primary binding responsible for the initiation of DNA degradation. It could also be due to the formation of partially inactive molecular aggregates (see G_{200} sephadex results Ch. 1) or a form of competition for multiple interaction points per binding site in the cell envelope normally occupied by only one colicin molecule similar to high substrate inhibition of certain enzymes.

TABLE 2 ii

Effects of colicins E_2 and E_3 on nucleic acid stability in E. coli

	% acid solubilisation after 2h incub.
(^3H) thymine labelled *E. coli* ROW	
exponential phase + colicin E_2	65
stationary phase + colicin E_2	10
exponential phase + colicin E_3	5
(^3H) thymine labelled *E. coli* ROW/E	
exponential phase + colicin E_2	7
(^3H) uridine labelled *E. coli* ROW	
exponential phase + colicin E_2	12

Colicins E_2 and E_3 were used at molecule per bacterium ratio of 10^4.

However since the bacterial chromosome may not be the primary site of colicin E_2 action (Luria, 1970) a more severe effect on the membrane at high colicin concentrations may interfere with the induced DNA degradation.

DNA degradation starts to plateau at approx. 30 ng colicin E_2 per ml (10^4 colicin molecules per bacterium), Fig. 2 v, i.e. all the primary binding sites are saturated assuming all the colicin molecules added are specifically bound to bacteria. This figure is slightly higher than the 2-3000 sites proposed by Maeda and Nomura (1967) and 1100 sites proposed by Mayr-Harting but varying experimental conditions appear to cause discrepancies.

Colicin induced DNA degradation in bacteria harvested from cultures in the stationary phase occurred after a lag of 30-60 min and the subsequent rate of breakdown was reduced (Table 2 ii). This may be consistent with the observations of Shortman and Lehman (1964) that cell nuclease activity was maximal during rapid growth and minimal during stationary phase. Colicin E_3 did not induce DNA degradation in the sensitive strain (*E. coli* ROW) and the resistant strain (*E. coli* ROW/E) was only slightly affected by colicin E_2 at 30 ng/ml (Table 2 ii). This confirms that DNA degradation is a specific effect of colicin E_2 and not the result of contaminants.

The radioactivity remaining in the acid insoluble material after incubation of the labelled cells with colicin E_2 was shown to be in DNA by similar methods to those previously described and Cs_2SO_4 isopycnic centrifugation (Fig. 5 xi).

(ii) RNA

It was originally reported by Nomura (1963) that colicin E_2 did not induce RNA breakdown in sensitive bacteria, but Nose *et al.*,

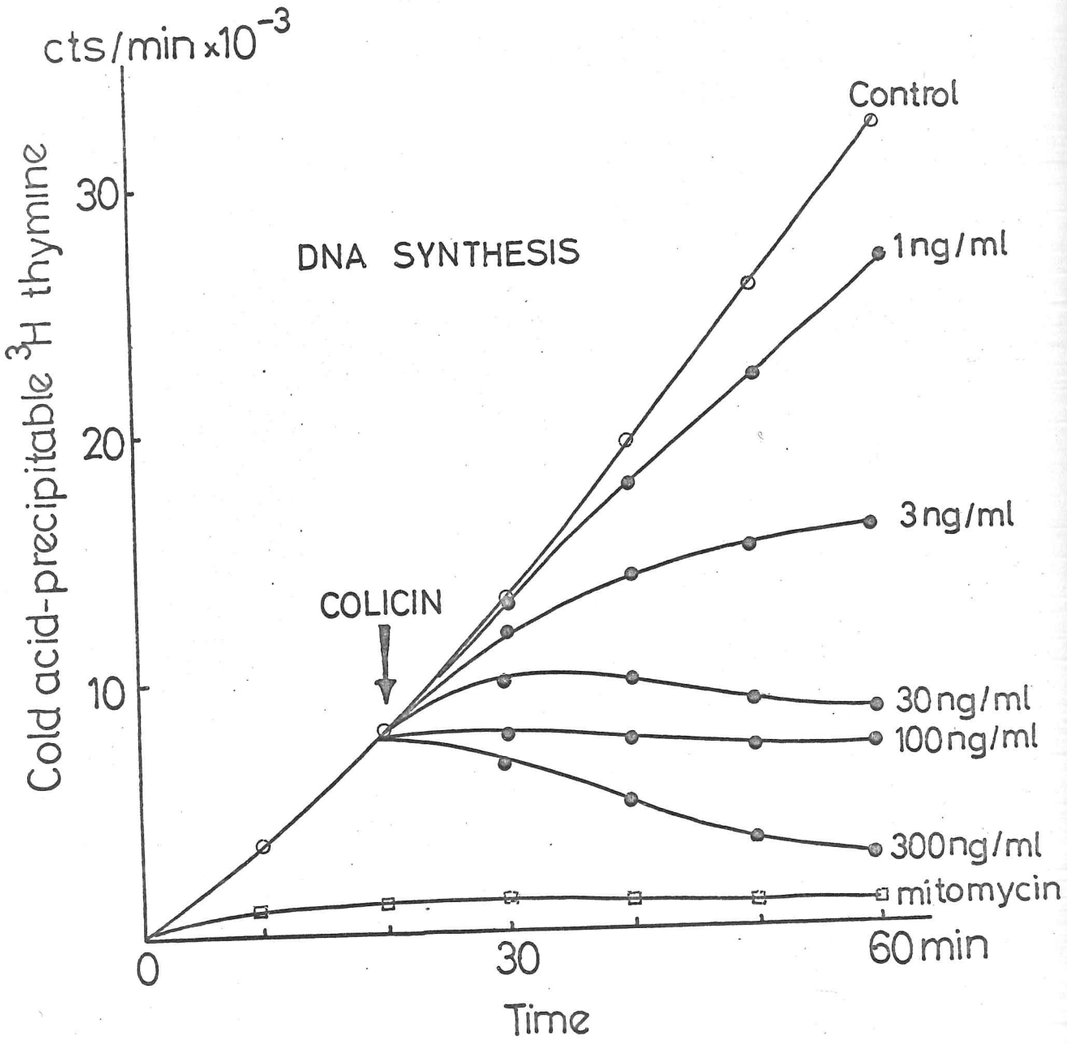


FIG. 2 vi Effect of colicin E2 on (^{3}H) thymine incorporation

Exponentially growing cells of E.coli ROW were incubated at an initial cell density of 10^8 bacteria perml in M9 medium containing 1μCi (^{3}H) thymine per ml. Colicin E2 was added after 20min and samples were taken . The incorporation of radioactivity in to cold-acid precipitable material was determined. Mitomycin (5μg/ml) was added at zero time.

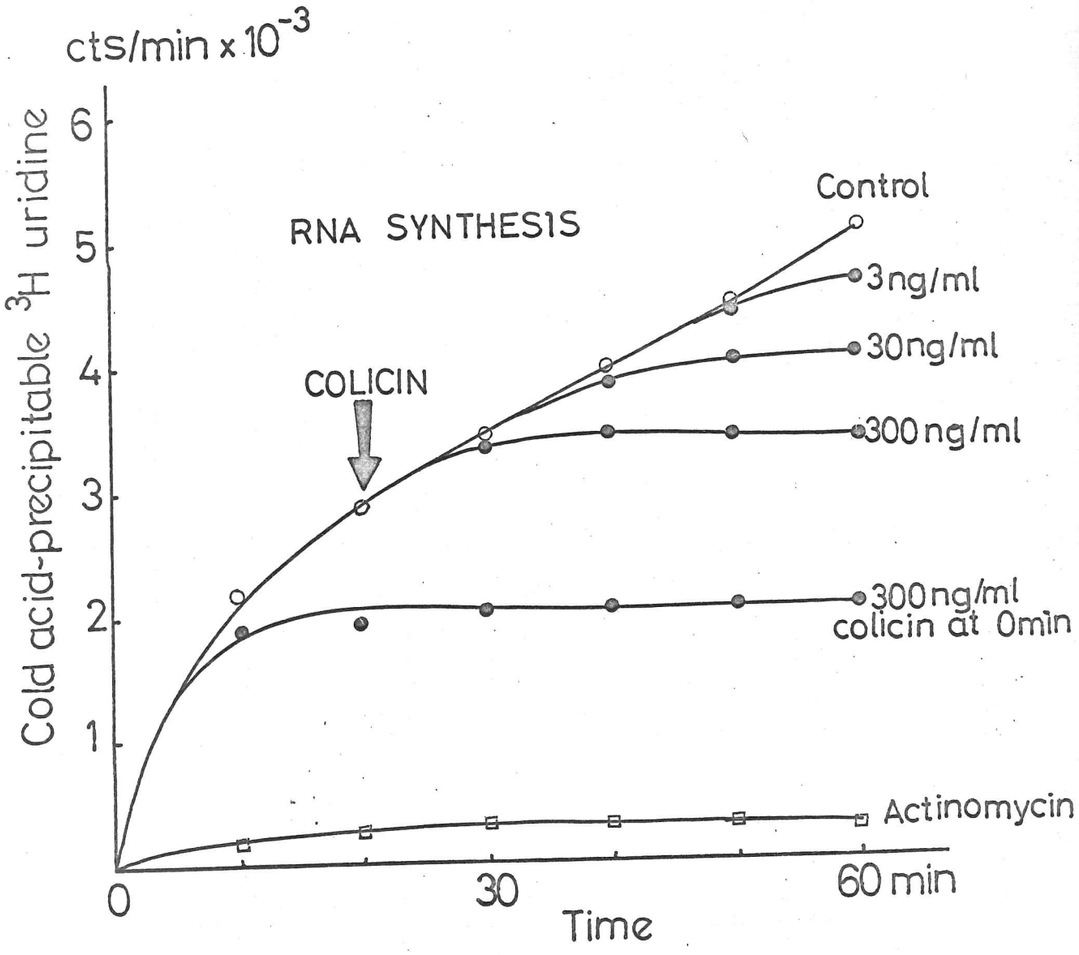


FIG. 2 vii Effect of colicin E2 on (^{3}H) uridine incorporation

Exponentially growing cells of E.coli ROW (10^{8} bacteria perml) were incubated with 1µCi (^{3}H) uridine per ml and treated with colicin E2 at the times indicated. Cold-acid precipitable radioactivity was determined at intervals. Actinomycin D (100µg/ml) was added to EDTA pretreated cells at time zero.

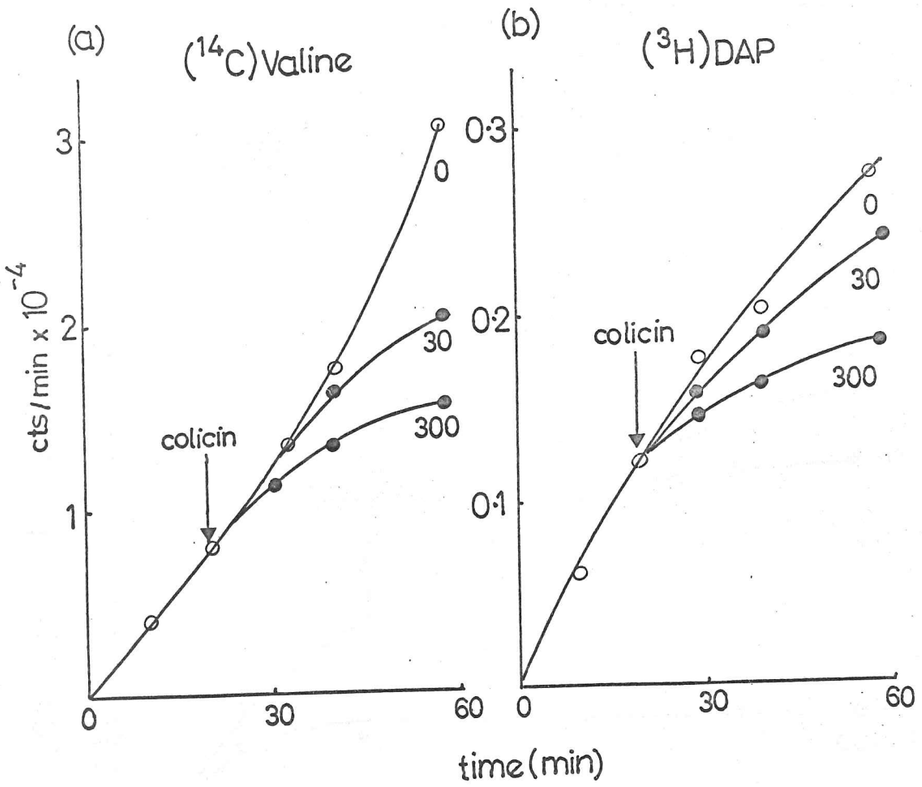


Fig. 2 viii Effect of colicin E2 on (^{14}C) valine and (^{3}H) diaminopimelic acid incorporation.

Exponentially growing cells of E.coli ROW were incubated at a density of 10^{8} bacteria per ml with (a) 0.1 µCi (^{14}C) valine perml , and (b) 0.5 µCi (^{3}H) DAP per ml . Colicin E2 (concn. in ng/ml indicated by numbers beneath the curves) was added after 20min. Hot (a) and cold (b) acid precipitable radioactivity was determined at intervals.

(1966) and Nose and Mizuno (1968) reported that colicin E_2 induced a specific degradation of ribosomal RNA. In these studies (Table 2 ii) no RNA degradation was detected during the first hour of colicin incubation and only 12% degradation occurred after 2h.

(c) Effect of colicin E_2-P9 on macromolecular synthesis in vivo

(i) Incorporation studies

The effects of colicin E_2 on the in vivo synthesis of DNA, RNA, protein and mucopeptide are shown in Figs. 2 vi, 2 vii and 2 viii. In all cases synthesis was followed by studying incorporation of labelled precursor.

The incorporation of labelled thymine, uridine, valine and DAP into macromolecules was affected by colicin E_2 by differing amounts according to the concentration. At 3 ng colicin E_2 per ml DNA synthesis was significantly inhibited, whilst RNA synthesis only showed an effect after a lag of approx. 30 min. Protein and mucopeptide synthesis were relatively unaffected at this colicin concentration after 40 min incubation, consistent with the formation of filamentous cell forms (Plate 2 i(b)).

At 30 and 300 ng colicin E_2 per ml net DNA synthesis was inhibited almost immediately whereas RNA synthesis was inhibited only after a lag period of 10-20 min. Protein and mucopeptide synthesis showed an almost immediate effect but continued at a reduced rate.

The interpretation of these results is complicated by the concomitant DNA degradation (Fig. 2 iv) since the products of DNA breakdown may lower the specific activity of the nucleotide pool. Comparison of the effects of colicin E_2 on DNA synthesis and breakdown in Figs. 2 iv and 2 vi must take account of the different cell densities, so that 1 ng colicin E_2 per ml in Fig. 2 iv is equivalent to 3 ng/ml in Fig 2 vi, i.e. approx. 300 colicin molecules per bacterium.

TABLE 2 iii

Effect of colicin E_2 on net synthesis of cellular macromolecules

	DNA	RNA	Protein
	(µg/mg dry weight at zero time)		
(a) Growing cells			
Control (initial)	33	120	408
" (2h incub.)	88	320	1840
Colicin E_2 (2h incub.)			
mols/bacterium $5x10^1$	74	296	1472
$5x10^2$	40	148	760
$5x10^3$	31	140	520
$5x10^4$	28	132	456
(b) Cells under conditions of limited growth			
control (initial)	28	111	440
" (2h incub.)	38	134	720
Colicin E_2 (2h incub.)			
mols/bacterium $5x10^1$	37	128	690
$5x10^2$	27	117	610
$5x10^3$	14	98	560
$5x10^4$	12	84	510

These results are consistent with those obtained by Reeves (1968) but differ from the results of Holland (1968) in the extent of inhibition of DNA synthesis at higher colicin concentrations.

(ii) Total macromolecular content

The effect of colicin E_2 on macromolecular synthesis in exponentially growing cells and in young cells under conditions of little growth was investigated by measurement of total cell nucleic acid and protein. The results are shown in Table 2 iii where the amount of colicin is given by the number of molecules per bacterium in order to enable comparison. The exponentially growing cells were incubated with colicin at a density of 2.5×10^8 bacteria per ml whilst the other cells were harvested at a density of 2.5×10^8 bacteria per ml during exponential growth, resuspended at 10^9 bacteria per ml and incubated for 10 min prior to addition of colicin.

Under conditions of cell growth (Table 2 iii(a)) there was little detectable overall DNA degradation even at colicin:cell ratios of 5×10^4 suggesting a competition between DNA synthesis and breakdown (see Figs 2 iv and vi). Total RNA increased very little in the presence of colicin which together with the results of Fig. 2 vii and Table 2 ii suggests a certain degree of RNA degradation (Nose et al., 1966). Net protein synthesis was also found to be inhibited under conditions of cell growth, however the amount of protein synthesised in the control after 2h incubation was disproportionately high compared with control DNA and RNA syntheses. This was probably due to the cells going into stationary phase towards the end of the incubation time.

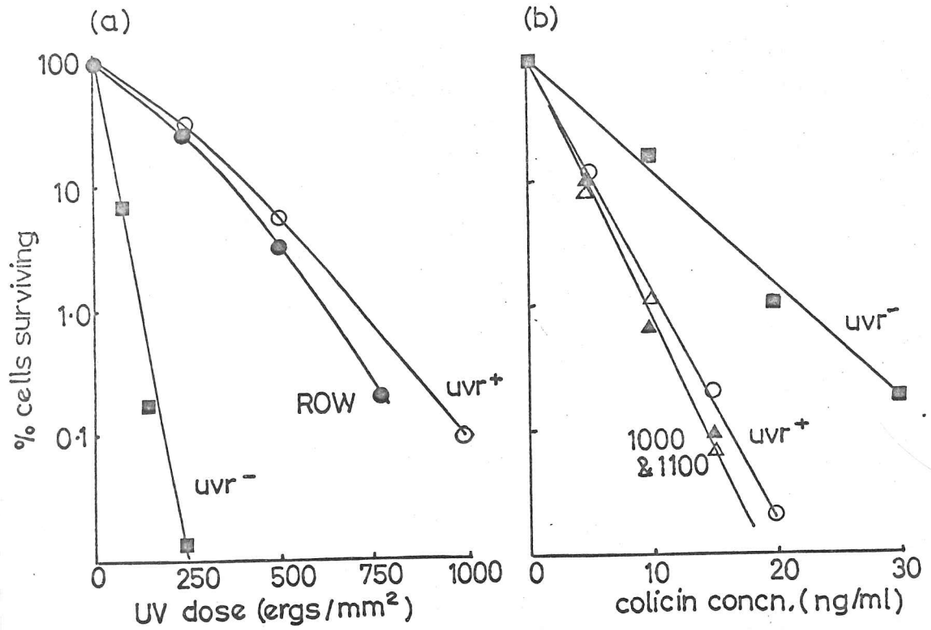


FIG. 2 ix UV and colicin survival curves for E.coli K12 mutants

Exponentially growing cultures of the strains of E.coli K12 indicated were treated with (a) ultraviolet irradiation and (b) colicin E2. The percentage of cell survivors was determined by serial dilution and plating on nutrient agar.

Under conditions of limited cell growth (Table 2 iii(b)), DNA degradation was significantly increased on incubation with colicin E_2, suggesting that DNA synthesis was not now competing so effectively with the degradative processes. RNA was also found to have a net degradation, although small in comparison with DNA. These results differ from the results obtained with stationary phase cells in Table 2 ii as the cells used in Table 2 iii(b) were relatively young at the time of colicin addition and presumably still contained significant nuclease activity (Shortman and Lehman, 1964.) Under these conditions protein synthesis in control cells was limited but still subject to inhibition by colicin E_2.

(d) Effect of colicin E_2 on the viability and nucleic acid stability of (i) UV sensitive and (ii) endonuclease I deficient mutants of E. coli K12

(i) UV sensitive mutant

Threlfall and Holland (1968) have produced genetic evidence that enzymes involved in repairing UV damaged DNA may also be involved in colicin E_2 induced DNA degradation. In order to test this hypothesis, the effects of colicin E_2 on a UV sensitive strain of E. coli K12 were compared with the effects on the parent strain. The two strains were characterised as described in Methods 2 and the UV irradiation survival curve is shown in Fig. 2 ix(a). The effects of colicin E_2 on the survival and DNA degradation of the parent uvr^+ strain were similar to the results reported for E. coli ROW. However the mutant uvr^- strain was shown to be more resistant to colicin E_2 induced cell death (Fig. 2 ix) although not to the same extent as the colicin adsorption resistant mutant E. coli ROW/E (see Fig. 2 ii). There was also a reduction in the extent of DNA degradation in the uvr^- strain, (Table 2 iv). These observations are therefore consistent with the above hypothesis.

TABLE 2 v

Nuclease Activity of E. coli strains

	% DNA cold-acid soluble after 30 min		
	Nuclease(-RNase)	Nuclease(+RNase)	Endonuclease I
Strain 1000	10	15	5
Strain 1100	13	67	54

(^{3}H) DNA was incubated with crude cell extract before and after RNase treatment. 100% radioactivity = 10800-11,300 cts/min. The above percentages are the average of duplicate determinations.

TABLE 2 iv

Colicin E_2 induced DNA degradation

Strain	% DNA degraded after 2h
E. coli ROW (CL 142)	53
ROW/E (CL 145)	3
uvr^+ (AB 1157)	49
uvr^- (AB 1885)	23
1000	50
1100	54

Colicin was used at a concentration of 3 ng/ml at a cell density of 3.10^7 bacteria per ml.

(ii) Endonuclease deficient mutant

It has been reported that endonuclease I is associated with the bacterial membrane and may be situated in the periplasmic space (Cordonnier and Bernardi, 1965; Nossal and Heppel, 1966). It has also been implicated in certain phage exclusion phenomena (Dr. M.R. Lunt, 1970; personal communication). It was of interest to investigate the effect of colicin E_2 on an endo I^- mutant since colicin E_2 is believed to bind to the inner membrane (see General Introduction). The endo I^- mutant 1100 and its parent strain 1000 were characterised by assaying the nuclease activity in cell extracts induced by RNase (see Methods 2) and the results are shown in Table 2 v. The effects of colicin E_2 on the survival and DNA degradation of strains 1000 and 1100 were similar (Fig.2ix(b) and Table 2 iv).

These results suggest that although the enzymes involved in DNA repair processes are necessary for colicin induced DNA degradation, endonuclease I is not required, (see Obinata and Mizuno, 1970).

CHAPTER 3

In vitro activity of colicin E_2-P9

INTRODUCTION

All published work so far reported with colicins has almost entirely concentrated on the *in vivo* activity. The central dogma which explains the allosteric nature of colicin action (Nomura, 1963; Changeux and Thiery, 1967) has precluded investigation of direct colicin interaction with biochemical targets and development of possible *in vitro* systems. In 1963 Nomura investigated the possibility that colicins interacted directly with the bacterial chromosome. It was first shown that trypsin reversed colicin action and therefore colicin molecules remained near the cell surface. In addition it was demonstrated that the ability of colicin K to inhibit production of T_4 phage in infected *E. coli* was independent of the number of phage chromosomes per bacterium, i.e. there was not a stoichiometric relationship between the colicin K molecules and phage chromosomes. From this evidence it was concluded that colicin E_2, which was known to be unlike colicins E_3 and K in the poor reversal of its inhibitory action by trypsin, did not interact directly with its biochemical target - the bacterial chromosome. In 1964 Nomura supported this claim with the finding that incubation of colicin E_2 with radioactively labelled DNA resulted in no increase in cold-acid soluble material. However all this proved was that colicin E_2 had no exonucleolytic activity under the conditions of the experiment; details of which were not given.

In 1966 Maeda and Nomura prepared radioactively labelled colicin E_2 and showed that, after disrupting cells that had been incubated with labelled colicin E_2, and treating with DNase, over 80% of the radioactivity was associated with the cell envelope fraction. They argued that any colicin associated with DNA in the cell would

appear in the soluble fraction after the above treatment. It was however pointed out (Maeda and Nomura, 1966) that a small fragment of DNA may remain attached to the membrane and colicin E_2 could complex with this (see Ch. 6).

Penetration of the cytoplasmic membrane therefore seems unlikely, but results with radioactive colicin E_2 and trypsin (Maeda and Nomura 1966) show that after colicin is attached to cells only 60% of the radioactivity is removed by trypsin digestion.

In 1967 Konisky and Nomura demonstrated that colicin E_3 had no effect *in vitro* on its biochemical target - the ribosome. On raising the colicin:ribosome ratio to an unphysiological 200, an inhibition of only 13% was observed using a poly U protein synthesising system. *In vivo* a colicin-ribosome ratio of 0.1 (equivalent to approx. 2×10^3 colicin E_3 molecules per bacterium) produces almost total ribosomal inhibition.

The published work has therefore not completely excluded the possibility that colicin E_2 binds to DNA rendering it more susceptible to nuclease attack or that it has endonucleolytic activity and it was decided to reinvestigate *in vitro* colicin:DNA interactions. Possible direct *in vivo* interactions are subsequently dealt with in Chs. 5 and 6.

METHODS 3

(a) Extraction and purification of radioactively labelled and unlabelled DNA from *E. coli* ROW

An overnight culture of *E. coli* ROW was diluted with fresh medium and incubated aerobically until the growth was exponential. The culture was diluted tenfold with fresh M9 medium containing final concentrations of 200 µg deoxyadenosine per ml in addition to 1 µCi

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(methyl-^{3}H) thymine (0.1 Ci/mmol) per ml or 0.025 µCi (2-^{14}C) thymidine (5.6 mCi/mmol) per ml. Unlabelled DNA was prepared in greater bulk, (5 l of culture compared with 100-500 ml for labelled cells).

The culture was incubated aerobically for 5-6 generations after the second dilution until a cell density of 5.10^{8} bacteria per ml was reached. The bacteria were harvested by centrifugation, washed twice with lysis medium (0.15 M NaCl; 0.1 M EDTA) at 4° and resuspended at a cell density of 1×10^{10} - 4×10^{10} bacteria per ml in lysis medium at 20° containing 500 µg lysozyme per ml. After 10 min incubation, sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% (w/v) and incubation continued at 37° for 15 min to complete lysis. In addition to breaking up the cell membranes, SDS also inhibits DNase activity (Marmur, 1961). The opaque viscous cell lysate was treated with 500 µg pronase per ml and the incubation continued overnight at 37°. After pronase digestion the lysate was clear and a further volume of 25% (w/v) SDS was added to give a final overall concentration of 1.0%. In order to dissociate DNA from residual protein complexes the ionic strength was increased by adding 5 M sodium perchlorate to a final concentration of 1M. The nucleic acid was separated from the cell debris by gently shaking the lysate mixture with an equal volume of chloroform containing 2% (v/v) n-octanol. The emulsion was broken by centrifugation at 10,000g for 15 min. The upper aqueous phase was removed using an inverted 10 ml pipette and propipette so as to minimise shear. Care was taken not to contaminate this phase with any interfacial material.

The aqueous phase was gently overlayered with 2 volumes 96% (v/v) ethanol and the nucleic acid (DNA and RNA) precipitated at the interface was removed by spooling on a thick glass rod. The

precipitated nucleic acid was quickly dissolved in 5-30 ml "dilute buffer" (0.01 M tris-HCl pH 8.0, 0.01 M NaCl and 0.5 mM EDTA) depending on original culture volumes. This nucleic solution will subsequently be referred to as stage I purified DNA.

RNA was removed from the stage I purified DNA by treating with 100 µg RNase A per ml (DNase free) and 1 unit RNase T_1 per ml for 3h at 37°. M tris-HCl pH 7.9 and 4 M NaCl were then added to the nucleic acid preparation to give final concentrations of 0.05 M and 0.2 M respectively. The residual protein and digested RNA were removed by shaking with an equal volume of water saturated redistilled phenol (or freshly made up AnalaR phenol) at pH 8.0. The 2 phases were separated by centrifugation, the upper aqueous phase removed and DNA precipitated with ethanol. The precipitated DNA was slowly dissolved in $SSC/_{10}$ (SSC is 0.15 M NaCl containing 0.015 M trisodium citrate) or "dilute buffer". The DNA solution was extracted four times with equal volumes of ether to remove traces of phenol and stored over chloroform at 0-4°. This will be referred to as stage II purified DNA. The procedure lasted 2-3 days. Disposable polythene gloves were worn when handling DNA to eliminate nuclease contamination.

In a bulk preparation a yield of 30 mg stage II DNA was obtained from 4 g dry weight of cells. This DNA had a $260/_{280}$nm optical density ratio of 1.96-2.01 and a melting temperature (Fig. 3 iii(a)) of 63-66° in $SSC/_{10}$ (method described later). The melting curve had a completely flat base line indicating the absence of RNA and a hyperchromicity of 25-30%. The labelled DNA's had specific activities of 3-6 x 10^3 cts/min/µg for (^3H) and 1.5-2.0 x 10^3 cts/min/µg for (^{14}C). The radioactivity was determined as cold-TCA precipitable counts on Oxoid membrane filters using BBOT:

toluene scintillant. The molecular weight of stage II DNA was approx. 10^7 determined by the sucrose density-gradient method (see Ch. 5). Protein contamination was less than 1% as determined using the methods of Lowry et al., (1951). DNA concentration was determined spectrophotometrically using the 1% (w/v) extinction coefficient value at 260 nm ($E_{260}^{1\%}$) of 200 (Mandel and Marmur, 1968) for pure preparations and the diphenylamine method (Burton, 1956) for preparations containing RNA or protein.

(b) Preparation of radioactively labelled T_4 phage and T_4 DNA

The method used was based on that of Thomas and Abelson (1966).

Exponentially growing cells of E. coli B at a cell density of 5×10^8 bacteria per ml in phage medium were washed and resuspended at the same cell density in fresh phage medium containing 200 µg deoxyadenosine per ml. The resuspended cells were infected with T_4 phage at a multiplicity of 5; 10 min after infection (2-^{14}C) thymidine (specificactivity 5.6 mCi/mmol) or (methyl-^{3}H) thymine (specific activity 0.1 Ci/mmol) was added to the medium at a final concentration of 0.05 µCi/ml or 2 µCi/ml respectively. Incubation was continued at 37° for a further 2.5h. To complete cell lysis, the culture was aerated for several minutes with 1/50 volume chloroform. The culture lysate was centrifuged twice in a SW41 rotor at 6,000 rev/min for 10 min at 5° in a Beckman L2-HV ultracentrifuge to remove cell debris. The supernatant fraction was further centrifuged at 21,000 rev/min for 25 min. The phage pellet was slowly resuspended in 1/50 of the original culture volume of 0.1 M phosphate buffer pH 7.0 containing 0.1 M NaCl. Using 50 ml original culture, the phage suspension (1 ml) contained 6×10^{12} plaque forming units using the soft agar technique of Adams (1959), i.e. corresponding to a burst size of 240.

Radioactively labelled phage DNA was extracted from the phage suspension by gently rolling with an equal volume of water saturated phenol for 30 min. The water saturated phenol was freshly made up from solid AR. phenol and was neutralised by shaking with an excess of 0.1 M phosphate pH 7.0. The phenol layer was separated from the aqueous layer by chilling in ice and centrifuging. The phenol layer was removed using a Pasteur pipette. The phenol rolling technique was repeated twice for 5 min each. The aqueous phase containing DNA was then extracted 5 times with an equal volume of aqueous ether to remove phenol. The final yield of phage DNA was approx. 200 µg (25% yield) from 6 x 10^{12} phage units using the specific absorbancy for glucosylated DNA at 260 nm of 0.0181 $cm^2/\mu g$ (Rubenstein et al., 1961). The specific activities were 3,000 cts/min/µg for (^{14}C) and 12,000 cts/min/µg for (^{3}H).

(c) Spectroscopic measurement of DNase activity

The assay mixture consisted of 0.05 M tris-HCl buffer pH 7.0 or 8.0, 5 mM $MgSO_4$, 20 µg stage II DNA per ml (dialysed against 0.05 M tris-HCl buffer) and 0.4 µg pancreatic DNase I per ml in a total volume of 2.5 ml. DNase I was freshly made up at 10 µg/ml in tris-HCl buffer pH 7.0 or 8.0 containing 1 mg serum albumin per ml and stored at 4° until required. The reaction occurred in a water jacketed quartz spectrometer cell. The temperature was kept at 30° by a Haake constant temperature device. The increase in optical density at 260 nm was measured against time using a Cary 15 recording spectrophotometer. A full scale deflection was equivalent to 0.1 OD unit.

On addition of DNase I to the DNA mixture the optical density increase was linear after 1 min and continued linearly for a minimum of 7 min. The enzyme activity is expressed in terms of optical density increase (measured over a 5 min period from 1-6 min) per min.

The stock colicin E_2 solution was dialysed against 0.05 M tris-HCl buffer pH 7.0 or pH 8.0 before use.

(d) Viscosity measurements of DNA

All viscosity measurements of DNA solutions were carried out using a simple Ostwald viscometer in a constant temperature water bath at 30° (Leith, 1963). The viscosity of a liquid was taken to be proportional to the time taken for its meniscus to travel under gravity between 2 points above a vertical capillary. The liquid volume was 5 ml and contained 500 µg stage II DNA per ml (stock solution dialysed against 0.05 M tris-HCl buffer pH 8.0), 0.05 M tris buffer pH 8.0 and 5 mM MgSO4.

The flow time for water was 27.4 ± 0.1 sec. The flow times for DNA solutions were highly reproducible (time error ± 0.1 sec) for constant viscosities. The DNA solutions were equilibrated in the viscometer for at least 30 min, during which the flow time did not alter by more than 0.2 sec. On addition of DNase I flow times were taken at 3-5 min intervals for 30-40 min.

Reduced viscosity η_{red} is defined as follows:

$$\eta_{red} = \frac{\eta_{rel} - 1}{c}$$

(Eigner 1968)

(units of decilitre per gram)

η_{rel} or relative viscosity = $\eta/\eta_o = tp/t_o p_o$

η and η_o are the actual viscosities of the sample and its solvent t and t_o are the flow times and p and p_o are the densities.

c is the concentration of DNA in the solvent in g/100 ml.

The intrinsic viscosity ($[\eta]$) is the reduced viscosity extrapolated to zero concentration (to eliminate molecular interactions) under conditions of zero shear gradient and stress (Newtonian liquid). ($[\eta]$) can be related to molecular weight.

(e) Thermal denaturation of DNA

All melting curves on DNA samples were performed in a teflon stoppered quartz 1 cm cell using a Cary 15 recording spectrophotometer. DNA samples (2.5 ml) had an optical density of 1.2-1.3 at 260 nm and 20°. The sample temperature was regulated using a Haake constant temperature bath and raised at a rate of 0.5°/min from 25° to 90°. The DNA solvent used was $SSC/_{10}$ or 10 mM phosphate pH 7.0 (Marmur and Doty, 1962). Cooling was performed by flushing the Haake constant temperature device with cold tap water through the cooling coil.

The $\sigma_{2/3}$ value or dispersion of the thermal transition (Mahler et al., 1966; Mandel and Marmur, 1968) was derived from the Cary 15 traces and corresponded to the temperature difference between 17% and 83% absorption rise

(f) Optical Rotatory Dispersion

All measurements were carried out at 20° in a Bendix-Ericsson Polarmatic 62 Recording Spectropolarimeter using a 250-watt Xenon lamp. Samples were placed in 10 mm or 2 x 5 mm quartz cells and the instrument scanned from 320 to 220 nm (32-45 wave nos.). The base line noise was approx. 0.3 millidegrees (m°) and the sample noise was approx. 1.0 m°. The noise level was too high below 220 nm to make any accurate measurements. Each spectrum was performed in triplicate and the mean of the points plotted. The scanning speed was approx. 1 wave no. per min and instrument range setting was 100. The sample volume was approx. 0.5 ml in a 10 mm cell and contained 50 µg nucleic acid per ml (OD_{260} = 1.0) in $SSC/_{10}$ solvent.

The combined ORD spectrum of colicin and DNA (separate) in Fig. 3 vi was obtained using 2 x 5 mm cells in series with twice the control concentration of colicin in one cell and twice the concentration of DNA in the other cell. The colicin and DNA solutions were then mixed and returned to the 2 cells for the mixed spectrum.

(g) Sucrose density gradient centrifugation

(i) Detection of endonuclease activity

5-20% (w/v) sucrose density gradients were made up in 0.1 M NaCl-0.01 M EDTA-0.01M tris-HCl pH 8.0 (NET buffer, Smith and Hanawalt, 1967) for neutral gradients and in 0.9 M NaCl-0.1 M NaOH 5 mM EDTA (Studier, 1965) for alkaline gradients. After incubating 25 µg (^{14}C) DNA with 50 µg colicin E_2 or 1 ng DNase I (plus 20 µg serum albumin) in 0.5 ml 10 mM phosphate buffer containing 5 mM $MgCl_2$ for 5-30 min at 37°, the reaction was stopped by adding 0.5 ml ice-cold 2 x concentrated NET buffer for neutral gradients or 0.5 ml ice-cold 0.5 M NaOH-10 mM EDTA for alkaline gradients. In addition to stopping the DNase reaction in neutral samples, EDTA was necessary to prevent precipitation of alkaline samples due to the presence of Mg^{++}. 0.2 ml treated DNA solution (approx. 10,000 cts/min) was layered on to a 4.8 ml sucrose gradient and centrifuged in a SW50 rotor at 40,000 rev/min and 20° for 3h using a Spinco Model L2-HV ultracentrifuge. Duplicate determinations were carried out in each case. 10 drop samples (0. 15 ml) were collected from a pinhole punched in the bottom of the centrifuge tube. Samples were mixed with 0.3 ml water and 3 ml Triton x 100:BBOT-toluene mixture (1:2) and their radioactivity determined using a Packard Tri-Carb scintillation spectrometer. Sedimentation values were approximated from results obtained in Ch. 5 and the weight average molecular weights of the DNA samples determined using the formulae of Studier (1965).

(ii) Detection of colicin binding to DNA

5-20% (v/w) sucrose density gradient was made up in 10 mM NaCl-1 mM phosphate buffer pH 7.1 (Felsenfeld et al., 1963). 50 µg colicin E_2 was incubated with 10 µg (^{3}H) DNA in 0.5 ml NaCl-

phosphate buffer pH 7.1 for 10 min at 20°. 0.2 ml DNA solution (approx. 20,000 cts/min) was layered onto 4.8 ml sucrose gradient and centrifuged in a SW50 rotor at 40,000 rev/min and 4° for 3h using a Spinco L2-HV ultracentrifuge. The gradients were fractionated and the radioactivity and colicin activity determined. Colicin activity was measured by the growth inhibition zone method (described in Ch. 1) after tenfold dilution of the sucrose fraction.

(h) Isopycnic centrifugation

50 µg colicin E_2 was incubated with 10 µg (^3H)-DNA in 0.5 ml 10 mM phosphate buffer pH 7.0 for 10 min at 37°. 0.2 ml DNA solution was mixed with stock CsCl or Cs_2SO_4 solutions in 10 mM tris-HCl buffer pH 8.0 containing 10 mM EDTA so that the final densities after adjustment with tris-EDTA buffer using the refractive index method of Szybalski (1968) were 1.7100 g/ml for CsCl or 1.4250 g/ml for Cs_2SO_4. The mixture was overlayered with liquid paraffin and centrifuged to equilibrium (45-70h) at 44,000 or 32,000 rev/min using a SW-50 or Ti-50 rotor in a Spinco Model L2-HV ultracentrifuge. The tube contents were fractionated (see Ch. 5) and the radioactivity measured.

Radioactively labelled DNA's from Micrococcus lysodiekticus and T_4 phage were included with the colicin treated DNA for reference. E. coli DNA was denatured by heating to 100° for 10 min in 10 mM phosphate buffer pH 7.0, followed by rapid cooling in ice.

A similar system was used on the Beckman Spinco Model E analytical ultracentrifuge at 20° using Kel-F centrepieces for the standard and 1° wedge cells. Each cell received 0.7-0.8 ml solution containing approx. 0.5 µg of each DNA. Photographs were taken after 16-20h with UV optics and tracings were made using a Joyce-Loebl recording microdensitometer.

(i) Protein:DNA complex binding to Millipore filters

The methods used were based on those described by Jones and Berg (1966) for RNA polymerase and Yuan and Meselson (1970) for restriction endonuclease. RNA polymerase fraction IV (Chamberlin and Berg, 1962) was used as a standard (see next section).

2 incubation systems were investigated containing in a total volume of 0.25 ml; (i) 10 µmols tris-HCl pH 8, 1 µmol $MgCl_2$ and 3 µmols mercaptoethanol; (ii) 2.5 µmols phosphate buffer pH 7.0. Both systems contained 12.5 µg stage II (^3H) labelled DNA per 0.25 ml of incubation mixture (60,000 cts/min). After incubation of each system with either RNA polymerase or colicin E_2 for 5-10 min at 37°, the mixture was diluted to 2 ml with ice cold 10 mM tris-HCl buffer pH 8 containing 50 mM NaCl (system (i)) or 10 mM phosphate buffer pH 7 (system (ii)) and filtered through a Millipore membrane (type HA, plain, white, 2.4 cm diameter). The membrane filters were presoaked for 15 min in the diluting buffer. After filtration the membrane was washed with 2 x 5 ml of the dilution buffer under very gentle suction. The membrane filters were dried and the radioactivity determined by liquid scintillation counting. System (i) was described by Jones and Berg (1966) whilst system (ii) duplicates the conditions used in thermal denaturation of DNA.

The effect of ionic strength on the stability of the DNA: protein complex was determined by increasing the concentration of NaCl in the phosphate dilution buffer. This saline-phosphate buffer was used for dilution after incubation of DNA with protein and for presoaking and washing the filters. The initial incubation was performed in the absence of saline.

(j) RNA polymerase assay

The system used for assaying RNA polymerase contained in a total volume of 0.25 ml; 10 µmols tris-HCl pH 7.9; 1.0 µmol MgSO4; 0.25 µmols $MnCl_2$; 0.1 µmols each ATP, CTP and UTP; 0.1 µmols (^{14}C) GTP (0.36 mCi/mmol); 3 µmols mercaptoethanol; 50 µg stage II DNA and 3.5 µg RNA polymerase (Biopolymers Inc., 2000 units * per mg fraction IV (Chamberlin and Berg, 1962)). The enzyme was stored at -20° in glycerol.

After incubation at 37° for 30 min the reaction was stopped by transferring the tubes to a boiling water bath and heating for 5 min in order to denature the nucleic acid and aid acid precipitation (Waring, 1964). The assay mixture was then cooled in ice and 0.1 ml (2 mg/ml) crude yeast nucleic acid added to aid cold acid precipitation of newly synthesised RNA. 0.35 ml 10% (w/v) TCA was added and the cold-acid precipitate was filtered on Oxoid membranes after 10 min, washed and the radioactivity determined.

(k) In vitro protein synthesis

(i) Preparation of active cell extract

The methods were based on those due to Nirenberg and Matthaei (1961) and R.N. Hill (1969; personal communication). All operations were carried out between 0° and 4°. 20 g of freeze dried cells of E. coli MRE 600 were suspended in 25 ml TAMM buffer * containing 2 µg DNase per ml and broken by blending with 60 g acid washed ballotini in a MSE microblendor at full speed for 10 min. The broken cells were centrifuged at 2000 rev/min for 1 min in a MSE 18 centrifuge using two 50 ml centrifuge tubes. The dark supernatant

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* 1 unit is defined as the amount of enzyme which catalyzes the incorporation of one nmol of total nucleotide per 15 min incubation at 37° with native E. coli DNA as primer.

* TAMM buffer; 20 mM tris-HCl pH 7.8, 100 mM NH_4Cl, 20 mM magnesium acetate and 6 mM mercaptoethanol.

fluid was retained and the two pellets resuspended in 2 x 5 ml TAMM buffer. The cell debris was again centrifuged and the supernatant fractions combined. After treatment with a further 2 µg DNase per ml at 4° for 10 min, the cell supernatant fraction was centrifuged for 15 min at 11,500 rev/min. The supernatant and top pellet fractions were removed and further centrifuged at 16,000 rev/min for 30 min. The clear supernatant fraction was dialysed for 16h against 3 x 3 l TAMM buffer after which time a sediment had formed which was removed by centrifugation at 11,500 rev/min for 15 min. The final clear cell extract had a volume of 5-10 ml and an OD_{260} of approx. 500.

(ii) Assay system

The poly U directed in vitro protein synthesising system contained in a total volume of 0.25 ml: 5 µmols tris-HCl pH 7.8; 4 µmols magnesium acetate; 25 µmols NH_4Cl; 1.5 µmols mercaptoethanol; 0.25 µmols ATP; 0.01 µmols GTP; 2.5 µmols creatine phosphate; 10 µg creatine kinase; 10 µg poly U; 2 nmols (^{14}C) phenylalanine (75 mCi/mmol) and 5.0 OD_{260} units * of cell extract.

After incubation at 37° for 15 min the reaction was stopped with 1.5 ml 5% (w/v) TCA. The precipitate was heated at 90° for 15 min to remove (^{14}C) phenylalanine attached to tRNA, cooled to room temperature and filtered on glass fibre filters. The filters were washed, dried and the radioactivity measured.

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* OD_{260} units; unit of material which in a 1 ml volume and 1 cm light path will have an optical density of 1.0 at 260 nm.

RESULTS 3

There are a number of possible *in vitro* interactions of colicin E_2 with DNA and these were studied by investigating (a) deoxyribonuclease activity, (b) DNA destabilising activity, (c) DNA binding activity and (d) action on *in vitro* macromolecular synthesis.

(a) Deoxyribonuclease activity

DNase activity was investigated by incubating DNA with colicin E_2 and examining the DNA for; (i) a decrease in cold-acid insoluble nucleotide material, (ii) an increase in absorption at 260 nm, (iii) a decrease in viscosity and (iv) a decrease in sedimentation value on neutral and alkaline sucrose density-gradients. Pancreatic DNase I was used as a standard throughout. This enzyme is a "double hit" endonuclease (Studier, 1965) and the final products of the degradation are predominantly di- and tri- nucleotides (Laskowski, 1966).

(i) Effect of colicin E_2-P9 on cold-acid insoluble nucleotide material *in vitro*

This was the only *in vitro* method used by Nomura (1964) for colicin E_2 and although the experimental details were not included in his paper, it was a useful starting point. The experiment involved incubating colicin E_2 or DNase with a variety of radioactively labelled DNA substrates. Certain nucleases in *E. coli* (Lehman, 1967; Richardson, 1969) are known to be inhibited by RNA (endonuclease I) activated by DNA denaturation (endonuclease II and exonuclease I) or decrease of DNA molecular weight (exonuclease IV). Most nucleases also need Mg^{++} for activation. 4 parameters were therefore varied; DNA molecular weight, presence of Mg^{++}, physical state of DNA and presence of RNA. These were chosen to enable detection of exonucleo-

TABLE 3 i

Effect of colicin E_2 on cold-acid precipitable nucleotide material in vitro

DNA	cold-acid insoluble radioactivity (cts/min) control	+colicin E_2	+DNase I
(^{14}C) DNA (MWt. 1.5×10^7)			
Stage II-Mg^{++}	18310	18570	17130
+Mg^{++}	18170	18320	270
Stage II heat denat.* +Mg^{++}	18110	18070	570
(^{14}C) DNA Stage I(+RNA)+Mg^{++} (MWt 2.2×10^7)	19110	18920	910
(^{3}H) DNA (MWt. 2×10^6)			
Stage II + Mg^{++}	33520	32910	420

10 µg (^{14}C) or (^{3}H) DNA was incubated with 50 µg colicin E_2 or 1 µg DNase I (plus 50 µg albumin) in 1 ml 50 mM tris-HCl pH 8.0 containing 5 µmols MgCl2 or 5 µmols EDTA for 30 min at 37°. The reaction was stopped by adding 1 ml ice-cold 10% (w/v) TCA. After 30 min at 4° the precipitate was filtered through glass fibre discs, washed, dried and the radioactivity determined.

* DNA was denatured by heating to 100° in tris-HCl buffer for 10 min followed by rapid cooling in ice.

lytic activity or massive endonucleolytic activity similar to DNase I under specific conditions. The effects of more specific activators such as nucleoside triphosphates and S-adenosyl methionine (exonuclease II and VI, endonuclease III) and of pH were not investigated.

The results are shown in Table 3 i and confirm those of Nomura (1964). Colicin E_2 was demonstrated to exhibit no nuclease activity as detected by this method under these conditions. The molecular weights of the DNA samples used were determined by centrifugation on neutral sucrose density-gradient as described in Methods 3 and Ch. 5. The (^{3}H) DNA was reduced in size by shearing in a hypodermic syringe needle. DNase I was active on all DNA preparations in the presence of Mg^{++}. The increased residual radioactivity after DNase I degradation of stage I DNA was probably due to acid precipitation of RNA increasing the retention of radioactive nucleotide material on the filter.

Stage I purified nucleic acid isolated from cells labelled with (^{3}H) uridine was also tested with colicin E_2. No decrease in cold-acid precipitable radioactivity was detected. Colicin E_2 was therefore assumed to have no RNase activity in vitro.

(ii) Effect of colicin E_2-P9 on the OD_{260} of DNA and DNase I activity

An alternative method of assaying DNase I is to determine the increase in absorption at 260 nm of the DNA sample (Kunitz, 1950). This method has been frequently used for investigating inhibitory effects of certain antibiotics on DNase activity (Sarkar, 1967). Although the pH optimum of this enzyme is approx. 7.0 (Laskowski, 1966 and own results) experiments are usually performed within a range of

TABLE 3 ii

Spectroscopic determination of nuclease activity

		CONDITIONS ..... DNA buffer				ΔOD260/minx10^3
		Mg^{++}	Colicin	DNase	Other	
pH 8.0	1	+	-	-	-	0
	2	+	-	+	-	7.8
	3	-	-	+	-	0
	4	+	+	-	-	0
	5	-	+	+	-	0
	6	+	+	+	-	10.8
	7	+	+($\frac{1}{2}$conc)	+	-	9.6
	8	+	+ denat.[1]	+	-	8.1
	9	+	-	+	DAU[2]	0.3
	10	+	-	+	ACT[2]	1.7[3]
pH 7.0	11	+	-	+	-	18.2
	12	+	+	+	-	19.8
	13	+	+	-	-	0

[1] Colicin E_2 heat denatured at 100° for 10 min prior to incubation.

[2] Daunomycin and actinomycin D concn. 20 µg/ml.

[3] After a lag of 4 min.

The incubation mixture contained 20 µg DNA per ml, 0.05 M tris-HCl buffer pH 8 or pH 7 and when indicated 5 mM $MgSO_4$, 0.4 µg DNase per ml and 30 µg colicin E_2 per ml. The increase in OD_{260} was determined as described in Methods 3. The rates were averages of triplicate determinations.

2 pH units above or below this optimum owing to the greater reproducibility of results and the easier detection of inhibitory or stimulatory effects.

DNA solutions were incubated with varying combinations of Mg^{++}, colicin E_2 and DNase and the increase in OD_{260} per min determined (Table 3 ii). Under these conditions colicin E_2 exhibited no nuclease activity (tubes 4 and 13), however on pre-incubating DNA with colicin for 5-10 min at pH 8.0, the rate of DNase induced increase in DNA absorption at 260 nm was enhanced by 38% (tube 6). The molecular ratio of colicin to DNA under these conditions (Table 3 ii) was 250 and at half this ratio the enhancement was 24% (tube 7), however above 250 molecules of colicin per DNA molecule there was no significant rate increase. This enhancement could be an artefact and not due to a specific DNA-colicin E_2 interaction. The artefact could arise if addition of colicin lowered the pH value of the DNA sample or altered the ionic environment, since it is known that Mn^{++} has a significant effect on DNase I action (Melgar and Goldthwait, 1968.) However the pH value did not change on addition of colicin to DNA in tris-HCl buffer pH 8.0 and incubation of DNA with colicin and DNase in the absence of Mg^{++} resulted in no detectable degradation (tube 5). In addition heat activated colicin E_2 had almost no effect.

The effects of colicin E_2 were only on the rate of DNA degradation by DNase I and not the absolute amount which corresponded to approx. 35% increase in OD_{260} after 2h incubation. The enhancement effect of colicin E_2 on DNase activity at pH 8.0 was probably the result of an interaction with the DNA substrate rather than the enzyme, owing to compatibility of the colicin:DNA molar ratios with

subsequent results. However the latter possibility was not completely excluded and could involve a protection of the enzyme at adverse pH values. The enhancement effect was reduced at pH 7.0 (tubes 11 and 12) probably due to the pH effect on the enzyme.

Preincubation of DNA with daunomycin and actinomycin D (tubes 9 and 10) resulted in different degrees of DNase inhibition. Daunomycin (Kersten et al., 1966) a well characterised intercalating drug almost totally inhibited DNase I whereas actinomycin D only inhibited DNase I action by 78% during the first 10 min. This actinomycin D result was consistent with results of Sarkar (1967) and may reflect protection of only guanine rich regions of DNA (Kirk, 1960), see Ch. 4.

In addition to these results it was observed that there was a 5-10% hyperchromic effect on mixing colicin E_2 with DNA. The interpretation of this observation may be related to the results with ORD.

(iii) Effect of colicin E_2-P9 on DNA viscosity in vitro

If colicin E_2 were a limited endonuclease the above experiments would not be expected to produce positive results. Measurement of DNA viscosity however provides a sensitive method for the detection of double-strand cleavages (Schumaker et al., 1956). Single-strand breaks are not detected by a significant change in viscosity except near the melting temperature (Hays and Zimm, 1970).

The results are shown in Fig. 3i where the "reduced" viscosity (for definition see Methods) is plotted against time. 0.5 µg DNase I per ml reduced the DNA viscosity to that of water within 2 min (see Leith, 1963) and at a concentration of 0.02 µg/ml

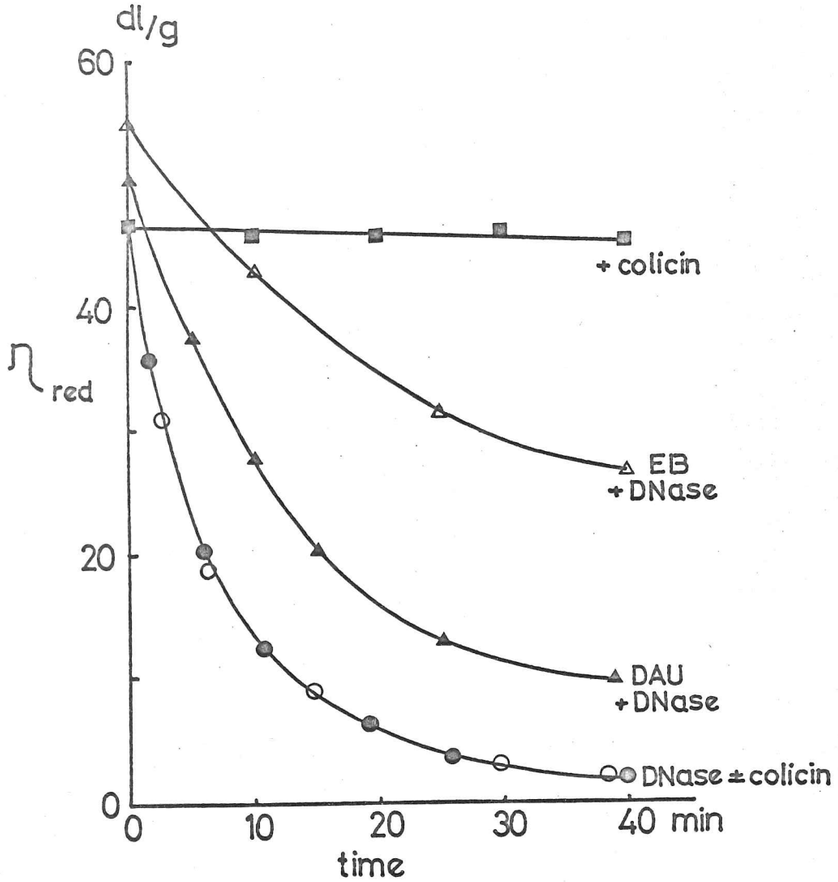


FIG. 3 i Effect of colicin E2 and intercalating drugs on DNA viscosity and the activity of DNase

The reduced viscosity (η_{red}) of E.coli DNA (500µg/ml) was determined using an Ostwald viscometer at 30°. DNase (0.02µg/ml) was added at time zero in the presence of daunomycin (10µg/ml), ethidium bromide (25µg/ml) and colicin E2 (50µg/ml) as indicated and the fall in vicosity plotted against time. The effect of colicin alone on DNA viscosity was also determined.

DNase was found to produce a measurable decrease in viscosity over 30 min. The DNA sample was always allowed to equilibrate for 30 min in the viscometer. The reduction in DNA molecular weight as a result of shear in the capillary was negligable since after 10 successive flow time determinations the flow time had decreased by only 1 sec (approx. 1%). Incubation of the DNA sample with 50 µg colicin per ml for 40 min did not result in a significant change in viscosity. There was no stimulation or inhibition of the control DNA degradation with DNase I on addition of 0.02 µg DNase per ml to colicin treated DNA.. These results would indicate that under these conditions colicin E_2 had no double strand endonuclease activity and did not activate DNase I directly or indirectly by rendering the DNA more susceptible to attack. Intercalating drugs daunomycin and ethidium bromide inhibited the DNase induced viscosity decrease of DNA (see Ch. 4) after first increasing the DNA 'stiffness' (Waring, 1966).

The Oswald viscometer is not ideal for DNA molecular weight determination since the shear gradient can be very significant for high molecular weight samples (Eigner, 1968). However the stage II DNA fragments used have an approx. molecular weight of 10^7 (Fig. 3 ii) and if it is assumed that the shear gradient and concentration effects are negligible the reduced viscosity approximates to the specific viscosity. The specific viscosity may then be related to the molecular weight using the data of Eigner and Doty (1965) from which a value of 8×10^6 daltons is obtained.

(iv) Effect of colicin E_2-P9 on the sedimentation value of DNA

The experiments so far have excluded exonuclease and double strand but not single strand endonuclease activity for colicin E_2. Single strand snips of the DNA helix are only detected after separation

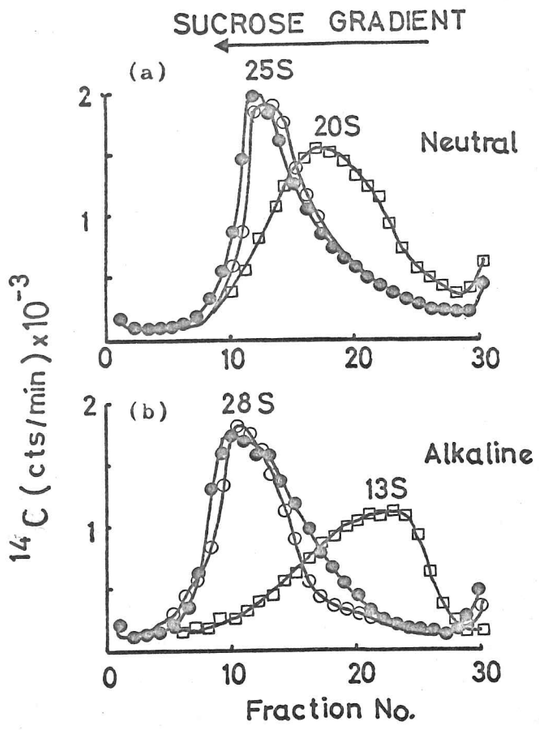


FIG. 3 ii Neutral and alkaline sucrose density-gradient profiles of(^{14}C) labelled DNA incubated with colicin E2 and DNase in vitro.

25µg (^{14}C)-DNA from E.coli was incubated with colicin E2 ●—● (50µg) and DNase I □——□ (1ng) at 37°for 30 and 5 min respectively. (DNA control ○——○) Samples were centrifuged on 5-20% (w/v) neutral and alkaline sucrose density-gradients in a SW50 rotor for 90 min at 40,000g. Sedimentation values were determined from FIG.5ii.

of the DNA strands by alkaline denaturation. Hence a change in DNA molecular weight is detected on an alkaline but not on a neutral sucrose density gradient (Studier, 1965).

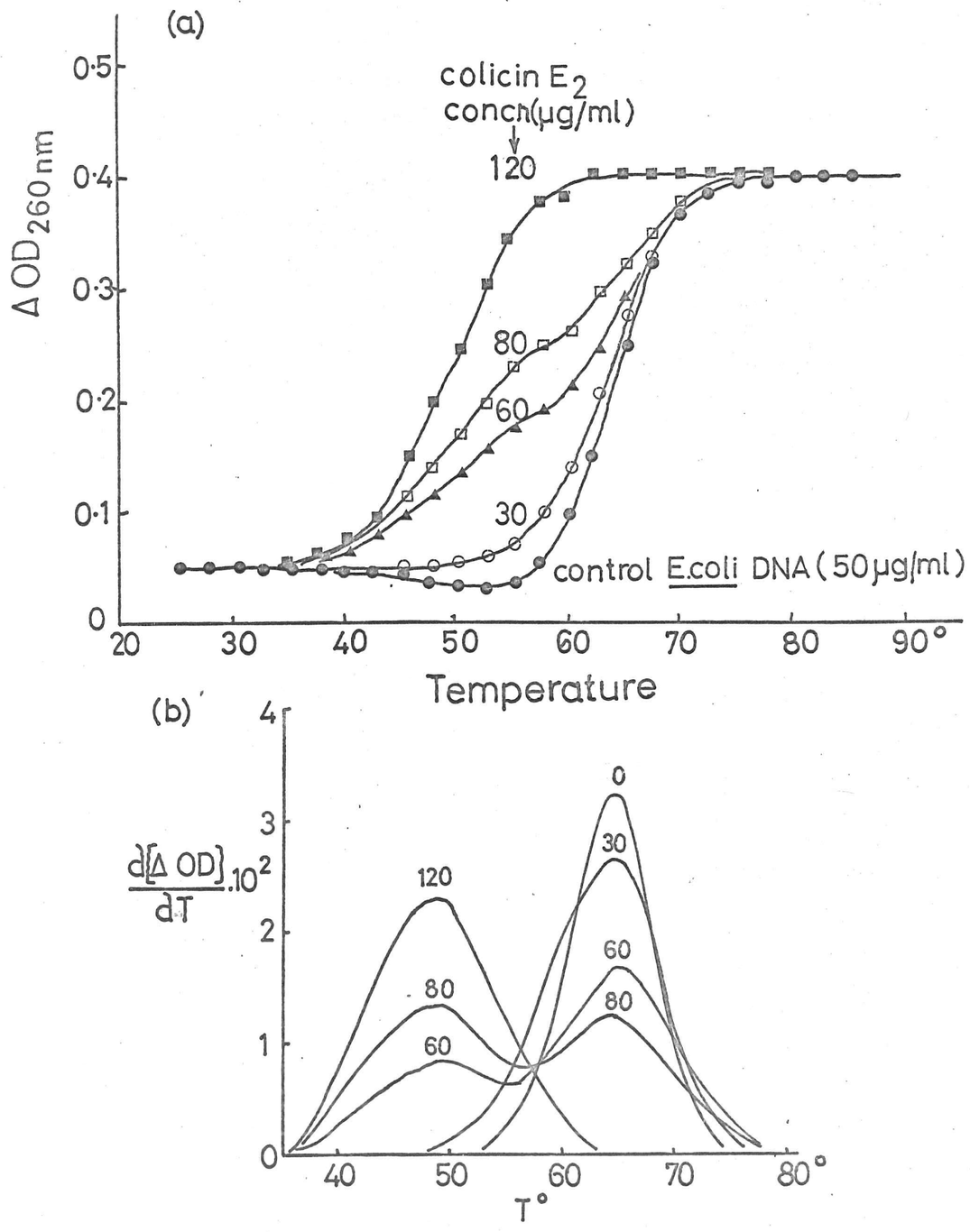
The sedimentation profiles of stage II DNA after incubation with colicin E_2 for 30 min or DNase I for 5 min on neutral and alkaline sucrose density-gradients are shown in Fig. 3 ii. There was no significant change in sedimentation coefficient of colicin treated DNA compared with the control on both types of gradient (25S neutral, 28S alkaline corresponding to a duplex molecular weight of 10^7). (^{14}C) labelled DNA was used owing to the spontaneous appearance of single strand lesions in high specific activity (^{3}H) labelled DNA after approx. one week's storage (Thomas and Abelson, 1966; and own observations). DNase I treated DNA however showed a greater reduction in sedimentation value on the alkaline gradient than on the neutral gradient consistent with its known mode of action of snipping single strands of DNA before double strand breakage, i.e. double hit mechanism as opposed to single hit mechanism of DNase II and E. coli endonuclease I (Studier 1965; Young and Sinsheimer, 1965; Melgar and Goldthwait, 1968).

It was therefore concluded from this section that colicin E_2 exhibited no exo- or endonucleolytic activities in vitro. However it was shown that under certain conditions colicin E_2 enhanced the rate of DNase attack on DNA suggesting a more subtle indirect action in vitro than the direct nuclease action initially proposed for investigation.

FIG. 3 iii Effect of colicin E2 on the thermal denaturation of DNA

(a) Melting profiles of stage II E.coli DNA (50µg/ml) in SSC/10 after treatment with concentrations of colicin E2 indicated.

(b) Derivative curves of the melting profiles in (a). The numbers correspond to the colicin concentration in µg/ml.



(b) DNA destabilising activity

Although colicin E_2 was shown to have no nuclease activity, it was possible that it affected the 3-dimensional structure of DNA. This possibility was studied by investigating the effect of colicin E_2 on (i) the thermal stability, (ii) the ORD spectrum and (iii) the buoyant density of DNA.

(i) Effect of colicin E_2-P9 on the thermal stability of DNA

Destabilisation of DNA can be detected by a lowering of the melting temperature (T_m) or a change in shape of the melting profile on thermal denaturation. The slope of the profile ($\sigma_{2/3}$) can also be used as an indication of DNA molecular inhomogeneity and DNA-protein interaction, (Doty et al., 1959; Marmur and Doty, 1962; Mandel and Marmur, 1968).

The effect on DNA melting profiles and their derivative curves of adding increasing amounts of colicin E_2 to stage II DNA in $SSC/_{10}$ is shown in Fig. 3 iii. The melting temperature and shape of the profile were unaffected by colicin E_2 at concentrations of less than 30 µg/ml. However at 30 and 40 µg colicin E_2 per ml the melting profile base line showed a premature increase at 50°. At 60 and 80 µg colicin per ml the profile shape was significantly altered with the $\sigma_{2/3}$ value increasing by approx. 4 times (Fig. 3 v). It was also apparent that the profile was composed of two melting curves which were more clearly defined in Fig. 3 iii(b). One corresponded to the control T_m value of approx. 65° while the other indicated the presence of DNA with a melting temperature of about 49°. On further increasing the colicin concentration to 120 µg/ml the $\sigma_{2/3}$ value decreased again (Fig. 3 v) and the profile was no longer biphasic. The DNA was now entirely converted to a lower melting temperature.

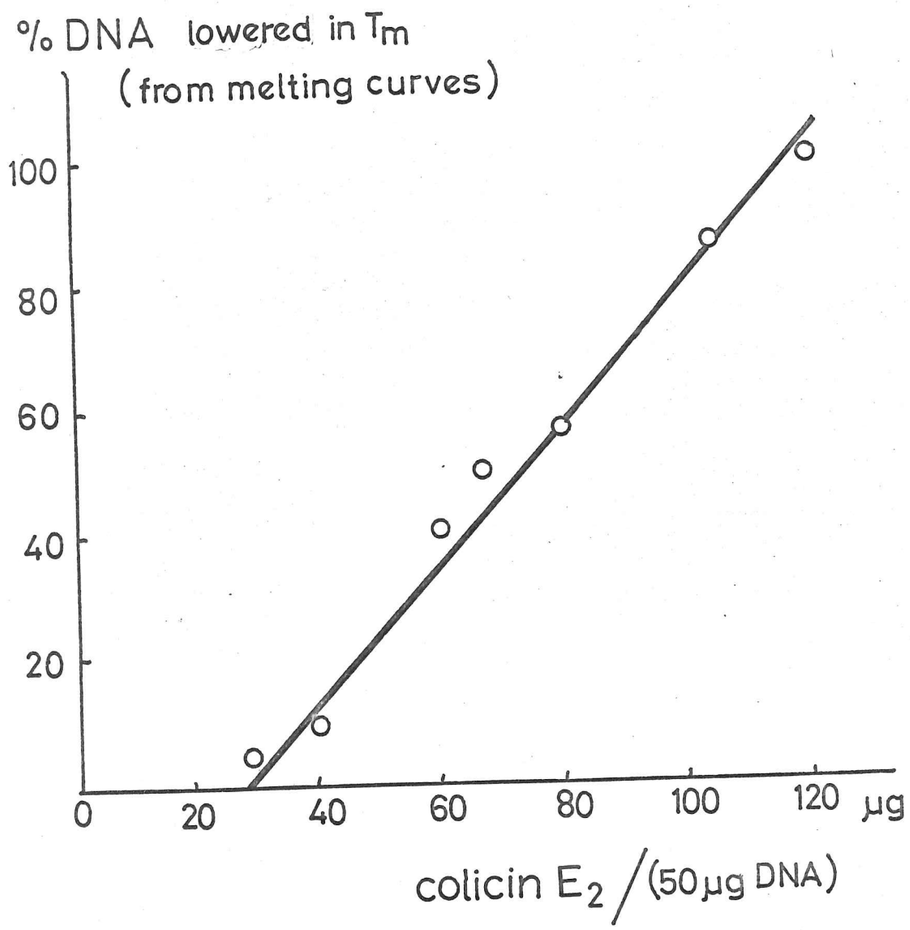


FIG. 3 iv Biphasic analysis of melting profiles of colicin E2 treated DNA

The percentage DNA lowered in melting temperature was derived from the hyperchromic effects of the melting curves in FIG. 3 iii and is plotted against the colicin E2 concentration.

These results (Fig. 3 iii) resemble those obtained on binding histone (Shih and Bonner, 1970.a) or clupeine (Inoue and Ando, 1966) to DNA except that colicin E_2 lowered the melting temperature whereas histone and clupeine raised its value. Pancreatic ribonuclease has been demonstrated to destabilise DNA but only below 60°, above 60° the denatured ribonuclease stabilises DNA (Felsenfeld et al., 1963).

If the amount of destabilised DNA was expressed as a percentage of the total DNA using the hyperchromicity of the melting profiles, it was found that the percentage destabilisation increased linearly with concentration of colicin E_2 above a threshold concentration of approx. 30 µg/ml (Fig. 3 iv). Since the DNA molecular weight was approx. 10^7, the molecular ratio of colicin to DNA at the threshold concentration was approx. 100 and at 100% destabilisation was approx. 400. It would appear that colicin E_2 bound to DNA under the above experimental conditions with the result that DNA was less stable to thermal denaturation.

The melting profiles were also carried out in 10 mM phosphate buffer pH 7.0 (stock colicin buffer) and similar results were obtained (Table 3 iii). Colicin E_2 was denatured by extensive dialysis (Reeves, 1963; and own observations) so this second method was often preferred. The action of colicin E_2 on the DNA melting profile was also unaffected by 10 mM phosphate buffer pH 8.0 which would suggest that the pH effect on DNase activation by colicin E_2 (Table 3 ii) was due to the enzyme and not the colicin:DNA interaction.

Heating colicin E_2 in phosphate buffer in the absence of DNA produced turbidity at approx. 70° which was due to aggregation of denatured protein. No protein aggregation occurred however in the presence of DNA on heating to temperatures as high as 100°. This

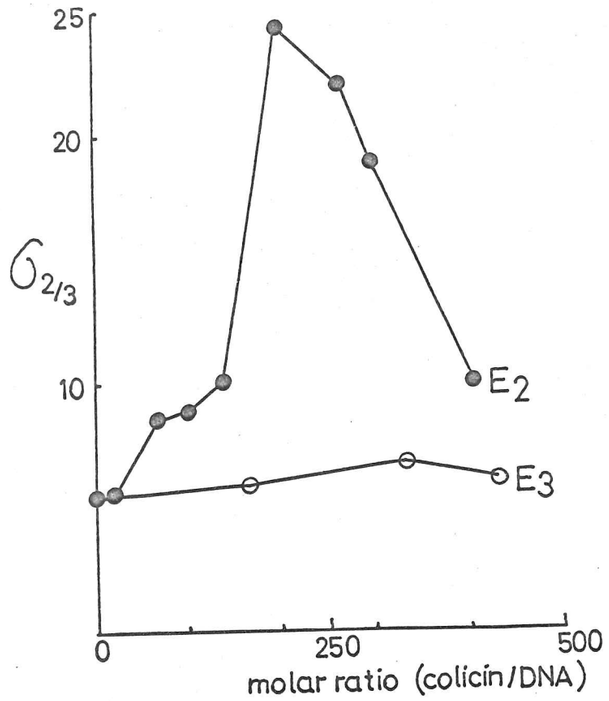


FIG. 3 v Effect of colicins E2 and E3 on the $\sigma_{2/3}$ values of DNA melting profiles.

The $\sigma_{2/3}$ value or the temperature interval between 17 and 83% hyperchromicity was derived from melting profiles as described in FIG. 3 iii and is plotted against the colicin/DNA molar ratio assuming molecular weights of 6.10^4 and 10^7 respectively.

observation indicated a protection of colicin E_2 by DNA against thermal denaturation and aggregation. Similar results were obtained with chymotrypsinogen by Bobb (1966).

These results were reproducible using different colicin E_2 preparations, although there were slight variations in the quantity of colicin required to destabilize 50% of the same DNA preparation, i.e. 70 $\pm$ 7 µg protein per ml. The results were not however quite so reproducible using different E. coli ROW DNA preparations since the decrease in T_m by colicin E_2 varied from 5° to 15° and the biphasic nature of the profile sometimes became less distinct. The change in $\sigma_{2/3}$ values was reasonably constant, i.e. at 60 ug colicin E_2 per ml, $\sigma_{2/3}$ varied from 23.2 to 18.1 for the same colicin preparation. The reason for this variability was not due to a change in molecular weight of the DNA since all preparations were $10^7 \pm 2 \times 10^6$ daltons and Marmur and Doty (1962) have shown that the T_m of calf thymus DNA was independent of its molecular weight within the range 6×10^5 to 8×10^6 daltons. Below this range Crothers et al., (1965) have found a decrease in T_m and increase in profile slope. However phenol (0.1 - 1.0 mM) was found to greatly enhance the lowering of the DNA melting temperature with colicin E_2 although the T_m and $\sigma_{2/3}$ values for DNA alone were not affected (Table 3 iii). Phenol also did not affect the aggregation temperature of colicin E_2 without DNA or the $\sigma_{2/3}$ value for the colicin E_2-DNA complex. In the presence of phenol at concentrations greater than 10 mM continuous DNA melting was observed from 40° to 80° in the control. EDTA (5 mM) reduced the interaction between colicin E_2 and DNA since neither $\sigma_{2/3}$ or T_m were altered significantly in the presence of colicin (Table 3 iii).

TABLE 3 iii

DNA melting effects

	DNA (50 µg/ml)	Buffer	Protein (60 µg/ml)	Other Additions	T_m (control)	ΔT_m	$\sigma_{2/3}$
1	*E.coli* ROW (stage II)	SSC/10	-		64		5.6
		"	E_2			-15	23.1
2	"	10mM PO_4	-		70		5.8
		"	E_2			-17	22.3
3	"	10mM PO_4	-	EDTA(1mM)	69		5.0
		"	E_2	"		-4	9.1
4	" (after dialysis)	SSC/10	-		63		5.8
		"	E_2			-4	20.8
5	"	SSC/10	-	phenol(0.1mM)	63		6.0
		"	E_2	"		-18	18.3
6	*E.coli* ROW/E (stage II)	10mM PO_4	-	"	71		6.2
		"	E_2	"		-17	19.1
7	Calf thymus	10mM PO_4	-		66		10.2
		"	E_2			-3	16.1
8	"	10mM PO_4	-	Mg^{++}(4mM) phenol(0.1mM)	75		11.7
		"	E_2	"		-15	17.2
9	*E.coli* ROW (stage II)	10mM PO_4	E_3			+3	6.1
10	"	10mM PO_4	BSA			+5	6.5

T_m is the DNA melting temperature of the DNA without protein

ΔT_m is the difference between the melting temperatures of protein treated DNA and control DNA.

$\sigma_{2/3}$ is the temperature transition between 17 and 83% hyperchromicity.

Extensive dialysis of the DNA against SSC/10 reduced the decrease in melting temperature with colicin E_2 to approx. 5°. It would therefore appear that for the maximum effect of colicin E_2 on DNA stability trace amounts of phenol must be present. As phenol was present in the *in vitro* system it is questionable whether such results occur *in vivo*. It may reflect an association of colicin E_2 with DNA only in hydrophobic regions of the cell, i.e. the membrane. Non-physiological substances such as methanol and ethanol have been widely used for investigating *in vitro* protein synthesis (Munro and Marcker, 1967) and Nagai *et al.*, (1969) have found that the peptide antibiotic bleomycin (Takita *et al.*, 1968) unlike closely related phleomycin lowered the melting temperature of DNA only if the DNA was pretreated with 1 mM mercaptoethanol or dithiothreitol. However under the conditions used in the colicin E_2 experiments, mercaptoethanol (1-10 mM) was observed to increase $\sigma_{2/3}$ and decrease T_m slightly for control DNA, see Bode (1967).

The effect of colicin E_2 on T_m was specific since a number of other proteins including bovine serum albumin, colicin E_3 and hydroxylapatite fractions A, II and IV (Fig. 1 iv) increased the T_m value of *E. coli* DNA (Table 3 iii). In addition thermal denaturation, trypsin digestion and age inactivation of colicin E_2 abolished all effects.

The interaction of colicin E_2 with DNA's from *E. coli* ROW/E, *E. coli* col^+ E_2-P9 and calf thymus (Sigma) was also studied and no significant difference was detected between the DNA from the colicin resistant or immune strain of *E. coli* and the sensitive strain *E. coli* ROW (Table 3 iii). It is therefore unlikely that the specificity of colicin E_2 action results from specific binding sites on the bacterial DNA. Calf thymus DNA was observed to interact with

colicin E_2 less efficiently than E. coli DNA. The lowering of the melting temperature was enhanced by 5 mM Mg^{++} and by phenol but the $\sigma_{2/3}$ effect was small under all conditions (Table 3 iii). The less responsive effect of calf thymus DNA may have been due to a different method of extraction and purification or may reflect some specificity for the source of DNA.

The percentage hyperchromicity of E. coli DNA on thermal denaturation was unaffected by the presence of colicin E_2 and phenol but low (25-30%) in comparison with that observed for calf thymus DNA (40%) (cf. Mandel and Marmur, 1968). This would suggest that E. coli DNA extracted and purified by the procedure described in Methods 3 was slightly denatured and may explain why colicin E_2 interacted more effectively with E. coli DNA. A 5% initial hyperchromic effect was noted on simply mixing colicin E_2 with DNA (see Results 3(a) ii).

Renaturation of heat denatured DNA by slowly cooling the heated sample was slightly inhibited by colicin E_2. The decrease in hyperchromicity was reduced from 40% of the total effect in the control to approx. 25% in the DNA sample treated with 80 µg colicin E_2 per ml.

(ii) Effect of colicin E_2-P9 on the ORD spectrum of DNA

Although colicin E_2 affected DNA thermal stability, the melting profiles did not provide information on possible changes in DNA structure at room temperature and subsequent studies with ORD were designed to investigate this possibility.

ORD measures the change in optical rotation of a molecule with wavelength and has been frequently used for determining changes in nucleic acid and protein secondary structure, (Adler and Fasman, 1968; Yang and Samejima, 1969; Gratzer and Cowburn, 1969). If colicin E_2 denatured or altered the base stacking of DNA in vitro

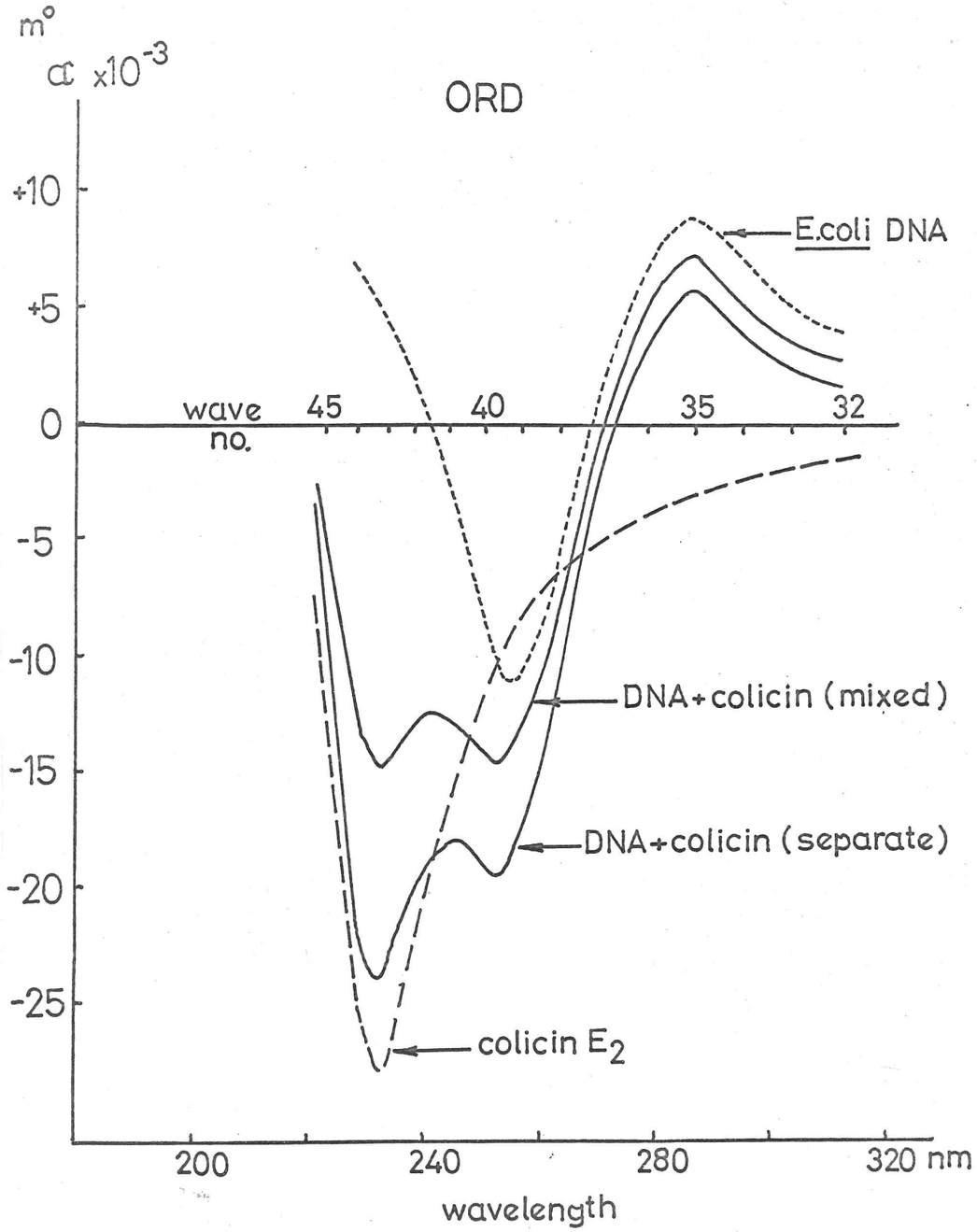


FIG. 3 vi ORD spectra of DNA and colicin E2

A 10mm light path was used and the DNA and colicin concentrations were 50 and 70ug/ml respectively in SSC/10.

it should be detected by this method. McPhie and Gratzer (1966) examined the ORD of ribosomes from E. coli, and concluded from their finding that the spectrum could be represented as the sum of the constituent protein and RNA ORD spectra, i.e. isolated ribosomal RNA has the same secondary structure as RNA in the ribosome. The opposite was found with phage nucleic acids (Maestre and Tinoco, 1965). This was probably due to a packing effect on the DNA inside the virus protein coat. Oriel (1966) found that histone protein in high salt (1 M NaCl) had the same helical content (approx. 20%) as in the nucleohistone complex and Tuan and Bonner (1969) found that the DNA in the complex was denatured to some extent, probably supercoiled. Interactions of DNA with small molecules such as acridine (Blake and Peacocke, 1966), steroidal diamines (Mahler et al., 1968) and metalions (Cheng, 1965) have all been detected using ORD. Steroidal diamines were interesting in that low concentrations increased the 290 nm DNA peak whereas high concentrations decreased the peak. This was consistent with the increase and subsequent decrease of DNA T_m values (Mahler et al., 1966). In contrast Hg^{++} ions completely inverted the whole ORD spectrum of DNA (Cheng, 1965).

The ORD spectra of stage II DNA from E. coli ROW and of colicin E_2 are shown in Fig. 3 vi and were characterised by a peak and trough at 287nm and 255 nm for native DNA (specific rotations equal to + 2150 and - 2250 respectively) and a trough at 232 nm for colicin protein (specific rotation equal to - 4060, see Ch. 1). The specific rotation $(\alpha)_\lambda$ is derived from the observed rotation, α using the formula;

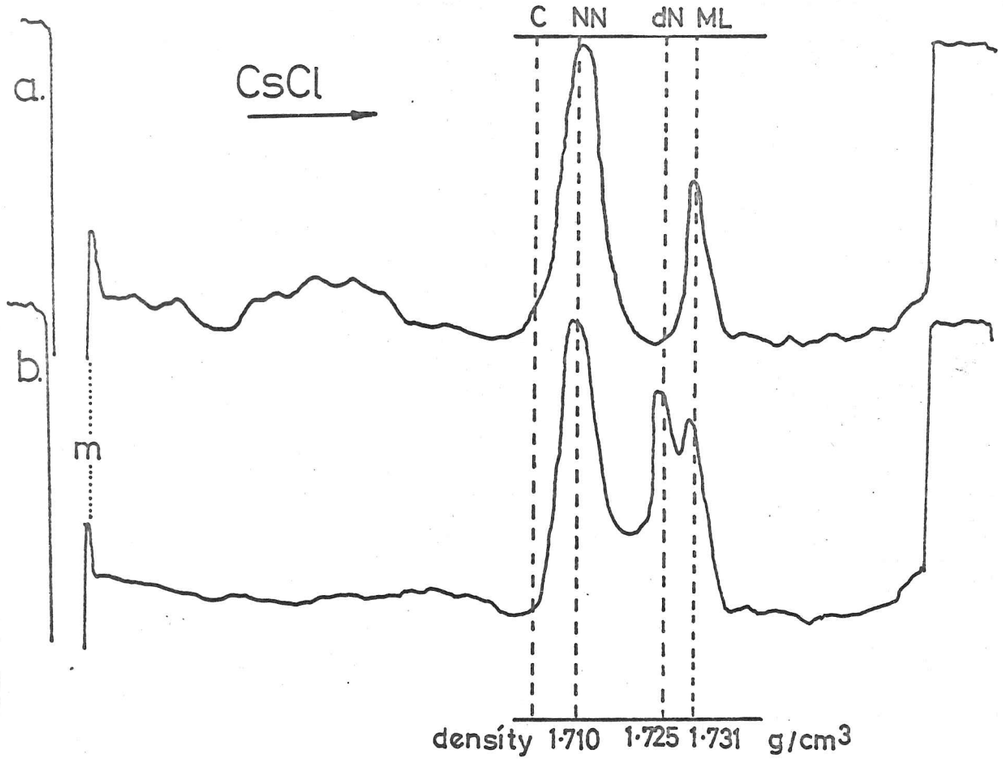
$$(\alpha)_\lambda = \frac{\alpha}{l.c}$$

(degrees. cm^2/decagram)

....Yang and Samejima, (1969)

l is the light path in decimeters and c is the concentration of material in g/ml.

The effect of mixing colicin E_2 with DNA in $SSC/_{10}$ on the combined ORD spectra is also shown in Fig. 3 vi and comparison of this with the addition spectrum of colicin and DNA indicated a decrease in the 232 nm protein and 255 nm DNA troughs. The DNA peak at 287 nm however slightly increased but there was no significant spectral shift consistent with denaturation of the nucleic acid. The result suggested that the protein molecule lost some of its helicity; $(m')_\lambda$ calculations indicated that the colicin helical content decreased from 16.2% to 5.7% and the changes in magnitude of the nucleic acid peaks would be consistent with this. Phenol (0.1 mM) did not significantly affect the spectra except to increase the noise level due to the wider slit width needed. Similar results were obtained with calf thymus DNA but it is impossible at present to state that the observed effect was entirely due to a decrease in helical content of colicin E_2. This apparent change in helicity could be explained by colicin E_2 binding to DNA and consequently altering its ionic environment due to the close proximity of the DNA phosphate groups (Tuan and Bonner, 1969). This phenomenon could also explain the 5-10% hyperchromic effect on mixing colicin E_2 with DNA at room temperature since the total hyperchromic effect on thermal denaturation of the DNA was unaffected by colicin and this would not be expected if it were DNA that caused the initial hyperchromicity. Therefore spectroscopic analysis and ORD suggest that it was colicin that changed in secondary structure on interacting with DNA and not DNA itself.



Densitometer Tracings (cell magnifn. 10·25)

FIG. 3 vii CsCl isopycnic centrifugation of E.coli DNA after incubation with colicin E2 in vitro.

(a) contained stage II native E.coli DNA incubated with colicin E2 and M.lysodiekticus DNA as marker (ML).

(b) contained control native (NN) and heat denatured (dN) E.coli DNA together with ML marker. C is the point of isoconcentration and m is the meniscus.

Centrifugation was in a Spinco model E ultracentrifuge for 20h at 44,000 rev/min. UV photographs were taken when equilibrium was attained.

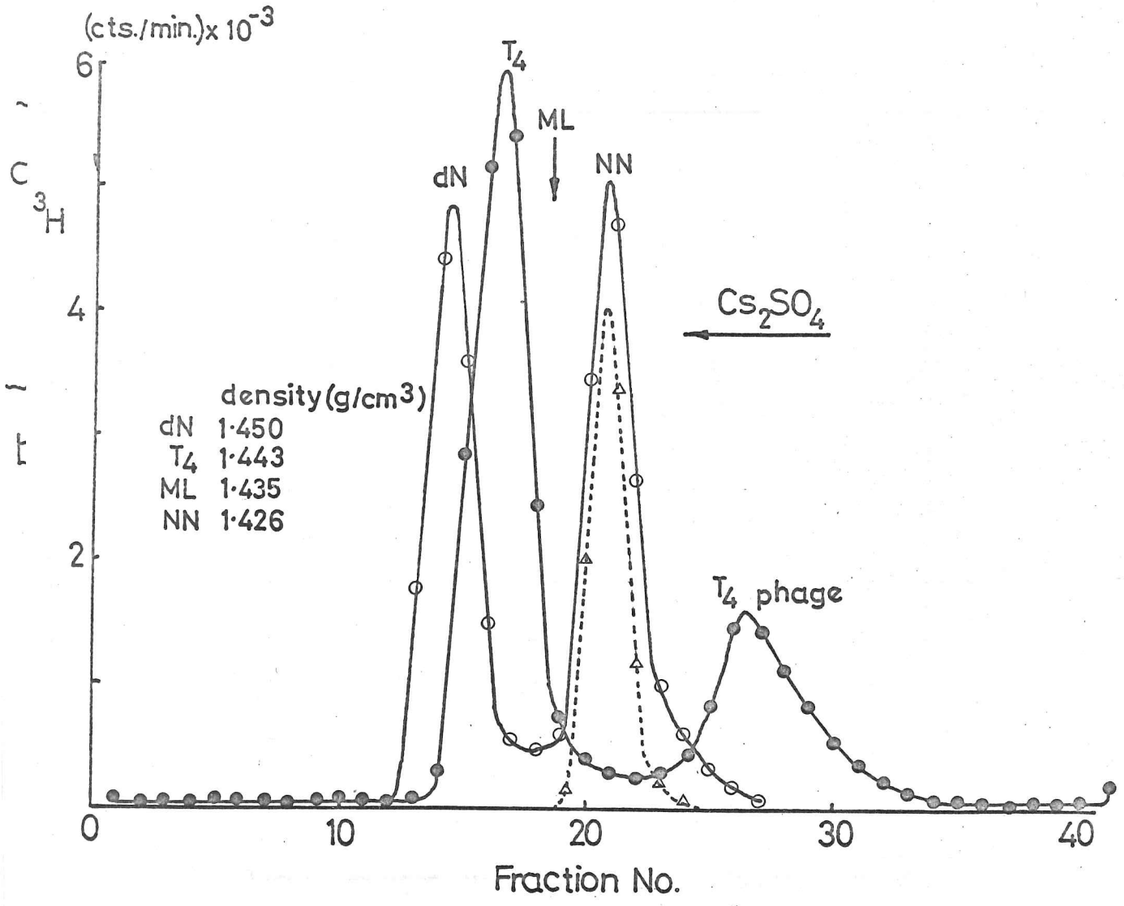


FIG. 3 viii

Cs_2SO_4 isopycnic centrifugation of E.coli DNA after incubation with colicin E2 in vitro.

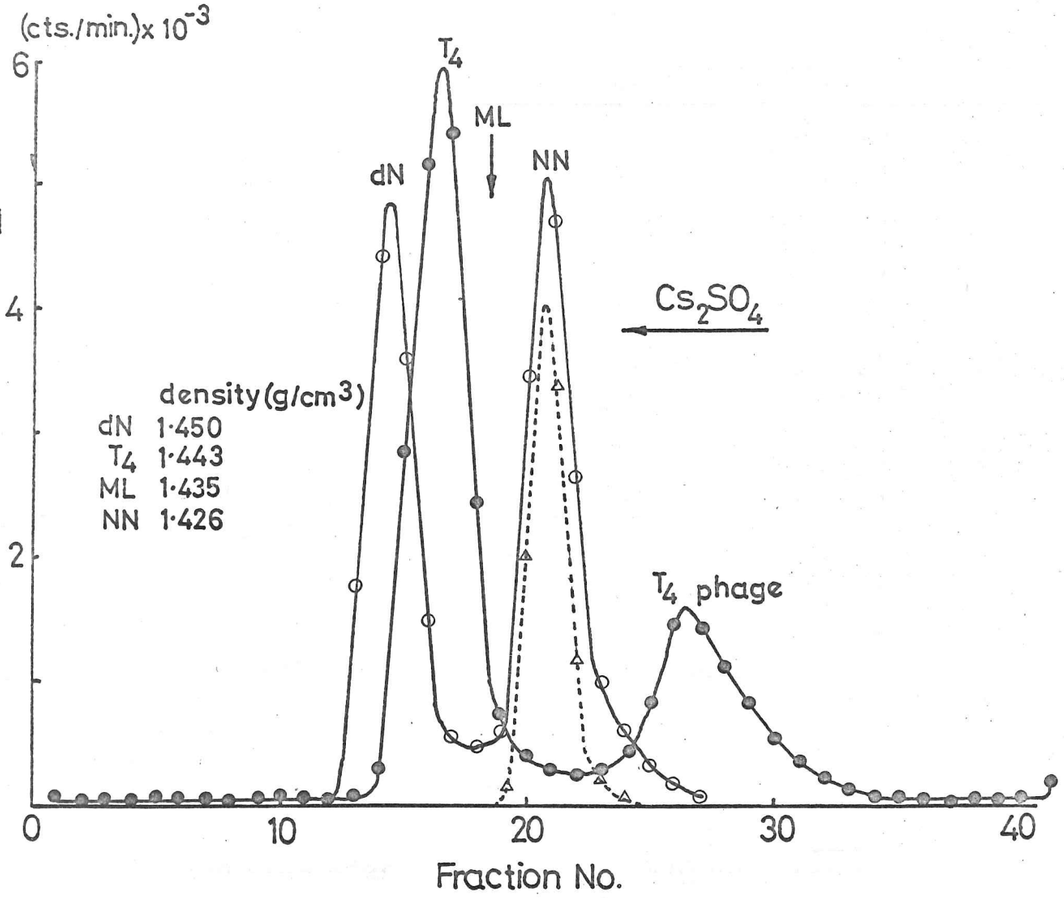
(^3H) labelled E.coli DNA was treated with colicin E2 Δ-----Δ and centrifuged to equilibrium with (^{14}C) labelled heat denatured E.coli DNA in Cs_2SO_4 at 32,000 rev/min in a Ti-50 rotor in a Spinco model L2-HV ultracentrifuge for 70 h. The abbreviations are the same as in FIG. 3 vii .

(iii) Effect of colicin E_2-P9 on DNA buoyant density

The buoyant density of a DNA molecule is dependent not on molecular weight but on base composition, particularly in CsCl (Mandel et al., 1968), and in certain circumstances on secondary structure. Denaturation of DNA by heat or alkali and UV irradiation of DNA cause characteristic increases in buoyant density (Szybalski, 1968). CsCl and Cs_2SO_4 distinguish between these two types of alteration in secondary structure. In CsCl the buoyant density increase for UV damaged DNA is greater than for heat denatured DNA, whereas in Cs_2SO_4 the opposite is true, (Opara-Kubinska et al., 1963). Isopycnic centrifugation has also been used for investigation of protein-DNA interactions, (e.g. phage; Weigle et al., 1959).

The effect of incubation of DNA with colicin E_2 on DNA buoyant density in CsCl and Cs_2SO_4 is shown in Figs. 3 vii and viii. In both caesium salts the DNA density was completely unaffected by colicin pretreatment. Owing to the smaller density increase in CsCl on heat denaturation of DNA, the centrifugation was performed in an analytical ultracentrifuge and UV photographs taken during the run. This gave better resolution than the more convenient method of using a Ti-50 rotor. T_4 DNA and T_4 phage were included (Fig. 3 viii) to demonstrate the effect of protein binding to DNA on buoyant density. The T_4 DNA had a greater density than E. coli in Cs_2SO_4 owing to the contribution of glucosyl groups. In CsCl E. coli DNA had a greater density than T_4 DNA.

There was no evidence from these results that colicin E_2 damaged DNA or bound to DNA sufficiently to cause a change in buoyant density. This latter result however was expected owing to the extremely high ionic conditions during centrifugation and to the fact that CsCl has been used for dissociating ribosomal proteins from ribosomal subunits (Stachelin and Meselson, 1966).



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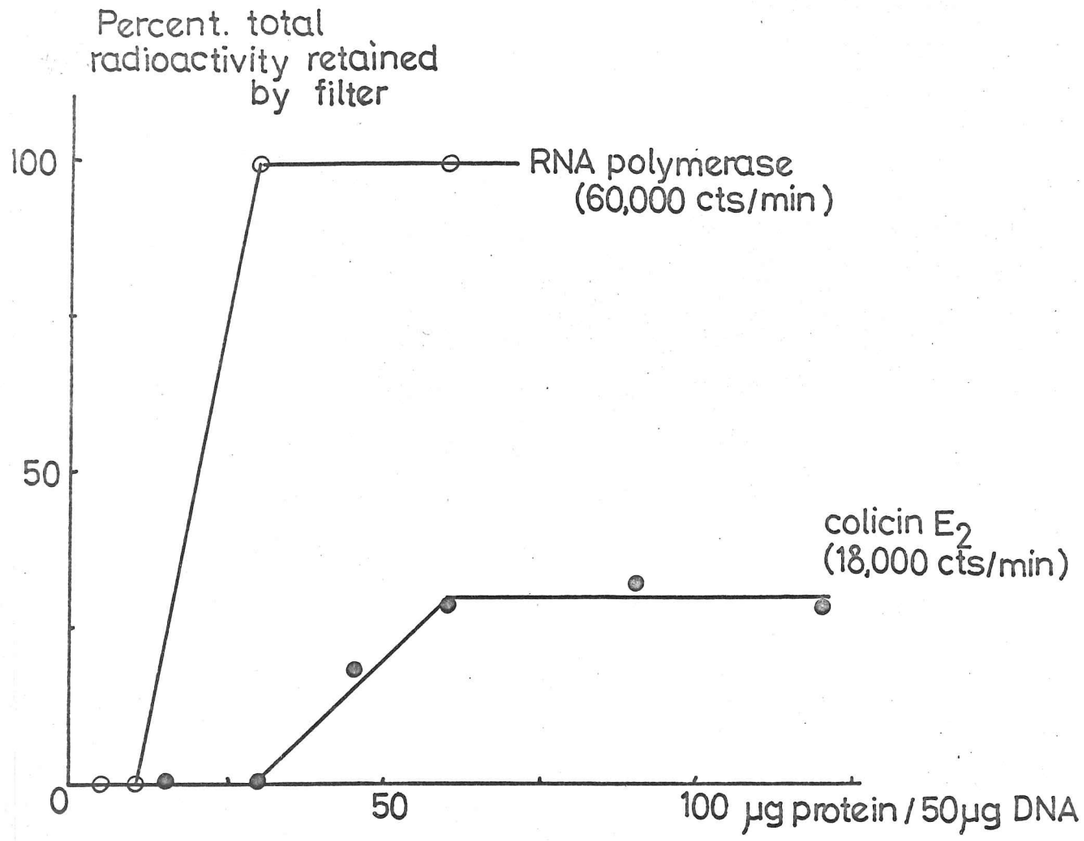


FIG. 3 ix

Protein:DNA complex retention by nitrocellulose filters.

(^{3}H) labelled E.coli DNA was incubated with colicin E2 or RNA polymerase for 5-10 min; the mixtures were passed through nitrocellulose Millipore filters and washed with buffer. The percentage radioactivity retained by the filters is plotted against the amount of protein per 50ug DNA to facilitate comparison with FIG. 3 iii .

(c) DNA binding activity

The binding of colicin to E. coli DNA has already been inferred from its effects on DNase activity, DNA thermal denaturation and ORD. A more direct method for investigating DNA-protein binding has been used by Jones and Berg (1966). This relies on the fact that Millipore membrane filters selectively retain DNA-protein complexes whilst allowing DNA and protein to pass through. This method has been used with considerable success with RNA polymerase (Jones and Berg, 1966) lactose repressor (Riggs et al., 1968) and restriction endonuclease (Yuan and Meselson, 1970). In addition amino-acyl tRNA binding to ribosomes has been followed using this method (Yanus and Berg, 1967). A second simpler method involved centrifugation of the "complex" under conditions of low ionic strength (Felsenfeld et al., 1963) on a sucrose density gradient and assaying the fractions for specific protein activity in the region of the DNA band.

(i) Effect of colicin E_2-P9 on retention of (^{3}H) labelled DNA by membrane filters

The incubation conditions of colicin E_2 and DNA were those used for investigating thermal denaturation of DNA so that colicin-DNA molecular binding ratios could be compared directly. RNA polymerase was chosen as a convenient standard and the incubation conditions were those described by Jones and Berg (1966), although the alternative colicin standard phosphate buffer did not significantly affect its DNA binding despite the absence of Mg^{++} and mercaptoethanol. Under the filter washing conditions used 2-5% total DNA radioactivity was retained in the absence of protein by the filter using either buffers. The amount of DNA-protein complex retained by the filter was progressively reduced if after incubation, the complex was allowed

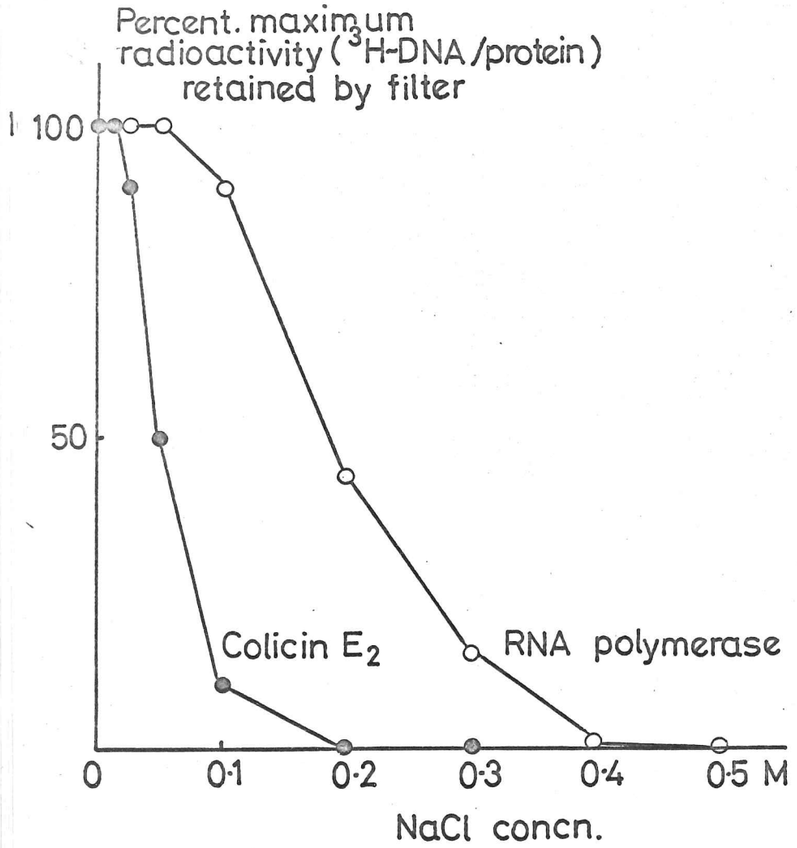


FIG. 3 x

Effect of NaCl on DNA:protein complex retention by nitrocellulose filters.

(^{3}H) labelled DNA and protein were incubated together under optimum binding conditions as described in FIG. 3 ix and diluted with buffer containing NaCl at the concentrations indicated. The mixture was filtered and washed with buffered saline. The radioactivity retained by the filter is expressed as a percentage of the maximum retention obtained for each protein in FIG. 3 ix .

to remain for more than 10-15 min in the cold dilute buffer. This limited the number of incubations that could be performed at any one time owing to the necessarily slow rate of filtration and washing. The effects of colicin E_2 and RNA polymerase on the retention of (^{3}H) labelled DNA are shown in Fig. 3 ix. RNA polymerase had a much higher affinity for DNA under the conditions used and at a concentration of 30 µg/ml caused 100% retention of the DNA. The maximum retention of DNA in the presence of colicin E_2 was 30% at a concentration of 60 µg/ml. This corresponded to the colicin concentration in Fig. 3 v that produced the maximum $\sigma_{2/3}$ value with similar amounts of DNA. The ionic stability of the protein-DNA complex was determined by diluting the standard incubation mixture after formation of the complex with increasing concentrations of NaCl in the appropriate buffers. This buffered saline was also used in the washing procedure. The results are shown in Fig. 3 x. The colicin-DNA complex was far more labile to increasing ionic strength than the RNA polymerase-DNA complex. Between 10 and 20 mM NaCl, colicin started to dissociate from DNA and at 50 mM NaCl 50% of the complex had dissociated. This extreme salt lability of the colicin complex would argue against any specific DNA interaction and also any direct in vivo colicin action on the bacterial chromosome under intracellular ionic conditions. However this method only measures the effect of salt on membrane filter retention and it is assumed that a decrease in retention is equivalent to complex dissociation. It could be that the colicin-DNA complex passes through the membrane filter in high ionic strength buffers without necessarily dissociating. In support of direct in vivo colicin action on DNA it could be argued that colicin only interacts with DNA at the chromosomal-membrane attachment point under comparative hydrophobic conditions.

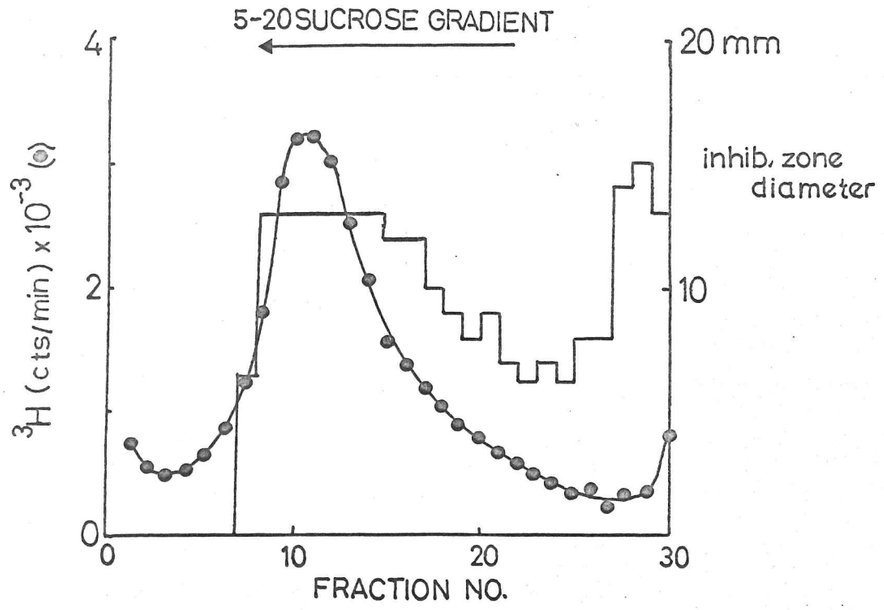


FIG. 3 xi

Sucrose density-gradient centrifugation of colicin E2 treated DNA under conditions of low ionic strength.

(^{3}H) labelled DNA from E.coli was incubated with colicin E2 in 1mM phosphate- 10mM NaCl pH 7.1 and centrifuged through a 5-20% (w/v) sucrose gradient(made up in the same buffer) for 90min at 40,000 rev/min in a SW50 rotor. The tube contents were fractionated and the fractions tested for growth inhibitory activity as shown in PLATE 1 iv and the cold-acid precipitable radioactivity determined.

Phenol (1.0-0.1 mM) did not affect either colicin or RNA polymerase binding to DNA. The background retention of native DNA alone was unaffected by prior filtration of colicin E_2 or RNA polymerase. The mechanism of retention of the DNA-protein complexes was not due to formation of large molecular aggregates since centrifugation of the complexes on sucrose gradients produced no dramatic increase in DNA sedimentation value (see Results 3 (c)ii and Jones and Berg, 1966).

The molecular binding ratios of RNA polymerase and colicin E_2 at 30 and 60 µg/ml to DNA (10^7 daltons) at 50 µg/ml were calculated to be 15 and 200 assuming the molecular weight of the polymerase to be approx. 5 x 10^5 (Travers and Burgess, 1969) and its purity to be 100% at 2000 units per mg (Chamberlin and Berg, 1962). The colicin-DNA molar ratio was therefore consistent with the results obtained in earlier sections.

(ii) Association of colicin E_2-P9 activity with DNA after sucrose density-gradient centrifugation

The ability of colicin E_2 to bind to DNA was also tested directly by an adaptation of the method of Felsenfeld et al., (1963). This method relied on the isolation of DNA after incubation with colicin by sucrose density-gradient centrifugation in a low ionic strength buffer. The gradient fractions were tested for colicin activity and if DNA binding had occurred the activity profile corresponded to the DNA profile.

The DNA radioactivity and colicin E_2 biological activity profiles after sucrose gradient centrifugation are shown in Fig. 3 xi. Owing to the inaccuracy of bioassay no molecular binding ratios were calculated, but it was demonstrated that colicin activity was associated with the DNA peak thus confirming the observations of DNA thermal denaturation and filter retention experiments regarding

TABLE 3 iv

DNA dependent RNA polymerase activity

		nmol GMP incorp.	% incorp.
Complete system	1. 5 µg DNA	2.2 (1021 cts/min)	100
	2. 50 µg DNA	4.4	
Additions to system 1.			
	-enz.	-	0
	-DNA	-	1
	+ACTD(2.5 µg)	-	1
	+colicin E_2 (5 µg)	1.7	78
	+colicin E_2 (10 µg)	1.6	74
	+heat denat. colicin E_2	2.2	101
	+1 µmol phosphate (pH 8)	1.2	57

The complete system (total volume 0.25 ml) is described in Methods 3. DNA samples were incubated with colicin at 37° for 10 min prior to addition of RNA polymerase. All assay incubations were for 30 min and the amount of (^{14}C)-GMP incorporated into the cold-acid precipitable fraction determined. The figures quoted are averages of 2 duplicate determinations and the variation was not more than ± 4%.

DNA-colicin *in vitro* binding. The *in vivo* activity of colicin, as determined by survival curves for *E. coli* ROW and DNA degradation, was completely unimpaired by preincubation with DNA, but this was partially expected owing to the ionic conditions of the culture medium. A similar diffuse colicin activity profile was obtained after sucrose gradient centrifugation with heat denatured DNA but the relative affinities of colicin for native and denatured DNA were not determined owing to the high degree of variability and lack of precision in the experiments.

(d) Effect of colicin E_2-P9 on *in vitro* macromolecular synthesis

Colicin E_2 was found to have inhibitory effects on the synthesis of DNA, RNA and protein *in vivo* at high colicin molecule to cell ratios (Ch. 2 and Nomura, 1963). As a final investigation into the *in vitro* activity of colicin E_2 it was decided to investigate the effects of colicin E_2 on RNA and protein synthesis *in vitro*.

(i) *In vitro* RNA synthesis

The binding of colicin E_2 to DNA described in Results 3 (c)i would suggest an inhibition of RNA polymerase activity by colicin E_2 owing to a competition for DNA binding sites. Shih and Bonner (1970.*a* and *b*) have shown however that histones and polyamines inhibit DNA template activity of RNA polymerase not by prevention of enzyme binding but by inhibition of helix uncoiling. Therefore it could also be argued from the DNA thermal denaturation data (Results 3 (b)i) that colicin E_2 would enhance DNA template activity. In order to resolve this inconsistency DNA was pretreated with colicin E_2 and incubated with RNA polymerase and nucleoside triphosphates. The amount of RNA synthesis was determined by the incorporation of (^{14}C) GMP into cold-acid precipitable material (Table 3 iv). 5 µg of stage II *E. Coli* DNA was used in the test

TABLE 3 v

Poly U directed Poly-phe synthesis

Additions	nmols (^{14}C) Phe	% incorp.
None	0.12 (22,210 cts/min)	100
- poly U	-	5
- cell sap	-	2
+ colicin E_2 (10 µg)	0.12	97
+ colicin E_3 (10 µg)	0.11	92
+ CAP (25 µg)	0.04	37

The complete system (total volume 0.25 ml) is described in Methods 3. The cell sap ribosomes were incubated with colicin E_2 at 37° 10 min prior to addition of (^{14}C)-phenylalanine. All assay incubations were for 15 min and the (^{14}C)-phenylalanine incorporated into the hot-acid precipitable fraction determined. Preincubation of the cell sap at 37° for 10 min in the control had no effect.

systems as this resulted in approximately half the maximum rate of RNA synthesis for the given concentration of enzyme and this was most sensitive to any change in template activity (see Waring, 1964). Under conditions of excess template (50 µg DNA) the maximum activity rate of RNA polymerase was 4.4 nmol GMP incorporated into cold-acid precipitable material in 30 min. The specific activity of the enzyme was therefore 2500 units per mg (see Methods 3).

Duplication of phosphate buffer conditions used in thermal denaturation and binding studies was prohibited owing to the inhibitory action of phosphate on the assay system (Table 3 iv).

Colicin E_2, at protein-DNA molar ratios of approx. 150 and 300, reduced the incorporation by about 25%. This effect was reduced and subject to wide variation if the enzyme and colicin were added to the DNA template at the same time. This would indicate that colicin E_2 partially blocked RNA polymerase activity in vitro but this effect was not consistent with the more effective inhibition of RNA synthesis observed in vivo.

(ii) In vitro protein synthesis

The poly U directed polyphenylalanine synthesising system was essentially the same as that described by Konisky and Nomura (1967). The control system was characterised by having maximum activity between 15 and 20 mM Mg^{++}. There was little activity below 10 mM and above 30 mM Mg^{++}. The effects of colicins E_2 and E_3 on polyphenylalanine synthesis at 16 mM Mg^{++} is shown in Table 3 v, and it was concluded that neither colicin produced a significant inhibition to account for its in vivo effects. However it was noted that chloroamphenicol was only partially effective as an inhibitor of this poly U system.

DISCUSSION 3

Investigations into the *in vitro* activity of colicin E_2-P9 may be divided into those that produced negative results and those that produced positive results. Negative results were obtained by investigations into possible direct nuclease activity and direct interaction with *in vitro* macromolecular synthesis, although a slight inhibition of RNA synthesis was detected. The interpretation of these experiments would be that colicin E_2 needed a whole cell environment to influence molecular synthesis and that the mode of action of colicin E_2 cannot be simply explained by hypothesising a membrane permeable nuclease. Together with results on magnesium ion depletion (Ch. 4), this would further eliminate another hypothesis of DNA leaking out to a colicin nuclease in the periplasmic space, (assuming non-penetration of the inner cell membrane by the colicin).

The positive results were probably all direct consequences of colicin binding to DNA *in vitro*. Despite the strict dependence on the ionic strength of the incubation buffer, suggesting that binding would not occur *in vivo*, the colicin:DNA molecular binding ratios were remarkably consistent for three different experiments, i.e. DNase activation (250), thermal destabilisation of DNA (100-400) and colicin-DNA complex retention by millipore filters (100-400). These ratios for a DNA molecular weight of 10^7 may indicate that there is a colicin molecule bound every 10^5 daltons, i.e. every 200 nucleotide pairs. It is interesting to compare this with the report of Sadron (1968) that there are local regions of untwisting or distortion every 5×10^5 daltons along native DNA strands. Sekine *et al.*, (1969) have used this to explain the binding ratios of ribonuclease A to DNA which were similar to those observed for colicin E_2. Histone I has also been shown to bind to DNA at a

molar ratio of approx. 400 (Shih and Bonner 1970.a). Other well-characterised protein-DNA interactions have been observed at lower molar ratios; 15-30 for RNA polymerase (Jones and Berg, 1966) and 70 for chymotrypsinogen (Bobb, 1966). The significance of the interaction of colicin E_2 with DNA is questionable since all the other DNA-protein complexes mentioned are considerably more stable with respect to increasing ionic strength, i.e. more specific.

The lowering of DNA melting temperature is difficult to explain since there was no apparent change in DNA secondary structure on binding colicin as detected by ORD, even in the presence of phenol. It is possible however that colicin E_2 shows a greater affinity for the denatured rather than the native structure of DNA, although there was no direct convincing evidence of this. Ribonuclease (Felsenfeld et al., 1963) and bleomycin (Nagai et al., 1969) are the only proteins together with colicin E_2 so far observed to lower DNA melting temperatures. Various other agents have been found to destabilize DNA such as nucleosides (Ts'o et al., 1962 and 1964) steroidal diamines (Mahler et al., 1968) and more drastic agents such as formamide, perchlorate and urea (Marmer et al., 1963) but protein destabilisation of DNA has interesting possibilities with regard to in vivo control of transcription and replication.

Colicin E_2 itself was observed to undergo a form of denaturation on associating with DNA due to the close proximity of the DNA phosphate groups and this change in the ionic environment of DNA may explain the DNase activation effects and the increase in $\sigma_{2/3}$ values on DNA melting (see Peacocke and Walker, 1962).

The positive effects of colicin E_2 *in vitro* were not pursued too vigourously after discovering the effect of increased ionic strength on DNA-colicin complex stability and the enigma of the phenol effect on DNA melting in the presence of colicin. The direct action of colicin E_2 on DNA may unfortunately be an artefact and not at all related to its *in vivo* action since it assumes inner membrane penetration and all other evidence argues against this, (see General Introduction).

CHAPTER 4

The effects of other chemical agents on colicin E_2-P9 induced DNA degradation *in vivo*

INTRODUCTION

A previous investigation (Nomura, 1963) into the effects of other chemical agents on colicin E_2 action *in vivo* has shown that the induced DNA degradation is interferred with by inhibitors of energy metabolism such as 2:4 dinitrophenol and colicin K, but unaffected by protein synthesis inhibitors such as chloramphenicol. Reynolds and Reeves (1963) also showed that under certain conditions trypsin reversed the lethal effect of colicin E_2, increasing the number of cell survivors. However trypsin was less effective with colicin E_2 compared with other colicins such as K, E_1 and E_3, (Nomura and Nakamura, 1962). Nomura (1963) concluded from this and other unpublished evidence that degradation of cell DNA induced by colicin E_2 did not utilise newly synthesised deoxyribonucleases but was dependent on energy production and DNA synthesis whilst the colicin molecule remained near the cell surface. This may suggest a possible involvement of Kornberg's DNA polymerase (Kornberg, 1969; Richardson, 1969) which is known to be dependent on nucleoside triphosphates and to have exo- and perhaps endonucleolytic activities (see Ch. 5).

It was therefore decided to investigate the effects of other chemical agents on this phenomenon with the view to elucidating the mode of action of colicin E_2. Various inhibitors of DNA synthesis and DNA intercalating drugs were studied in addition to agents affecting cell membranes. It has already been observed *in vitro* that certain DNA intercalaters inhibit deoxyribonuclease action, (Leith, 1963; Sarkar, 1967). The colicin E_2 system therefore also provided a

novel and useful means for investigating the effect of induced deoxyribonuclease activity on intercalated DNA *in vivo*.

METHODS 4

(a) Pretreatment of (^{3}H) labelled cells with antibacterial agents

The DNA of exponentially growing cells of *E. coli* ROW was labelled with (methyl-^{3}H) thymine as described in Ch. 2. The labelled cells were washed and resuspended with fresh medium at a cell density of 3×10^{7} bacteria per ml. The cells were preincubated for 10 min with growth inhibitory concentrations of various antibacterial agents before addition of the appropriate concentration of colicin E_2. 1 ml aliquots were taken at intervals and mixed with 1 ml ice cold 10% (w/v) TCA. The precipitate was filtered and the radioactivity determined as described previously.

This procedure applied to the preliminary investigations with dinitrophenol (DNP), chloramphenicol (CAP), mitomycin (MIT), phleomycin (PHL), novobiocin (NOV), acriflavin (AC), ethidium bromide (EB), daunomycin (DAU) and nogalamycin (NOG). The concentrations used are given in the text.

The more detailed investigations with actinomycin D (ACT. D), ethidium bromide, phenethyl alcohol (PEA), 5-bromouracil (BU) and trypsin are described in the text.

(b) Isopycnic centrifugation of BU-DNA

In order to show that 5-bromouracil was incorporated into the DNA of *E. coli* ROW under the conditions used, it was necessary to show an increase in bouyant density of the extracted cell DNA in CsCl.

The DNA of *E. coli* ROW was uniformly labelled with (methyl-^{3}H) thymine and the exponentially growing cells were washed and resuspended at a cell density of 10^{8} bacteria per ml with fresh medium

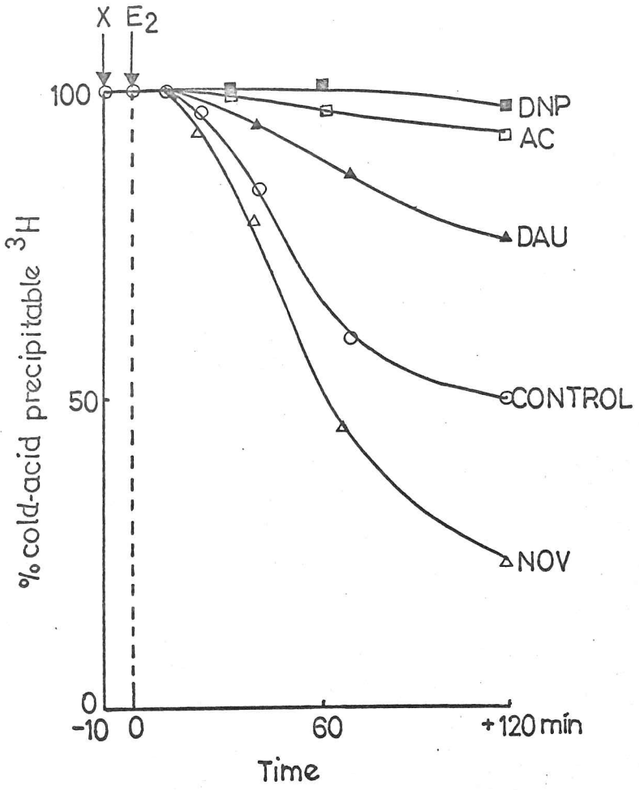


FIG. 4 i

Effects of antibacterial agents on colicin E2 induced DNA degradation.

(^{3}H) thymine labelled cells of E.coli ROW (3.10^7 bacteria per ml) were preincubated for 10min in the presence of the agents (X) indicated . Concentrations are given in TABLE 4 i. Samples were taken at intervals after addition of colicin E2 (3ug/ml) and the cold-acid precipitable radioactivity determined.

containing 5 µg 5-bromouracil per ml, 200 µg deoxyadenosine per ml and 1 µg (methyl-^{3}H) thymine per ml (specific activity 1 Ci/mmol). After incubation for specified times, 10 ml aliquots were mixed with 10 ml NET buffer pH 8.0 containing 10 mM KCN (NET-CN) at 4° to stop further incorporation. The cells were harvested by centrifugation and resuspended in 1 ml NET-CN buffer pH 8.0 at 20° and incubated with 200 µg lysozyme per ml for 15 min. The cells were then lysed by the addition of sarkosyl detergent (final concentration 1% (w/v)) and digested with pronase at 100 µg/ml for 15 min at 37°. 0.6 ml lysate was mixed with 5.4 ml NET buffer pH 8.0 and added to 7.8 g solid CsCl. The mixture was overlayered with liquid paraffin in a 13 ml centrifuge tube and centrifuged to equilibrium in a Ti-50 rotor at 30,000 rev/min for 60h at 20°. The gradients were fractionated and the cold-acid precipitable radioactivity in the fractions determined (see Ch. 5).

RESULTS 4

(a) Effects of preincubation of E. coli ROW with antibacterial agents on colicin E_2-P9 induced DNA degradation

In order that stimulation as well as inhibition of colicin induced DNA degradation could be detected it was necessary to use a concentration of colicin E_2 that did not induce excessive DNA degradation in the control. 3 ng colicin E_2 per ml was therefore used (10^3 molecules per cell) since this induced 50 ± 5% degradation of cell DNA after 2h incubation.

Preliminary investigations involved a simple preincubation of cells with various agents for 10 min followed by incubation in the presence of colicin (Fig. 4 i). Mitomycin (Reich et al., 1961) and phleomycin (Falaschi and Kornberg, 1964) were used as examples of antibiotics that would specifically inhibit DNA synthesis (see Ch. 2) but do not intercalate. Phleomycin has also been shown to

TABLE 4 i

Effect of chemical agents on colicin E_2 induced DNA degradation

Preincubation Treatment	concn. (µg/ml)	% DNA degradation after 2h	
		- colicin E_2	+ colicin E_2
Control		0	48
Mitomycin C	1	2	43
	20	15	41
Phleomycin	1	3	50
	20	19	47
Acriflavin	100	0	7
Ethidium Bromide	100	0	19
Daunomycin	100	0	25
Nogalomycin	100	2	31
EDTA	(1 mM)	15	41
Actinomycin D	50	18	32
(- Mg^{++}		0	49)
Novobiocin	100	0	78
Chloroamphenicol	100	0	48
(- Casamino acids		0	53)
Dinitrophenol	(2 mM)	2	4

inhibit exonuclease I *in vitro* (Falashi and Kornberg, 1964) but not endonuclease I. DNA isolated from cells treated with mitomycin C was degraded as well as untreated DNA by exo and endonuclease *in vitro*, in contrast to DNA treated with intercalating drugs (Sarkar, 1967). Mitomycin and phleomycin were used at 1 µg/ml (close to the M.G.I.C.) and 20 µg/ml. There was no significant difference in colicin E_2 induced DNA degradation between control cells and cells preincubated with either mitomycin or phleomycin, (Table 4 i and Fig. 4 ii). However there was a slight degradation (10-20%) at the higher concentrations of the two antibiotics in the absence of colicin E_2 after 2h incubation.

Four intercalating drugs were selected; acriflavin, ethidium bromide, daunomycin and nogalamycin (see Waring, 1968; Kersten *et al.*, 1966). Owing to their charged nature, high concentrations (100 µg/ml) were needed to overcome the permeability barrier of gram-ve bacteria. The inhibitory effect of such intercalaters on certain deoxyribonucleases by binding to the DNA substrate *in vitro* has been investigated by Leith (1962) and Sarkar (1967) and was described in Ch. 3. However very little has been reported of their *in vivo* effect on DNase activity. Acriflavin and ethidium bromide produced the most significant results for inhibiting colicin E_2 induced DNA degradation (Table 4i and Figs. 4 i and 4 ii). Daunomycin and nogalamycin had slight inhibitory effects but still allowed approx. 30% DNA degradation on incubation of cells with colicin. This was probably due to a decreased permeability effect. All drug concentrations were growth inhibitory and preincubations were performed in the dark. Interpretation of the acriflavin results was complicated by (i) extensive colour quenching of the cold-acid precipitates when measuring the radioactivity by liquid scintillation and (ii) the

possibility that acriflavin not bound to DNA may bind to colicin or certain DNases. The first difficulty was partially overcome by adding acriflavin to colicin control samples just before mixing with cold TCA. However the results still showed a considerable variability. The second difficulty was partly eliminated by washing acridine pre-treated cells with fresh medium and then treating with colicin. Similar results to unwashed cells were observed, but this in itself did not eliminate the possibility of the drug binding to the nucleases. The results with the other intercalaters which are known not to bind so avidly to protein would however discount this as the cause of the inhibitory effect. The results with ethidium bromide were examined in greater detail later.

Actinomycin D has been well characterised as an inhibitor of RNA synthesis (Kirk, 1960) but its method of binding to DNA has been uncertain owing to the minor groove H bonding theory of Reich (review, 1966) and the intercalation theory of Müller and Crothers (1968). It was therefore interesting to see whether ACT D resembled the other intercalaters studied in inhibiting colicin induced DNA degradation. Sarkar (1967) has shown that ACT D inhibited DNase action *in vitro* and Reiter *et al.*, (1966) have shown that repair of UV damaged DNA was inhibited by ACT D *in vivo*.

EDTA pretreatment (Leive, 1965) was necessary to increase the permeability of E. coli towards ACT D. This was performed as described in Ch. 2 and the cells treated with 50 µg ACT D per ml for 10 min before incubation with colicin E_2. The results are shown in Table 4 i. The EDTA treatment caused the cells to be "leaky" giving rise to the apparent DNA degradation in the EDTA controls with or without ACT D . Colicin induced DNA degradation was inhibited by

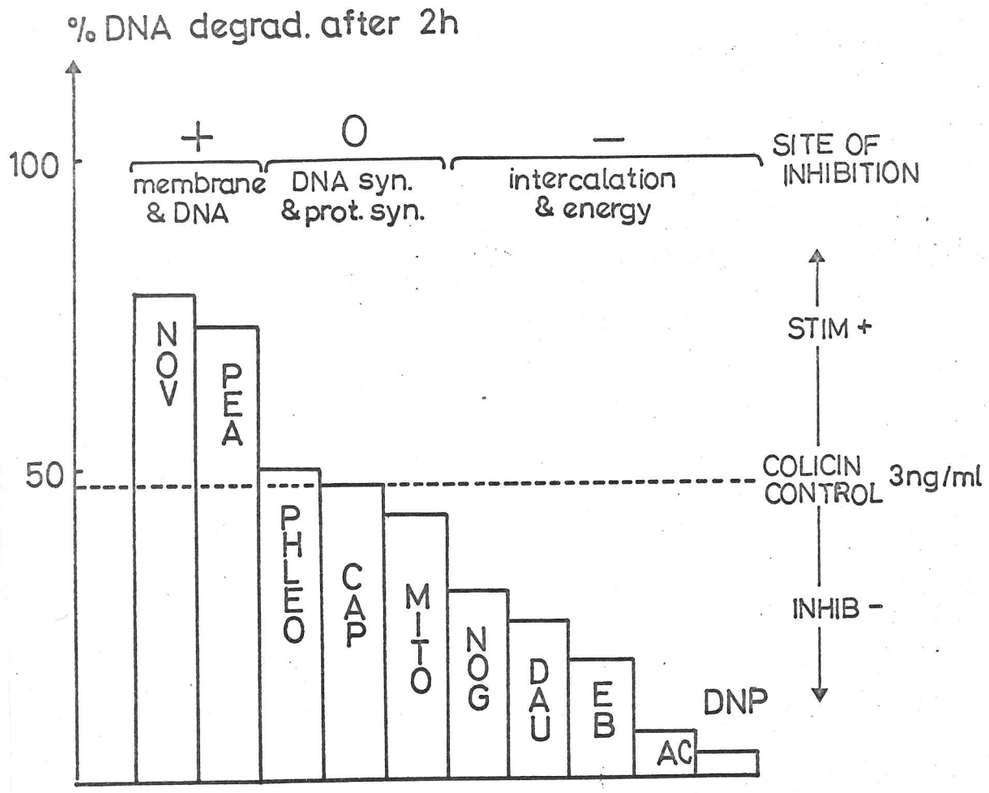


FIG. 4 ii

Histogram of the effects of antibacterial agents on colicin E2 induced DNA degradation.

The percentage DNA degradations after 2h incubation of cells in the presence of colicin E2 and the appropriate pretreatments with with antibacterial agents were determined as described in FIG.4 i all except the pretreatment with phenethyl alcohol which is desribed in FIG. 4 iv. These results are also presented in TABLE 4 i .

ACT D pretreatment but exact determinations were complicated by the "leaky" controls. This result would put ACT D in the same catagory as the intercalators described above. If the cells were not pretreated with EDTA, ACT D was without effect on cell growth.

Suspension of exponentially growing cells in a magnesium deficient medium 10 min prior to colicin addition had little effect on the DNA degradation although the magnesium deficient control cells were unable to grow. From this result and the results with EDTA it is not possible however to determine whether colicin E_2 induced DNA degradation was Mg^{++} dependent, but it does indicate that if the nuclease system is Mg^{++} dependent then degradation does not occur in the periplasmic space as has been suggested for DNA degradation in phage exclusion phenonomena (Dr. M.R. Lunt, personal communication 1970).

Novobiocin (Smith and Davies, 1967) is a known membrane-active antibiotic and inhibits DNA metabolism in E. coli. Pre-incubation of cells with 100 µg novobiocin per ml was found to have labilising effect towards subsequent colicin induced degradation of DNA (Table 4 i and Figs. 4 i and 4 ii). This was the first case of an agent increasing the biochemical effect of colicin E_2. β Phenethyl alcohol (Lark and Lark, 1966) has similar antibacterial properties and its effect on colicin action was investigated in a later section.

The results of Nomura (1963) with chloramphenicol and dinitrophenol were confirmed and are shown in Table 4 i. In this connection amino acid starvation for 90 min (Lark and Lark 1966) prior to colicin attack had little effect on the subsequent DNA degradation and was independent of whether amino acids were restored on incubation of cells with colicin (Table 4 i).

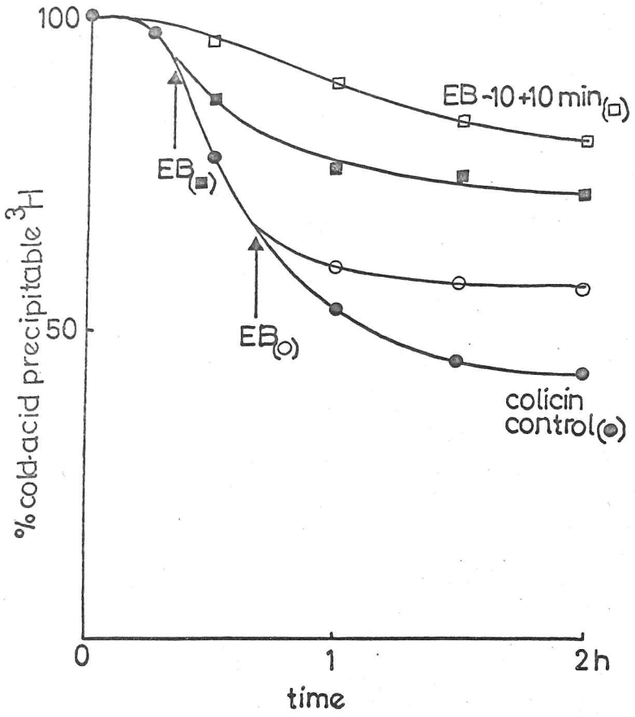


FIG. 4 iii

Effects of ethidium bromide on colicin E2 induced DNA degradation.

(^{3}H) thymine labelled cells (3.10^7 bacteria per ml) were treated with colicin E2 (5ng/ml) at time zero and ethidium bromide (100µg/ml) at the times indicated. Aliquots were taken at intervals and the cold-acid precipitable radioactivity determined. (□) are the averages of the points obtained by adding ethidium bromide to cells at -10,-1, 0, +5 and +10min .

(b) Effect of ethidium bromide on colicin E_2-P9 induced DNA degradation

Ethidium is a well characterised intercalating drug (Crawford and Waring, 1967) and was selected for a more detailed examination of the inhibitory effect of intercalaters on colicin action since it was almost as effective as acriflavin but did not suffer from the disadvantages outlined above. On addition of TCA to ethidium bromide treated cells, the bright red colour of the dye changed to pale yellow and this was removed on washing the membrane or glass fibre filter with 1% (v/v) acetic acid.

Since this drug inhibited colicin E_2 induced DNA degradation when added prior to colicin, it was of interest to see whether addition of this drug to cells simultaneously with or subsequent to colicin E_2 had any effect on the induced DNA degradation.

The results are shown in Fig. 4 iii. This time a concentration of 5 ng colicin E_2 per ml was used to increase the colicin control DNA degradation. If the time of colicin addition to bacteria was at 0 min, then additions of EB at -10, -1, 0, +5 and +10 min had equal inhibitory activity on the induced DNA degradation. After the "lag phase" (10-15 min see Ch. 2), DNA degradation to acid soluble material was occurring at the time of drug addition. Despite this, ethidium bromide inhibited the overall degradation and even after addition of EB at +40 min degradation of the DNA was inhibited and ceased by +60 min. These results suggest that (i) ethidium bromide inhibition of colicin E_2 action during the preincubation period (-10 min to 0 min) was not the result of the drug binding to colicin or an alteration in the cell surface thus preventing colicin binding to the cell, and (ii) ethidium bromide penetration of the cell and binding to DNA was unaffected by prior treatment of the cell with colicin E_2.

(c) Effect of phenethyl alcohol on colicin E_2-P9 induced DNA degradation

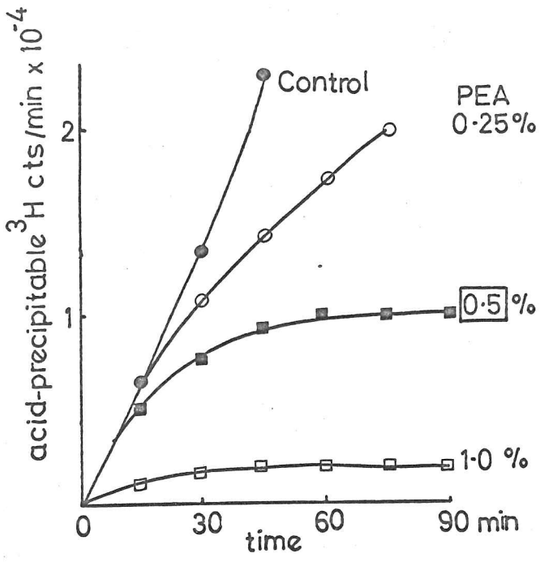
β Phenethyl alcohol (PEA) has been well characterised as a selective inhibitor of DNA synthesis (Berrah and Konetzka, 1962). Treich and Konetzka (1964) also showed that PEA only inhibited DNA synthesis after the bacterial chromosome had completed a cycle of replication, i.e. inhibited initiation of a new cycle. The point on the chromosome at which replication ceased with PEA was the same point at which replication ceased with amino acid starvation. It was also the point from which a premature cycle of replication was initiated by thymine starvation (Lark and Lark, 1966). Initiation of a new replication cycle appeared to need two proteins. One was sensitive to inactivation by PEA but its synthesis resistant to chloramphenicol; the other was unaffected by PEA but its synthesis was inhibited by chloramphenicol (Lark and Lark, 1966). These proteins may well correspond to the structural and initiator proteins hypothesised by Jacob et al., (1963).

In addition to affecting DNA synthesis, PEA appears to damage the cell membrane and it has been suggested by Treich and Konetzka (1964) and Lark and Lark (1966) that this is the cause of the disruption of DNA replication and that the chromosome may have become detached from its replication point on the membrane. In view of the effects of PEA on DNA metabolism and membranes in E. coli it was clear that it would be useful in studying the mode of action of colicin E_2.

The concentration of PEA required for a specific effect on bacterial DNA synthesis is determined to some extent by the growth conditions (Lark and Lark, 1966) and this was studied for E. coli ROW.

Effect of Phenethyl Alcohol (PEA)

(a)



(b)

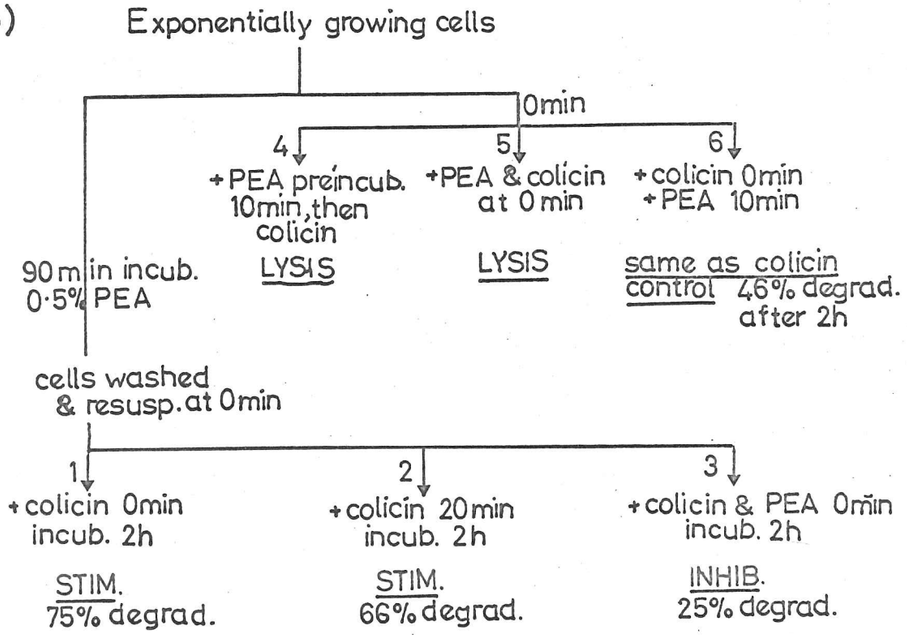


FIG. 4 iv

(a) Effect of PEA on (^{3}H) thymine incorporation by E.coli ROW

Exponentially growing cells (2.10^8 bacteria per ml) were incubated with (^{3}H)thymine and phenethyl alcohol at the concentrations indicated. Samples were taken and the cold-acid insoluble radioactivity determined.

(b) Scheme for PEA and colicin E2 treatment of cells.

(^{3}H)thymine labelled cells were incubated with 0.5% PEA for 90min, washed and resuspended at 3.10^7 bacteria per ml at time zero. Colicin E2 (3ng/ml) was added at times indicated (tubes 1-3). PEA was added to 0.1%to tube 3. Tubes 1-3 were incubated for a further 2h after E2 addition. Tubes 4-6 contained 2.10^8 bacteria per ml, 0.5% PEA and 20ng colicin E2 per ml as indicated.

The results are shown in Fig. 4 iv(a). The incorporation of (^{3}H) thymine into cold-acid precipitable material was not immediately inhibited by 0.5% (v/v) PEA and continued for approx. 50 min. 1.0% produced an effect too rapidly whereas 0.25% failed to completely inhibit DNA synthesis after 90 min incubation.

Exponentially growing cells of E. coli ROW previously labelled with (^{3}H) thymine were incubated with 0.5% PEA for 90 min by which time all DNA synthesis ceased. The cells were washed and diluted as described in Fig. 4 iv(b). At intervals after addition of colicin E_2, aliquots were taken, cold-acid precipitated and the radioactivity determined. The amounts shown in Fig. 4 iv(b) are the percentage degradations of DNA after 2h incubation of treated cells with colicin. If the PEA treated cells were incubated with colicin in the absence of PEA (tubes 1 and 2) there was a stimulation of colicin induced DNA degradation over the control. This stimulatory effect was most pronounced when the cells were treated with colicin immediately after removing the PEA (tube 1, Fig. 4 v). If however the cells were allowed to recover slightly before addition of colicin (tube 2), the stimulatory effect decreased (Fig. 4 v). Incubation of cells however with colicin in the presence of PEA after 90 min PEA pretreatment (tube 3) caused an inhibition of DNA degradation compared with the colicin control. These results may be interpreted in two ways; either the PEA directly inactivated the colicin or some partial recovery of the PEA treated cells was necessary for colicin to act (i.e. DNA/membrane interaction).

The possibility that colicin E_2 was directly inactivated by PEA was investigated by mixing the two together before addition to the cells (tube 5). On addition of the mixture to growing cells there was rapid lysis. Preincubation of the cells for 10 min

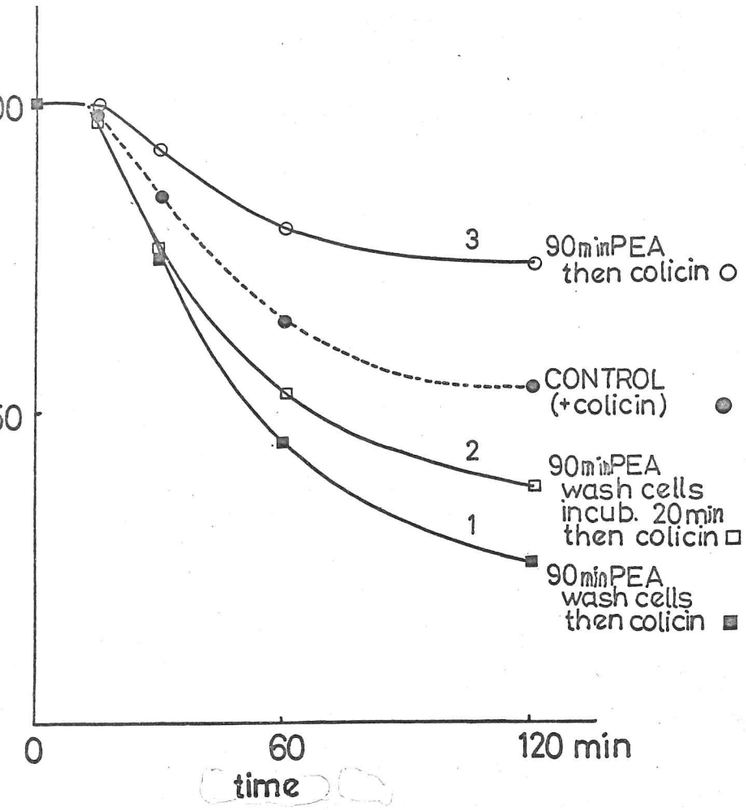


FIG. 4 v

Effect of PEA on colicin E2 induced DNA degradation.

(^3H) thymine labelled cells were preincubated with PEA and treated with colicin as described in FIG. 4 iv(b) . Samples were taken at intervals after the addition of colicin (time 0) and the cold-acid precipitable radioactivity determined. The numbers above the curves are the tube numbers in FIG. 4 iv(b).

with PEA also brought about lysis on addition of colicin (tube 4). This phenomenon of lysis had been reported by Lark and Lark (1966) when PEA treated cells were incubated with 5-fluorouracil. This therefore left the question of direct interaction partially unresolved, but since PEA did not lyse the bacteria in the absence of colicin E_2, the colicin presumably was at least partially active.

If PEA was added to the cells after the addition of colicin, there was no lysis (tube 6) and the DNA degradation was unaffected with respect to the colicin control (unlike the results with ethidium bromide). It would therefore appear that colicin stabilised the cell envelope (see Nose et al., 1970; and Chs. 5 and 6).

(d) Effect of colicin E_2-P9 on 5-bromouracil-DNA in vivo

Incorporation of the base analogue 5-bromouracil into bacterial DNA has long been known to increase sensitivity to UV and Xray irradiation. Aoki et al., (1966) have shown with UV irradiation that there was excessive DNA degradation and prevention of the normal repair processes in cells labelled with bromouracil. Lett et al., (1970) however found an inhibition of the repair exonuclease with Xray irradiation. Since the repair enzymes have been implicated in colicin induced DNA degradation(Ch. 2 and Threlfall and Holland, 1968) it was of interest to investigate the effect of bromouracil-DNA on colicin action.

With all the other chemical agents studied it was possible to demonstrate their effect on cells by simply testing for growth inhibition. Incorporation of bromouracil into cellular DNA could be demonstrated only by isopycnic centrifugation.

FIG. 4 vi

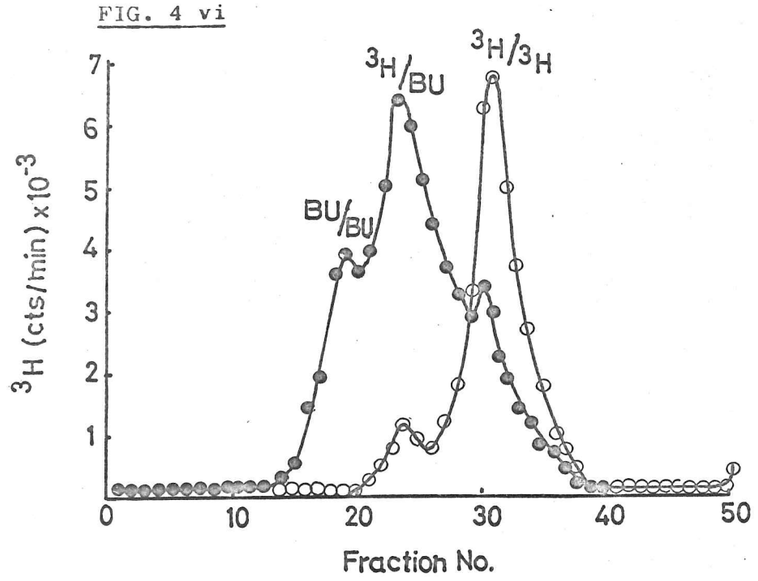


FIG. 4 vii

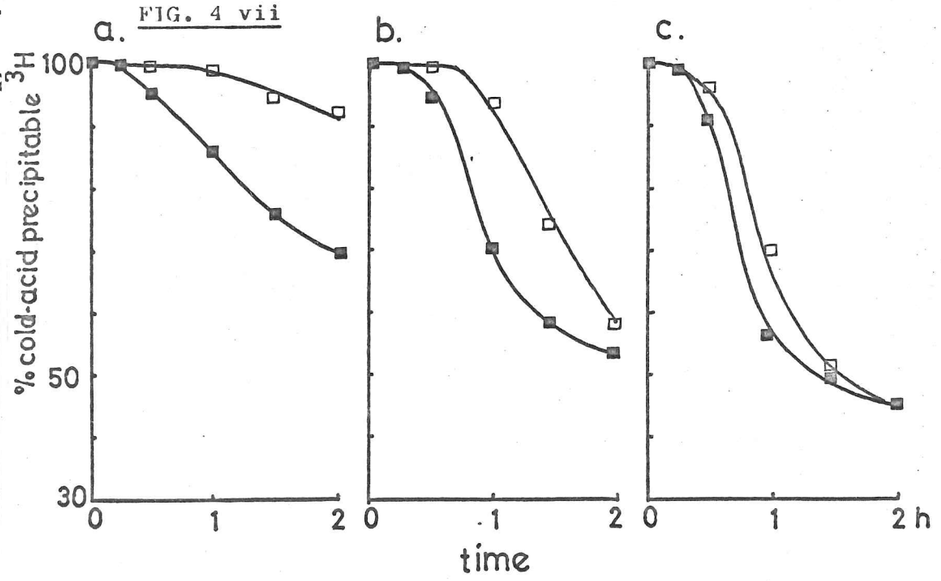


FIG. 4 vi Isopycnic centrifugation of 5-bromouracil labelled DNA.

(^{3}H) thymine labelled cells of F.coli were incubated with (^{3}H) thymine and 5-bromouracil for 5min O—O and 30min ●—●. The cells were lysed and centrifuged to equilibrium in CsCl at 30,000 rev/min in a Ti-50 rotor . The fractions were cold-acid precipitated and the radioactivity determined.

FIG. 4 vii Action of colicin E2 on BU-DNA in vivo.

Exponentially growing cells incubated with 5BU and (^{3}H)thymine for 2-3 generations were washed , resuspended at a cell density of 3.10^{7}bacteria per ml and incubated with colicin E2 □—□ at (a)1ng/ml (b) 3ng/ml and (c) 5ng/ml . Control cells ■—■ were incubated with colicin but not bromouracil.

The results of incorporating 5-bromouracil into E. coli for 5 and 30 min are shown in Fig. 4 vi and indicate linear uptake with time so that after 5 min, approx. 12% of the DNA appeared as a BU/^{3}H hybrid consistent with an observed cell doubling time of 40-45 min. The appearance of radioactivity in a third peak (BU/BU) indicated that (^{3}H) thymine was competing with the BU and that there was more than one site of replication per bacterium (Caro 1970). Cells that had incorporated BU gave the same minimum growth inhibitory concentration (MGIC) with colicin E_2 as control cells. The procedure with colicin E_2 was first to incorporate BU in the presence of (^{3}H) thymine into DNA of exponentially growing cells for 2-3 generations. The cells were then washed and resuspended at a cell density of 3 x 10^7 bacteria per ml with fresh medium containing 1-5 ng colicin E_2 per ml. Aliquots were taken at intervals and the cold-acid precipitable radioactivity determined. The percentage radioactivity was plotted against time of incubation with colicin (Figs. 4 vii(a), (b) and (c)) instead of recording the degradation after 2h, since here the effect of BU was on the overall shape of the degradation curves rather than the 2h degradation point. With very low concentrations of colicin the BU effect was most pronounced and was the opposite of that found with UV induced DNA degradation, i.e. inhibition instead of stimulation (Fig. 4 vii(a)). When the colicin concentration was increased, the inhibitory effect decreased, so that at 3 ng colicin per ml (Fig. 4 vii(b)) the 2h degradation was almost equal to the control value but the lag phase was still greater than in the control. At 5 ng colicin per ml (Fig. 4 vii(c)) and greater concentrations there was no detectable effect. The results with colicin therefore appear to resemble those of Lett et al., (1970) but are difficult to interpret.

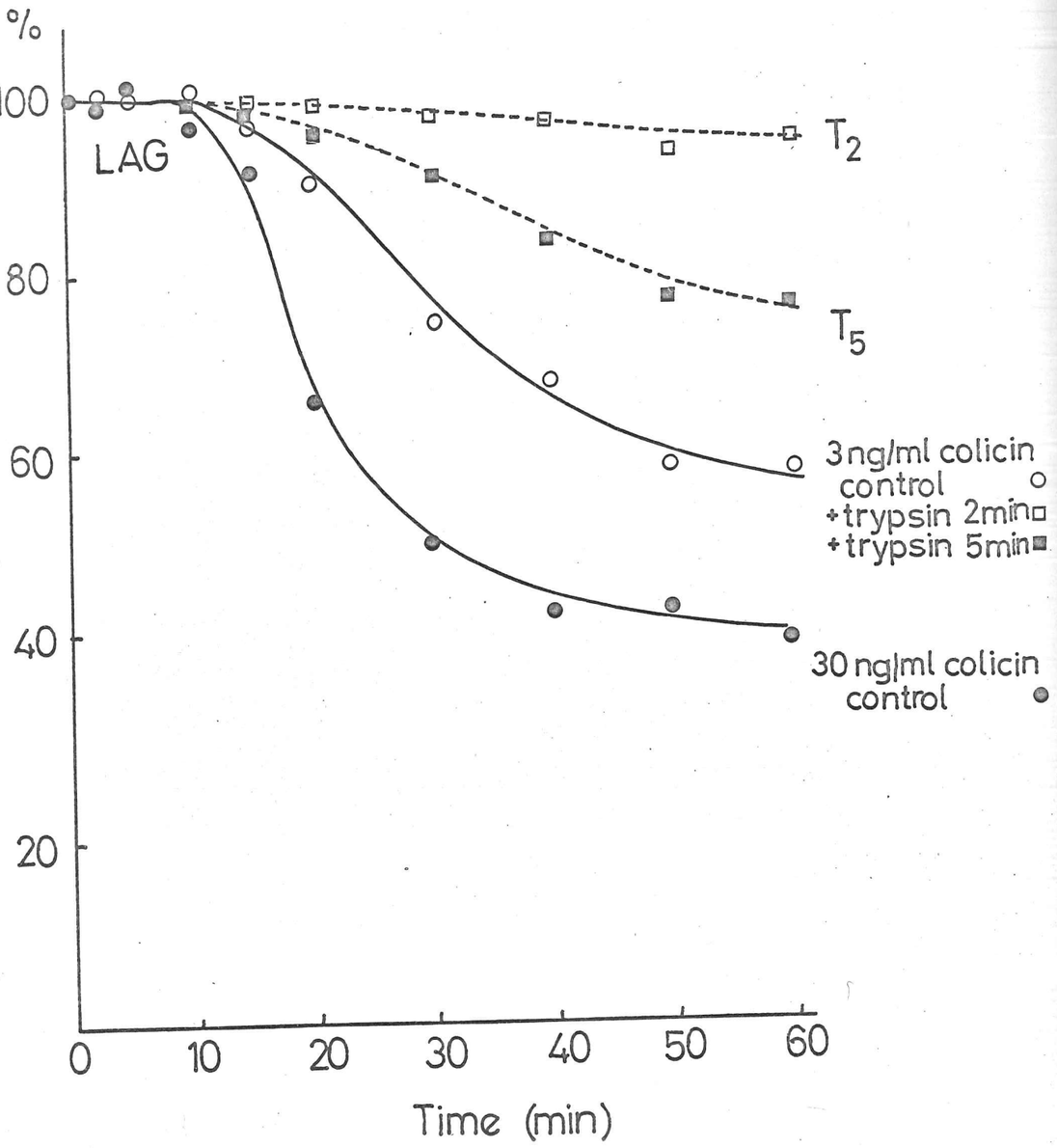


FIG. 4 viii Effect of trypsin on colicin induced DNA degradation.

(^{3}H) thymine labelled cells of E.coli ROW were incubated with colicin E2 at 3ng/ml or 30ng/ml. In addition trypsin (2mg/ml) was added to cells treated with 3ng colicin E2 per ml after 2min (T_2) and 5min (T_5) . Aliquots were taken at intervals and the cold-acid precipitable radioactivity determined.

(e) Effect of trypsin on colicin E_2-P9 induced DNA degradation

The results of Reynolds and Reeves (1963 and 1969) on the recovery of colicin treated cells with trypsin have shown that the survival of colicin treated cells could only be maintained if trypsin was allowed to digest the protein antibiotic within the first 15-20 min incubation with colicin and then only if dinitrophenol was added at the same time as trypsin. This recovery process necessitated the use of high trypsin concentrations (Reynolds and Reeves, 1963 and 1969) and even then a considerable fraction of radioactively labelled colicin E_2 was found attached to the cell envelope (Maeda and Nomura 1966).

Addition of trypsin (2 mg/ml) during the first 10 min incubation of cells with 3 ng colicin per ml reduced the extent of DNA degradation (Fig. 4 viii) and if added 2 min after the addition of colicin completely prevented degradation as detected by the cold-acid precipitate method. After 10 min incubation with colicin the subsequent degradation of cellular DNA was unaffected by trypsin even though Maeda and Nomura (1966) have shown that the ability of trypsin to remove colicin from cells was independent of the colicin incubation time. The effects of trypsin on cells incubated with 30 ng colicin per ml were unreproducible and varied widely with conditions. This was probably due to the more rapid DNA degradation. The significance of these results was investigated in Ch. 5.

DISCUSSION

Colicin E_2 induced DNA degradation was inhibited by; (1) combining the substrate DNA with an intercalating drug so that nuclease action was directly prevented; (2) blocking the production of energy and (3) inactivation of the colicin molecule at its receptor site before there was any detectable acid-solubilisation of DNA. The results with ethidium bromide indicated that penetration

of the cell membrane and intercalation with DNA was not significantly affected by colicin E_2. This would suggest that there was no direct interaction of colicin with DNA *in vivo* similar to histone "coating" of the chromosome. Bobb (1968) has however shown that actinomycin D will interact with chymotrypsinogen-DNA complex. Binding of ethidium bromide to DNA also appeared to be independent of the state of degradation of the DNA.

Inhibition of degradation of the bacterial chromosome by energy deprivation may indicate the specific nature of the nucleases involved but could also indicate an energy dependent membrane conformational change which was necessary for initiation of nuclease attack on DNA. An energy dependent transport process of colicin molecules across the membrane is difficult to reconcile with trypsin reversal and the radioactively labelled colicin experiments of Maeda and Nomura (1967), see Ch. 3.

The inhibition of BU incorporated DNA to act as a substrate for limited colicin E_2 induced degradation may reflect an inability of the induced exonuclease to degrade phosphodiester links to this base analogue (see Lett *et al.*, 1970). However this one result would suggest a difference in the enzymes used for degradation of UV and mitomycin damaged DNA (Aoki *et al.*, 1966) and the nuclease enzymes induced by colicin E_2. This apparently contradicts the findings of Threlfall and Holland (1968) and the results in Ch. 2. However growth inhibition experiments indicated that there was no difference in sensitivity between control cells and cells that had incorporated BU. This could mean that the cold-acid solubilisation of DNA was not necessary for the lethal effect of colicin E_2 but the experiments are far from conclusive.

Colicin action on DNA was unaffected by prior inhibition of protein or DNA synthesis which suggested that colicin E_2 did not initiate the synthesis of new nucleases or protein activators of latent cell nucleases and did not need active DNA synthesis for DNA degradation to occur. This last result contradicted the findings of Nomura (1963) for which there was no convincing evidence. The results with amino acid starvation supported the effects of chloramphenicol, mitomycin and phleomycin since after 90 min starvation all protein and nucleic acid synthesis ceased and yet colicin induced DNA degradation was unaffected. The result with phleomycin and mitomycin indicated that exonuclease I (Falashi and Kornberg, 1964) and exonuclease II and IV (Kornberg, 1969) were not involved in the colicin induced acid-solubilisation of DNA.

The effects of novobiocin and phenethyl alcohol were most interesting in that they potentiated the biochemical effect of colicin E_2. Both these antibacterial agents are believed to be membrane active (Smith and Davies, 1967; Lark and Lark, 1966) but differ from colicin E_2 in that they labilise the membrane whereas colicin E_2 has a stabilising effect on whole cells and spheroplasts (Nose et al., 1970; and see Ch. 6). An increased permeability of the cell membrane toward colicin molecules could be envisaged in support of a direct interaction of DNA and colicin E_2, but an effect on DNA-membrane association is far more likely since both novobiocin and phenethyl alcohol affect DNA metabolism and colicin E_2 could act at the DNA-membrane attachment site.

CHAPTER 5

Sedimentation analysis of DNA degradation products resulting from the action of colicin E_2-P9 on E. coli

INTRODUCTION

Colicin E_2 induced DNA degradation has been measured using the disappearance of radioactive DNA from the cold-acid precipitable fraction of treated cells (Nomura, 1963; Holland, 1968; and Chs. 2 and 4). A variety of time lags have been reported (Nose and Mizuno, 1968) depending on (a) the concentration of colicin; (b) the sensitive strain of E. coli used; and (c) the growth conditions employed prior to colicin attack (see Ch. 2). This lag has been interpreted as the time needed for the adsorbed colicin molecule to "transmit" its lethal effect to its particular biochemical target through the membrane complex (Nose and Mizuno, 1968).

Genetic studies suggest that the enzymatic system involved in colicin induced DNA degradation may be related to that used in the excision-repair process occurring after UV irradiation (Threlfall and Holland, 1968; and Ch. 2). If this is the case, the first stage of colicin attack would be the breakdown of cellular DNA by an endonuclease. The loss of radioactivity from labelled DNA present in a cold-acid precipitate may not therefore be a satisfactory method for following the initial events on addition of colicin E_2 to cells. It was decided to look for the appearance of single and double strand breaks in the DNA by analysis of lysates from colicin treated cells on neutral and alkaline sucrose density-gradients. Similar work has been carried out by Obinata and Mizuno (1970) but their results and those presented here differ and will be discussed later.

It was also of interest to determine whether DNA isolated from colicin treated cells was denatured or damaged in any way so as to explain colicin initiated DNA degradation. Obinata and Mizuno (1970) have reported that the activity and sub-cellular localisation of nucleases in E. coli was unaltered by colicin E_2 and have suggested that "an alteration in the state of DNA" may be responsible for initiation of degradation.

METHODS 5

(a) Preparation of labelled cell lysates for gradient analysis

The DNA of an exponentially growing culture of E. coli ROW was labelled with (^3H) thymine and the cells treated with colicin E_2 at 3 and 30 ng/ml (i.e. 10^3 and 10^4 colicin molecules per bacterium) as described in Methods 2. At intervals after addition of colicin E_2 to the labelled bacteria 0.5 ml and 5.0 ml aliquots were taken. The total cold-acid precipitable radioactivity was determined by mixing the 0.5 ml sample with 0.5 ml 10% (w/v) TCA as described in Methods 2. The 5 ml sample was diluted with 15 ml ice-cold lysis medium (0.15 M NaCl - 0.1 M EDTA pH 8.0) containing 2 mM dinitrophenol to stop further DNA degradation. The diluted bacteria were harvested by centrifugation at 4°, washed and resuspended in 1 ml lysis medium containing 200 µg lysozyme per ml. After incubation at 20° for 10-15 min, 0.5% (w/v) SDS and 100 µg pronase per ml were added and the incubation continued for a further 20-30 min at 40° to free the (^3H)-DNA from membrane and protein complexes and to eliminate any deoxyribonuclease activity (Marmur, 1961). 0.2 ml DNA preparation was layered directly on to a sucrose gradient using the wide end of a 1 ml pipette to eliminate excessive shear. The amount of sample corresponded to 0.4-1.0 µg.DNA (40,000 cts/min).

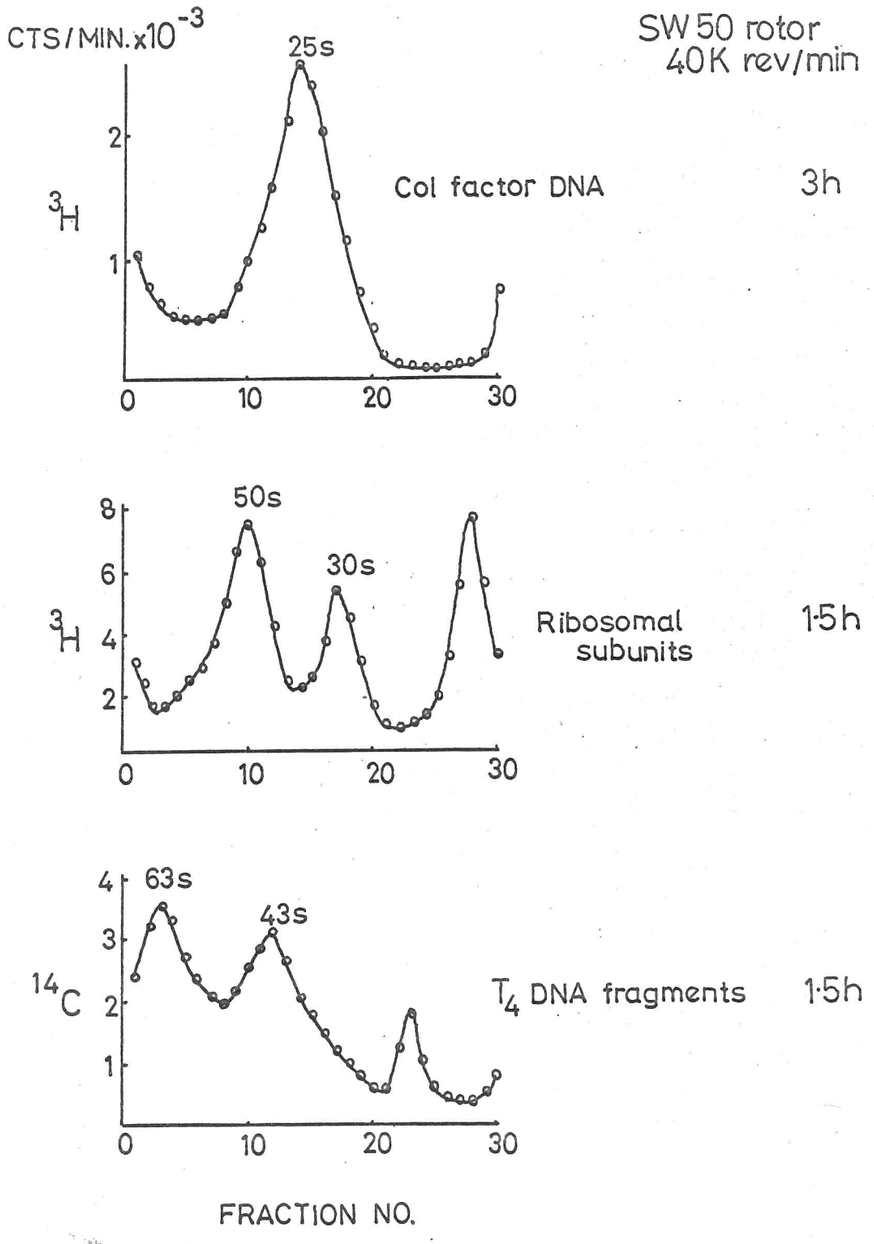


FIG. 5 i Standard sedimentation on sucrose density-gradients.

Col. factor DNA, ribosomal subunits and T_4 DNA fragments were prepared as described in METHODS 1,3 and 5, and centrifuged through 5-20% (w/v) neutral sucrose density-gradients as indicated. After fractionation, the cold-acid precipitable radioactivity of the samples was determined.

(b) Sucrose density-gradient analysis

5-20% (w/v) sucrose density-gradients were made up in NET buffer pH 8,0 (Smith and Hanawalt, 1967) for neutral gradients and in 0.9 M NaCl - 0.1 M NaOH (Studier, 1965) for alkaline gradients. The neutral gradient had a volume of 4.8 ml on which was layered 0.2 ml cell lysate whilst the alkaline gradient had a volume of 4.7 ml on which was layered 0.1 ml 0.5 M NaOH followed by 0.2 ml cell lysate. After preparation, the alkaline gradients were allowed to stand for 30 min at 20° prior to centrifugation in order to allow denaturation of the DNA to take place (McGrath and Williams, 1967).

The gradients were centrifuged for 1.5 to 3h at 40,000 rev/min at 20° in a Spinco Model L2-HV ultracentrifuge using a SW-50 rotor. 10 drop samples (approx. 0.15 ml) were collected from a pinhole punched into the bottom of the tube. Initially the samples were mixed with 1.5 ml 5% (w/v) TCA and 0.1 ml of a solution of 1 mg crude yeast nucleic acid per ml at 4°. After 30 min at 4° the cold-acid precipitates were collected by filtration on Oxoid membrane filters, washed and the radioactivity determined. However identical results were obtained by simply mixing the samples with 0.3 ml water and 3 ml triton X-100:BBOT-toluene mixture (1:2) as described in Ch. 3. Recovery of labelled material from gradients varied between 75 and 85% of the input radioactivity (Table 5 i).

(c) Calculation of sedimentation values and molecular weights of the DNA fragments

The sedimentation values of the (^{3}H) labelled DNA fragments were calculated using the formula of Nomura et al., (1960) and the values obtained were checked using the following markers of known sedimentation coefficient (see Fig. 5 ii):

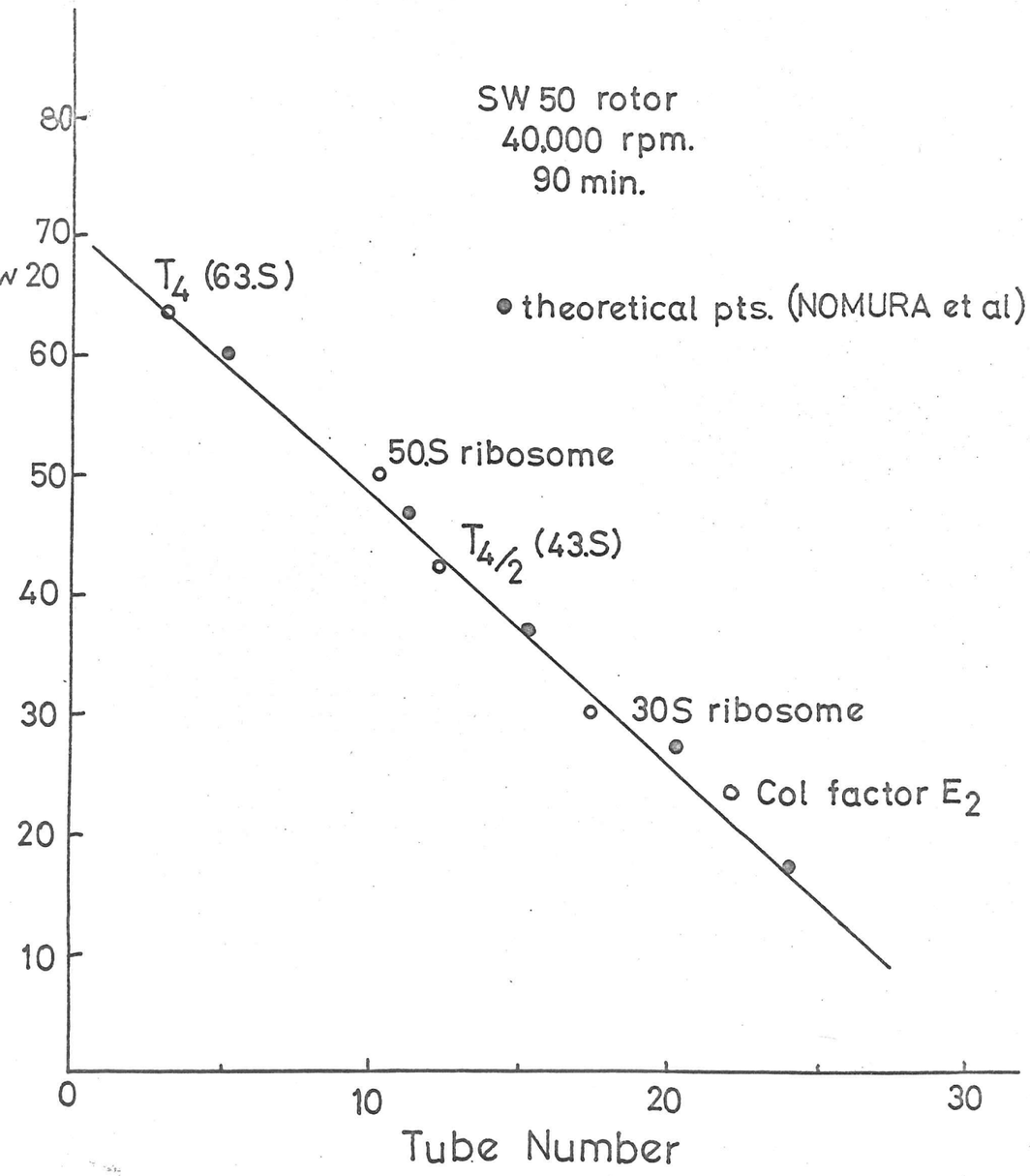


FIG. 5 ii

The filled circles are the theoretical points (Nomura et al., 1960) and the open circles are the empirical points obtained from FIG. 5 i . The relationship of sedimentation value and fraction or tube number is only applicable for gradients prepared and centrifuged as described in METHODS 5 and above .

(^{14}C) thymidine labelled T_4 phage DNA (preparation described in Methods 3), its fragments having sedimentation values of 63S, 43S and 30S (Burgi and Hershey, 1963); (^{3}H) thymine labelled colicinogenic factor E_2-P9 DNA (preparation described in Methods 1) sedimentation value 25S (Bazaral and Helinski, 1968); and 50S and 30S ribosomal subunits from sonicated E. coli ROW labelled with (^{3}H) uridine (Methods 2, sonicated cell extract centrifuged at 30,000 g for 15 min and supernatant fluid stored at -20°). The T_4 DNA fragments and Col. factor DNA were centrifuged on neutral sucrose gradients as described above whilst the ribosomal subunits were centrifuged in a 5-20%(w/v) sucrose gradient made up in 10 mM tris-HCl-25 mM KCL-0.1 mM magnesium acetate pH 7.0 (Fig. 5 i).

The sucrose density-gradients were shown to be linear by adding a radioactive label to the 20% sucrose and preparing the gradient with unlabelled 5% sucrose solution. The centrifuge tube was pierced and the radioactivity of the samples determined using the triton-toluene-BBOT method. The distance moved by a DNA sample was assumed to be directly proportional to the number of drops between the peak maximum and the meniscus since the difference in viscosity between 5% and 20% sucrose made a variation of less than 3% in drop size. The number of drops collected per gradient was 300 $\pm$ 5 after correction for the effect of SDS in the last few fractions.

The weight average molecular weights of the DNA fragments were calculated from their sedimentation coefficients, (Doty et al., (1958); Rubenstein et al., (1961); Burgi and Hershey (1963); Studier, (1965)) see Fig. 5 iii.

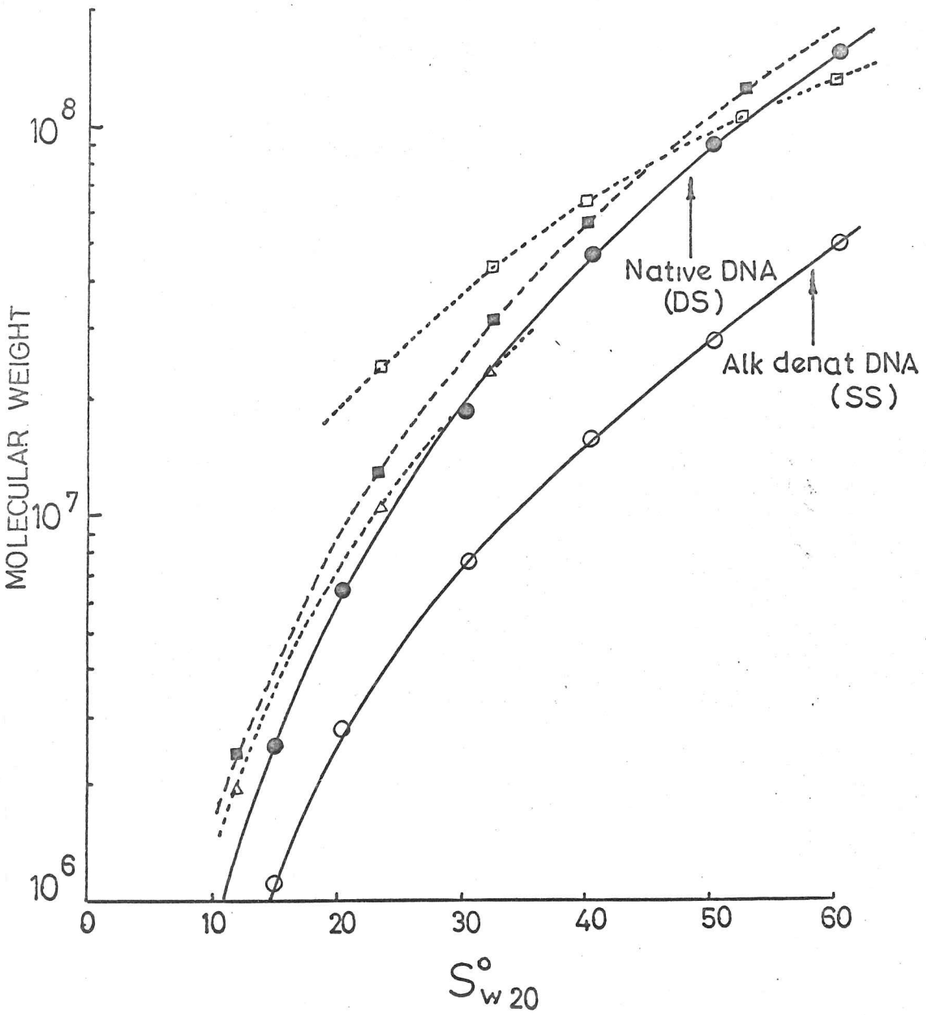


FIG. 5 iii

Relationship between DNA molecular weight and sedimentation coefficient.

The plots shown are derived from the formulae of ;

△——△ Doty et al. (1958) for native DNA 1-10.10^6 daltons

□——□ Rubenstein et al. (1961) for native DNA 30-160.10^6 daltons

■——■ Burgi & Hershey (1963) for native DNA 15-30.10^6 daltons

●——● Studier (1965) for native DNA 1-100.10^6 daltons

○——○ " " " alkaline denatured DNA

(d) Isopycnic centrifugation

SDS forms a precipitate with CsCl and was therefore replaced by 1% (w/v) sarkosyl solution in the preparation of (^3H) labelled cell lysate for CsCl and Cs_2SO_4 equilibrium centrifugation. The lysate was mixed with a solution of CsCl or Cs_2SO_4 in 0.01 M tris-HCl buffer pH 8.0 containing 0.01 M EDTA for neutral gradients or in 0.01 M tris-0.1 M K_3PO_4 buffer pH 12.5 containing 0.01 M EDTA for alkaline gradients (Smith and Hanawalt, 1969). The final densities were adjusted to 1.7100 g/ml for CsCl or 1.4250 g/ml for Cs_2SO_4 (or as indicated in the text) by the refractive index method described by Szybalski (1968). 4 ml of the mixture containing approx. 1 µg (^3H) labelled DNA was overlayered with 1 ml liquid paraffin and centrifuged to equilibrium (45-60h) at 32,000 or 44,000 rev/min at 20° in a Spinco Model L2-HV ultracentrifuge using the SW50 rotor. The tube contents were fractionated to give 50 x 5 drop samples by piercing the tube bottom with a needle connected to a LKB, peristaltic pump operating at 80 ml/h to decrease the flow rate. This method was used for nitrocellulose tubes. Polyallomer tubes (used for alkaline centrifugation) were however difficult to pierce and the tube contents were pumped out from above using a cannula (Methods 1).

The sample radioactivity was determined by either cold acid precipitation or the triton-toluene-BBOT method described above. There was no significant difference between the two methods.

(e) Caesium sulphate/Hg^{++} isopycnic centrifugation

This method was based on that described by Corneo et al., (1970). 1.0 ml cell lysate (dialysed against 5 mM borate buffer pH 9.5) was mixed with 3.7 ml 65% (w/w) Cs_2SO_4 stock solution (in H_2O), 0.4 ml 0.1 M borate buffer pH 9.5, 0.15 ml 1 mM $HgCl_2$

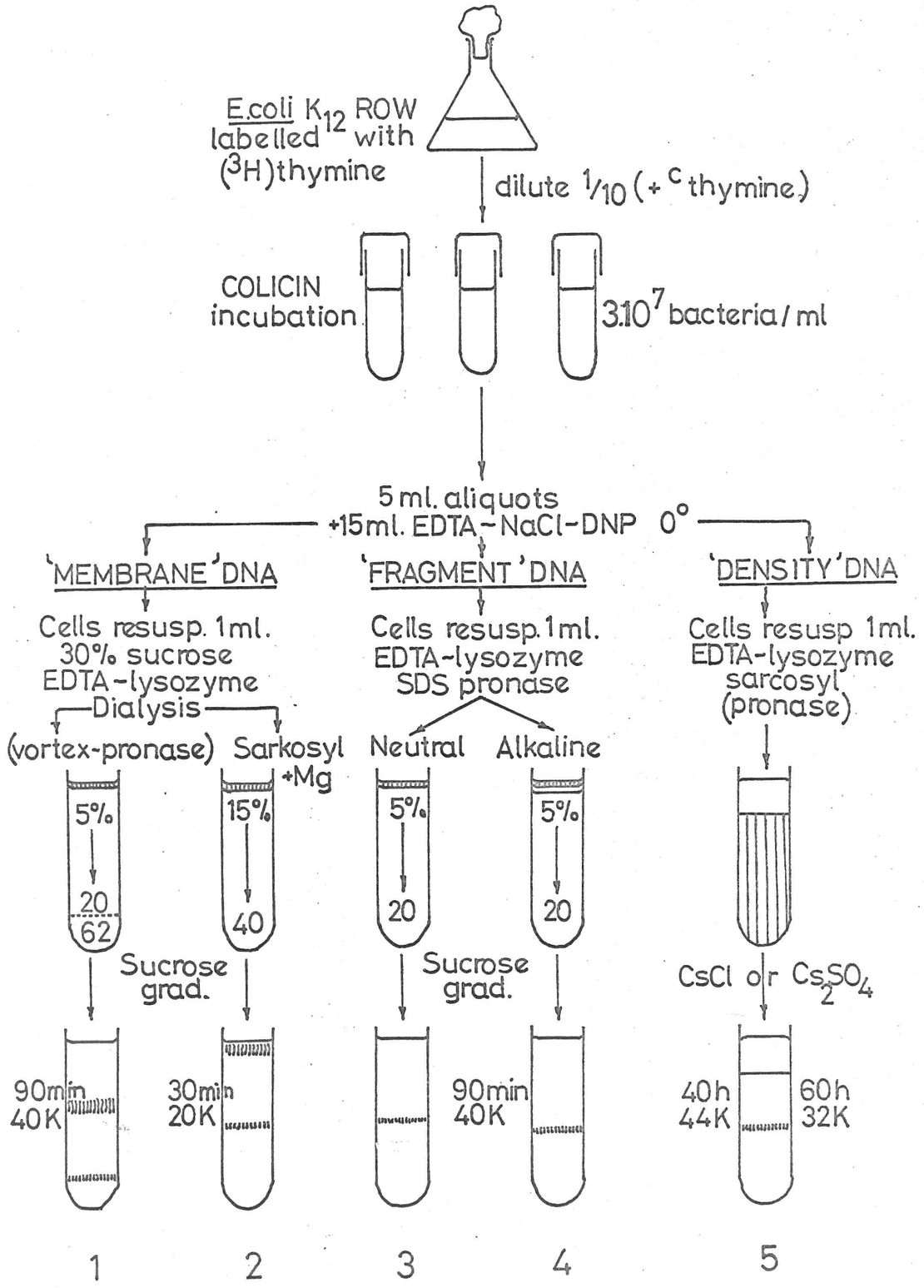


FIG. 5 iv Scheme for investigating DNA membrane association, fragmentation and density.

For tubes 3,4 and 5 see METHODS 5; for tubes 1and2 see METHODS 6.

and 2.75 ml glass distilled water. The final density was 1.4625 g/ml. The 8 ml mixture was overlayed with liquid paraffin so as to fill the tube and centrifuged to equilibrium (70h) at 32,000 rev/min in a Ti-50 rotor at 20°. The tubes were pierced and the contents fractionated using a peristaltic pump as described above. The 0.2 ml samples were cold-acid precipitated with yeast nucleic acid as carrier and the radioactivity determined.

RESULTS 5

Initially it was intended to investigate the physical integrity of DNA from cells incubated with colicin by lysing spheroplasts directly on top of the sucrose density-gradient, in this way causing a minimum shear of the released DNA. However after E. coli ROW had been incubated in the presence of colicin E_2 for a few minutes it proved extremely difficult to form spheroplasts and once formed these spheroplasts showed an increased resistance to lysis; similar effects have been reported with colicin E_2 by Nose et al., (1970) and with other anti-bacterial agents by Cundliffe (1967.b). This was probably the result of changes in the cell envelope and may be related to the appearance of long filamentous forms of E. coli after prolonged colicin treatment, (Ch. 2). The alternative approach of treating preformed E. coli spheroplasts with colicin was also unsuccessful owing to the reduced sensitivity at the high sucrose concentrations used and the implicit damage to the bacterial cell envelope (Šmarda, 1965; Beppu and Arima, 1967).

It was therefore decided to use conventional methods of cell lysis and a schematic representation of the methodology used in Chs. 5 and 6 is shown in Fig. 5 iv. For examination of DNA-membrane association a different method of cell lysis was used (Fig. 5 iv, tubes 1 and 2) and this will be discussed in Ch. 6. The method

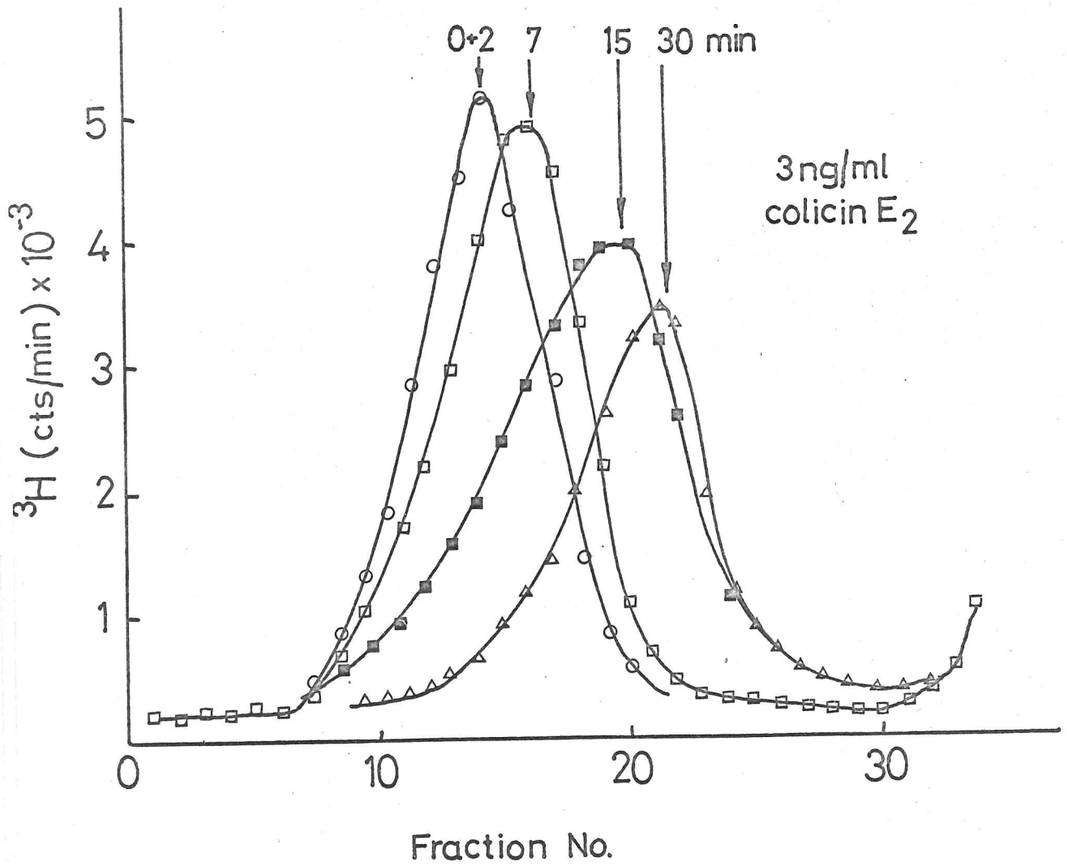


FIG. 5 v

Sedimentation analysis of DNA from (^{3}H) thymine labelled cells of E.coli after treatment with colicin E2.

The labelled bacteria were incubated with colicin E2 (3ng/ml) for the times indicated above the peaks, harvested by centrifugation lysed and centrifuged for 90min as described in FIG. 5 ii through neutral (FIG. 5 v) and alkaline (FIG. 5 vi) 5-20% (w/v) sucrose density-gradients. The 30 and 60 min peaks differ only in magnitude.

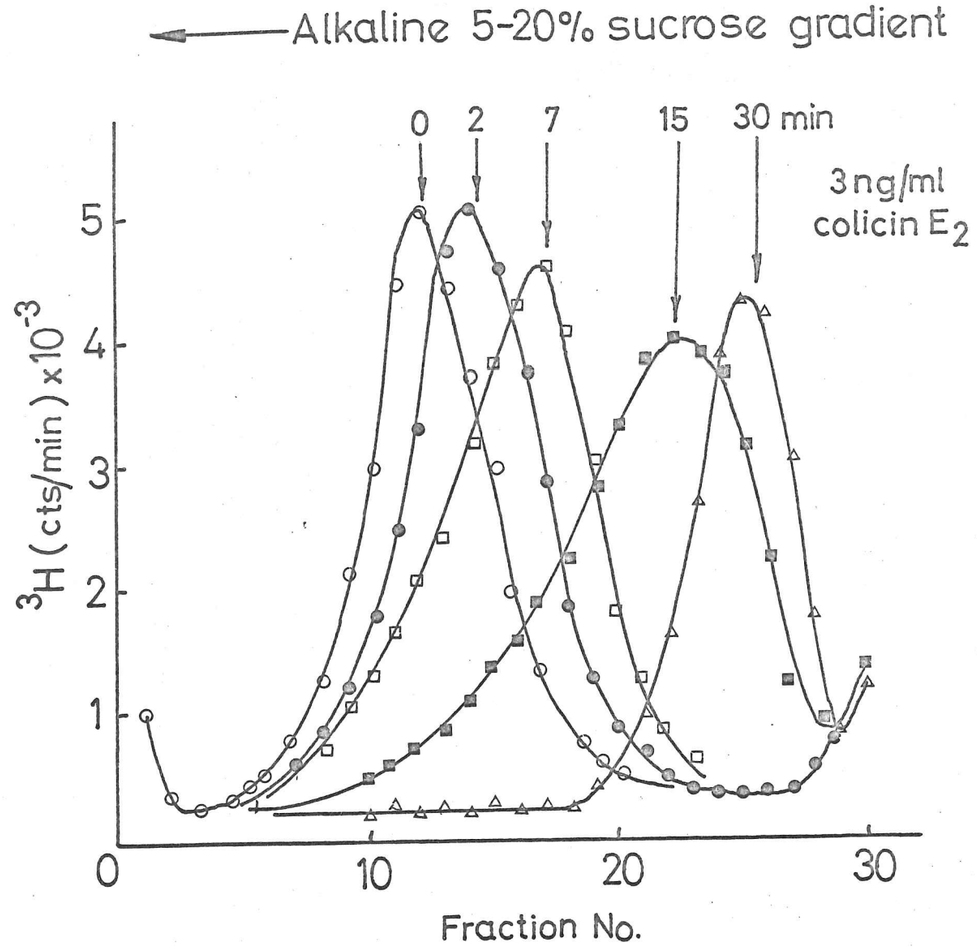


FIG. 5 vi

DNA profiles in alkaline sucrose (3ng colicin E2 perml)

For conditions see FIG. 5 v .

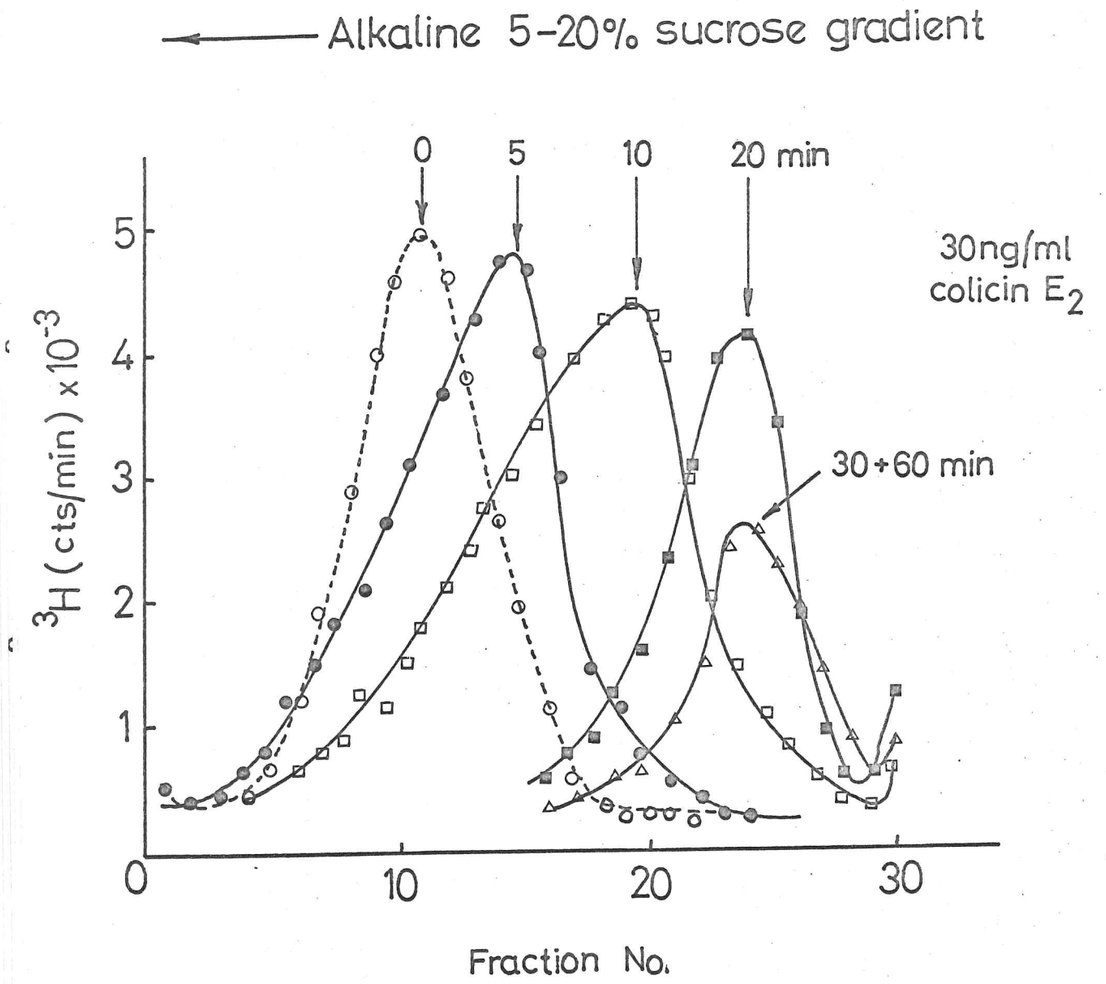


FIG. 5 vii

DNA profiles in alkaline sucrose (30ng colicin E2 per ml)

(^3H) thymine labelled cells of E.coli were incubated with colicin E2 for the times indicated above the peaks. The control DNA was centrifuged for 90min whereas the DNA samples from colicin treated bacteria were centrifuged for 3h. The 30 and 60 min peaks differ only in magnitude.

employed for investigating "Fragment" DNA gave reproducible sedimentation values of 40S and 47S for control DNA in neutral and alkaline gradients respectively, both of which corresponded to an original double stranded DNA of molecular weight 5×10^7 using Studier's equation (see Fig. 5 iii). This approximates to values obtained by Sakabe and Okazaki (1966), Smith and Hanawalt (1967) and Nomura et al., (1962). Under the ionic conditions used single stranded DNA (denatured) had a slightly higher sedimentation value than double stranded DNA (native) (Studier, 1965). The amount of DNA used per gradient was always less than 1 µg in order to eliminate concentration effects due to aggregation, DNA/wall interaction in the centrifuge tube and rotor speed (Burgi and Herschey, 1963; Studier, 1965).

(a) Analysis of DNA from cells incubated with colicin E_2-P9 using sucrose density-gradients

Cultures of E. coli ROW prelabelled with (^3H)-thymine were treated with colicin E_2, sampled at intervals and after lysis the DNA was analysed using both neutral and alkaline sucrose density-gradients. The results are shown in Figs. 5 v, vi and vii. The sedimentation values of both native and denatured DNA were progressively reduced on incubation with colicin E_2 (3 ng/ml) over a period of 30 min, but there was a difference in that the reduction in the case of denatured DNA was detected after 2 min incubation (Fig. 5 vi) whereas no differences from the control sample were observed in the native DNA until 7 min after the addition of colicin E_2 (Fig. 5 v). This alkaline shift was more pronounced at higher colicin concentrations (30 ng/ml) Fig. 5 vii. The difference between the results on the two types of gradients after 2 min indicated the presence of single strand but not double strand scissions in the DNA helix.

TABLE 5 i

Relationship between acid precipitable counts and total peak counts in neutral and alkaline sucrose density-gradients

1	2	3	4		5	6
Colicin E_2 (3ng/ml) incubation time	Acid precipitable counts in 0.2ml lysate before centrifugation[a]	% control counts for column 2	Total counts recovered in main peak after centrifugation[b]		% recovery	% control counts for column 4
			pH 8	pH 12		
0 min	40130	100%	32890	33010	83%	100%
2 min	40500	101%	32130	31200	78%	96%
7 min	40110	100%	31580	31630	79%	95%
15 min	38910	97%	32090	32010	83%	97%
30 min	30120	75%	24130	23870	80%	73%
60 min	23640	59%	19590	18700	81%	58%

a. Calculated from cold TCA precipitable material in 0.5 ml of the original cell suspension before lysis.

b. Profiles from which these values were derived are shown in Figs. 5 v and 5 vi.

After 5-10 min incubation the sedimentation value of native DNA from colicin treated bacteria was progressively reduced but was still greater than that of the alkaline denatured DNA from the same bacteria after 60 min. The recovery of radioactivity in the DNA preparation after gradient centrifugation is shown in Table 5 i. Radioactivity was not lost from the DNA during the first 15 min incubation of the bacteria with colicin E_2, yet it was clear from Fig. 5 vi that there was an appreciable molecular weight inhomogeneity in the DNA strands which manifested itself as an increase in peak width and assymetry in addition to a decrease in sedimentation value.

A plot of the DNA molecular weights against time of incubation of cells with colicin E_2 is shown in Fig. 5 viii. The molecular weights of single strand DNA prepared from cells incubated in the presence of colicin E_2 at a concentration of either 3 or 30 ng/ml fell rapidly with no detectable lag, whereas reduction of the molecular weight of native DNA occurred only after a lag of 5-10 min. The concentration of colicin E_2 in the incubation medium not only influenced the rate of DNA fragmentation but also its extent. The molecular weight of DNA isolated from cells treated with colicin for 30 min at a concentration of 30 ng/ml had a native molecular weight of 2×10^6 compared with 10^7 for DNA from cells incubated in the presence of 3 ng colicin E_2 per ml. Although there was this difference in molecular weights, the number of single strand scissions per duplex fragment was between 1 and 3 for both colicin concentrations and the whole process of single and double strand snipping was largely over by 30 min.

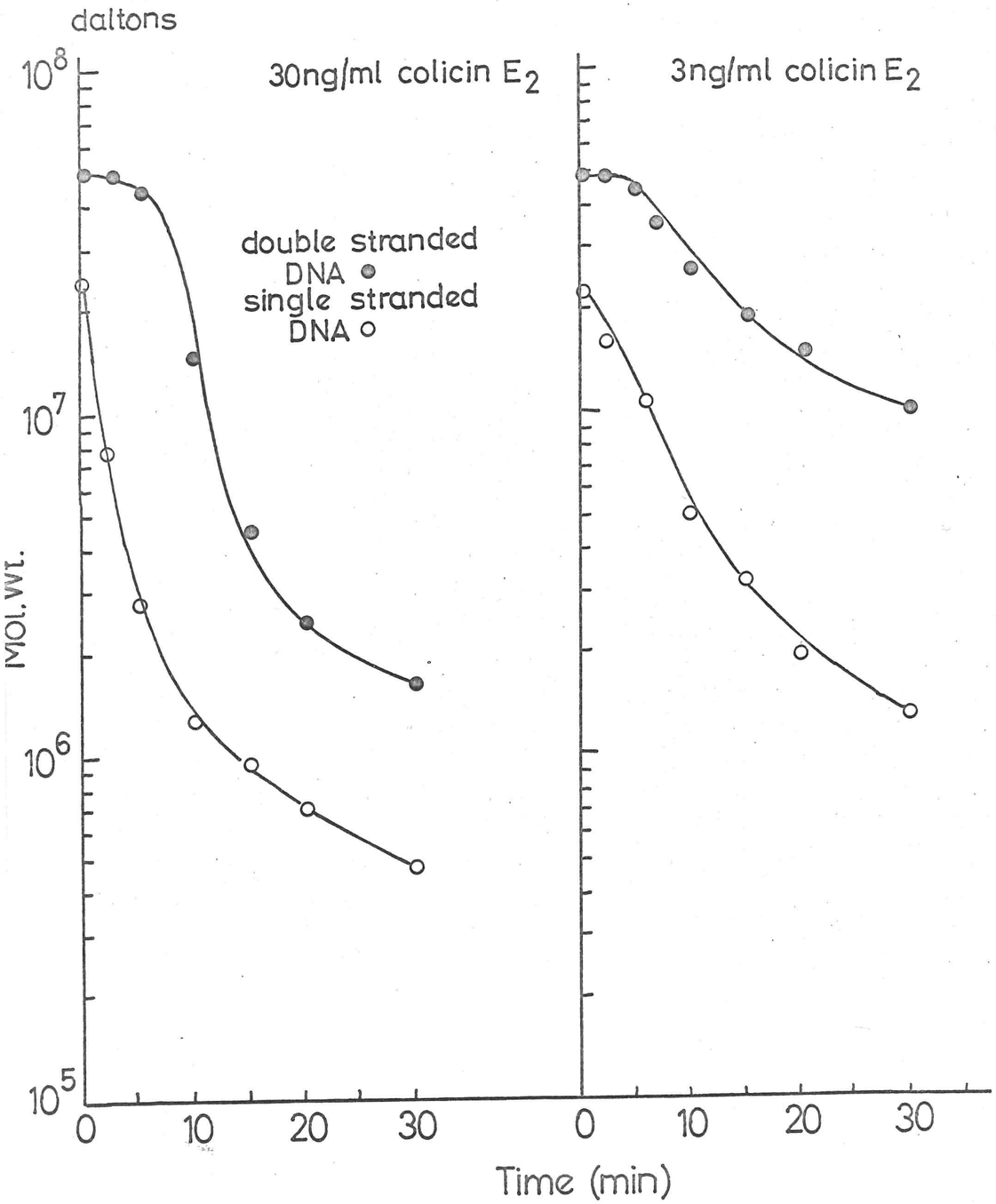


FIG. 5 viii Decrease in molecular weight of DNA from cells of E.coli after incubation with colicin E2.

Cells were incubated with colicin E2 at the concentrations indicated. The sedimentation values of single and double stranded DNA were determined after alkaline and neutral sucrose centrifugation, (FIGS. 5 v,vi &vii). Molecular weights were obtained using the formula of Studier (1965).

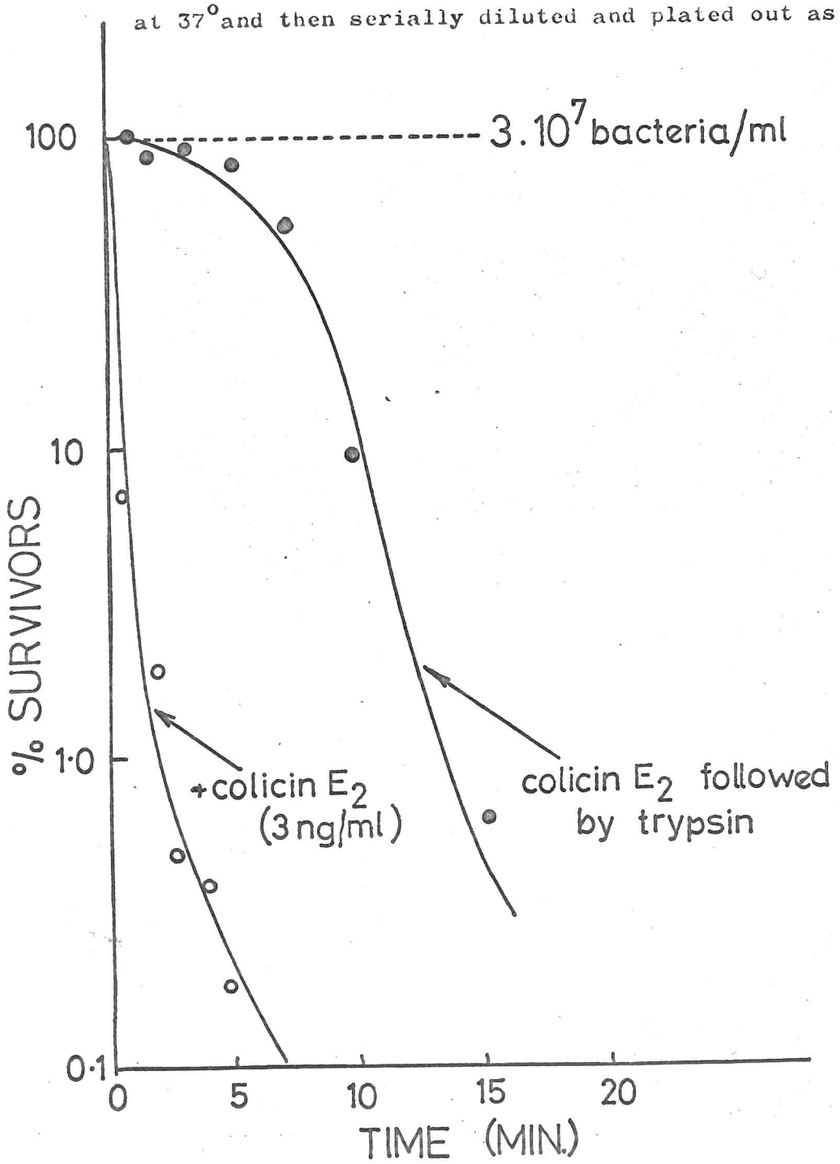
The decrease in cold-acid precipitable radioactivity from (^{3}H) thymine labelled cells incubated with colicin E_2 occurred after a lag of 10-15 min and was largely over after 1h (Fig. 2 iv), with the result that only 50-30% of the DNA remained acid precipitable for 3-30 ng colicin per ml. It was interesting to compare this with the observation that the DNA fragment molecular weight after 20-30 min incubation of cells in the presence of colicin E_2 was $2 \times 10^6 - 10^7$ daltons (Fig. 5 viii) and that no fragments of DNA with molecular weights between this value and that of cold-acid soluble fragments (i.e. approx. 5×10^3 daltons) were detected. This observation must indicate that once the exonuclease has attached itself to a particular piece of DNA, the breakdown to small acid-soluble oligonucleotides was extremely rapid and similar to that found for T_4 phage induced host chromosomal degradation (Kutter and Wiberg, 1968).

(b) Lethal process in colicin E_2-P9 induced cell death

It was apparent from the studies reported so far that DNA degradation induced by colicin E_2 could be divided into three stages; stages I and II being limited single and double strand endonuclease steps and stage III the rapid exonuclease step. It was therefore necessary to determine which was the "lethal step" after which the effects of colicin E_2 could not be reversed by trypsin (see Ch. 3). The method for trypsin treatment was similar to that described by Reynolds and Reeves (1963) and is given in the legend to Fig. 5 ix. The shape of the graph of the viability of cells incubated with colicin E_2 (without subsequent trypsin treatment) against time indicated the rate of adsorption of colicin molecules to the cell surface. However if at the times indicated the colicin treated cells were incubated with trypsin before dilution, the survival

FIG. 5 ix Effect of trypsin on the viability of E.coli treated with colicin E2

Exponentially growing cells of E.coli were incubated with colicin E2 (3ng/ml) at a density of 3.10^7 bacteria per ml and serially diluted at intervals to prevent further adsorption of colicin molecules and hence determine the number of bacteria still able to produce colonies on nutrient agar. Appropriate dilutions were plated out in triplicate O—O. Similar colicin treated samples were incubated with trypsin (2mg/ml) and 2:4 dinitrophenol for 20min at 37°and then serially diluted and plated out as before, ●—●.

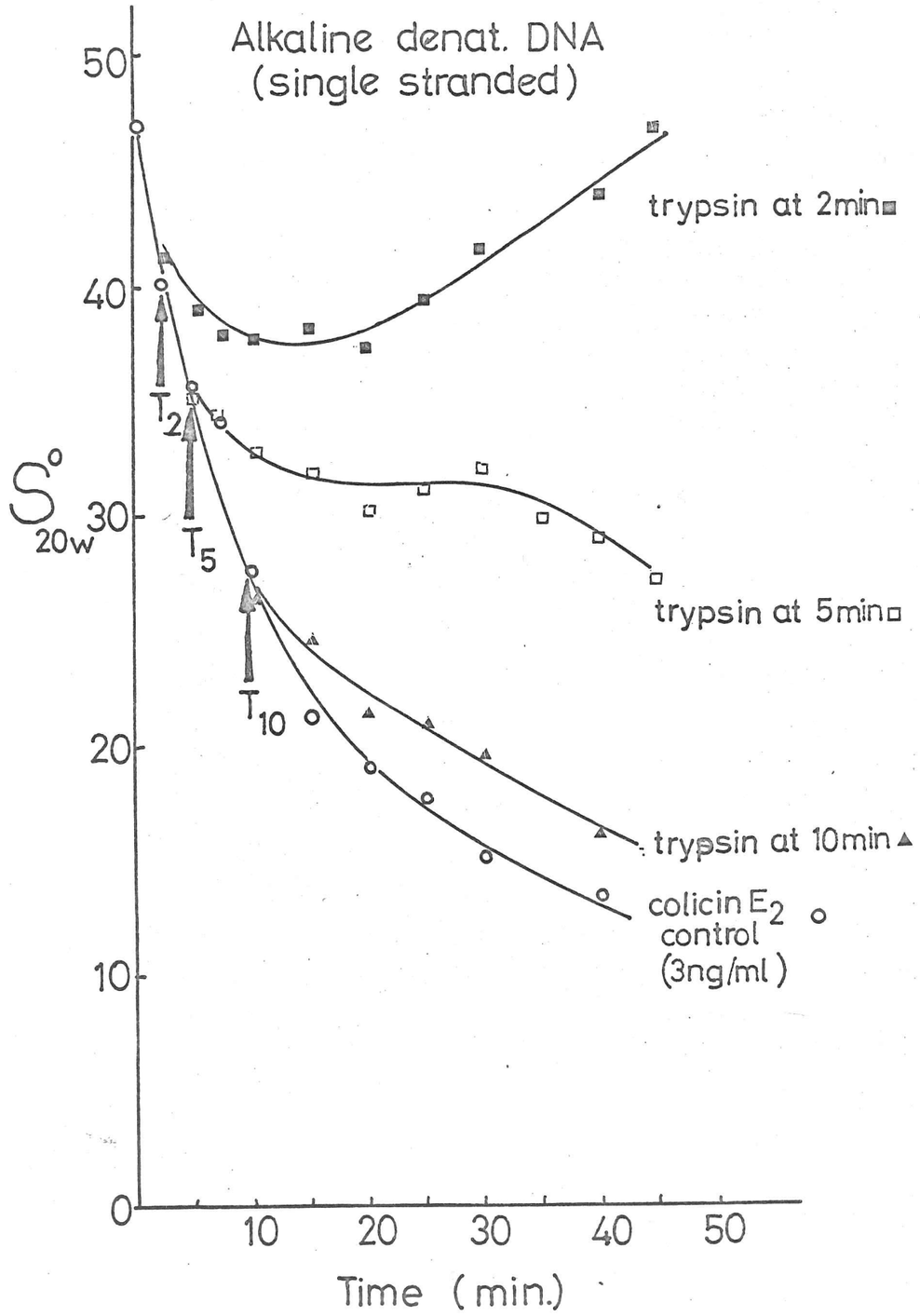


curve differed from the control in that there was a period during which trypsin apparently reversed the effect of colicin E_2. A comparison of these survival curves with those showing the temporal decrease of molecular weight of native DNA from bacteria incubated with colicin E_2 (Fig. 5 viii) would indicate that once double strands breaks had occurred in DNA, the lethal effect could not be reversed by trypsin.

(c) Trypsin induced repair of colicin E_2-P9 damaged DNA

If the lethal process in colicin induced cell death was the production of double strand breaks in the chromosome then it must be possible for cells to repair the single strand lesions produced initially. Addition of trypsin during the first 10 min to a culture of colicin treated bacteria reduced the extent of DNA degradation to acid-soluble fragments and if added 2 min after the addition of colicin, completely prevented any exonuclease activity (see Fig. 4 viii). The repair process in the presence of trypsin was followed by analysing cellular DNA using alkaline-sucrose density-gradient centrifugation (Fig. 5 x). A concentration of 3 ng colicin E_2 per ml was used because under these conditions there was a slightly longer and more distinct time lag before the appearance of double strand breaks in the DNA. The high trypsin concentrations used were similar to those prescribed by Reynolds and Reeves (1963 and 1969). There was a lag between the addition of trypsin and the cessation of colicin E_2 activity and if colicin action was not stopped before the appearance of double strand breaks then the exonucleolytic process was initiated and continued even after trypsin digestion of colicin.

FIG. 5 x Effect of trypsin on colicin induced fragmentation of single stranded DNA from E.coli.



(^{3}H) thymine labelled cells were incubated with colicin E2 (3ng/ml), after 2,5 and 10 min trypsin (2mg/ml) was added as indicated. Samples were taken at intervals, lysed and the DNA denatured and centrifuged on alkaline sucrose density-gradients.

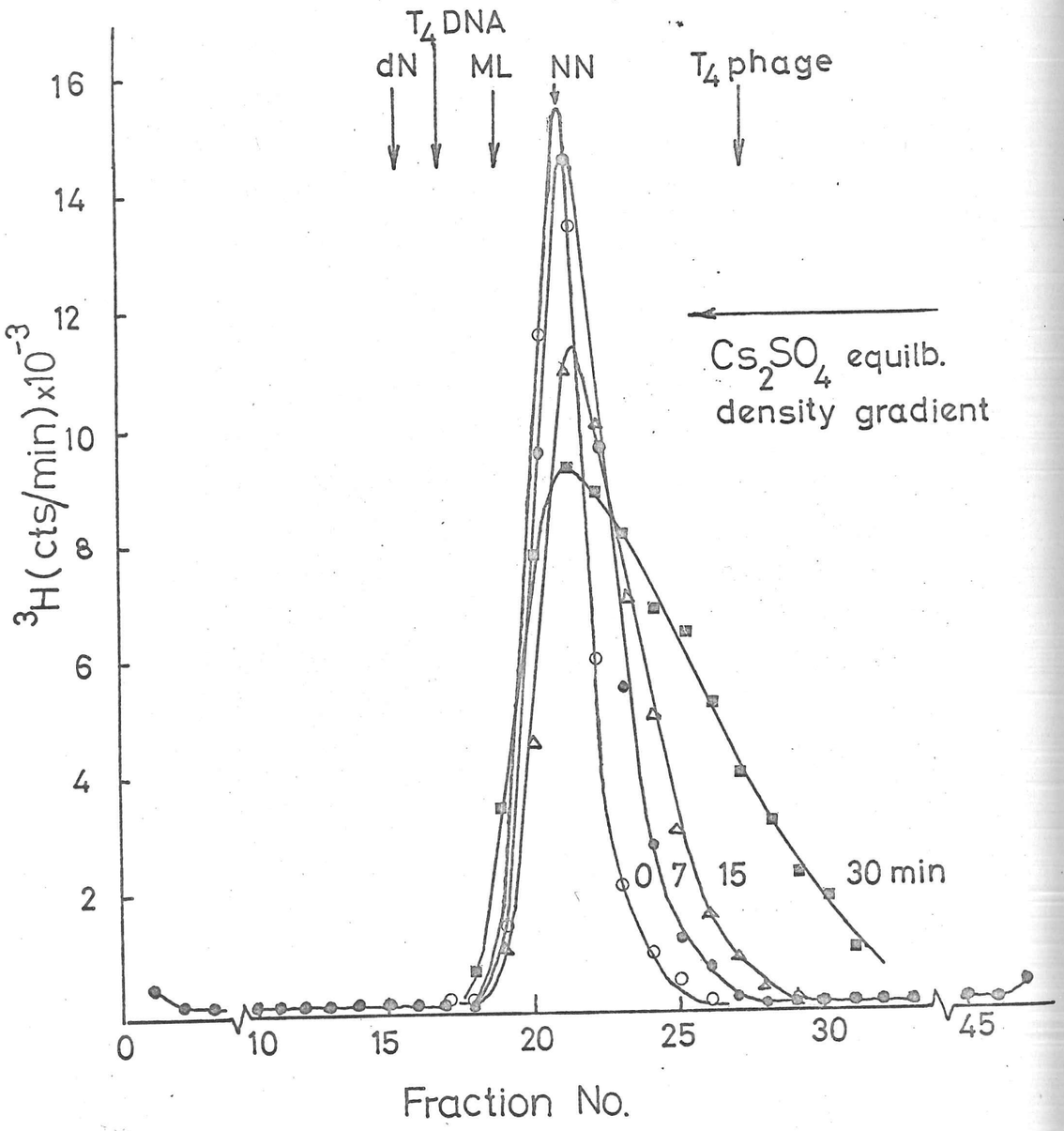
When trypsin was added 2 min after the addition of colicin E_2 to E. coli, the sedimentation value fell for a further 5-10 min, although at a reduced rate compared with the colicin treated control, and did not begin to increase until about 20 min after addition of trypsin. By 50 min the sedimentation value of the DNA was similar to the initial value (see McGrath and Williams (1966) for repair of UV damaged DNA). Addition of trypsin to the culture after 5 min incubation in the presence of colicin E_2 did not completely inhibit stage II of colicin induced DNA degradation. There was an immediate reduction in the rate of change of the sedimentation value, followed by a period of no apparent degradation, probably a result of competition between degradative and repair processes occurring within the cell. After 30 min there was a further decrease in the sedimentation value.

Trypsin treatment after 10 min incubation with colicin E_2 had virtually no effect on DNA degradation. Comparable samples were analysed on neutral-sucrose density-gradients and double strand breaks were found in the preparations from the 5 min but not the 2 min trypsin treatment. It would appear probable that once double strand cleavages have occurred, recovery was impossible.

(d) Buoyant density of DNA from cells incubated with colicin E_2-P9

It was of interest at this stage to determine whether colicin E_2 altered the secondary structure of DNA in vivo and in so doing initiated nuclease attack. The theory behind this investigation has already been discussed with reference to in vitro studies (Ch. 3). DNA was isolated from cells incubated with colicin and centrifuged to equilibrium in neutral CsCl or Cs_2SO_4 as described in Methods 5 and Fig. 5 iv (tube 5). The radioactivity profile isshown in Fig. 5 xi for a Cs_2SO_4 gradient. There was no shift in mean density of the DNA

FIG. 5 xi Cs_2SO_4 isopycnic centrifugation of DNA from colicin E2 treated E.coli.



(^3H) thymine labelled cells were incubated with colicin E2 (3ng/ml) for the times indicated, harvested by centrifugation, lysed and centrifuged to equilibrium in Cs_2SO_4 pH 8.0 at 32,000 rev/min and 20°(initial density 1.4250g/ml). The arrows indicate the buoyant density positions of marker DNA's; for abbreviations see FIGS. 3 vii & viii .

fragment after colicin treatment a result consistent with the *in vitro* observations (Figs. 3 vii and viii). If pronase treatment of the cell lysate was left out there was still no detectable change in buoyant density of the DNA. Although this last observation eliminates the possibility of any strong protein-DNA interaction similar to phage it does not eliminate formation of more subtle protein-DNA complexes which would be dissociated under conditions of high ionic strength (see Ch. 3).

Although the mean DNA density was not altered by colicin E_2 the peak width increased and a skewness indicative of molecular weight and density inhomogeneity became apparent. This peak assymetry was most pronounced in Cs_2SO_4 and was investigated further since it could indicate a specificity of nuclease attack at GC rich regions leaving AT rich or lighter DNA fragments compared with control DNA. It could also be the result of increased density inhomogeneity as a result of a decrease in molecular weight as reported by Thomas and Pinkerton (1962) for small phage fragments and Sueoka (1959) and Doty *et al.*, (1959) for sonicated calf thymus and bacterial DNA. More recent investigations of this phenomenon have used the Hg-Cs_2SO_4 method of density analysis (Nandi *et al.*, 1965). Yamagishi (1970) found that *E. coli* DNA fragments with a molecular weight of approx. 10^6 varied in GC content from 39 to 56%, and that the AT rich fragments were more numerous.

The Hg-Cs_2SO_4 method relies on the preferential binding of Hg^{++} to AT regions in the DNA at pH 9.5 (see Gruenwedel and Davidson, 1966) and the results of such an experiment are shown in Fig. 5 xii. The mean density of control and colicin treated DNA was increased from 1.4260 g/ml to 1.5100 g/ml consistent with a Hg^{++}/DNA-phosphate molar ratio of 0.2 (Nandi *et al.*, 1965). The colicin treated DNA however gave an assymetric peak, but this time the skew

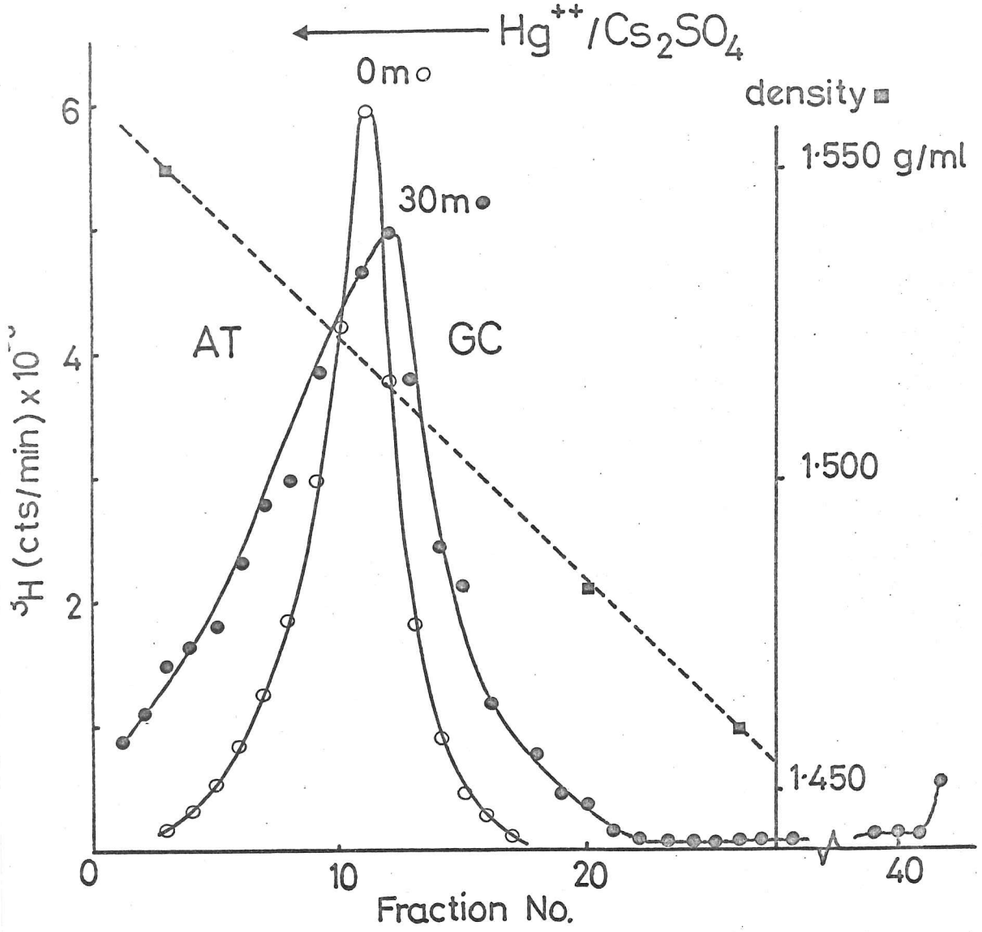


FIG. 5 xii

Hg-caesium sulphate isopycnic centrifugation of DNA from colicin E2 treated E.coli

Lysates of (^{3}H) thymine labelled bacteria treated with colicin E2 for the times indicated were mixed with Cs_2SO_4 pH 9.5 containing 20μM $HgCl_2$ and the DNA centrifuged to equilibrium at 32,000 rev/min and 20^0. The final density of the fractions was determined by the refractive index method of Szybalski (1968).

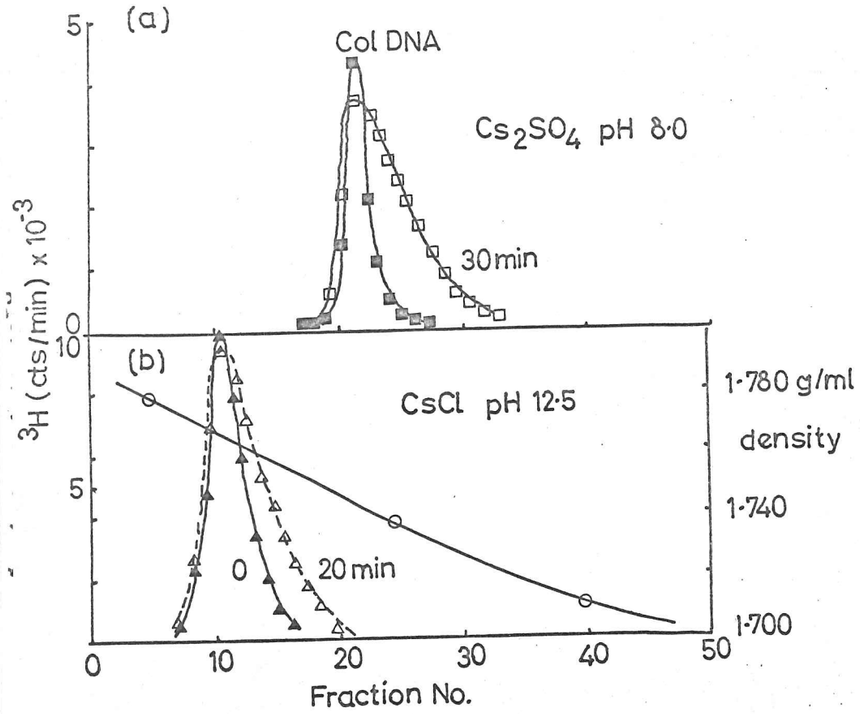


FIG. 5 xiii

Isopycnic centrifugation of (a) Col. factor DNA pH 8.0
(b) alkaline denatured E.coli DNA pH 12.5

(a) Col. factor DNA ■—■ (METHODS 1) was centrifuged in Cs_2SO_4 pH 8.0 together with DNA from cells treated with colicin E2 for 30 min □——□ .

(b) DNA from E.coli treated with colicin E2 for 0 and 20min was centrifuged in CsCl pH 12.5 at 32,000 rev/min. The density of the fractions was determined as in FIG. 5 xii .

was on the heavy side of the peak maximum consistent with the presence of AT rich fragments and the results of Yamagishi (1970). These findings eliminated the possibility of peak assymetry in Fig. 5 xi being due to preferential diffusion of small DNA fragments into the lighter Cs_2SO_4 or the result of artefacts arising from fractionation of samples after centrifugation or DNA/wall interactions in the centrifuge tube, etc. If nuclease attack were base specific it would be expected that using the Hg-Cs_2SO_4 method the degraded fragments would perhaps give rise to a distinct second peak heavier than the control (see Corneo et al., 1970). The results of Fig. 5 xii do not completely eliminate this possibility but together with similar results of DNA degraded by sonication it would suggest that the peak assymetries in Figs. 5 xi and xii were due to compositional inhomogeneity as a result of fragmentation of the bacterial chromosome.

Col. factor E_2 DNA has a molecular weight of 5×10^6 (Bazaral and Helinski, 1968) but was found to give an almost symmetrical profile after Cs_2SO_4 isopycnic centrifugation (Fig. 5 xiii(a)). Therefore simply reducing the molecular weight of homogeneous DNA would not cause assymetry, it is because DNA is heterogeneous with respect to its base composition that small fragments contain base compositions widely different from the chromosomal average. Isopycnic centrifugation under alkaline conditions showed an increase in density of DNA fragments from control and colicin treated bacteria, (Fig. 5 xiii(b)). There was no shift in mean density or the appearance of a second peak consistent with a single-strand base-specific nuclease.

Determination of B values

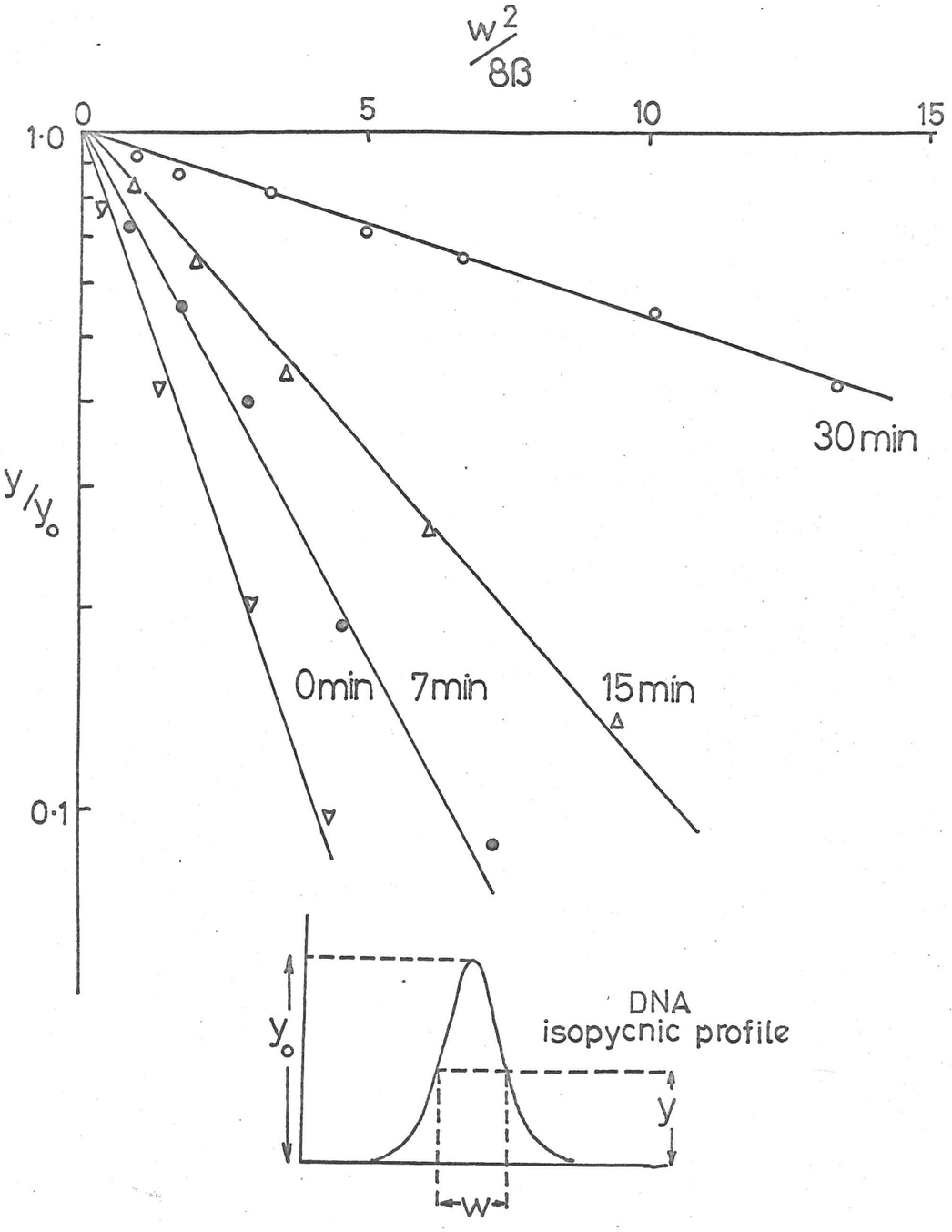


FIG. 5 xiv

By using the data in FIG. 5 xi, the peak width (w) squared is plotted against the fractional peak height (y/yo). β is a constant for the gradient conditions used (Thomas & Berns, 1961) and was found to 0.36 x 10^4 for Cs_2SO_4 at 32,000 rev/min in a SW50 rotor. The times refer to the cell incubation times with colicin, (FIG. 5 xi). The B value is given by the slope of the lines plotted.

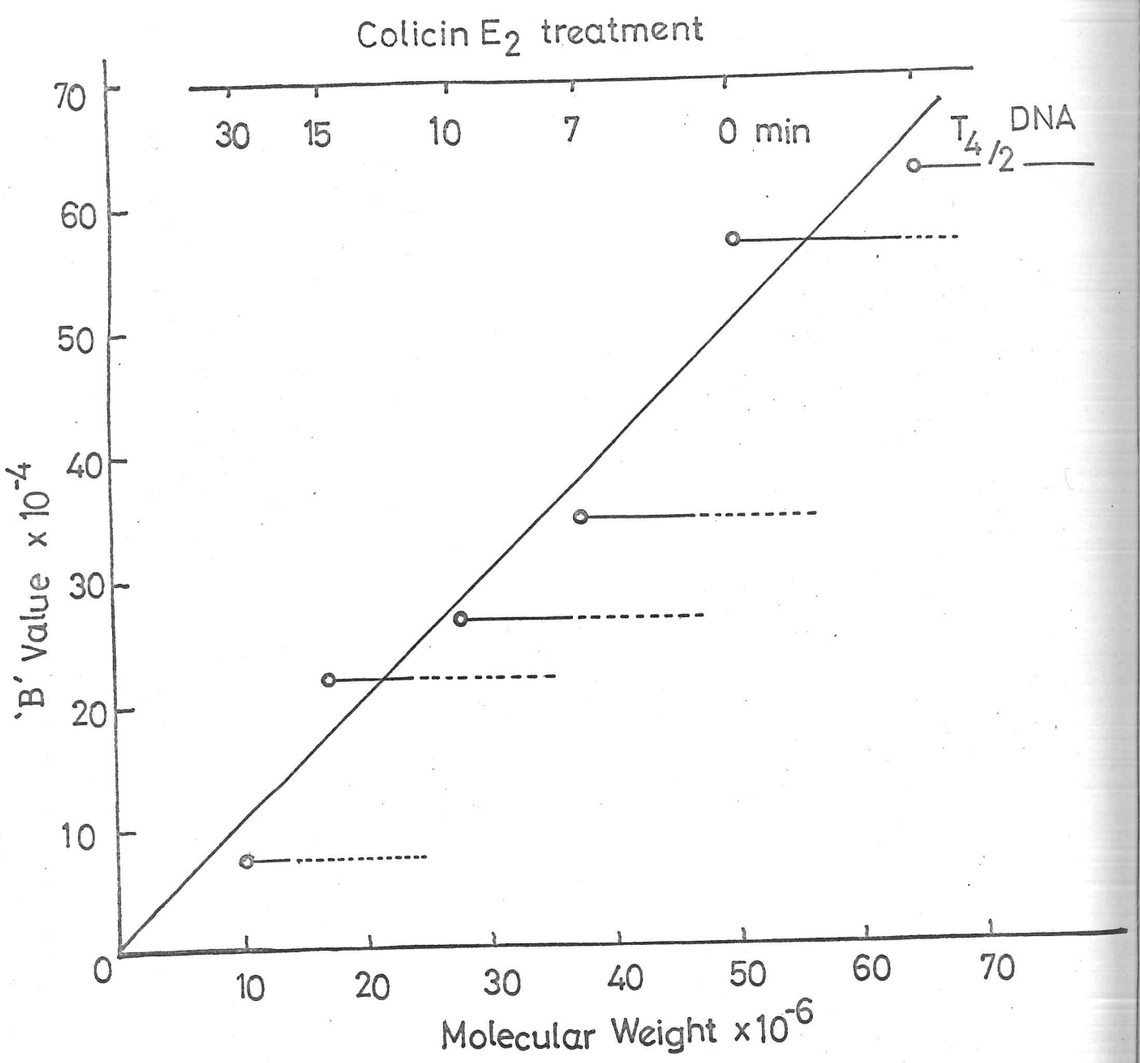


FIG. 5 xv Relationship between B values and Molecular Weights

The B values (FIG.5 xiv) are plotted against the corresponding DNA molecular weights calculated from sucrose density-gradient centrifugation (FIG. 5 viii) using Studier's equation (1965) (O). The lines from the open circles indicate the spread in estimation of molecular weight using the eqations of Burgi and Hershey (1963) ——— and Rubenstein et al. (1961) ------- . The only takes account of the Studier values. $T4/_2$ DNA was used as a standard

It was of interest to compare molecular weights of DNA fragments computed from sedimentation through sucrose density-gradients with peak widths obtained after isopycnic centrifugation using the method of Thomas and Berns (1961). Meselson et al., (1957) originally found that the peak variance was indirectly proportional to molecular weight. B values were determined by plotting the log of the fractional peak height against the peak-width squared as described in the legend to Fig. 5 xiv . The sedimentation molecular weights of the DNA fragments were then plotted against the B values (Fig. 5 x v) and an approximate linear relationship was obtained which provided a useful confirmation of the relative molecular weights assigned to particular DNA fragments isolated from colicin treated cells. This method of B value determination is only strictly applicable under conditions of peak symmetry and Gaussian distribution. However despite the marked peak assymetry a workable relationship between B values and molecular weights was obtained.

DISCUSSION

The results reported show that there were at least three distinct stages in colicin E_2 initiated degradation of E. coli DNA. Stages I and II involved the appearance of single and double strand scissions in the DNA which may result from the action of either two specific limited endonucleases or one double hit endonuclease similar to DNase I (see Ch. 3). These two stages overlapped to some extent although stage I was usually observed in isolation during the first few minutes of cell incubation with colicin. Stage III involved a rapid exonucleolytic attack on the products of stages I and II producing cold-acid soluble fragments without any observable inter-

mediates. Exonuclease activity was detected after a lag of 10 min and was largely over by 45-60 min, similar to T_4 phage induced degradation of host DNA where there is a lag of about 5 min and the whole process is over by 30 min after infection (Kutter and Wiberg, 1968). However the percentage of residual DNA left after this stage is only 12-15% with T_4 phage compared with 25-30% at optimal colicin concentrations. The initial lag phase with colicin may have been the result of exonuclease activity being initiated only when stage II DNA fragments were of a suitable size (about 10^7 daltons) or could have simply been due to a competition between repair and excision processes. The nucleotide pool of E. coli is resistant to a rapid "cold" chase (see Britten et al., 1964) and since it has been shown that DNA synthesis continues for some minutes after colicin attack at low colicin:cell ratios, (Ch. 2), the appearance of net exonuclease activity after a lag of 10 min may be apparent rather than real.

Stage I was not the lethal process associated with colicin induced cell death since the single strand lesions were repairable if the adsorbed colicin molecules were digested by trypsin. However stage II was irreversible and the extent of double strand breaks was indirectly related to the number of cell survivors.

Obinata and Mizuno (1970) also found that the appearance of double strand breaks was the lethal process of colicin E_2 attack but did not detect the separate initial appearance of single strand lesions and any consequent repair on digestion of colicin with trypsin. This was probably due to the fact that they used impure colicin preparations which were 10^2 - 10^3 more concentrated than reported above. The treatment of cell lysates for alkaline and neutral sucrose density gradients also differed in that the lysates for neutral gradients were subjected to a freeze-thaw and phenol

extraction procedure. The molecular weights of native DNA fragments could therefore be lowered relative to DNA obtained under more comparable conditions to alkali treated cells and thus mask any detection of a difference from twice the molecular weight of denatured DNA. The trypsin treatment used by Obinata and Mizuno (1970) was only with low trypsin concentrations after 5 min colicin incubation and it has been reported by Reynolds and Reeves (1969) that at such concentrations there was under certain conditions a potentiation of colicin activity.

Possible nucleases involved in colicin E_2 induced DNA degradation

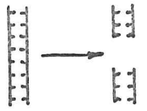
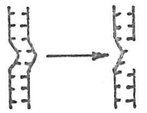
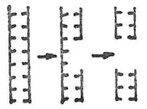
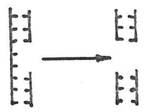
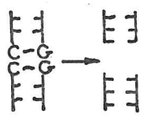
The appearance at the end of stage II of DNA fragments with molecular weights between 10^6 and 10^7 daltons may be related to the finding of Szybalski et al., (1966) that there is a cytosine-rich cluster per 10^6 - 10^7 daltons of E. coli DNA and that these may be the sites for initiation of transcription by RNA polymerase. Hence stage I and II could involve a cytosine-specific nuclease of the type described by Kutter and Wiberg (1968) and Opara-Kubinska et al., (1964). This may indirectly account for the apparent density heterogeneity of DNA fragments reported on isopycnic centrifugation since despite the apparent similarity between the above results on $Hg\text{-}Cs_2SO_4$ centrifugation and the results of Yamagishi (1970) the degree of fragment heterogeneity was comparatively high for a fragment molecular weight of approx. 10^7. Yamagishi found that above a molecular weight of 4×10^7, fragments containing the extreme base composition of 39% GC were not detectable, but below 2×10^7 daltons they were. However reduction of fragment size to 2×10^6 daltons was necessary for maximum release of 39% GC fragments. The molecular weight of the DNA fragments isolated from cells after

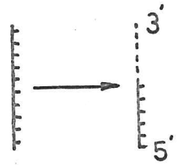
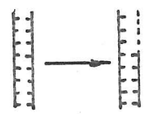
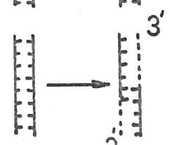
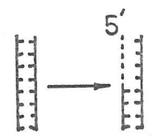
30 min incubation with colicin E_2 was confirmed as being approx. 10^7 from the B values. This would suggest that colicin E_2 induced DNA degradation released 39% GC fragments from the chromosome more readily than sonic disintegration and therefore involved an endonuclease with a certain degree of specificity. In other words the AT rich fragments do not arise because of a cytosine-guanine specific endo and exonuclease system depriving 50% GC fragments of cytosine and guanine but probably because the induced nuclease fragments the chromosome in such a way as to release pieces of DNA that are already hetrogeneous. A cytosine specific nuclease would therefore operate by selecting whole genes (Szybalski *et al.*, 1966) which were heterogeneous with respect to each other. If it were assumed that each fragment were homogeneous, removal of the whole cytosine rich cluster (approx. 100 nucleotide pairs) by a hypothetical nuclease would not produce the drop in % GC content observed above.

The involvement of a cytosine specific nuclease would explain why T_4 DNA present in colicin treated cells of *E. coli* is relatively resistant to degradation (Nomura, 1963) since here the cytosine is substituted by hydroxymethyl-cytosine and these clusters occur with lower frequency in T_4 than in *E. coli* (Szybalski *et al.*, 1966; Taylor *et al.*, 1967). However there is an objection to this comparison between phage and colicin induced breakdown of DNA in that colicin action is independent of protein synthesis whereas phage induced host degradation is inhibited by chloramphenicol (Ch. 4 and Nomura *et al.*, 1962). However although T_4 induced DNA degradation is dependent on phage specific protein synthesis, it does not necessarily follow that the nucleases used are coded for by the phage genome. It is possible that a phage coded protein is needed to activate a cellular nuclease.

FIG. 5 xvi

DNases of E.coli

ENDONUCLEASE	SUBSTRATE	ATTACK	COMMENT
I	native DNA		1 inhib.by tRNA. 2 outside membrane 3 not necess.in DNA syn.
★ II	UV damaged DNA or alkylated DNA		specific for DS. DNA REPAIR
★ III	'foreign' DNA		used in RESTRICTION needs ATP & SAM
★ IV	single strands of native DNA		used in RECOMBINATION (with endo II ?) large MWt fragments
★ T_4 CYTOSINE DNase	host DNA with C rich clusters		10^6-10^7 MWt fragments

EXONUCLEASE			
I	denatured DNA (single stranded)		3′→5′
II	native replicating DNA		1 3′→5′ 2 assoc. with DNA polym. 3 stim by 4 dNTPs REPLICATION
III	ONLY native DNA		NOT SS. DNA
★ IV	large oligo–nucleotides		NOT native or denat. DNA
VI	native replicating DNA		5′→3′ associated with DNA polym & exo II

★ NUCLEASES IN COLICIN ATTACK ?

Colicin E_2 has no endo or exonucleolytic activity *in vitro* (Ch. 3) and before discussing mechanisms whereby colicin E_2 initiates the degradation of *E. coli* DNA, it is worthwhile considering which of the DNases so far characterised may be involved (see Fig. 5 xvi and Richardson, 1969). Endonuclease I was originally an attractive candidate for the initial stages of DNA degradation since it is located in the region of the bacterial membrane (see Ch. 2 and Cordonnier and Bernardi, 1965) and is inhibited by tRNA (Lehman *et al.*, 1962). But because (1) there was no detectable degradation of RNA during the first hour of colicin induced DNA degradation (Ch. 2), (2) the DNA in an endonuclease I deficient mutant was degraded as efficiently after colicin attack as the wild type (Ch. 2 and Obinata and Mizuno, 1970) and (3) endonuclease I was specifically involved in a single hit cleavage of the DNA duplex (Studier, 1965; Melgar and Goldthwait, 1968), it is unlikely that colicin E_2 acts by removing tRNA and activating this enzyme.

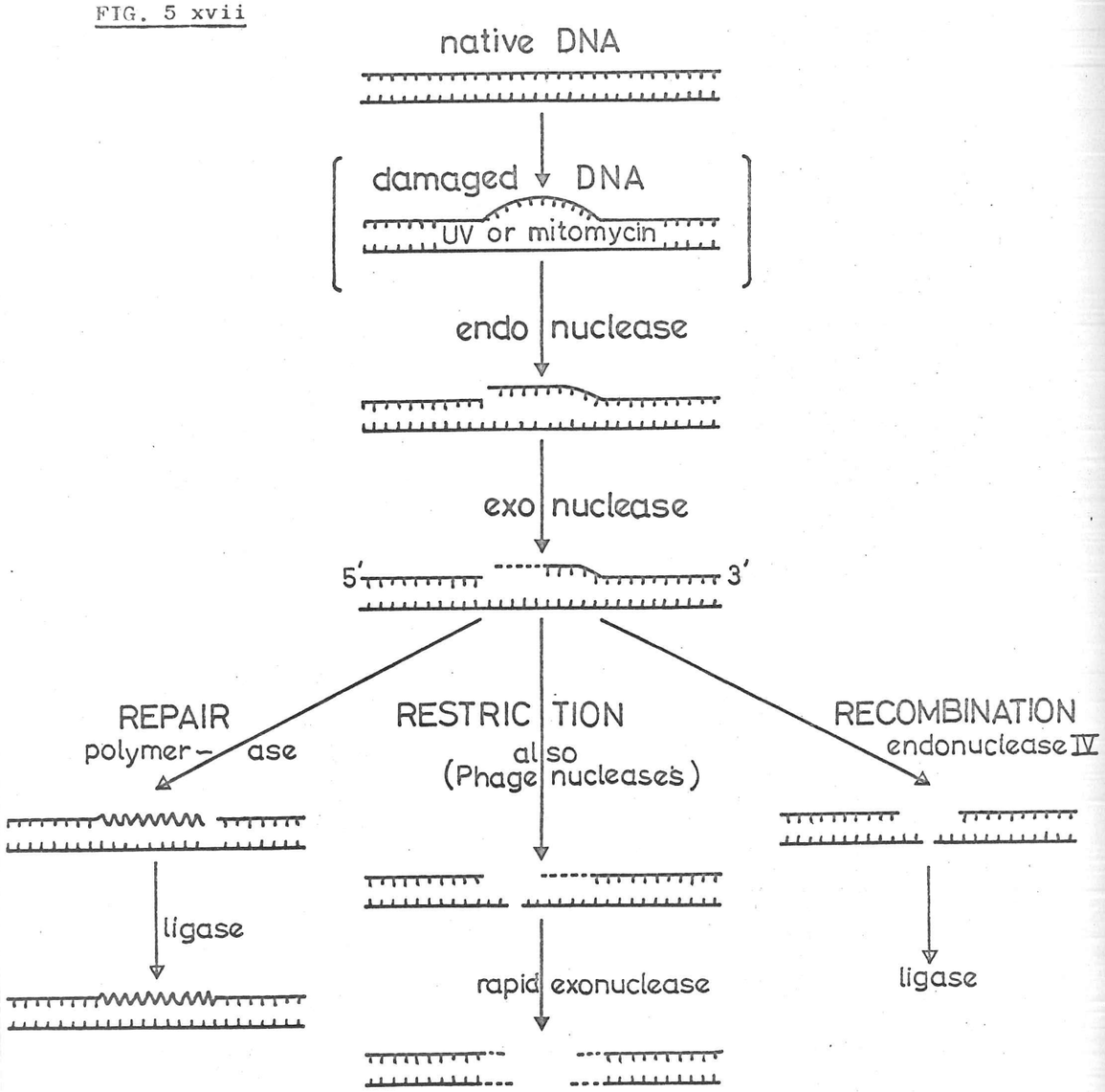
Endonuclease II (Hurwitz *et al.*, 1967) produces single strand scissions in native DNA and is not inhibited by tRNA. Together with endonuclease IV (Fig. 5 xvi) it appears to be involved in recombination of T_4 DNA (Sadowski *et al.*, 1968) and although it is synthesised on T_4 infection after host DNA degradation a similar activity has been found in non-infected *E. coli* (Friedberg and Goldthwait, 1968). This observation, together with the finding that certain UV sensitive and recombination deficient mutants of *E. coli* are resistant to colicin E_2 (Ch. 2 and Threlfall and Holland, 1968) gives support to the hypothesis that endonuclease II or a similar enzyme may be involved in stage I of colicin initiated breakdown of *E. coli* DNA. Enzymes falling into this catagory have been

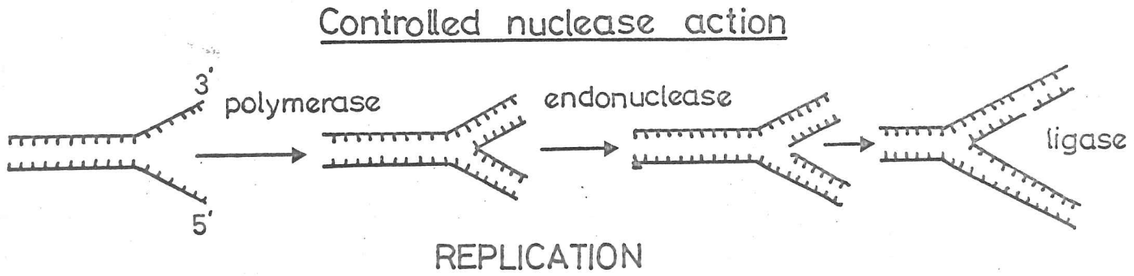
isolated from E. coli and M. lysodiekticus and have been implicated in the specific endonucleolytic degradation of UV damaged DNA (Takagi et al., 1968) and mitomycin C crosslinked DNA (Venitt, 1968).

Endonuclease IV specifically attacks single stranded DNA and is believed to fragment double stranded DNA after endonuclease II has cleaved single strands of the native DNA and these single strand snips have been opened up by a limited exonuclease, thus revealing localised regions of single stranded DNA in the duplex molecule (Sadowski et al., 1968). The fragments produced are large and have been reported as 10^4 nucleotide pairs long (6.10^6 daltons) for lambda phage (Hurwitz et al., 1967). However as mentioned earlier a cytosine specific nuclease is also possible in the initial stages.

Other possibilities are the restriction enzymes that enable bacterial cells of one strain to destroy DNA synthesised in foreign strains. In this context colicin E_2 may act by making native DNA appear foreign to its own cellular restriction mechanisms. Endonuclease III is a well characterised example and functions in the restriction of lambda phage (Meselson and Yuan, 1968). This enzyme is known to initiate single strand breaks and to be dependent on ATP, Mg^{++} and S-adenosyl methionine. It is well established that colicin E_2 induced DNA degradation is inhibited by metabolic inhibitors such as dinitrophenol (Ch. 4) and colicin K (Nomura, 1963). However exonucleases II and VI are activated by all four deoxynucleoside triphosphates (Richardson et al., 1964; Klett et al., 1968). An interesting experiment would be to investigate the effect of colicin E_2 on the Cairns mutant or DNA polymerase mutants that were defective in nuclease activity (Kornberg, 1969 and personal communication 1970).

FIG. 5 xvii





The results with phleomycin and mitomycin (Ch. 4) suggest that exonucleases I, II and VI are not involved in colicin induced DNA breakdown but this is entirely circumstantial.

The exonuclease involved in stage III could resemble exonuclease IV (Jorgensen and Koerner, 1966) which is known to degrade cold acid-insoluble oligonucleotides rapidly to mononucleotides whilst being relatively inactive on native or denatured DNA.

The work of Obinata and Mizuno (1970) has shown that there is no activation of known deoxyribonucleases or change in subcellular locations of these enzymes during colicin E_2 induced DNA degradation. There was even no significant increase in endonuclease activity in crude extracts of colicin E_2 treated E. coli 1100 (i.e. endonuclease I deficient). Colicin E_2 must therefore either alter the state of DNA, so that it is rendered susceptible to a potentially active nuclease system, or interfere with the delicately balanced cellular control mechanisms, so that nucleases normally active in E. coli, but only used under strict control in replication (exonucleases II and IV), recombination (endonucleases II and IV), repair (endonuclease II and an exonuclease) and restriction (endonuclease III), see Fig. 5 xvii, become uncontrolled and rapidly destroy the bacterial chromosome.

The results described with isopycnic centrifugation suggest that DNA was not denatured or damaged in a way similar to that caused by UV irradiation. The specific binding of DNA to membrane was therefore investigated in Ch. 6 as a further clue to the mode of action of colicin E_2 regarding an alteration in the state of DNA.

CHAPTER 6

Effect of colicin E_2-P9 on the association of DNA and the bacterial membrane

INTRODUCTION

In 1963 Jacob, Brenner and Cuzin postulated a functional association of the bacterial chromosome with the cytoplasmic membrane and this has now been substantiated in *E. coli* and *Bacillus subtilis* (Ganesan and Lederberg, 1965; Jacob, *et al.*, 1966; Smith and Hanawalt, 1967; Tremblay *et al.*, 1969; Knippers and Strätling, 1970. It therefore seemed logical to investigate the effect of colicin E_2 on the association of its biochemical target (DNA) with the cytoplasmic membrane. It could be hypothesised that colicin induced DNA degradation occurred as a result of an alteration in DNA/membrane association.

The methods employed for investigating DNA/membrane complexes have largely involved the separation of membrane attached DNA from bulk DNA by sucrose density-gradient centrifugation. The membrane associated DNA sediments faster and is collected either in the form of a pellet at the bottom of the centrifuge tube (Ganesan and Lederberg, 1965) or on a 60% (w/v) sucrose (Smith and Hanawalt, 1967) or CsCl (Knippers and Strätling, 1970) cushion which selectively collects membrane fractions at the interface between the cushion and the less dense linear gradient, but allows whole cells, spheroplasts and large cell fragments to sediment through and pellet at the bottom of the tube. Using these methods Ganesan and Lederberg (1965) and Smith and Hanawalt (1967) isolated cell membrane fractions associated with portions of DNA rich in newly synthesised regions of the molecule

The association of uniformly labelled DNA with the membrane fractions is dependent on treatment of cell lysates before centrifugation by vortex shearing or with pronase, (Smith and Hanawalt, 1967). Pronase is found to completely dissociate any DNA/membrane complex from B. subtilis (Ganesan and Lederberg, 1965) but has only a slight effect with E. coli (Smith and Hanawalt, 1967) except to "clean up" gradient profiles and to enrich the membrane fraction with newly synthesised DNA. Pronase also appears to render the DNA/membrane complex from E. coli more susceptible to dissociation by vortex shearing. It is important to emphasise here the difference between DNA that is specifically attached to membrane (presumably at the site of replication) and that which is non-specifically associated with a variety of membrane fractions and vesicles.

Tremblay et al., (1969) have used an entirely different method of fractionation involving the formation of magnesium sarkosyl crystal complexes with certain membrane fractions and their separation on sucrose density-gradient to give "M bands". By itself DNA will not complex with the crystals but will when in association with membrane. Using spheroplasts of B. megaterium and lysing them in the presence of sarkosyl, Tremblay et al., (1969) found that over 90% of the cell DNA was associated with the Mg-sarkosyl crystal fraction but only 10-30% of the cell membrane. It was shown that this 10-30% of the cell membrane was enriched with newly synthesised phospholipid and that DNA was associated with this portion of the membrane consistent with postulated association of the site of replication and cell division, (Jacob et al., 1963; Eberle and Lark, 1966).

The experiments described in this chapter examine the effect of colicin E_2 on the association of pulse and uniformly labelled DNA with cell membrane fractions using the two methods described above.

METHODS 6

(a) Labelling of cell DNA and treatment with colicin E_2-P9

The DNA of exponentially growing cultures of E. coli ROW was uniformly labelled with (methyl-^{3}H) thymine at 5-10 µCi/ml (specific activity 1 Ci/mmol) or (2-^{14}C) thymidine at 0.1 µCi/ml (specific activity 5.6 mCi/mmol) as described previously. For pulse labelling of DNA, (methyl-^{3}H) thymine at 10-20 µCi/ml (specific activity 13.75 Ci/mmol) was used. In some experiments double labelling techniques were used by first uniformly labelling cell DNA with (2-^{14}C) thymidine for 3-4 generations until the cell density was 3 x 10^8 bacteria per ml. The (^{14}C) labelled bacteria were harvested by centrifugation, washed and incubated with fresh medium containing 200 µg deoxyadenosine per ml but no thymidine for 10-15 min. The cells were then pulsed for 30-100 sec with the high specific activity (methyl-^{3}H) thymine. After labelling, the culture was diluted tenfold with fresh medium containing 100 µg unlabelled thymine per ml and 10 ng colicin E_2 per ml so that the cell density was 3 x 10^7 bacteria per ml. Under these conditions incorporation of high specific activity label into DNA continued for 1-2 min at a reduced rate in the control after dilution whereas the colicin incubated cells ceased incorporating label at a reduced rate approx. 30 sec after dilution (see Britten et al., 1964). 10 ml aliquots were taken at intervals and diluted with an equal volumes of ice-cold NET buffer containing 10 mM KCN (NET-CN). The cells were washed, resuspended in 1 ml NET-CN containing 30% (w/v) sucrose

and 1 mg lysozyme per ml and incubated for 15-30 min at 37° in order to form spheroplasts. Occasionally cells that had been incubated with colicin E_2 for longer than 30 min needed up to 1h lysozyme treatment for complete conversion to spheroplasts. The spheroplast suspensions were lysed by dialysis at 4° (Hanawalt and Ray, 1964) against successive changes of (i) NET/10 buffer pH 8.0 for the sucrose cushion method of Smith and Hanawalt (1967) or (ii) TMK buffer pH 7.0 (10 mM tris-HCl; 10 mM magnesium acetate; 100 mM KCl) for the "M band" method of Tremblay et al., (1969).

(b) Fractionation of DNA/membrane complexes using the sucrose cushion method

The cell lysates were treated with pronase and/or sheared as required (see text). Further dialysis against NET/10 buffer after treatment as described by Smith and Hanawalt (1967) was found to be unnecessary and aggregation of the DNA at higher salt concentrations (i.e. NET buffer) was minimal (see Studier, 1965).

Pronase (Calbiochem B grade) was prepared at 4 mg/ml in NET/10 buffer and used at 100 µg/ml at 37° for 15 min. Mild shearing of the cell lysate was achieved by vortex mixing in a 15 ml centrifuge tube on a Whirlimixer (Fison's, Loughborough UK.) for 30 sec.

The sucrose gradients were made up on NET/10 buffer and consisted of either 22 ml or 4.0 ml 5-20% (w/v) linear sucrose density-gradient on top of a 5 ml or 0.8 ml 62% (w/v) sucrose "cushion". 1.0 ml or 0.2 ml treated cell-lysate was layered on to the appropriate gradient and centrifuged in a SW-25 or SW-50 rotor on a Spinco L2-HV ultracentrifuge at 5-10° (see text). The tube contents were fractionated and the cold-acid precipitable radioactivity in the samples determined.

TABLE 6 i

Factionation of (^{3}H) thymine labelled DNA

Cell incubation	Lysate treatment	% radioactivity associated with membrane fraction	
		Uniform label	Pulse label
-	None	80-100	100
-	Pronase	40-50	100
+	"	41	24
-	Pronase+0.1% SDS	16	77
+	"	6	22
-	Pronase+0.5% SDS	0	1
-	Shear (vortex)	10	89
+	"	18	78
-	Pronase and shear	6	10
+	"	5	6
-	Shear and pronase	10	53
+	"	6	15

- control cells

+ cells incubated with colicin E_2 for 1h.

(c) Fractionation of DNA/membrane complexes using the "M band" method

After dialysis against TMK buffer, the lysate was treated with pronase (in TMK buffer) as described above and a 0.2 ml sample layered on to 0.1 ml 0.3% (w/v) sarkosyl detergent (sodium lauroyl sarcosinate NL) on top of a 4.8 ml 15-40% (w/v) linear sucrose density-gradient (prepared in TMK buffer). The lysate was gently stirred in with the detergent (final concentration 0.1% (w/v)) and crystals were seen to form. Occasionally preformed Mg-sarkosyl crystals were used and the results were unchanged. After formation of the Mg-sarkosyl membrane complex, the gradients were centrifuged for 30 min at 20° in a SW50 rotor at 20,000 rev/min. Centrifugation was almost to equilibrium since the bouyant density of the crystal complexes (corresponded to about 30% (w/v) sucrose. After deceleration, there was a thin white band half way down the tube. The gradients were fractionated into 30-31 x 8 drop samples using a peristaltic pump (80 ml/h) attached to a needle which pierced the bottom of the tube. This was necessary because of the viscosity of the M band and recovery of cold acid-insoluble radioactivity was only 40-50% of the layered lysate. However the amount of cells per gradient (60-100 µg dry weight) was considerably less than that used by Tremblay et al., (1969).

RESULTS 6

(a) Fractionation of DNA/membrane complexes from cells incubated with colicin E_2-P9 using the sucrose cushion method

Before studying the effect of colicin E_2 on the association of DNA and the bacterial membrane, various control systems were investigated to determine the pretreatment of cell lysates needed to obtain a satisfactory distribution between the DNA/membrane fractions and the unassociated cell DNA.

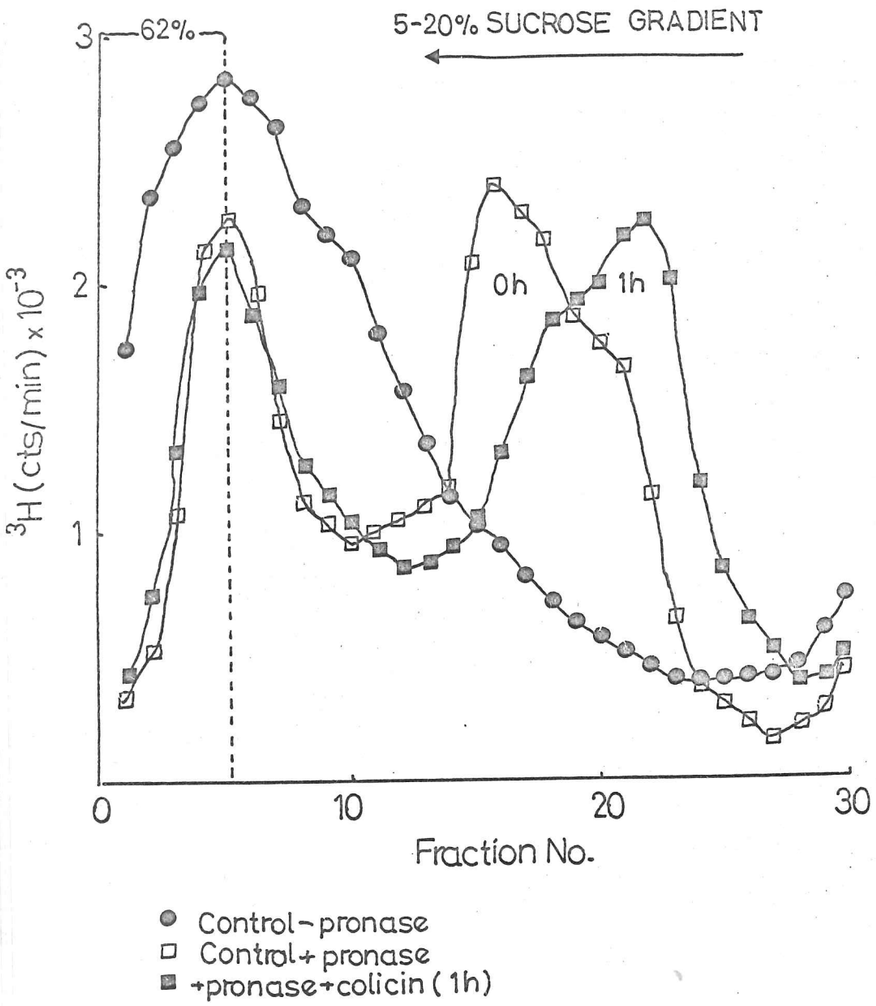


FIG. 6 i Effect of colicin E2 on the fractionation of uniformly labelled DNA after pronase treatment of the cell lysate.

(^{3}H) thymine labelled cells were incubated with colicin E2 for 0 h □——□ and 1 h ■——■ and the spheroplasts lysed by the dialysis method. The lysates were treated with pronase (except a control sample ●——●) and centrifuged through neutral sucrose gradients (containing sucrose cushions - indicated by the dashed line above) in a SW50 rotor for 2h at 40,000 rev/min and 5°. The tube contents were fractionated and the cold-acid precipitable radioactivity determined. Approx. twice the amount of lysate from the colicin treated cells as the control lysates was used .

If the cell lysate was centrifuged directly after dialysis, prelabelled bulk DNA was found in nonspecific aggregates with other cell fractions. DNA was distributed throughout the sucrose cushion region and tailed away into the linear gradient without the appearance of any distinct peaks (Fig. 6 i). Pronase pretreatment however separated the two types of DNA so that about 40-50% (Table 6 i) appeared as a distinct peak at the sucrose cushion interface, Fig. 6 i. This membrane associated DNA was susceptible to detergent treatment, so that 0.1% SDS released approx. 60% of the membrane bound DNA whilst 0.5% SDS caused total release (Fig. 6 ii). Pulse labelled DNA sedimented entirely with the membrane fractions after pronase treatment and was more resistant to release by 0.1% SDS (Table 6 i). Shearing alone reduced the membrane associated bulk labelled DNA to approx. 10% (Table 6 i) and increased the molecular inhomogeneity of the unassociated DNA with a consequent increase in peak width (Fig. 6 v). Pulse labelled DNA was relatively unaffected in its association with membrane by shearing but longer vortex treatment (i.e. greater than 2 min) reduced this to approx. 30%.

The result of combining the two treatments depended on the order in which they were given (Table 6 i). If pronase was added before shearing the association of pulse labelled DNA with membrane was considerably labilised, so that only 10% remained, after shearing, at the cushion interface. Bulk labelled DNA at the interface was reduced to 6%. Pronase digestion after shearing the lysate had virtually the same effect as shearing alone, except that pulse labelled DNA associated with membrane was reduced by approx. 40%.

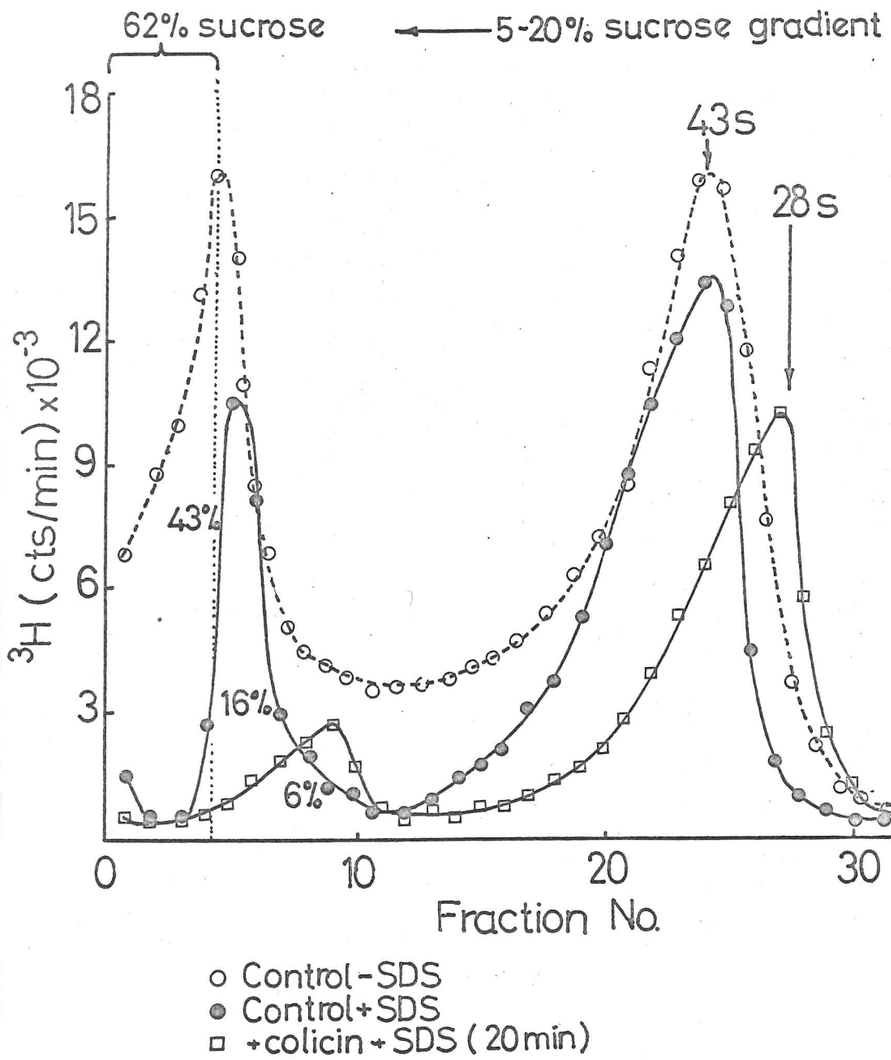


FIG. 6 ii

(^{3}H) thymine labelled cells were incubated with colicin E2 for 0min ●–● and 20min □–□ and the spheroplasts lysed by the dialysis method. The lysates were treated with pronase followed by 0.1% SDS (except a control sample which was only treated with pronase) and centrifuged for 4h in a SW25 rotor at 25,000 rev/min and 10^5. The percentage of the total cold-acid precipitable radioactivity found at the cushion interface is indicated under the appropriate peak. The S values were calculated from the equation of Nomura et al.(1962).

The first system used for studying the effect of colicin E_2 involved pronase treatment of the dialysed cell lysate. Bulk labelled DNA however showed virtually no difference from the control on incubation of cells with colicin for up to 90 min (Fig. 6 i). This negative result eliminated the possibility that bulk labelled DNA would become dissociated from the membrane because of a reduction in molecular size under these conditions, since after 30 min incubation with colicin E_2 the DNA fragment size after lysis was considerably reduced (Fig. 5 viii). Vortex mixing must therefore cause a reduction in membrane associated DNA by shearing the DNA off the membrane rather than just fragmenting the DNA molecules.

If however the dialysed lysate from colicin treated cells was subjected to mild detergent treatment (0.1% SDS) after pronase digestion the amount of uniformly labelled DNA remaining attached to membrane was reduced compared with the control (Fig. 6 ii). This labilisation only occurred after approx. 20 min incubation of cells with colicin E_2 and the small percentage of total DNA left associated with the membrane remained constant (5-8%) after 30 min incubation with colicin. It was also observed that under the experimental conditions used, the 0.1% SDS-resistant DNA/membrane complexes sedimented more slowly after incubation of cells with colicin, indicating a reduction of membrane fragment size (Fig. 6 ii).

The observations that cells did not readily form spheroplasts after incubation with colicin E_2 and that the spheroplasts once formed were more osmotically stable compared with control spheroplasts (Ch. 5 and Nose et al., 1970) led to an additional investigation into the effect of detergent lysis methods on DNA fractionaction. Cells incubated with colicin E_2 were lysed using the method described in Ch. 5, except that after EDTA-lysozyme treatment, the

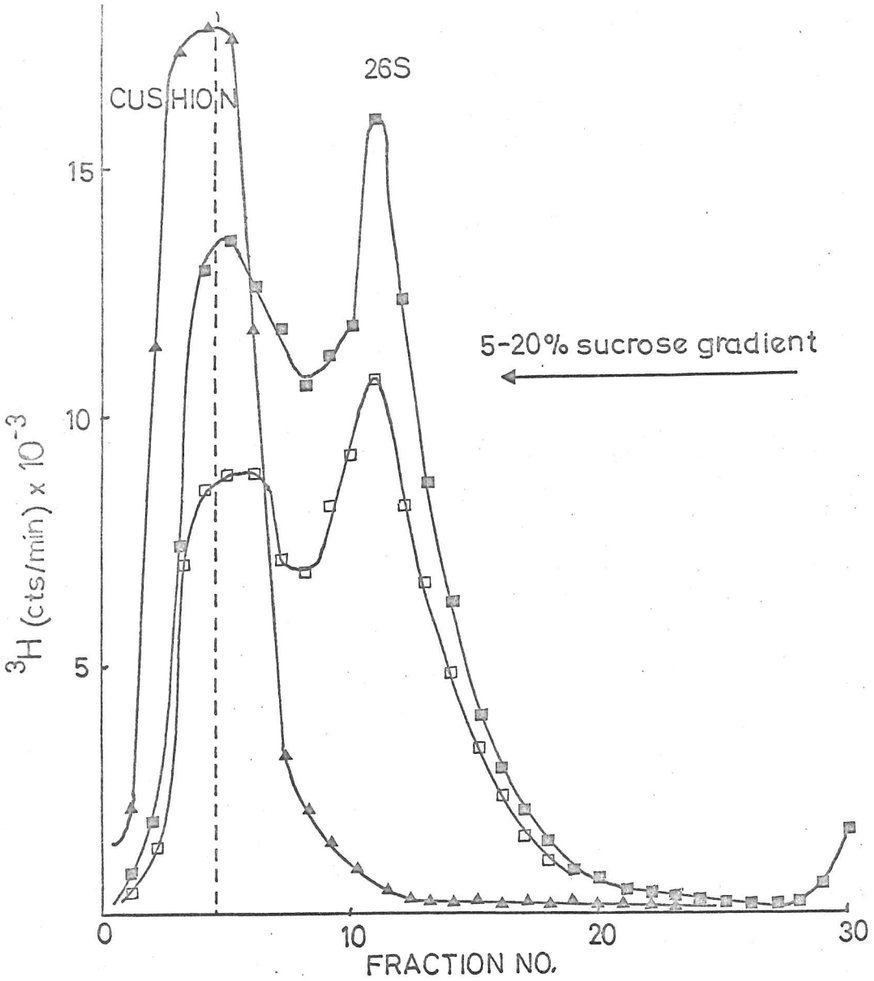


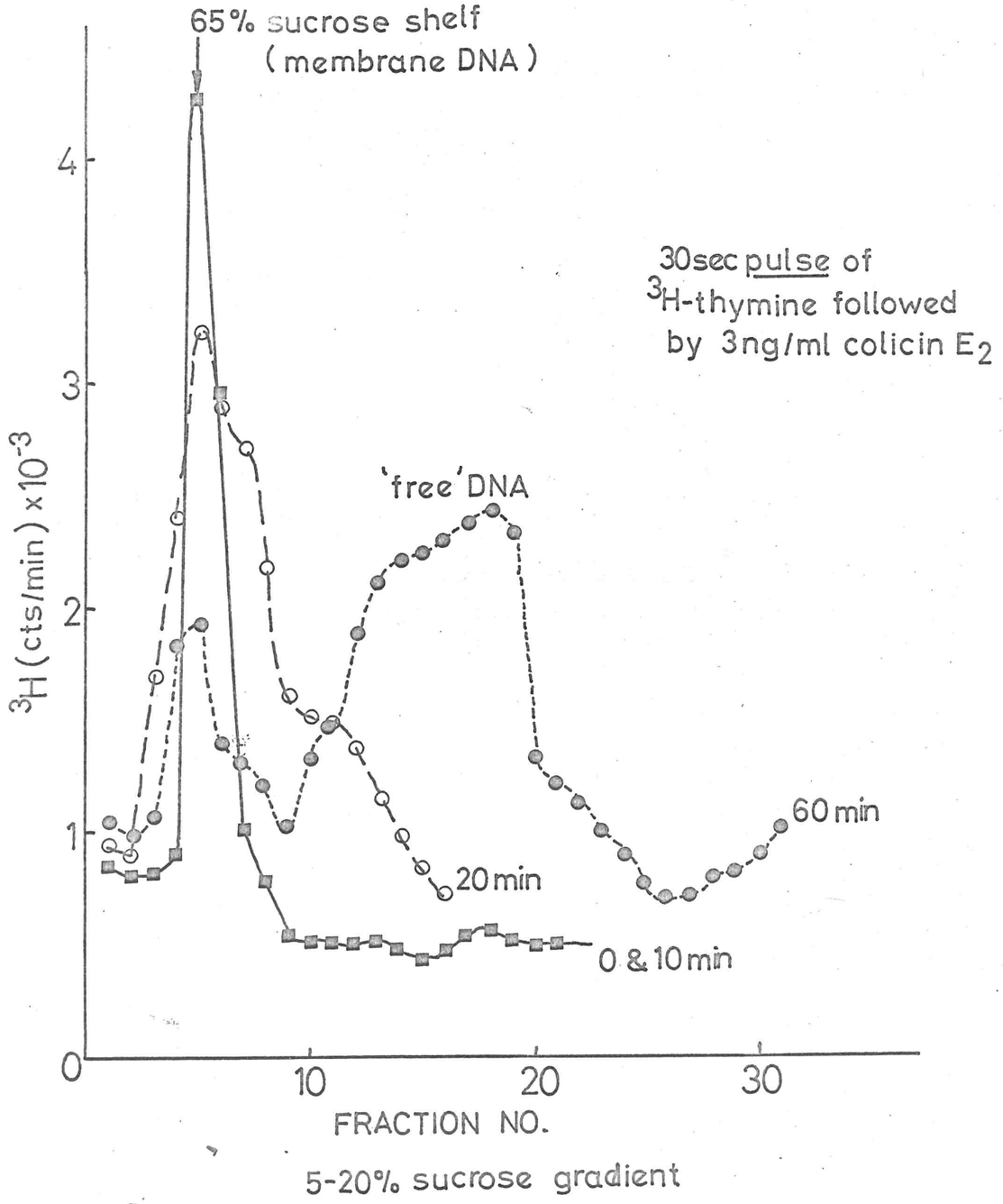
FIG. 6 iii

Effect of mild detergent lysis on the fractionation of uniformly labelled DNA from cells treated with colicin E2.

(^{3}H) thymine labelled cells were treated with colicin E2 for 0min ▲——▲ ; 30min ■——■ ; and 60 min □—□ and lysed as described in METHODS 5 except that after EDTA-lysozyme treatment the 'cells' were'titrated' with SDS to effect lysis (final concn. 0.05-0.1% (w/v) SDS) and pronase was omitted. The lysate was centrifuged for 16h in a SW25 rotor at 25,000 rev/min. Only about half the control radioactivity is shown.

FIG. 6 iv

Effect of colicin E2 on the fractionation of pulse labelled DNA after pronase treatment of the cell lysate.



(^{3}H) thymine pulsed cells were incubated with colicin E2 for the times indicated and the spheroplasts lysed by the dialysis method . The lysates were treated with pronase and centrifuged on the above gradient for 2h in a SW50 rotor at 40,000 rev/min. (Only one third of the radioactivity from the 0 & 10min cells is shown.)

cells were titrated with SDS, and pronase was not included. The lysates were analysed by the sucrose cushion method of centrifugation and the distribution of uniformly labelled DNA is shown in Fig. 6 iii. The control DNA formed a sharply defined peak at the cushion interface after long centrifugation. However after 30 min incubation with colicin a second peak was observed which had a sedimentation value between 20 and 30S (see Ch. 5). The percentage DNA released from membrane remained at approx. 50% but after 60 min incubation with colicin the DNA/membrane fragments sedimented slightly above the cushion (Fig. 6 iii). These observations confirmed the experiments described in Fig. 6 ii.

So far the experiments with colicin E_2 were carried out using uniformly labelled DNA, however by pulse labelling DNA it is possible to study the effects of colicin E_2 on the newly synthesised region of the bacterial chromosome. There are advantages in that it can be assumed that this region of the DNA will be more specifically associated with the membrane. The results obtained using pulse labelled bacteria and the dialysis method of lysis were quite different from those with steady state label (Fig. 6 iv and Table 6 i).

If after colicin incubation and cell lysis, pronase was added, it was found that the attachment of pulse labelled DNA to the membrane was labilised. Similar to the 0.1% SDS effect for uniformly labelled DNA, this labilisation effect was not observed until approx. 20 min after addition of colicin E_2 to the pulse labelled cells, when a broadening of the DNA peak at the cushion interface occurred. Eventually a distinct second peak was observed above the cushion after longer colicin incubation times (Fig. 6 iv), but this peak sedimented slightly faster than unassociated DNA from uniformly labelled cells under similar conditions.

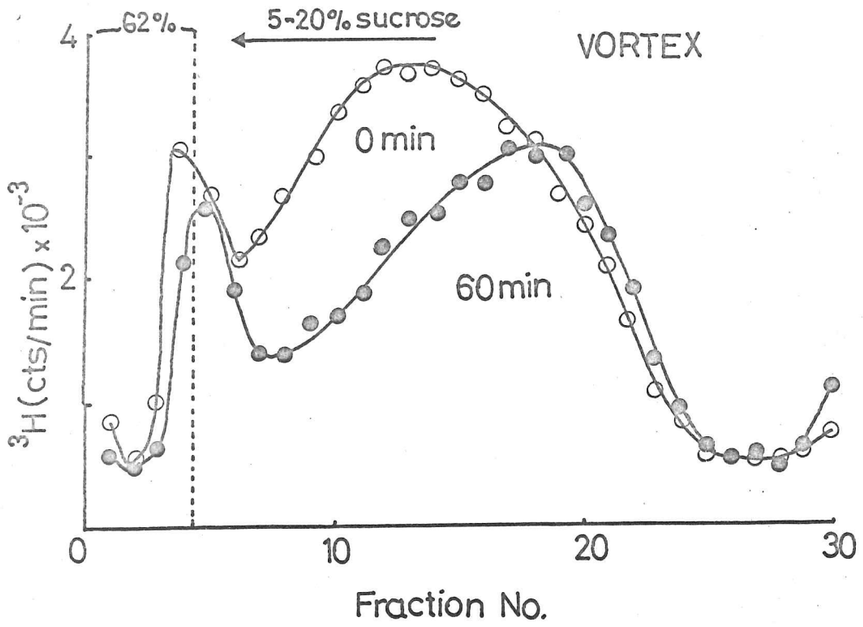


FIG. 6 v

Effect of shearing on the fractionation of uniformly labelled DNA from cells treated with colicin E2.

(^{3}H) thymine labelled cells were treated with colicin E2 for the times indicated and the spheroplasts lysed by the dialysis method. The lysates were vortex sheared for 30 sec and centrifuged for 3h in a SW50 rotor at 40,000 rev/min.

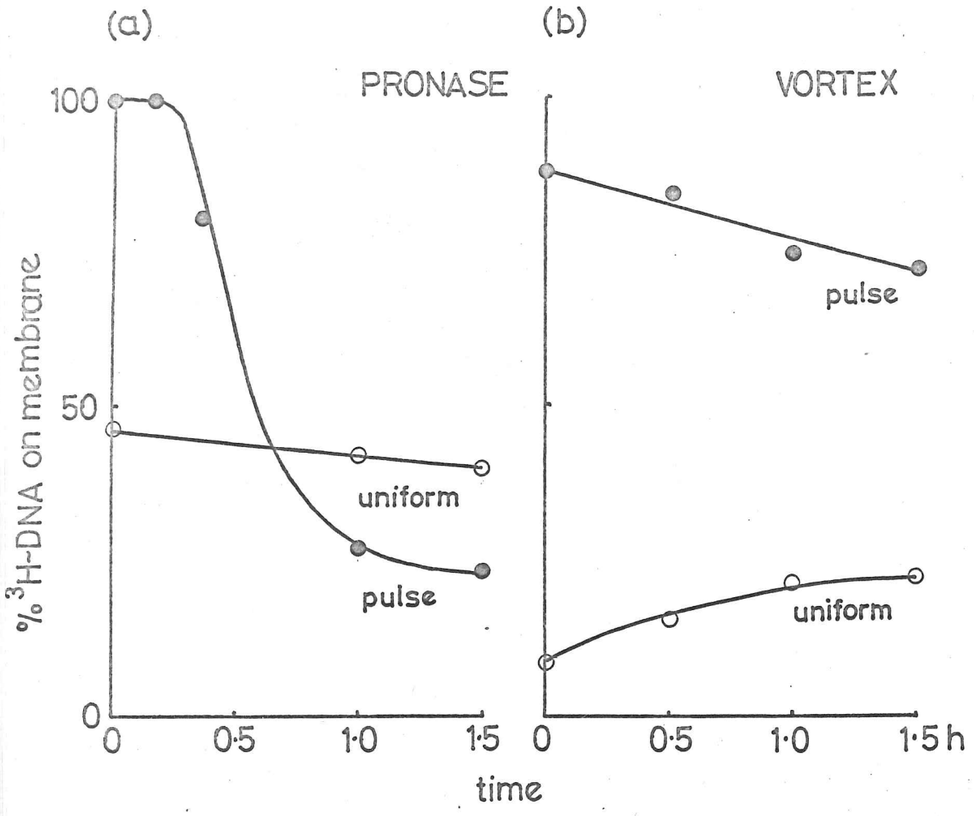


FIG. 6 vi

Effects of pronase and shearing on the percentage of pulse and uniformly labelled DNA associated with the membrane fraction of colicin E2 treated cells.

The percentage pulse or uniformly labelled DNA sedimenting at the sucrose cushion interface as described in FIGS. 6 i-v was determined after cells had been incubated with colicin E2 (3.10^3 molecules per bacterium) for the times indicated and the lysates subjected to pronase or vortex treatment.

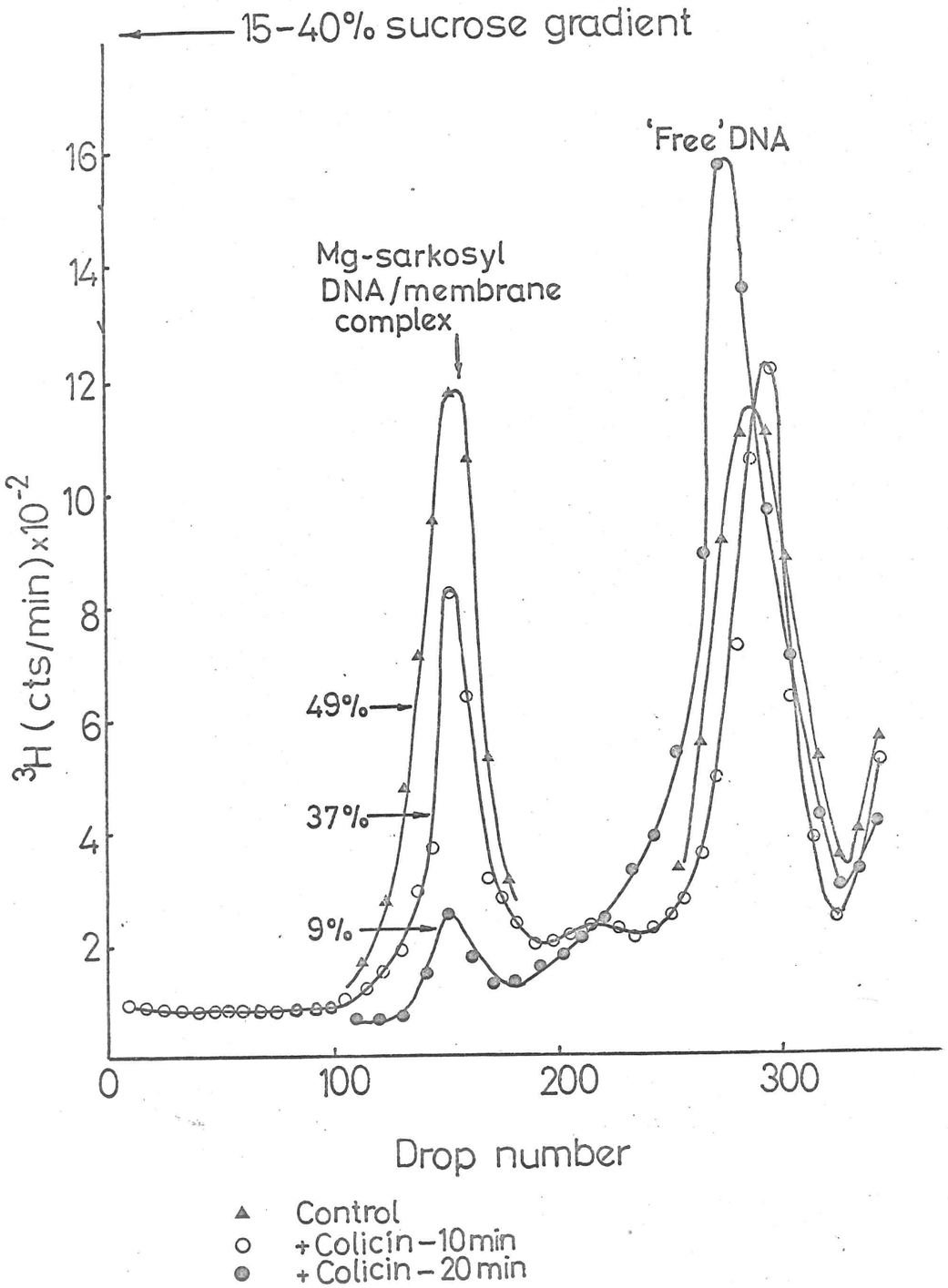
The decrease in percentage pulse labelled DNA associated with membrane on treatment of cells with colicin E_2 is shown in Fig. 6 vi(a). A residual 20-30% of the pulse labelled DNA remained attached to the membrane after colicin attack but again this dissociation of DNA from membrane appeared to be a secondary effect of colicin E_2 action, (see discussion).

If shearing of the lysates from colicin treated bacteria was substituted for pronase treatment, pulse labelled DNA remained associated with membrane (Table 6 i), however the percentage bulk labelled DNA associated with membrane after shearing increased with colicin E_2 incubation suggesting a partial protection of membrane associated DNA against colicin E_2 induced exonuclease attack, (Figs. 6 v and 6 vi(b); Table 6 i). If however pronase digestion was included in the lysate treatment after shearing then there was a slight decrease in membrane associated DNA after colicin attack (Table 6 i). This was probably the result of newly synthesised DNA being more readily detached from the membrane as a result of colicin attack owing to pronase labilisation of the attachment point. It therefore appeared that newly synthesised DNA was specifically attached to a part of the membrane instead of non-specifically associated with the total membrane fraction and was partially protected against colicin induced degradation to cold-acid soluble fragments. This hypothesis was tested more fully in section (c).

Pronase digestion before vortex shearing resulted in almost total depletion of bulk and pulse labelled DNA from the membrane fraction and colicin had no detectable effect on this (Table 6 i).

FIG. 6 vii

Effect of colicin E2 on the resolution of M band-DNA material



Cells were uniformly labelled with (^{3}H)thymine and incubated with colicin E2 for 0min ▲–▲ ; 10min O–O ; and 20min ●–● . The cell lysates were treated with pronase and mixed with sarkosyl on the top of the above gradient. Centrifugation was for 30min at 20,000 rev/min in a SW50 rotor.

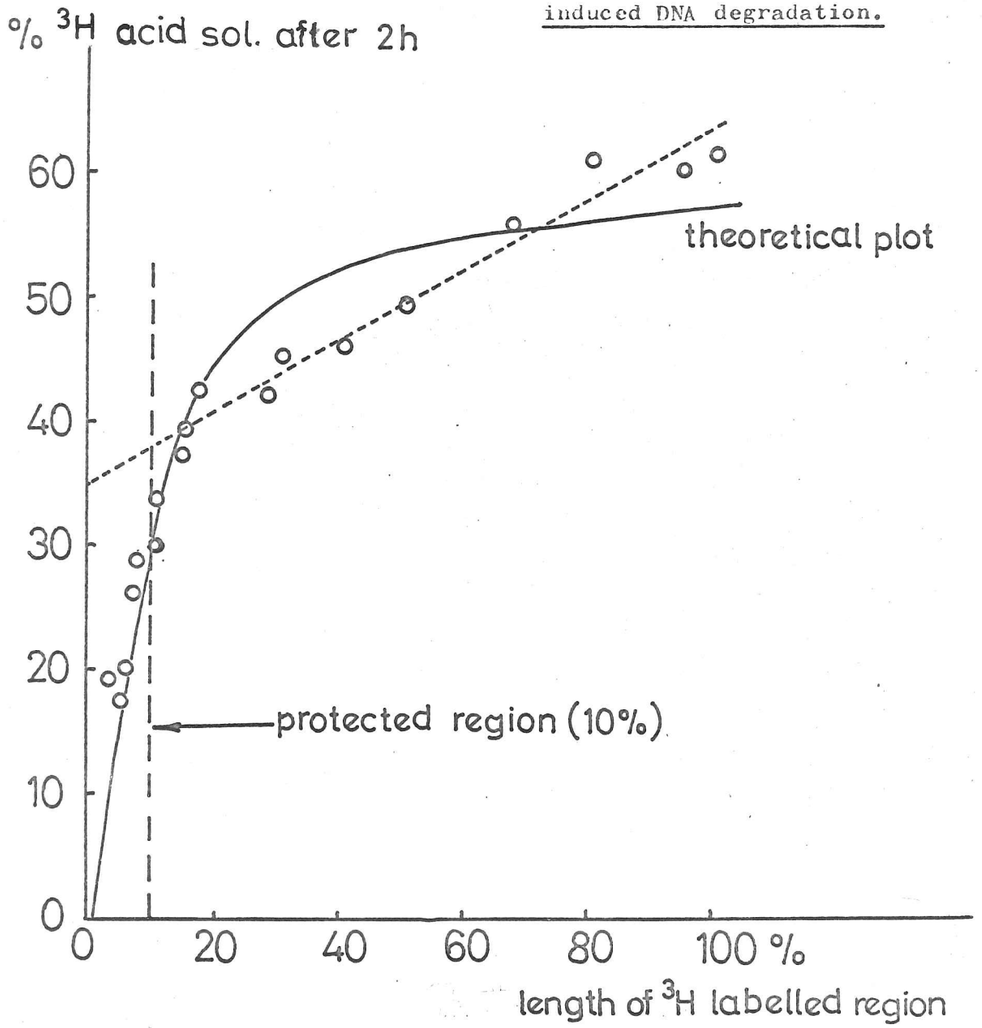
(b) Fractionation of DNA/membrane complexes from cells incubated with colicin E_2-P9 using the "M band" method

After investigating the effect of colicin E_2 on DNA/membrane association using the sucrose cushion method of Smith and Hanawalt (1967) it was decided to use the method developed by Tremblay et al., (1969) which relies on separation of membrane associated DNA from unassociated DNA by hydrophobic interaction of membrane phospholipid with the waxy magnesium sarkosyl crystals and not faster sedimentation of membrane fragments compared with unassociated DNA.

The sedimentation profiles of pronase treated dialysed lysates are shown in Fig. 6 vii. Recovery of cold-acid precipitable radioactivity was low owing to the viscosity of the M band and apparent sticking to the tube walls. The bulk labelled DNA present in the M band was approx. 50% of the total DNA in the control. This was much lower than that reported by Tremblay et al., (1969), probably due to the fact that the E. coli spheroplasts were osmotically lysed before pipetting on to the gradient and hence the DNA was subjected to a certain amount of shear. This distribution was obtained both when the lysate was mixed with preformed Mg-sarkosyl crystals and when mixed with free sarkosyl detergent which then formed crystals due to the Mg^{++} in the lysate buffer. Lysis of spheroplasts directly on the gradient (without pronase) using free sarkosyl detergent was unsatisfactory and produced smeared profiles. After colicin treatment little change was observed during the first 5 min. The percentage of complexed DNA approximated to the control values of 44-56%. After 10 min colicin treatment this percentage of complexed DNA began to decrease and by 20 min had dropped to a minimum of 6-10% which then remained constant with longer incubation times. These results may be related to those obtained by Nose et al., (1970) where the

FIG. 6 viii

Effect of DNA labelling time on the apparent colicin E2 induced DNA degradation.



The percentage cold-acid precipitable radioactivity lost after 2h incubation of cells with colicin E2 (3.10^3 molecules per bacterium) is plotted against the length of the labelled region in the DNA ie. the duration of the labelling time expressed as as a percentage of the cell doubling time (40min) . The empirical points are compared with the theoretical plot which assumes a 10% protected region of the chromosome (see text).

irreversible change in the cell membrane induced by colicin E_2 occurred after 5-10 min incubation. Interpretation of this result however was not unambiguous (see discussion).

(c) Effect of colicin E_2-P9 on newly synthesised DNA

Similar experiments to those described in section (a) were performed with DNA that had been uniformly labelled with (^{14}C) and pulsed with (^{3}H). It was shown that the (^{3}H) labelled DNA was more resistant than the (^{14}C) labelled DNA to degradation as measured by a decrease in cold-acid precipitable radioactivity, (Table 6 ii). The effect of increasing the duration of the labelling period with (methyl-^{3}H) thymine on the apparent percentage DNA degradation induced by colicin E_2 is shown in Fig. 6 viii. It was shown that all pulse label did in fact go into DNA and it is clear from these results that the exonuclease attack on the DNA fragments produced in stages I and II of colicin E_2 attack (Ch. 5) is not random since a random mechanism would predict that the apparent degree of DNA degradation would be independent of the fraction of DNA fragments labelled. These results also eliminate any mechanism similar to that proposed by Farmer (1968) for actinomycin D initiated degradation of DNA, where it was found that as the length of pulse time increased the degradation of radioactive DNA into cold-acid soluble material decreased.

The simplest interpretation of Fig. 6 viii was that the most recently labelled DNA, i.e. that attached more specifically to the membrane, was resistant to colicin E_2 induced exonuclease attack. The increase in the percentage membrane bound DNA after shearing lysates from uniformly labelled cells incubated with colicin E_2 (Fig. 6 vi(b)) is consistent with this interpretation.

Table 6 ii

Protection of newly synthesised DNA from colicin E_2 induced degradation

	Ratio of radioactivity ((^3H) pulse/ (^{14}C) uniform)			
Duration of (^3H) label	30 sec	100 sec	5 min	20 min *
Control	0.13	0.47	1.52	0.53
+ Colicin E_2 incub.				
45 min	0.21	0.72	1.78	0.57
90 min	0.27	0.89	2.15	0.63

* Cells pulsed with lower activity (^3H) thymine

TABLE 6 iii

Effect of PEA and amino-acid starvation on DNA-membrane association

	% uniformly labelled DNA associated with membrane (after pronase treatment)
Control	44
0.5% PEA (90 min)	36
- amino-acids (90 min)	46

If this is the explanation, then it should be possible to calculate the results that one would expect if (i) 10% of the total cell DNA was specifically attached to the membrane at the replication site; (ii) this region of DNA remained attached to the membrane after colicin induced fragmentation and was subjected to a gradual increase in probability of being degraded into acid soluble material from total protection at the point of replication to 60% degradation at the end of the membrane attached region, i.e. the overall average degradation of this protected region would therefore be 30%, and (iii) the rest of the cellular DNA which was not protected by any specific membrane association was degraded by 60%.

Therefore if 20% of the cell DNA is labelled and assuming one replication point, the first 10% will be degraded by 30% whilst the last 10% will be degraded by 60%. This gives an overall degradation of 45%. The theoretical curve thus obtained (Fig. 6 viii) shows a basic similarity with the experimental points but a true theoretical curve should take account of there probably being more than one replication point. Since the culture was not synchronous a fraction of the cells would divide during the pulse time and hence some label would occur in parental DNA strands. However this should not affect the above results since it is assumed from Ch. 5 that parental and daughter DNA strands are degraded equally once a particular fragment has been selected by the exonuclease. Attempts at cell synchrony by amino acid starvation were unsuccessful (see Lark, 1966).

A simple test of the hypothesis would be to pulse a short region of the bacterial DNA and chase this with unlabelled thymine for varying times before incubation with colicin. The hypothesis would predict that as the pulsed DNA moved out from the protected

region it would become immediately degraded at the maximum rate of 60%. However pulse/chase experiments with E. coli are notorious in that the internal nucleotide pool does not equilibrate rapidly with exogenous precursor (see Britten et al., 1964). A further complication was that the pulsed DNA would move out from one protected region and into another if there were multiple replication points per chromosome (see BU incorporation results, Ch. 4 and Caro, 1970). This apparently simple experiment was therefore unsuccessful.

DISCUSSION

The evidence presented in previous chapters and by Obinata and Mizuno (1970) suggests that colicin E_2 may work by initiating an alteration in the state of DNA. As colicin E_2 is known to bind specifically to or near to the bacterial inner membrane, a possible point of attack could be the site(s) at which the DNA is associated with the membrane.

The results from the experiments just described on the isolation of membrane associated DNA are difficult to interpret because of distinguishing between first the specific binding of DNA to a small region of membrane at the replication point and the non-specific random association of DNA with membrane subfractions and vesicles due to isolation techniques; and second the effect of colicin specifically acting on the site of chromosome attachment to the membrane and the effect of DNA fragmentation on the apparent degree of DNA associated with membrane fractions.

The membrane association of pulse labelled DNA may be taken as an approximate indication as to the specific attachment and this was shown to be relatively resistant to pronase, shearing and mild detergent treatment in E. coli ROW. However pronase treat-

ment considerably labilised the apparent attachment of the pulse labelled region to membrane when cell lysates were subsequently subjected to vortex treatment. The rapid decrease in membrane association of pulse labelled DNA after 10 min incubation of cells with colicin E_2 (Fig. 6 vi(a)) shows a distinct resemblance to the appearance of double strand snips in DNA described in Ch. 5 (Fig. 5 viii). Since there is probably only one attachment site on the membrane associated with replication of the chromosome and the actual size of the residual DNA stub left after sonication is less than 1% of the total chromosome (Dr. M. Daniels, 1970; personal communication) the effect of colicin on membrane association of pulse labelled DNA may result entirely from fragmentation of the DNA. However the length of the pulsed region used in the above experiments was in the order of 1% of the chromosome as estimated from the pulse time expressed as a percentage of the cell doubling time. It could therefore also be argued that incubation of the cells with colicin labilised the DNA/membrane attachment point to pronase treatment in contrast to vortex treatment of the cell lysate before incubation with pronase. The similarity in kinetics of double strand snipping and DNA/membrane dissociation may be due to their both being the result of an initial as yet unknown phenomenon responsible for the initiation of colicin induced DNA degradation rather than the latter being the result of the former. The results of Nose et al., (1970) on the time course of bacterial spheroplast membrane stabilisation by colicin E_2 would support this hypothesis.

The results on the apparent protection of newly synthesised DNA against colicin induced exonucleolytic degradation implies that the chromosome remains attached to membrane after colicin attack unlike the proposed effect of phenethyl alcohol (Ch. 4 and Treich and Konetzka 1964). Preliminary investigations with phenethylalcohol

did not support the chromosome/membrane detachment theory however (Table 6 iii). This protection of DNA which is specifically bound to the membrane is contrary to the results of Farmer (1968) with actinomycin D induced exonucleolytic degradation of the chromosome daughter strands and Hanawalt and Ray (1964) who found that newly synthesised DNA was more susceptible to shear and denaturation. No explanation is offered at this stage except to say that colicin E_2 probably alters the state of DNA by labilising its specific attachment to membrane towards pronase but in addition the newly synthesised DNA at this site is protected against colicin induced exonuclease. This alteration in the state of DNA may be the result of some subtle membrane conformational change induced by colicin (Changeux and Thiery, 1967). This is particularly relevant to the results of Ch. 4 on phenethyl alcohol sensitization of cells to colicin action, since this membrane-active antibacterial agent has been suggested to interferewith the structural or initiator proteins at the replicon site (Lark and Lark, 1966).

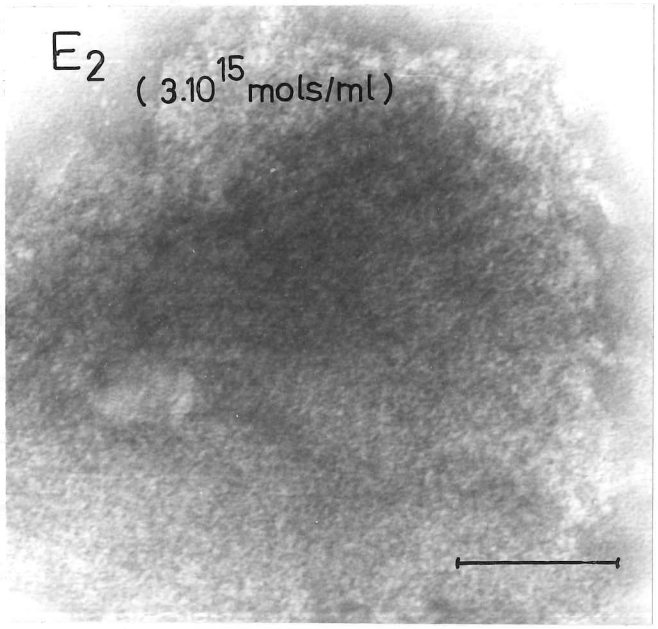
The results with uniformly labelled DNA can be largely explained from the behaviour of pulse labelled DNA and the effect of colicin E_2 on the membrane. Pronase treatment does not affect the non-specific association of DNA fragments with membrane, but this association is considerably labilised with respect to detergent irrespective of pronase pretreatment (Figs. 6 i, ii and iii). This reflects a change in the membrane consistent with the results discussed above. It is interesting however to contrast this membrane fragment labilising effect toward detergent with the stabilising colicin effect on whole spheroplast membranes reported by Nose et al., (1970) and (Ch. 5); and on whole cell membranes with respect to PEA (Ch. 4).

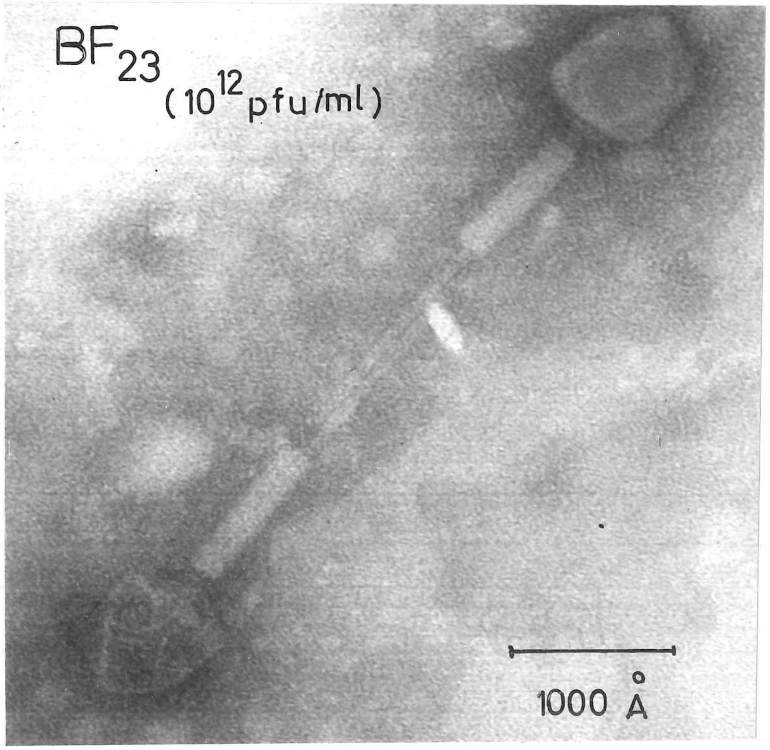
The apparent relative increase in membrane bound bulk labelled DNA after vortex treatment of the lysate from colicin treated cells is consistent with the protection phenomenon of newly synthesised DNA. Non-specific association of DNA with membrane after cell lysis appears to be very sensitive to shearing and partially sensitive to pronase treatments. Membrane bound DNA after vortex treatment therefore behaves like specifically bound pulse labelled DNA and is protected against colicin induced degradation. The relative increase in membrane associated DNA after incubation of cells with colicin is of course abolished if pronase is added to the lysate, since this causes release of the protected regions.

The results of the sarkosyl crystal experiments may be interpreted as reflecting a decreased association of bulk labelled DNA with membrane because of DNA fragmentation. The effect could also be due to a colicin induced alteration in DNA/membrane attachment or the fact that only growing membrane will complex with the detergent crystals (Tremblay et al., 1969). However the formation of filamentous cell forms with colicin E_2 would indicate that cessation of membrane synthesis was not a rapid effect.

APPENDIX

Purified colicin E_2 preparations were investigated for physical similarities to defective phage components by electron microscopy (see Bradley and Dewar, 1966). There were no observable phage-like particles and a representative picture of the amorphous appearance of colicin E_2 is shown in the plate below. Phage BF23 is shown for comparison since this phage is believed to use the same receptor sites as the E group colicins (Fredericq, 1957). The negative staining and electron micrographs were done by Dr. D. Ellar.





CONCLUSIONS

The results reported in this thesis show that the mode of action of colicin E_2 cannot be explained by any of the familiar mechanisms of direct interaction reported for other antibiotics. Colicin E_2 kills sensitive strains of E. coli by a single hit process; (one hit corresponding to approx. 100 colicin molecules per bacterium), whilst apparently remaining near the cell surface. It induces a three stage degradation of the bacterial chromosome, the lethal event being the endonucleolytic cleavage of both DNA strands (Ch. 5). The initial single strand scissions are repairable on removal of colicin activity from the cell surface by trypsin digestion.

The initiation of nuclease attack on the chromosome by colicin E_2 must be the result of a more primary biochemical interaction, since colicin E_2 possesses no nuclease activity in vitro. The primary biochemical action of colicin E_2 could involve (i) an activation or decontrol of a specific endo- exo-nuclease system or (ii) a change in state of the DNA so that the chromosome is rendered susceptible to already active nucleases.

The first possibility is in keeping with the membrane transmission system (Holland 1967.a) which proposes that colicin interferes with the delicately-balanced cellular control mechanisms at the membrane level, so that nucleases normally present in E. coli but only used under strict control in replication, recombination, repair and restriction become uncontrolled, (analogous to cancer in animal cells) and rapidly destroy the bacterial chromosome. Direct activation of nucleases was not observed by Obinata and Mizuno (1970) but a decontrol process is still very much a possibility.

The second possibility can encompass a variety of interactions of DNA with colicin E_2 so as to alienate the chromosome from its vital nuclease systems or it could involve certain colicin initiated changes in environment of DNA so that macromolecules such as membrane and protein normally necessary for the correct functioning of the chromosome are altered in their relationship with DNA. The results reported in Chs. 3 and 5 show that DNA is not denatured by colicin E_2 *in vitro* or *vivo* although direct interaction of colicin with DNA is suggested in Ch. 3, but only under non-physiological ionic conditions. These results together with the trypsin reversal effect studied in Ch. 5 suggest that colicin does not penetrate the cytoplasmic membrane. A colicin induced alteration in the specific attachment of DNA to membrane could initiate nuclease attack but the results reported in Ch. 6 suggest that this is probably only a secondary effect.

The effects of other chemical agents on colicin E_2 induced exonucleolytic degradation indicate that no new nucleases are synthesised in response to colicin attack but that the nuclease system involved is inhibited by intercalating drugs. The effects of phenethyl alcohol and novobiocin are interesting in that they both interfer with membranes and DNA metabolism and hence in some way stimulate colicin induced DNA degradation.

The primary effect of colicin E_2 appears to be on the membrane and probably involves very subtle changes that will only be satisfactorily investigated using more sophisticated techniques such as electron spin resonance (Calvin *et al.*, 1969) and the development of an *in vitro* membrane fraction system in which the effects of colicins may be more specifically localised and observed. In connection with this last possibility interesting effects have

been observed by Freer et al., (1968) and Dr. D. Ellar (1969; personal communication) on the physical appearance of membrane preparations after treatment with certain toxins and membrane active agents. The recent work of Bhattacharyya et al., (1970) on isolated membrane vesicles and colicins also presents interesting possibilities.

A complete explanation of the mechanism of colicin action may have to wait for an advance in basic methodology and the molecular biology of membranes and their associated mysterious "control circuits". The difficulties hampering research into animal cell differentiation and cancer may be related to those limiting a more fundamental investigation of colicin action. In both cases research is at present limited primarily to an investigation of effects rather than causes.

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SUMMARY

The protein antibiotic colicin E_2-P9 was isolated after mitomycin C induction from a colicinogenic strain of Escherichia coli K12 and purified using ion exchange chromatography on DEAE and CM sephadex followed by hydroxylapatite. It was characterised using polyacrylamide-gel electrophoresis, sedimentation analysis, G200 sephadex and ORD. Its primary biochemical effect was induction of DNA degradation in a sensitive strain of E. coli K12. This degradation was inhibited by dinitrophenol, bromouracil and certain intercalating drugs but stimulated by novobiocin and phenethyl alcohol. Chloramphenicol, mitomycin and phleomycin had little effect. Cell macromolecular synthesis was only affected at high colicin E_2 concentrations.

Colicin E_2 induced exonucleolytic degradation of DNA was detected after a lag of 10-15 min. Single strand scissions were formed in DNA within the first few minutes incubation of bacteria with colicin, followed by double strand breaks after about 7 min. The single strand lesions were repairable on removal of colicin from the cell surface with trypsin prior to the appearance of double strand breaks in the DNA. The production of double strand lesions was the irreversible lethal event in colicin E_2 initiated cell death. After 30 min incubation of cells with colicin, endonuclease activity ceased and the DNA fragment size had been reduced to 10^6-10^7 daltons. Using caesium chloride and sulphate isopycnic centrifugation the DNA during this time was shown not to be denatured although density inhomogeneity of the fragments was implied from the peak skewness.

Newly synthesised DNA was less sensitive to colicin induced exonucleolytic degradation but its association with the membrane fraction was labilised on incubation of cells with colicin. Uniformly labelled DNA was more readily released from the membrane after colicin attack if the cell lysate was treated with mild detergent, but this was a secondary effect of colicin action.

In vitro studies have shown that colicin E_2 has no exo- or endonucleolytic activities, however it does bind to native DNA in low ionic strength buffers as shown by complex binding to nitrocellulose filters, DNA melting curves and ORD measurements. Colicin E_2 also lowered the DNA melting temperature under certain conditions, whilst its own thermal denaturation was prevented by DNA. ORD measurements and isopycnic centrifugation indicated that colicin E_2 does not denature DNA in vitro. Studies with in vitro colicin treated DNA as a substrate for DNase and RNA polymerase have shown a slight stimulation and inhibition respectively.