

GENETICAL AND PHYSIOLOGICAL STUDIES IN  
ESCHERICHIA COLI K-12 USING CLOROBIOCIN,  
AN INHIBITOR OF DNA GYRASE

Thesis submitted for the degree of Doctor  
of Philosophy at the University of Leicester

by

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TO MY MOTHER AND FATHER



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## Chapter 1

### INTRODUCTION

This thesis is concerned with the effects of the antibiotic clorobiocin on physiological processes which occur in Escherichia coli K-12. Clorobiocin inhibits primarily DNA synthesis, apparently due to its effect on the enzyme DNA gyrase. The first part of this Introduction reviews some aspects of our present knowledge of DNA replication in E.coli, including a brief discussion of DNA replication in vitro. The second part is concerned with inhibitors of DNA replication, including a description of the activities of antibiotics structurally similar to clorobiocin. In the final part, the various factors which may affect the tertiary structure of DNA are reviewed, with particular emphasis on the activities of the enzyme DNA gyrase.

## I. THE MECHANISM OF DNA SYNTHESIS

### I.(a) DNA Replication in E.coli

The structure of DNA as proposed by Watson and Crick (1953) immediately suggested a relatively simple replication system. The two polynucleotide chains of DNA are linked by hydrogen bonds between their purine and pyrimidine bases to form a double helical structure. In consequence of this base pairing the sequence of nucleotides on one strand is complementary to the other and therefore each strand may serve as a template for the synthesis of the other. Thus DNA can be accurately copied by breakage of the hydrogen bonds between the bases and subsequent pairing and polymerisation of nucleotides on daughter strands.

The chromosome of Escherichia coli is a circular double-stranded molecule of DNA of molecular weight  $2.5 \times 10^9$ . It is contained within the nucleoid, a multi-folded structure containing, by weight, 10% protein, 10% RNA and 80% DNA in a supertwisted form (Stonington and Pettijohn, 1971). The structure of the nucleoid and the role of supertwisted DNA will be discussed later.

#### (i) The Genetics of DNA Replication in E.coli

Chromosomal replication, although conceptually simple, is in fact a very complex process requiring many different enzymes and substrates. This is reflected by the many different mutants in, for example, E.coli and bacteriophage T4 which are defective in either the initiation or the subsequent elongation steps of replication. It should be noted that this "initiation" event in E.coli refers to the formation of a pair of

replication forks at a specific site on the chromosome, termed the origin. This should not be confused with the initiation of Okazaki pieces (see later). In contrast, "elongation" refers to the movement of the replication fork(s) around the chromosome. Approximately 20 loci have been implicated in DNA replication in E.coli; the individual mutants and their phenotypes are summarised in Table 1.1 and the majority of these will not be considered in detail here as they have been reviewed elsewhere (Gross, 1972; Wechsler, 1978). Recently, many mutants defective in DNA synthesis were isolated by a computerised automatic screening procedure (Sevastopoulos et al., 1977). This study has so far only produced two new mutants (see Table 1.1) but may yet prove useful in the isolation of other novel DNA synthesis mutants.

Loci involved in chromosomal replication are generally identified by the isolation of mutants which exhibit either (1), a conditional lethal phenotype (usually temperature sensitivity) and an altered pattern of DNA synthesis at the non-permissive temperature or (2), resistance to antibiotics that interfere with DNA synthesis.

(1) Conditional lethal mutants defective in replication fall into two phenotypic classes. (a) Those defective in initiation of replication do not cease replication immediately upon a shift to the non-permissive temperature, but continue to replicate those chromosomes already initiated before the temperature shift. Mutants in this category include dnaA and dnaC (reviewed by Wechsler, 1978). Two additional mutants of this type which are not well characterised are dnaI and dnaP (see Wechsler, 1978). (b) Conditional lethal mutants defective in the elongation of previously initiated replication forks usually cease DNA replication immediately upon

Table 1.1

Loci Involved in DNA Replication in *E.coli* K-12

- (a) Genetic nomenclature and map position are according to Bachmann et al. (1976).
- (b) It has been proposed that the nalA and cou loci be renamed gyrA and gyrB respectively (Hansen and von Meyenburg, 1979).
- (c) Reference A is Wechsler and Gross (1971).
- (d) The reader is also referred to the updated *E.coli* K-12 linkage map (Bachmann and Low, 1980).
- (e) It should be noted that where a gene product is involved in elongation, this does not necessarily rule out a role in initiation (and vice versa).

| <u>Mutant</u><br><u>designation</u> (a) | <u>Map</u> (a)<br><u>position (min)</u> | <u>Gene product or</u><br><u>process affected</u><br><u>by mutation</u> (e) | <u>Reference</u> (c)   |
|---|---|---|--|
| dnaA                                    | 82                                      | Initiation  | A. Hirota <u>et al.</u> (1968)                               |
| dnaB                                    | 91                                      | Elongation  | A. Hirota <u>et al.</u> (1968),<br>Carl (1970)               |
| dnaC                                    | 99                                      | Elongation and<br>initiation  | A. Carl (1970)   |
| dnaE                                    | 4                                       | Elongation; DNA<br>polymerase III   | A. Gefter <u>et al.</u> (1971)                               |
| dnaG                                    | 66                                      | Elongation; DNA<br>primase  | A. Rowen and Kornberg<br>(1978)                              |
| dnaI                                    | 37-44                                   | Initiation in<br><u>E.coli</u> B/r  | See Wechsler (1978),<br>Beyersmann <u>et al.</u> (1974)      |
| dnaL                                    | 96                                      | Elongation  | Sevastopoulos <u>et al.</u> (1977)                           |
| dnaM                                    | 76                                      | Elongation  | Sevastopoulos <u>et al.</u> (1977)                           |
| dnaP                                    | 84                                      | Elongation;<br>possibly membrane<br>associated                              | Wada and Yura (1974)   |
| dnaZ<br>(originally<br>dnaH)            | 10                                      | Elongation; sub-<br>unit of DNA poly-<br>merase III<br>holoenzyme           | Filip <u>et al.</u> (1974),<br>Wickner and Hurwitz<br>(1976) |
| polA                                    | 85                                      | DNA polymerase<br>I   | de Lucia and Cairns<br>(1969), Gross and Gross<br>(1969)     |

| <u>Mutant</u><br><u>designation</u> (a) | <u>Map</u> (a)<br><u>position (min)</u> | <u>Gene product or</u><br><u>process affected</u><br><u>by mutation</u> (e) | <u>Reference</u> (c)  |
|---|---|---|---|
| polB                                    | 2                                       | DNA polymerase II   | Campbell <u>et al.</u> (1974)                                 |
| lig                                     | 51                                      | DNA ligase  | Gottesman <u>et al.</u> (1973)                                |
| cou (b)<br>(gyrB)                       | 82                                      | Elongation and<br>initiation. DNA<br>gyrase subunit B                       | Ryan (1976), Gellert<br><u>et al.</u> (1976a), this<br>thesis |
| nalA (b)<br>(gyrA)                      | 48                                      | Elongation. DNA<br>gyrase subunit A   | Hane and Wood (1969),<br>Sugino <u>et al.</u> (1977)          |
| ssb                                     | 90                                      | Elongation. DNA<br>binding protein  | Meyer <u>et al.</u> (1979)                                    |
| dna-517                                 | 82                                      | Probable <u>gyrB</u><br>allele  | A. Projan and<br>Wechsler (1978)                              |
| rpoB                                    | 89                                      | Initiation. $\beta$<br>subunit of RNA<br>polymerase                         | Lark (1972),<br>Bagdassarian <u>et al.</u><br>(1977)          |
| rep                                     | 83                                      | Elongation  | Lane and Denhardt (1975)                                      |
| sdr <sup>c</sup>                        | 5-8                                     |   | Kogoma (1978)   |



a shift to the non-permissive temperature. Examples of this type of mutant are dnaE, dnaB and dnaG. However, mutations affecting elongation need not be lethal to the cell : mutants defective in the rep function (Lane and Denhardt, 1975) and many mutants defective in the lig and polA genes (Gottesman et al., 1973; Kuempel and Veomett, 1970) have reduced fork movement or slow joining of replication intermediates without necessarily imposing temperature sensitivity on the host.

(2) Isolation of mutants resistant to antibiotics which interfere with DNA synthesis has unfortunately not proved particularly fruitful until recently. No antibiotics have been identified as specifically inhibiting E.coli DNA polymerases or processes involved in initiation of replication or segregation of daughter nuclei. The antibiotic rifampicin inhibits RNA polymerase by binding to the enzyme (Wehrli et al., 1968) and mutants in the  $\beta$  subunit which confer resistance to rifampicin have been useful in studying the regulation of this important enzyme (see review by Scaife (1976)). Sensitivity to rifampicin has, however, indicated a requirement for RNA polymerase activity in the initiation of chromosomal replication (see later).

Novobiocin and nalidixic acid are two unrelated antibiotics which inhibit DNA synthesis in E.coli (Smith and Davis, 1967; Goss et al., 1965; see part II). Mutants resistant to these antibiotics have helped to identify their target enzyme, DNA gyrase (Gellert et al., 1976a; Sugino et al., 1977). This rather special case will be discussed in detail in parts II and III.

(ii) The Use of Small Replicons to Study DNA Replication

The significance of some important mutants and their contribution to our knowledge of DNA synthesis will be outlined later when individual steps in the replication process are discussed. However, much of our knowledge of DNA replication has come not from studies with chromosomal DNA, but from studies with the smaller replicons of phage and plasmids. This is primarily due to the ease of isolation and handling of these small DNA molecules. It is appropriate at this point therefore to discuss briefly the properties and structures of these elements.

Plasmids, ranging in molecular weight from  $2 \times 10^6$  to  $10^8$  (reviewed by Clowes, 1972), can be separated from chromosomal DNA in crude extracts as covalently closed circular molecules of double-stranded DNA by caesium chloride density gradient centrifugation in the presence of ethidium bromide. The physical basis for this separation will be outlined in section III, however an important point to note is that plasmid DNA isolated in this way is negatively supercoiled (see part III).

Bacteriophage DNA can be isolated from purified phage particles with relative ease. Some large phages e.g.  $\lambda$ , P1 and T4 contain a double-stranded linear DNA molecule which circularises upon infection and replicates in this form, whilst other phage DNA e.g. T7 remains linear throughout the replication cycle. In contrast to plasmids, these large phages code for many of the enzymes necessary for their own replication. Studies of infected cells have led to the identification and characterisation of specific polymerases, ligases, primases and DNA binding proteins. However, the bacteriophages that have provided the most information about the biochemistry of DNA replication are the single-

stranded circular DNA phages. In particular ØX174, and the closely related phage G4, when used as templates for in vitro DNA replication dependent upon wild-type E.coli extracts, have led to the identification and purification of many proteins involved in the replication process. This has been achieved by complementation of extracts from temperature sensitive dna mutants (see Wickner, 1978).

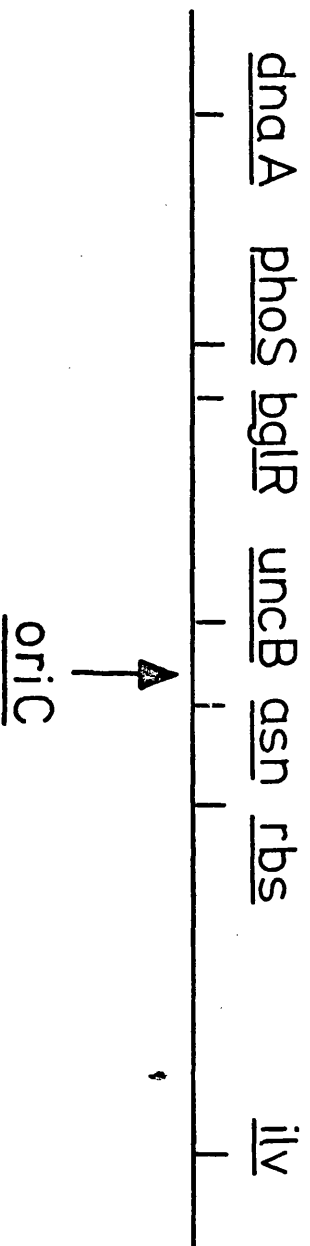
(iii) The Origin and Direction of Replication in E.coli

Initial studies indicated that chromosomal replication in E.coli was bidirectional, proceeding from a fixed origin positioned between 74 and 83 minutes, close to the ilv locus (Masters and Broda, 1971; Hohlfield and Vielmetter, 1973; Louarn et al., 1974). By hybridisation studies using strains containing the bacteriophage Mu as a prophage inserted into one of four different genes in the vicinity of the ilv locus, Fayet and Louarn (1978) were able to conclude that oriC lay between bglR and rbs (see Figure 1.1). Von Meyenburg et al. (1978) isolated various  $\lambda$  transducing phages carrying chromosomal markers in this region, and found that some  $\lambda$  asn phages would replicate in E.coli  $\lambda$  lysogens by virtue of antonomous replication from an origin distinct from the  $\lambda$  origin and therefore presumably from oriC. It was concluded that oriC lay between uncB and asn. A restriction fragment from this  $\lambda$  asn replicon (Messer et al., 1978) or from restricted total E.coli DNA (Yasuda and Hirota, 1977) when ligated to a second, non-replicating fragment coding for ampicillin resistance has enabled the origin to be studied in detail. The nucleotide sequence of a 422 base pair segment of chromosomal DNA from the oriC - containing restriction fragment has been determined; this sequence appears to contain all the information necessary for efficient

Figure 1.1

Order of Genes around the Origin of Replication of E.coli

This diagram represents the E.coli chromosome in the vicinity of the origin of replication (oriC) at approx. 82 minutes. The concluded order is taken from Fayet and Louarn (1978) and von Meyenburg et al. (1978).



initiation of replication (Sugimoto et al., 1979; Meijer et al., 1979).

The direction of replication, usually from a fixed origin, has also been studied in other bacteria. In both Salmonella typhimurium and Bacillus subtilis replication appears to be bidirectional (Fujisawa and Einsenstark, 1973; Wake, 1974). In phage  $\lambda$  (Schnos and Inman, 1970) and the mini-F plasmid (Eichenlaub et al., 1977) electron microscopic analysis of replicating molecules has shown that replication is bi-directional, whilst in phage P2 (Schnos and Inman, 1971) and the small plasmid ColE1 (Inselburg, 1974) replication is unidirectional. As indicated above, not all replicons are circular; phage T7 DNA is replicated bidirectionally from an origin positioned approximately 17% from one end of the linear molecule (Dressler et al., 1972). Some naturally occurring R factors e.g. R6K, and plasmid chimeras generated by in vitro recombinant DNA techniques have more than one origin of replication, although only one is normally used (Crosa et al., 1978; Timmis et al., 1974).

#### I.(b) Events at the Replication Fork

This section will outline the events which occur at DNA replication forks. No one system has been responsible for the discovery of all the known activities, and I will summarise only the data which describes the important steps in the replication process.

##### (i) Polymerases Involved in DNA Replication

Three enzymes have been isolated from E.coli which direct the synthesis of DNA from a complementary strand as template. These enzymes are termed DNA polymerases I, II and III (abbreviated polI, II and III)

and are identified genetically by the loci polA, polB and dnaE respectively (see Table 1.1). All three enzymes extend chains in the 5' to 3' direction only and require a primer molecule with a free 3'OH group base paired to a single-stranded DNA template before polymerisation can commence. The three polymerases differ markedly in their detailed properties; for example with respect to their turnover number,  $K_m$  for triphosphates and number of copies per cell (reviewed by Kornberg, 1974). On the other hand all three enzymes contain a 3' to 5' exonuclease activity but in addition polI contains a 5' to 3' exonuclease activity.

Attempts to demonstrate an absolute requirement of the polymerase activity of polI for growth have failed. However, polA mutants are sensitive to UV light (Gross and Gross, 1969) and are slow to join up Okazaki pieces (Kuempel and Veomett, 1970); and the polA1 mutation is lethal when in combination with the non-lethal temperature sensitive lig-4 mutation (Gottesman *et al.*, 1973). An absolute requirement for growth has been shown only for the 5' to 3' exonuclease activity of polI, by the isolation of temperature-sensitive conditional lethal polAex mutants (Konrad and Lehman, 1974; Olivera and Bonhoeffer, 1974). Thus although the polymerase activity of polI may be important in DNA replication, the most convincing role for polI appears to be an involvement in repair synthesis, and perhaps the removal of RNA primers from, and the subsequent joining together of Okazaki pieces (see later). However, replication of plasmids ColE1 and ColE2 is absolutely dependent upon polI, both *in vivo* and *in vitro* (Kingsbury and Helinski, 1973; Staudenbauer, 1976b) although this appears limited to the synthesis of "early" 6s DNA fragments

(see Tomizawa, 1978). The role of polII remains obscure and will not be discussed further.

It is now accepted that polIII provides the polymerase activity at the replication fork. E.coli strains carrying conditional lethal dnaE mutations rapidly cease DNA synthesis upon a shift to the non-permissive temperature (Weschler and Gross, 1971) and elongation of single-stranded DNA phages and ColE1 DNA in vitro all require a functional polIII (Wickner et al., 1972; Staudenbauer, 1976b). Studies with  $\phi$ X174 DNA replication in vitro have in fact shown that the active polIII polymerase in this system is associated with a multimeric complex termed the holo-enzyme (Wickner et al., 1973). This holoenzyme is apparently composed of polIII\*, consisting of polIII (dnaE gene product) and at least two other proteins (one of which is the dnaZ gene product) and co polIII\*, a protein cofactor necessary for polymerase activity (see Kornberg, 1978).

#### (ii) Discontinuous Synthesis

As outlined above, DNA polymerases will only extend chains in the 5' to 3' direction. Since double-stranded DNA consists of two anti-parallel chains, synthesis of at least one strand presumably therefore has to be discontinuous. Studies by Okazaki et al. (1968) have shown that after alkaline gradient sedimentation of pulse labelled E.coli DNA in vivo, most of the newly synthesised DNA is in the form of short chains (Okazaki fragments) with a sedimentation coefficient of 10S, corresponding to 1000-2000 nucleotides. These fragments can be chased into mature 38S fragments in wild-type strains, but accumulate in polA and lig mutants (Keumpe and Veomett, 1970; Gottesman et al., 1973).



Contradictory evidence exists as to whether one or both chains are synthesised discontinuously in E.coli. Okazaki et al. (1968) and Sternglanz et al. (1976) observed only one size class of fragment (10S) consistent with discontinuous synthesis on both chains. However Louarn and Bird (1974) found that DNA synthesis in E.coli produced two discrete size classes of DNA. Synthesis in the 5' to 3' direction (the "leading strand") was continuous, producing mature fragments ( $>53S$ ), while synthesis in the 3' to 5' direction was apparently discontinuous producing short 10S fragments. In addition, Louarn and Bird found that synthesis on both strands was discontinuous in polA mutants. This controversy is still unresolved, but many workers interpret the results to indicate discontinuous synthesis on both strands, but that the leading strand may be synthesised in fewer larger fragments which may be ligated together faster than the shorter fragments produced by lagging strand synthesis (Alberts and Sternglanz, 1977; Denhardt, 1978; Kwoh et al., 1979). This uncertainty is confused further by doubts over the authenticity of many Okazaki fragments as genuine replication intermediates (Tye et al., 1977).

#### (iv) Primer Synthesis

Elongation of DNA by a discontinuous mechanism raises the problem of the nature of the primer, since no DNA polymerase can initiate chains de novo. RNA polymerase, on the other hand, does not require a primer and will transcribe single-stranded DNA forming an RNA chain with a 3'OH terminus which potentially could act as a primer for DNA polymerase III. Early evidence for the existence of Okazaki pieces in E.coli containing 5' ribonucleotides has proved to be spurious and much effort has been

spent in determining whether or not DNA chains are indeed initiated by an RNA primer. Ogawa et al. (1977) and Miyamoto and Denhardt (1977) reported the presence of RNA-terminated DNA replication intermediates in polAex mutants in vivo, but could find little or no evidence for them in wild-type strains. One attractive solution is to envisage that RNA primers are rapidly removed from DNA by the polI exonuclease before being joined together by the combined activities of polI and DNA ligase. Until low molecular weight DNA fragments can be unambiguously identified as true Okazaki pieces the contribution of non-nascent DNA molecules (Denhardt et al., 1979) will continue to hinder the search for 5' ribonucleotide terminated DNA replication intermediates.

Confirmation of the existence of RNA primers in E.coli comes indirectly from the study of the dnaG function of E.coli. This function is essential for growth, and a temperature shift in a dnaG mutant results in abrupt cessation of DNA synthesis (Wechsler and Gross, 1971). Primer synthesis in the phages G4 and  $\phi$ X174 has been studied both in crude extracts and with purified proteins in vitro. A simple primer system has been demonstrated for G4 which, in vitro, only requires DNA binding protein (also termed helix destabilising protein, see part III), ATP, ribo or deoxyribonucleosides and the dnaG gene product (Zechel et al., 1975). Subsequent DNA synthesis requires the DNA polymerase III holoenzyme and deoxyribonucleosides. The dnaG protein synthesises oligonucleotides which contain both dNMP and rNMP residues, and is therefore a special kind (also rifampicin resistant) of RNA polymerase (Bouché et al., 1975) more correctly termed DNA primase.  $\phi$ X174 has a more complex priming reaction requiring, in addition to those proteins of the G4 system, dnaB protein,

dnaC protein and other replication factors termed X, Y and Z (Wickner and Hurwitz, 1974) or i, n and n' (Scheckman et al., 1975). In these priming reactions, the DNA binding protein is thought to bind single-stranded DNA and, perhaps by inducing the formation of secondary structures, promote recognition of DNA by the DNA primase. A role for the dnaB protein as a "mobile promoter" has been proposed by Kornberg (1978); it is hypothesised that dnaB protein binds to single-stranded DNA coated with DNA binding protein and migrates along the DNA in a 3' to 5' direction. DNA primase recognises conformational changes induced by the presence of the dnaB protein and subsequently initiates primer synthesis. The immediate cessation of DNA synthesis in vivo in dnaB mutants at the restrictive temperature is clearly consistent with a role for the dnaB protein at the replication fork. The cellular roles of dnaC protein and the replication factors X, Y and Z as deduced from in vitro studies are unclear, although the dnaC protein is required for initiation of chromosomal replication in vivo (see later).

#### (v) Elongation of DNA Chains In Vitro

In this section I will describe a few interesting features of in vitro DNA replication in bacteriophages. Perhaps the simplest elongation reaction described is the conversion of single-stranded phage DNA to the duplex circular (RF) form. After priming, the only host components necessary for this reaction with M13, G4 or  $\phi$ X174 DNA are DNA binding protein and DNA polymerase III holoenzyme (Hurwitz and Wickner, 1974; Wickner and Kornberg, 1973; see also Kornberg, 1978). DNA polymerase and DNA ligase are required for conversion of the resulting nicked circle into a covalently closed form.

Replication, both in vivo and in vitro, of  $\phi$ X174 RFI DNA requires two additional proteins, the  $\phi$ X174 cisA protein and the E.coli rep protein. Eisenberg et al. (1977) have shown that the cisA protein nicks the viral (+) strand at a specific site; the protein is then attached to the 5' end of the DNA, presumably by a covalent bond. The cisA protein will only nick the supercoiled (RFI) form of the covalently closed duplex; the relaxed (untwisted) RFIV form is not a suitable substrate (Ikeda et al., 1976) unless it is converted to the supercoiled form by DNA gyrase (Marians et al., 1977). The cisA protein has been observed to nick and recircularise viral strands upon completion of synthesis of a unit length of DNA by a modified "rolling circle" type of replication (Eisenberg et al., 1977). This DNA synthesis is most likely a covalent extension from the 3'OH terminus formed at the nick generated by the cisA protein.

The E.coli rep function was identified by Denhardt et al. (1967) who isolated a mutant that could not replicate  $\phi$ X174 RFI DNA. The rep protein has been purified; it is a 68KD polypeptide which enzymically melts a DNA duplex to separate DNA strands with the hydrolysis of ATP (Scott et al., 1977; Yarranton and Gefter, 1979). Scott et al. have shown that, in the absence of DNA synthesis, the combined action of rep protein, cisA protein and DNA binding protein will completely unwind duplex RFI  $\phi$ X174 DNA. This, combined with the evidence that E.coli rep mutants replicate their chromosomal DNA more slowly than wild-type strains (Lane and Denhardt, 1975) suggests that the rep protein is involved in vivo in strand separation ahead of the replication fork. This will be discussed further in part III.

I.(c) The Cell Cycle of *E.coli* : Initiation of DNA Replication and  
Cell Division

In this section I will discuss briefly the cell cycle of *E.coli*, with particular reference to the initiation of chromosome replication and cell division. The cell cycle of *E.coli* may be described in terms of two parameters : a period, C, during which the chromosome is replicated and a subsequent period, D, which precedes cell division (Cooper and Helmstetter, 1968). The values of C and D remain essentially constant over a wide range of growth rates (Cooper and Helmstetter, 1968; Helmstetter and Pierucci, 1976).

(i) Initiation of DNA Replication

The rate of DNA synthesis in a bacterial culture is controlled by the rate of initiation of chromosomal replication. Each new round of replication is thought to be initiated when the ratio of cell mass to number of chromosome origins reaches a critical value, the initiation mass (Donachie, 1968). The process of initiation halves this value by duplicating the origin; subsequent cell growth then increases the ratio until, after one mass doubling or generation time, it again reaches the critical value. Several authors have proposed models to explain how the cell might titrate its mass/origin ratio (Helmstetter *et al.*, 1968; Pritchard *et al.*, 1969; Sompayrac and Maaløe, 1973). In these models the period of growth between successive initiations is regarded as the time taken to accumulate some positive regulatory factor, or to dilute out an inhibitor of initiation. Recent evidence in this area has been reviewed by Pritchard (1978).

Initiation of DNA replication requires both de novo protein and RNA synthesis since starvation of a culture for essential amino acids or the addition of chloramphenicol or rifampicin allows the continued synthesis of DNA from existing forks but no new forks are initiated (Maaløe and Hanawalt, 1961; Lark, 1972). The requirement for RNA synthesis appears to be restricted to immediately before, or at initiation, whereas the proteins necessary for initiation may be synthesised earlier in the cell cycle (Lark, 1972). Messer et al. (1975) reported the isolation of a specific RNA species, termed o-RNA (origin RNA), which was apparently synthesised during initiation and was regulated by the dnaA gene product. However, this o-RNA does not hybridise with the oriC-Ap plasmid (Messer, W., personal communication) and its role, if any, in the initiation process is not clear.

The two genes dnaA and dnaC are required for initiation of chromosomal replication (see Table 1.1), although their functions are by no means clear. The dnaC protein has been purified and appears to interact with the dnaB protein in the replication of single-stranded phages in vitro (Wickner, 1978). The dnaA protein has not yet been purified.

Mutations at the dnaA locus have been reported to be either recessive (Wechsler and Gross, 1971; Wehr et al., 1975; Gotfried and Wechsler, 1977) or dominant (Beyersmann et al., 1974; Zahn et al., 1977) depending upon the particular allele and strain background employed. The block to initiation in both dnaA and dnaC mutants is reversible; upon a shift from the non-permissive to the permissive temperature, initiation of a new round of replication immediately takes place. Zyskind et al. (1977) have shown that in dnaA5 mutants, this reinitiation is sensitive to

rifampicin but not to chloramphenicol, whilst in dnaC mutants it is sensitive to neither. However Tippe-Schindler et al. (1979) found that reinitiation in such dnaA5 mutants was insensitive to rifampicin and they have suggested that such inconsistencies may be the result of strain differences. In any case the results with chloramphenicol suggest that the protein synthesis necessary for initiation can occur in the absence of synthesis of either the dnaA or dnaC gene products. Zyskind et al. (1977) found that the sensitivity to rifampicin described above was relieved by the introduction of an rpoB mutation, confirming the activity of RNA polymerase in initiation of replication. In addition, a direct interaction between the dnaA product and RNA polymerase is suggested by the isolation of suppressor mutations of dnaA which map within rpoB, the structural gene for the  $\beta$  subunit of RNA polymerase (Bagdassarian et al., 1977).

The results of Zyskind et al. which show reinitiation to be sensitive to rifampicin in dnaA but not dnaC mutants suggested that the dnaA protein may act before the dnaC protein. Kung and Glaser (1978) have constructed a dnaA (heat sensitive) / dnaC (cold sensitive) double mutant. When this strain was incubated at 41°C chromosome replication eventually ceased due to the inactive dnaA product, and could not be reinitiated upon a shift to 20°C where the dnaC protein was inactive. In the reciprocal experiment, a shift from 20°C to 41°C did allow reinitiation. These results reinforce the idea that the dnaA product acts before the dnaC protein.

It appears that a full understanding of the biochemical processes involved in the initiation of chromosomal replication in E.coli will only

be achieved by the development of an assay which will measure the initiation of DNA replication in vitro. It may prove possible to develop such a system using the oriC-Ap plasmid described earlier. In addition, the isolation and characterisation of novel mutants which are defective in initiation will almost certainly prove useful in studying the initiation process.

(ii) Cell Division

The processes of DNA replication and cell division must be tightly linked since each division event must accompany segregation of genomes to daughter cells. The observation of a fixed D period in E.coli (about 20 minutes, Cooper and Helmstetter, 1968) between termination of replication and cell division may reflect the timing of division from some stage of replication. Thus initiation of replication could activate a division C+D minutes later, or alternatively termination of replication could directly trigger division D minutes later. Indeed inhibition of DNA synthesis by thymine starvation, UV irradiation or nalidixic acid treatment results in a period of residual divisions lasting about 20 minutes (Helmstetter and Pierucci, 1968; Inouyi, 1971). The fractional increment of cells in such experiments is equal to the fraction of cells in the population expected to be in the D period at the time of treatment. Moreover Helmstetter and Pierucci found that those cells which carried on to divide were those cells which had completed a round of replication prior to treatment. These results have been taken as circumstantial evidence that termination of replication is a necessary condition for subsequent cell division.



However, such treatments which inhibit DNA synthesis also lead to the induction of a number of functions, including the inhibition of cell division, collectively termed the SOS system (Witkin, 1976; see part II). Thus it is possible that the observed inhibition of cell division is a consequence of the induction of the SOS system and not the direct result of a failure to terminate replication. In support of this hypothesis Inouyi (1971) found that the expression of the SOS functions was dependent upon a functional recA gene. Thymine starvation or nalidixic acid treatment of recA mutants resulted in extensive cell division leading to the production of DNA-less cells. In conclusion, it remains unclear whether the inhibition of cell division in rec<sup>+</sup> strains is the sole consequence of induction of the recA-dependent SOS system or if some factor based upon DNA replication e.g. termination, is necessary for subsequent cell division.

## II. INHIBITORS OF NUCLEIC ACID SYNTHESIS IN E.COLI

### II.(a) Mode of Action of Some Inhibitors of Nucleic Acid Synthesis

Inhibitors of DNA replication in E.coli may be divided into three categories : (1) those that bind to DNA, (2) those that compete with nucleotides for utilisation by the replication enzymes (nucleotide analogues) and (3) those that bind to a target enzyme. Inhibitors in the first category include actinomycin D (which is a preferential inhibitor of RNA synthesis), the acridine dyes e.g. acriflavin and proflavin, mitomycin C and bleomycin (see Kornberg, 1974). The acridines and actinomycin D intercalate between base paired nucleotides : the acridines induce frame-shift mutations in phage and in some strains of E.coli and Salmonella typhimurium during DNA replication. The acridines are also used to "cure" bacterial cells of resident plasmids, although the exact mechanism whereby this occurs is not clear. Bleomycin and mitomycin C inhibit DNA synthesis by covalent binding to the DNA template : mitomycin C cross links between DNA strands whereas bleomycin apparently binds to the 2-carbonyl position of thymine and at high concentrations causes DNA chain scissions in vitro and DNA breakdown in vivo.

The most common nucleotide analogues which inhibit DNA synthesis are the arabinonucleotides (reviewed by Cozzarelli, 1977). Two such compounds, 1- $\beta$ -D-arabinofuranocytosine (ara-C) and 9- $\beta$ -D-arabino-furanosyladenine (ara-A) are analogues of both ribonucleotides and deoxyribonucleotides, and inhibit the growth of a wide variety of

eukaryotic and prokaryotic cells, although bacteria are generally more resistant than eukaryotes. Arabinonucleotides may inhibit DNA synthesis either by competitive inhibition of DNA polymerase or by incorporation of the analogue into DNA and subsequent chain termination due to the lack of a suitable 3'OH terminus. It has been shown that in a polI mutant of Bacillus subtilis resistance to arabinonucleotides is achieved by mutational alteration of DNA polymerase III, thereby implicating this enzyme as the primary target for arabinonucleotide inhibitors (Rashbaum and Cozzarelli, 1976).

Nucleotide precursors may also bind to the target enzyme. One such group of inhibitors are the hydroxyphenylhydrazinopyrimidines. These analogues do not inhibit the growth of E.coli, but they have been shown to bind specifically to the DNA polymerase III of Bacillus subtilis (Mackenzie et al., 1973). This polymerase is resistant to these inhibitors in vitro when purified from a drug resistant strain, and many resistant mutants map at the polC locus, the structural gene for DNA polymerase III in this organism (reviewed by Cozzarelli, 1977).

Another extensively studied inhibitor of nucleic acid synthesis is rifampicin, an antibiotic which binds tightly to the  $\beta$  subunit of RNA polymerase (Wehrli et al., 1968). Rifampicin inhibits initiation, but not the elongation of RNA transcripts (Sippel and Hartman, 1968) indicating that nucleic acid-RNA polymerase complexes are less able to bind rifampicin than the free enzyme. Mutants resistant to rifampicin, which yield a rifampicin resistant RNA polymerase in vitro, map in the gene coding for the  $\beta$ - subunit of RNA polymerase (see Scaife, 1976).

Two other groups of antibiotics which inhibit DNA replication are exemplified by novobiocin and nalidixic acid. These compounds have recently been shown to inhibit an enzyme termed DNA gyrase (Gellert et al., 1976a). The mechanism of action of novobiocin and nalidixic acid will be discussed later and in part III.

## II.(b) Induction of SOS Functions

Many of the inhibitors of DNA replication described above also cause the induction of a number of processes in E.coli collectively known as "SOS" or "error-prone repair" functions (reviewed by Witkin, 1976). SOS functions include inhibition of cell division, induction of prophage  $\lambda$ , increased mutagenesis, DNA degradation and increased synthesis of a 40kD protein, termed protein X. It has recently been shown that protein X is the recA<sup>+</sup> gene product (Emmerson and West, 1977; Gudas and Mount, 1977; McEntee, 1977). The SOS functions are also induced by UV irradiation, X-rays and thymine starvation. Using synchronous cultures of E.coli B/r, Gudas and Pardee (1976) demonstrated that the induction of protein X by nalidixic acid was reduced in cells not replicating DNA. Similarly, nalidixic acid did not induce protein X in a dnaA mutant when replication had ceased at the non-permissive temperature. These results indicated that induction of the SOS functions was the result of inhibition of DNA replication; however protein X could be induced in the absence of replication by treatment of bacteria with bleomycin (Gudas and Pardee, 1976) or UV irradiation (Little and Hanawalt, 1977). Therefore the inhibition of fork movement per se is not a prerequisite for

the induction of the SOS functions. However, there does appear to be some correlation between the induction of protein X and the presence of acid-soluble DNA fragments resulting from DNA damage (Gudas and Pardee, 1976; Smith and Oishi, 1978), and Gudas and Pardee proposed that a DNA degradation product may be the signal for the induction of SOS functions.

Degradation of DNA in cells induced for SOS repair could arise from repair synthesis and concomitant exonuclease activity following strand scission caused by, for example, bleomycin or the excision of pyrimidine dimers introduced by UV light. Degradation may also result from preferential breakdown of newly synthesised DNA which is known to accompany nalidixic acid treatment and thymine starvation in B.subtilis (Ramareddy and Reiter, 1969; Reiter and Ramareddy, 1970).

The synthesis of the recA protein is thought to be controlled by a negatively acting repressor, the product of the unlinked lexA gene (Gudas and Pardee, 1975; Mount et al., 1972) and by the positive action of the recA protein (Emmerson and West, 1977). This hypothesis follows from the observations that induction of protein X is both lexA<sup>+</sup> and recA<sup>+</sup> dependent (Inouyi, 1971; Gudas and Pardee, 1975). The auto-regulatory role of the recA protein is also directly supported by the isolation of tif mutants (Castellazzi et al., 1972) which are constitutive for protein X synthesis at 42°C and carry a mutation which is allelic with recA (Gudas and Pardee, 1975; Castellazzi et al., 1977; Emmerson and West, 1977).

At present the precise role of the recA product in vivo is unclear, although several properties have been ascribed to the purified protein

in vitro. Roberts et al. (1978) demonstrated that purified recA protein caused the cleavage of purified phage  $\lambda$  repressor in vitro in an ATP-dependent reaction. RecA protein was also shown to exhibit a single-stranded DNA dependent ATP-ase activity (Ogawa et al., 1979; Roberts et al., 1979). Recently, Weinstock et al. (1979) have demonstrated that this ATP hydrolysis is associated with the renaturation, into a double-stranded form, of complementary single-stranded DNA molecules. Finally, Shibata et al. (1979) have shown that purified recA protein will catalyse the ATP-dependent pairing of superhelical DNA with homologous single-stranded DNA to form structures known as D-loops. This activity of the recA protein will be discussed in part III.

## II.(c) Permeability of the Bacterial Cell Envelope to Antibiotics

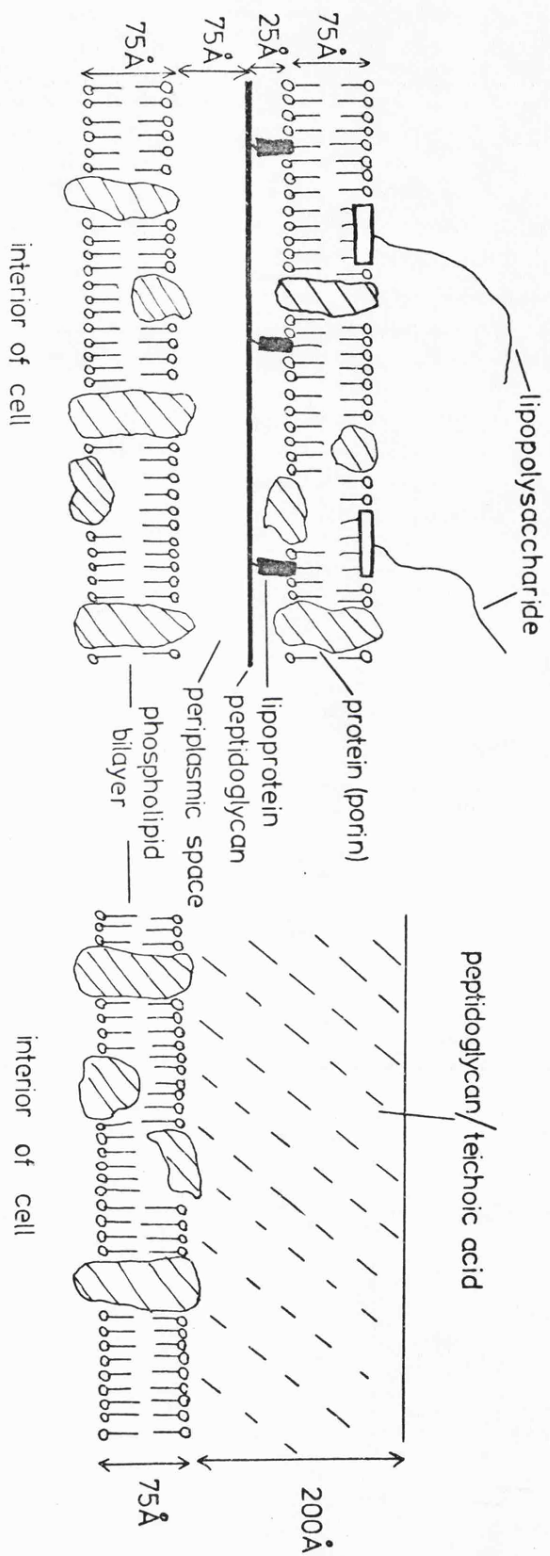
Antibiotics may be divided into two groups according to their activity against gram-positive and gram-negative bacteria. Many antibiotics e.g. neomycin, cycloserine and some penicillins are equally effective against both groups of bacteria, whereas others such as actinomycin D, rifampicin and erythromycin and novobiocin are much more active against gram-positive bacteria. The cell envelopes of gram-positive and gram-negative bacteria are illustrated in Figure 1.2. Both envelopes contain an inner membrane composed of protein and phospholipid and, external to this, a peptidoglycan layer. In gram-positives, the peptidoglycan layer is ~20 nm thick and is surrounded by a layer of teichoic acids, whereas in gram-negatives the monolayer of peptidoglycan is enclosed by a second membrane, the outer membrane. It is this outer

Figure 1.2

Diagrammatic Representation of the Membranes of Gram-Negative and Gram-Positive Bacteria

The membrane of a gram-negative bacterium (left) is composed of two membranes, an inner or cytoplasmic membrane and an outer membrane which are separated by a single layer of peptidoglycan.

The gram-positive bacterial membrane (right) is composed of a single membrane bilayer, surrounded by a dense layer of peptidoglycan containing teichoic acids.



GRAM - NEGATIVE

GRAM - POSITIVE



membrane, composed of phospholipid, protein and lipopolysaccharide (LPS) which appears to provide the permeability barrier to some antibiotics.

The structures of S.typhimurium and E.coli LPS are shown in Figure 13. Roantree et al. (1969) determined the sensitivity of various S.typhimurium "deep rough" mutants to several antibiotics and found that those mutants lacking 80-90% of the LPS, e.g. Ra or Rc mutants, were not usually sensitive. However those that had lost the next few residues, e.g. Rd<sub>1</sub>, Rd<sub>2</sub> or Re mutants, were extremely sensitive to some but not other antibiotics. In support of these findings Tamaki et al. (1971) found that E.coli mutants isolated as supersensitive to novobiocin had incomplete LPS chains although their exact composition was not determined.

Nikaido (1976) determined the partition coefficient of many antibiotics and dyes and observed that those compounds which were ineffective against gram-negative bacteria, but whose activity was increased in deep rough mutants, were usually hydrophobic. In contrast, the majority of compounds whose activity was unaffected by changes in LPS structure were quite hydrophilic. Nikaido concluded that at least two pathways exist for the diffusion of small compounds across the outer membrane; one for hydrophilic compounds and one for hydrophobic compounds.

Stein (1967) has suggested that hydrophobic molecules cross a membrane bilayer first by dissolving in the hydrophobic interior of the membrane, diffusing through the thickness of the hydrocarbon bilayer and then crossing the membrane by partitioning into the aqueous phase. By measuring the influence of various conditions e.g. the effect of temperature on the diffusion rate, Nikaido (1976) concluded that hydrophobic compounds penetrate through the outer membrane of deep rough mutants by a similar mechanism.

### Figure 1.3

#### Tentative Structure of *E.coli* K12 Lipopolysaccharide

The length of the oligosaccharide chain of some mutants of *E.coli* K12 (D21 series) and *S.typhimurium* (R series) are indicated. The structure of the LPS of *S.typhimurium* is similar except it contains an O antigen. Taken from Prehm et al. (1976) and Nikaido (1976).

|        |   |                                  |
|--------|---|----------------------------------|
| GlnNac | : | N-acetylglucosamine              |
| Glc    | : | glucose                          |
| Gal    | : | galactose                        |
| Hep    | : | heptose                          |
| Rha    | : | rhamnose                         |
| P      | : | phosphate                        |
| KDO    | : | 3-deoxy-D-manno-octulosonic acid |
| Etn    | : | ethanolamine                     |



The low permeability of the outer membrane to hydrophobic compounds suggests there is little or no phospholipid bilayer in the membrane which is accessible from the cell exterior. There are however two opposing views as to how this inaccessibility arises. Nikaido argues that there are no phospholipid bilayer regions in the outer membrane of wild-type strains, the outer leaflet being composed of lipid groups of the LPS and of protein. Some support for this comes from studies by Kaimo and Nikaido (1976) who have shown that in S.typhimurium the polar heads of the outer membrane are inaccessible in intact cells to reaction with external phospholipase C or with cyanogen bromide - activated dextran. In the deep rough (Rd and Re) mutants however, an increased level of phospholipid was observed which was now accessible to the external phospholipase C. In these mutants a decreased outer membrane protein to phospholipid ratio is observed and to complement this, different mutants with decreased levels of outer membrane proteins (Omp<sup>-</sup>) were shown to contain increased levels of phospholipid (Smit et al., 1975) which were now accessible to the phospholipase C. The increased permeability towards hydrophobic compounds could therefore be accounted for by an increased phospholipid to protein ratio in the outer membrane resulting in many phospholipid bilayer domains.

An alternative explanation is offered by van Alphen et al. (1977) who used phospholipases to probe the surface of a wide range of mutant strains which either have shortened LPS oligosaccharides or lack specific outer membrane proteins. While finding that phospholipase sensitivity was associated with an increase in phospholipid content, van Alphen et al. argued that this alone was not sufficient to cause

sensitivity and that in wild-type cells the outer leaflet of the outer membrane contains phospholipids which are masked by proteins and lipopolysaccharide.

Despite this controversy, it is clear that the structure and content of the outer membrane provides the permeability barrier to hydrophobic compounds. A hydrophobic pathway may therefore exist in mutants, but is largely absent in wild-type strains.

Hydrophilic compounds, on the other hand, enter the cell quite readily by an extremely efficient pathway. Payne and Gilvarg (1968) studied the uptake of oligopeptides in an amino acid auxotroph of E.coli. They found that small but not large oligopeptides could relieve auxotrophy and concluded that the cell envelope acted as a molecular sieve excluding larger molecules from passive pores. Subsequently, reconstitution experiments with purified envelope proteins and phospholipid vesicles have shown that some major outer membrane proteins, termed "porins", are able to form channels or pores through which hydrophilic compounds of less than 600 daltons may diffuse (Nakae, 1976a, b). Many mutants lacking porins have now been isolated and these are defective, for example, in the uptake of many sugars, amino acids, various ions and ampicillin (Bavoil et al., 1977; Beacham et al., 1977; Nikaido et al., 1977; Luktenhaus, 1977).

## II.(d) Novobiocin and Clorobiocin

Clorobiocin (18, 631 R.P.) is a naturally occurring compound produced by Streptomyces hygroscopicus, S. albocinereus and S. roseochromogenes var. oscitans (Mancy et al., 1970; Ninet et al., 1972). Clorobiocin is structurally similar to novobiocin and coumermycin A<sub>1</sub>, two other naturally occurring antibiotics (Figure 1.4). In this section I will review early work concerning the mode of action of these antibiotics and will attempt to correlate the results with recent evidence which almost certainly has revealed the primary mode of action of novobiocin and coumermycin A<sub>1</sub>.

The antimicrobial activity of clorobiocin is similar to that of coumermycin A<sub>1</sub> and novobiocin (Ninet et al., 1972; Godfrey and Price, 1972). Typical minimum inhibitory concentration (MIC) values for these compounds are shown in Table 1.2. All three antibiotics are 3,000 - 6,000 times more effective against Staphylococcus aureus (gram-positive) than against E.coli (gram-negative), and clorobiocin and coumermycin A<sub>1</sub> are 13 - 15 times more active than novobiocin in both cases. Since penetration of the gram-positive membrane would not be expected to be the limiting factor for novobiocin, this difference may reflect reduced activity of this antibiotic at the target level.

Two early observations demonstrated that novobiocin caused filamentation of gram-negative rods (Smith et al., 1956) and that inhibition of growth by the drug was readily reversed by the addition of magnesium ions (Brock, 1956). Brock (1967) reported that novobiocin also inhibited respiration, electron transport, ATPase activity, amino

Figure 1.4

The Structures of Novobiocin, Coumermycin A1 and Clorobiocin

Taken from Ryan (1976) and Ninet et al. (1972).

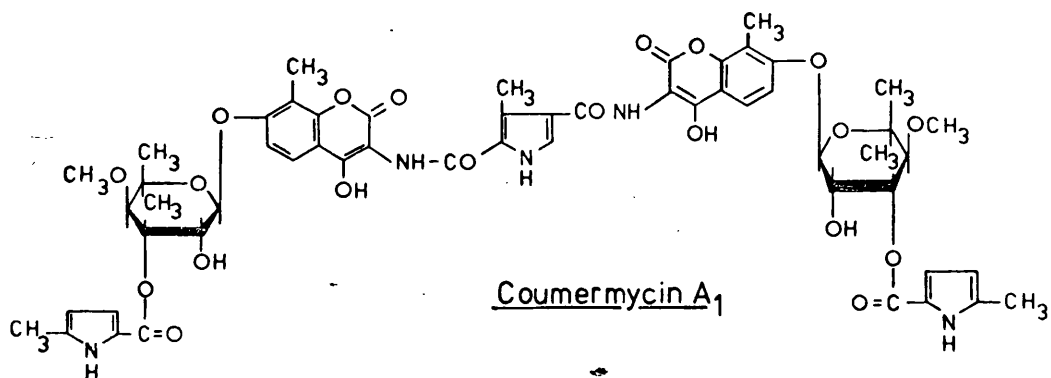
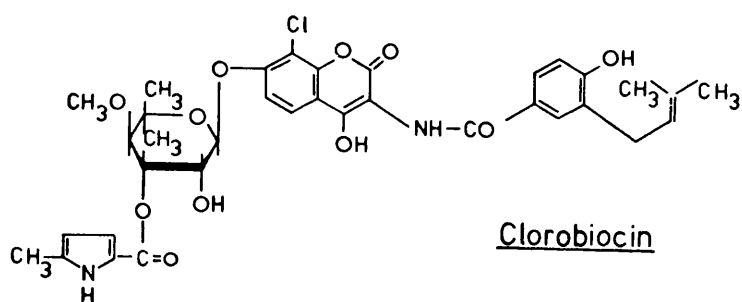
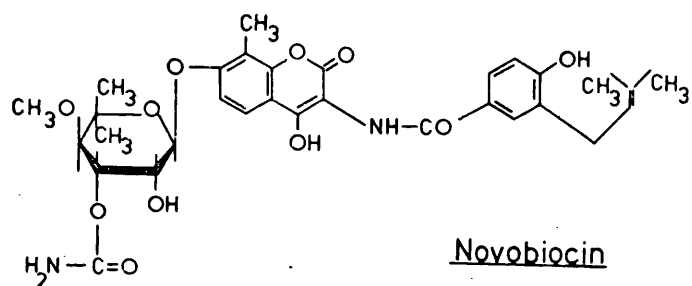




Table 1.2

MIC Values for Novobiocin, Clorobiocin and Coumermycin A1

| Compound    | <u>E.coli</u> K12 | <u>S.aureus</u> |
|-------------|-------------------|-----------------|
|             | JE1011            |                 |
| Novobiocin  | 250-500           | 0.08            |
| Clorobiocin | 32                | 0.006           |
| Coumermycin | 12.5              | 0.004           |

MIC (minimum inhibitory concentrations) are expressed in µg/ml and are defined as the lowest concentration of compound that inhibits overnight growth. Determined by two-fold dilutions in TS broth at 37°C. Data of May and Baker Ltd.

acid and acetate transport and the incorporation of amino acids into soluble t-RNA. With E.coli ML-35 novobiocin was also shown to cause the loss of intracellular material absorbing at 260 nm (Brock, 1956), however Smith and Davis (1967) found this effect was peculiar to ML strains and not observed with other E.coli strains. To account for these diverse effects, Brock (1967) proposed that novobiocin acted by sequestering magnesium ions. This hypothesis attempted to explain the effects of novobiocin by pointing out that a magnesium requirement was common to all the processes inhibited by novobiocin. It was also consistent with the antagonistic effect of magnesium on some effects of novobiocin, especially cell killing (Brock, 1967; Morris and Russel, 1969).

Evidence for a more specific effect of novobiocin was obtained by Smith and Davis (1967) who showed that in E.coli novobiocin primarily inhibited DNA synthesis and to a lesser extent RNA synthesis. Protein and cell wall syntheses were even less affected. Since nucleotide triphosphate synthesis and energy metabolism were not affected and DNA degradation was not observed, Smith and Davis concluded that novobiocin inhibited nucleic acid synthesis by direct action on the template-polymerase complexes. Michaeli et al. (1971) compared the mode of action of coumermycin A<sub>1</sub> with novobiocin in S. aureus. Coumermycin at 2 µg/ml rapidly inhibited both DNA synthesis and to a lesser extent RNA synthesis in a manner similar to novobiocin at 20 µg/ml. Although coumermycin treatment also caused the accumulation of nucleotide-bound precursors in a manner similar to penicillin, Michaeli et al. concluded that this was a secondary effect of the antibiotic. Ryan (1976) found

that coumermycin inhibited both DNA and RNA synthesis although once again RNA synthesis was less sensitive to the drug.

The hypothesis that novobiocin and coumermycin act primarily by inhibiting nucleic acid synthesis was strengthened by studies utilising both toluene-treated cells and cell extracts to study macromolecular synthesis. Staudenbauer (1975) found that novobiocin at 0.5  $\mu\text{g/ml}$  reduced ATP-dependent DNA synthesis in toluene-treated E.coli to 50% of the control value after 30 minutes. In contrast, ATP-independent DNA synthesis was not inhibited even at 100  $\mu\text{g/ml}$ . In this study novobiocin at 200  $\mu\text{g/ml}$  had no effect on RNA synthesis and only a slight inhibitory effect on protein synthesis. Under similar conditions Ryan and Wells (1976) found that DNA and RNA synthesis were 50% inhibited after 45 minutes by 0.035 and 0.6  $\mu\text{g/ml}$  coumermycin respectively. In crude cell extracts, replication of ColE1 plasmid DNA was reduced to 2% of the control level with 1  $\mu\text{g/ml}$  novobiocin (Staudenbauer, 1976a).

The target for novobiocin and coumermycin was finally identified by Gellert et al. (1976b). These workers had previously isolated and characterised a novel enzyme from E.coli termed DNA gyrase which catalysed the introduction of negative superhelical turns into covalently closed circular DNA molecules in vitro (Gellert et al., 1976a). This enzyme was found to be inhibited by 0.3  $\mu\text{g/ml}$  novobiocin in vitro while gyrase from a novobiocin resistant strain was insensitive to the antibiotic. A full discussion of DNA gyrase is presented in part III.

### III. FACTORS AFFECTING THE TERTIARY STRUCTURE OF DNA

#### III.(a) The Origin of Superhelical Turns in DNA

The intracellular organisation of DNA is thought to differ from a simple double-helical structure in a number of ways. Firstly, while still adopting the Watson-Crick double-helix, the DNA exists in the cell in a tightly compacted form termed the tertiary structure. It is reasonable to assume that the formation of this structure is an ordered process leading to defined tertiary forms of the DNA. Secondly, the DNA in vivo is rarely static but is constantly changing its form as a result of replication, transcription and recombination, all of which require the DNA to undergo strand separation. In this section I will define the basis of superhelicity and describe some activities that alter DNA conformation including enzymes which increase or decrease the superhelical density of DNA.

The most common form of DNA tertiary structure is the superhelix. DNA is defined as superhelical if the axis of the duplex follows a helical path in space, i.e. if the double-helix itself is wound into a helical form. Supercoiled DNA might arise by two different mechanisms. (a) Superhelical turns may result from the association of DNA with proteins inside the cell. Thus in nucleosomes, closed circular DNA is under a torsional constraint which is relieved by the adoption of superhelical turns when the proteins are removed in the course of purification of the DNA (Germond et al., 1975). Proteins may be associated with either linear or circular DNA in vivo to produce such torsionally constrained DNA.

(b) Superhelical turns may be an intrinsic property of a closed circular DNA molecule; this kind of superhelix may be generated by enzymes (see later) or a change in environmental conditions, but is not due to the presence of bound protein. Any closed circular molecule, or domain of a larger molecule containing intact strands bound by barriers which prevent the free winding of the helix, may adopt this form of superhelix. An example of the latter is a topological domain of the E.coli folded chromosome (Worcel and Burgi, 1972).

A closed circular DNA is described by three parameters (Vinograd et al., 1968). (1) The topological winding number ( $\alpha$ ), also called the linking number, is the number of times one strand winds around the other when the closed circle is forced to lie in a plane. This parameter is invariant unless one or both strands of the DNA are broken. (2) The helix winding number ( $\beta$ ) is the number of times one strand winds around the other in the unconstrained molecule, and is dependent upon ionic strength, temperature and pH.  $\beta$  is also reduced by an unwinding ligand such as ethidium bromide (Bauer and Vinograd, 1968). The difference between  $\alpha$  and  $\beta$  is a measure of the superhelicity of a DNA molecule. If  $\alpha > \beta$  the DNA will contain an excess of helical turns and is said to be positively supercoiled; if  $\alpha < \beta$  the DNA is considered negatively supercoiled. If  $\alpha = \beta$  the DNA is relaxed. The third parameter,  $\gamma$ , is given by

$$\gamma = \alpha - \beta$$

and is thus a measure of the number of superhelical turns in the molecule.

Superhelicity is often expressed as superhelical density, ( $\sigma$ ), the number of superhelical turns per 10 base pairs. Virtually all naturally

occurring DNA circles, at least when deproteinised, exhibit a superhelical density of approximately  $-0.05$ , i.e. one negative superhelical turn per 200 base pairs (see for example Wang, 1974; Benyajati and Worcel, 1976). It should be emphasised that superhelical turns are maintained in the closed circular molecule by the forces imposed by the double-helical structure of the DNA (Vinograd *et al.*, 1965). The superhelical density of a closed circular DNA is thus altered by any changes in environmental conditions which alter  $\beta$  or by chemical or enzymic changes that involve changes in either  $\alpha$  or  $\beta$ . As stated earlier, ethidium bromide is known to intercalate between base pairs, the binding of each molecule unwinding the helix by  $26^\circ$  (Wang, 1974). Thus in a molecule containing superhelical turns, the addition of ethidium bromide will cause the duplex to unwind and, since the system is closed, the stress introduced into the molecule is counteracted by the adoption of a form with an altered superhelical density. Since the ethidium bromide causes the helix to unwind, the superhelical turns introduced are positive in an attempt to stabilise the Watson-Crick base pairing. This is illustrated in Figure 1.5. Closed circular forms of DNA can only bind a limited number of ethidium bromide molecules as their superhelical density cannot increase indefinitely. Nicked species, however, may continue to unwind their duplexes to bind dye (see Figure 1.5). Bauer and Vinograd (1968) found that the uptake of ethidium bromide ( $> 100 \mu\text{g/ml}$ ) by closed circular SV40 DNA ( $\gamma = -13$ ) was 65% of that taken up by the nicked form. This differential binding of ethidium bromide is exploited in the separation of covalently closed circular DNA from nicked circular or linear DNA (Radloff *et al.*, 1967). Ethidium


Figure 1.5

Effect of Ethidium Bromide on the Conformation of Circular  
Duplex DNA

This diagram illustrates the conformational changes in closed circular duplex DNA effected by addition of ethidium bromide.

The negatively supercoiled, closed circular duplex molecule (a) loses negative supercoils on binding of ethidium bromide to form the relaxed isomer (b). Upon further binding, a positively supercoiled molecule is formed (c).

The nicked circular duplex (d) binds ethidium bromide but can accommodate the intercalation without adopting a significantly altered tertiary structure (e).

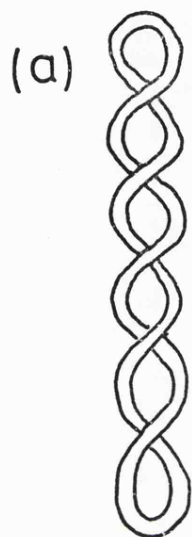
 = double stranded DNA

Et Br = ethidium bromide

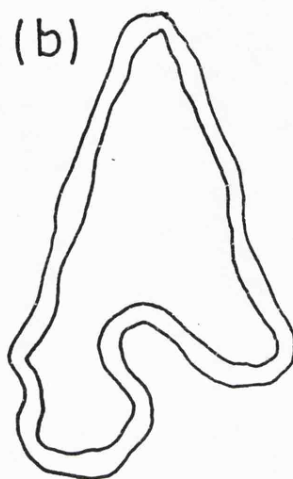
$\tau = -ve$

$\tau = 0$

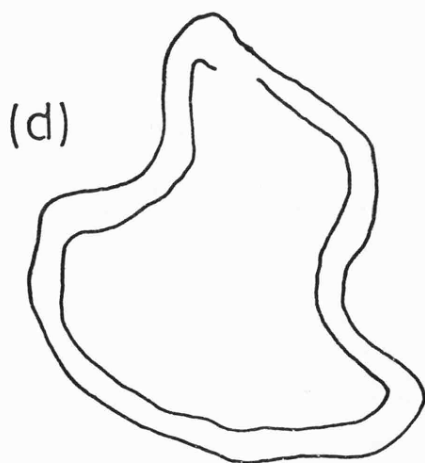
$\tau = +ve$



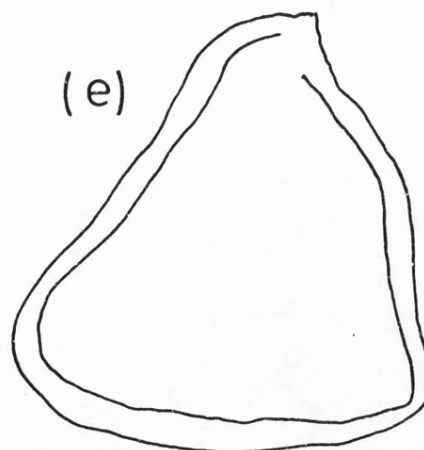
+Et Br  
→



+Et Br  
→



+Et Br  
→





bromide can also be used to determine the superhelical density of a closed circular molecule by the analysis of the sedimentation coefficient of the DNA in the presence of varying concentrations of the dye (Bauer and Vinograd, 1968).

There is a positive free energy ( $\Delta G$ ) associated with both negative and positive superhelicity, although the value of  $\Delta G$  per supercoil is far greater in the former (Bauer and Vinograd, 1970). The free energy associated with a negative superhelix may be used in any reaction that will reduce  $\beta$ , i.e. to unwind the helix. In the next three sections I will review the activities of proteins which affect the conformation of DNA, including those enzymes which alter the superhelical density of circular DNA molecules.

### III.(b) DNA Binding Proteins and Unwinding Enzymes

#### (i) Helix Destabilising Proteins

The helix destabilising proteins (HD - proteins) are by definition proteins that bind tightly and preferentially to single-stranded DNA (Alberts and Sternglanz, 1977). These proteins have also been termed DNA unwinding proteins, DNA melting proteins and DNA binding proteins. The prototype for this class of protein is the T4 gene 32-protein or T4 HD-protein. This protein, of molecular weight 35,000, lowers the melting temperature of double-stranded DNA by binding cooperatively to single-stranded regions (Alberts and Frey, 1970). Delius et al. (1972) visualised T4 HD-protein induced denaturation of  $\lambda$  DNA by electron microscopy and showed that the protein preferentially invaded A-T rich

regions within the molecule. Genetic analysis of gene 32 mutants has shown that the protein is essential for normal DNA replication, transcription and recombination in T4 (Kozinski and Felgenhauer, 1967; Tomizawa et al., 1966).

A protein with very similar properties has been isolated from uninfected E.coli by Sigal et al. (1972). Although correctly termed E.coli HD-protein, this protein is often referred to as E.coli DNA binding protein. The functional protein is a tetramer, with a subunit size of 20,000 daltons, which like T4 HD-protein lowers the melting temperature of duplex DNA and binds cooperatively to single-stranded DNA. Weiner et al. (1975) have shown that each tetramer could bind 32 nucleotides of single-stranded DNA but whereas the T4 HD-protein caused an extension in length, i.e. unfolding, of the single-stranded DNA (Delius et al., 1972) the E.coli HD-protein caused a 40% contraction in the length of such DNA (Sigal et al., 1972). This contradiction has not yet been resolved, but could reflect the differences in the substrates or conditions of the in vitro assays; alternatively it could reflect true differences in the functions of the proteins in vivo. As already mentioned, the E.coli HD-protein is required for efficient DNA synthesis in every in vitro replication system so far studied (see Wickner, 1978). Meyer et al. (1979) have isolated a temperature sensitive mutant of E.coli, designated ssb, which is defective in the E.coli HD-protein. The rate of DNA synthesis in the mutant is greatly reduced at 42°C, implying that the HD-protein is essential for elongation of replication forks in vivo. In addition the mutant will not support the growth of phage G4 at 42°C. HD-protein isolated from

this mutant is active in a reconstituted G4 DNA replication system in vitro at 30°C, but is inactive at 42°C with the result that DNA synthesis is reduced to less than 10% of that observed at the permissive temperature.

At this point it is useful to refer to a diagram illustrating the probable role of some of the proteins involved in DNA synthesis at the replication fork (see Figure 1.6). HD-proteins appear essential for replication; they are probably involved in maintaining localised regions of single-stranded DNA at the replication forks, but presumably are not the proteins responsible for separation of the duplex strands. For this activity, we look to the DNA unwinding enzymes.

#### (ii) DNA Unwinding Enzymes

Two enzymes have been isolated from E.coli which cause the separation of duplex DNA into single strands. The first, a 180,000 dalton protein termed DNA unwinding enzyme, was purified by Abdel-Monem and Hoffman-Berling (1976) and is a single-stranded DNA-dependent ATPase which will unwind long strands of DNA-DNA or RNA-DNA duplexes (Abdel-Monem et al., 1976).




Thus the enzyme derives the energy necessary for the unwinding of the duplex from the hydrolysis of ATP. This enzyme will not unwind linear duplexes with flush ends suggesting that an initial binding step to single-stranded DNA is a prerequisite for the reaction. Since the complete unwinding of a 6000 base pair region required 85 enzyme molecules, Abdel-Monem et al. (1976) suggested that many enzyme molecules are bound to single-stranded regions of the DNA to prevent renaturation, and that the enzyme molecules migrate along the single-stranded DNA (at the expense of ATP) to cause strand separation (see later). No mutants

## Figure 1.6

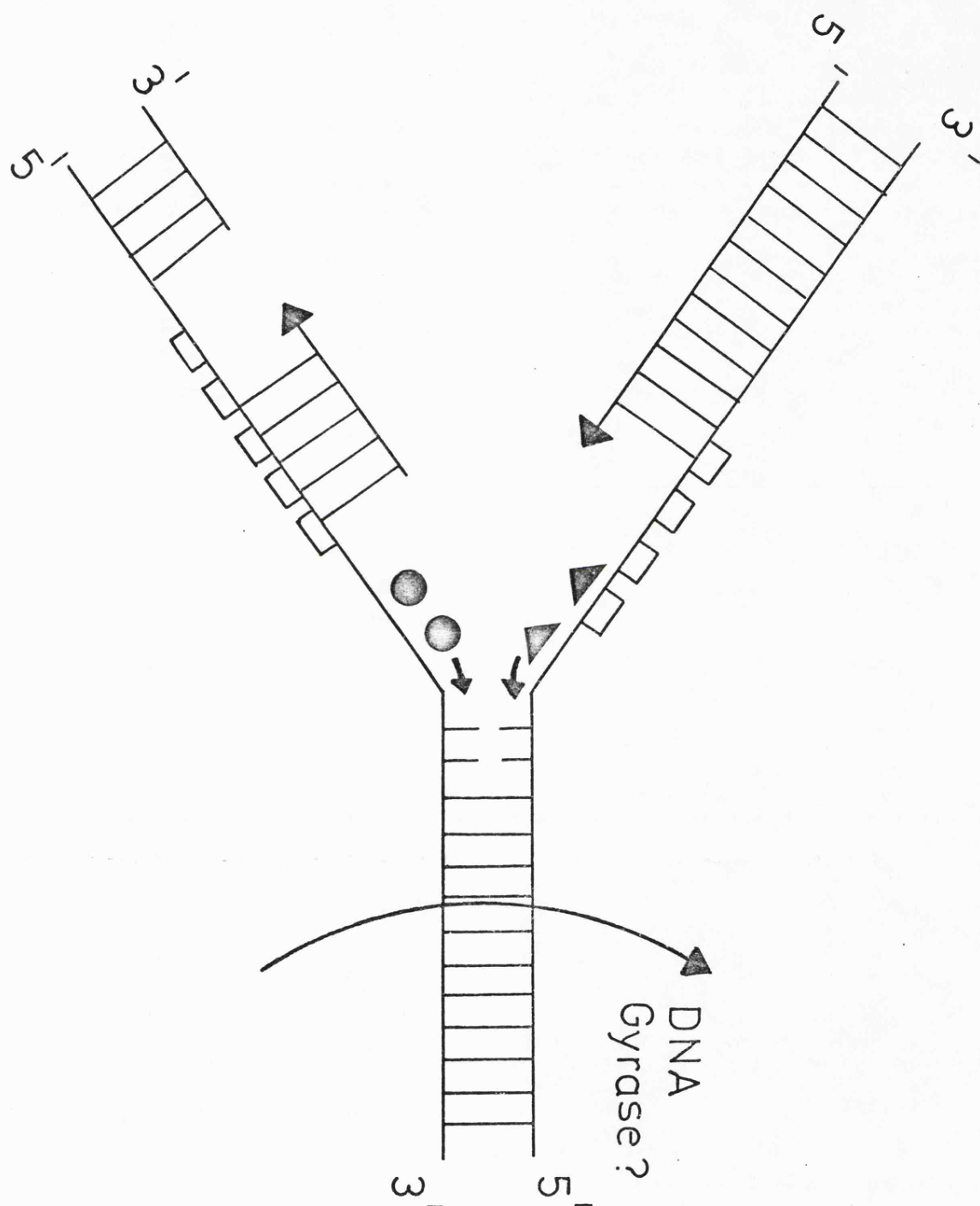
### The E.coli DNA Replication Fork : Possible Roles for Some DNA

#### Binding and Unwinding Proteins

This figure represents a partially replicated DNA molecule and indicates the possible location of some proteins involved in helix unwinding. The DNA unwinding enzyme and the rep protein are assigned to the lagging and leading strands respectively (for explanation, see text). DNA gyrase is assigned to the DNA in front of the replication fork, but this is only based on an assumed role in the generation of compensatory negative supertwists. See Chapter 8 for further discussion on this point.

-  DNA Binding Protein or E.coli HD-Protein
-  DNA Unwinding Enzyme (Abdel-Monem et al., 1976)
-  Rep Protein

Modified from Alberts and Sternglanz (1977)



defective in E.coli unwinding enzyme have been identified, and no in vitro DNA synthesis reaction has been shown to require this enzyme. The second DNA-unwinding activity to be discovered was that of the rep protein following the isolation by Denhardt et al. (1967) of an E.coli mutant which failed to support the replication of  $\phi$ X174 RFI DNA. As mentioned earlier,  $\phi$ X174 RFI DNA replication in vitro requires an active rep protein and this facilitated the purification of the rep protein by a complementation assay (Eisenberg et al., 1976). The rep protein is a 68,000 dalton ATPase which, together with  $\phi$ X174 cisA protein, E.coli HD-protein (DNA binding protein) and DNA polymerase III holoenzyme will allow duplex strand separation and viral strand synthesis in vitro. Scott et al. (1977) found that when DNA polymerase III holoenzyme was omitted from the reaction, the rep protein catalysed strand separation of the RFI to form single strands. This reaction was dependent upon ATP, DNA binding protein and the cisA protein. Thus in vitro the rep protein apparently unwinds the DNA duplex to facilitate polymerisation.

Yarranton and Gefter (1979) studied the rep protein catalysed unwinding of partial duplexes of  $\phi$ X174 DNA. These were constructed by annealing single-stranded DNA fragments (generated by restriction endonucleases) to full length linear  $\phi$ X174 DNA as shown in Figure 1.7. The two partial duplexes differed by the polarity of the strands with respect to the flush end of the larger duplex region. In the presence of DNA binding protein and ATP, the rep protein only catalysed the unwinding of the partial duplex which contained the 5' terminated free end in the centre of the molecule (structure B in Figure 1.7). Yarranton and

Figure 1.7


Activity of the Rep Protein on Partially Duplex DNA Molecules


This diagram indicates the inferred direction of movement of the Rep protein on DNA molecules as described by Yarranton and Gefter (1979). See text for further explanation.

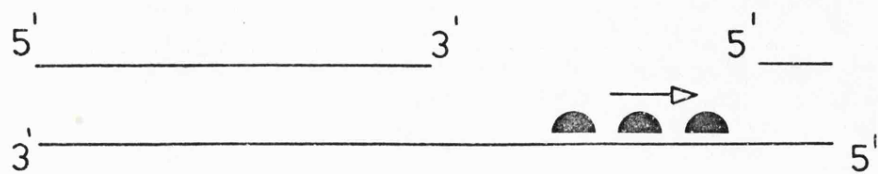
Structure A : Rep protein moves along the DNA in the 3' to 5' direction and does not cause significant unwinding of the duplex.

Structure B : Rep protein moves along the DNA in the 3' to 5' direction causing significant unwinding of the duplex.

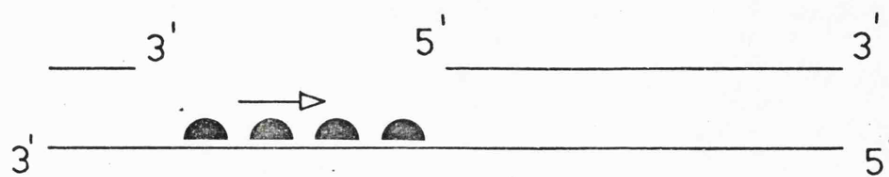
Structure C : Hypothetical structure of  $\phi$ X174 DNA during RF replication. The positions of Rep protein and DNA binding protein are consistent with the interpretation of movement of Rep as indicated in Structure B.

 = Rep protein

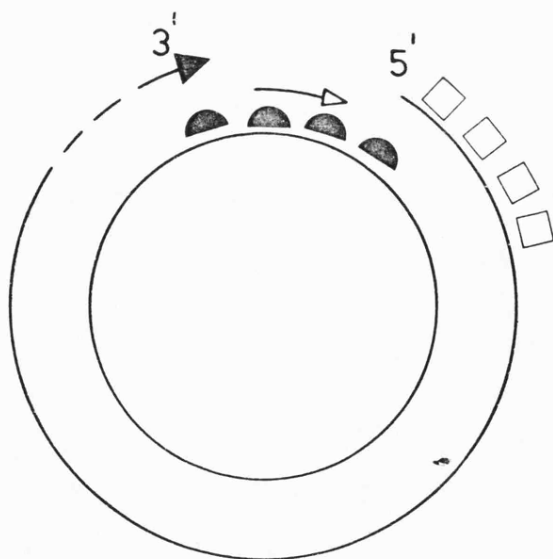
 = DNA binding protein



Structure A



Structure B



Structure C



Gefter concluded that the rep protein was able to bind to the single-stranded region of the partial duplex and translocate along in the 3' to 5' direction until the duplex was reached. The 5' end of the complementary strand was then displaced. This is in accordance with the structure of the cisA-mediated nicked  $\phi$ X174 DNA during replication (see Figure 1.7, structure C). This movement of the rep protein in the 3' to 5' direction is in apparent contrast with the direction of translocation of the E.coli DNA unwinding enzyme. Abdel-Monem et al. (1977) constructed partial duplexes similar to those of Yarranton and Gefter, and found that only those molecules containing a 3'OH in the centre of the duplex could act as substrates for the E.coli unwinding enzyme. Thus it is possible that two types of unwinding activity occur at the replication fork ; they could act simultaneously to unwind the duplex by translocating along strands of opposite polarity as visualised in Figure 1.6.

### (iii) Other DNA Binding Proteins

E.coli RNA polymerase is an enzyme which binds to DNA and causes a localised melting of the duplex prior to initiation of transcription. Estimates of the number of base pairs opened by RNA polymerase vary from 9-15 per molecule (see Siebenlist, 1979). Mangel and Chamberlin (1974) have shown that at 37°C, but not at 10-20°C, a stable complex is formed between RNA polymerase and T7 DNA which is capable of initiating RNA synthesis very rapidly upon addition of precursors. Hayashi and Hayashi (1971) found that the rate of transcription from similar complexes was greater when supercoiled  $\phi$ X174 DNA (RFI) was used in preference to the relaxed (RFII) form. Botchan et al. (1973) went on to show that the

initiation of transcription in vitro was more frequent from  $\lambda$  DNA templates which contained increasing numbers of negative superhelical turns. This in turn is due to the ease of melting supercoiled DNA (see earlier), perhaps in consequence of localised regions of unpaired double-helix (Dean and Lebowitz, 1971). It is possible that the degree of superhelicity of DNA in vivo is one way of regulating transcription, and this is borne out by changed levels of transcription as a result of inhibition of the supercoiling activity of DNA gyrase (see later and Discussion of this thesis).

As mentioned earlier, the recA protein catalyses the pairing of homologous single-stranded DNAs (Weinstock et al., 1979) and of superhelical DNA with complementary single-stranded DNA to form D-loops (Shibata et al., 1979). It is very unlikely that the recA protein is involved in DNA replication as strains containing deletions into at least part of the recA gene have been isolated (McEntee, 1977a). However, the recA protein is essential for generalised recombination. Its function in this process is probably to bind single-stranded DNA and to stabilise certain recombination intermediates, e.g. the D-loops of Shibata et al. However the winding of complementary single strands into a duplex by the recA protein implies that it may also function to keep strands together during recombination. Finally, the recA mediated formation of D-loops requires superhelical DNA; this is another example of the importance of the tertiary structure of DNA in recognition by proteins.

### III.(c) DNA Topoisomerases

DNA topoisomerases are enzymes that catalyse the increase or decrease in the superhelical density of a closed circular molecule. Since DNA subjected to topoisomerase activity remains in the altered conformation after the topoisomerase is removed, these enzymes must alter the topological winding number ( $\alpha$ ) rather than the helix winding number ( $\beta$ ). The only way  $\alpha$  can be changed is to break one strand, wind one end of it relative to the helix axis, and reseal the break. Thus DNA topoisomerases by definition must contain a nicking-closing activity and also, if necessary, an activity which will drive this helical winding or unwinding.

#### (i) E.coli $\omega$ Protein

The first enzyme with the capacity to relax closed circular superhelical DNA was discovered by Wang (1971) and termed the  $\omega$  protein. This enzyme, of molecular weight 110,000, reduces the number of negative supercoils in a closed circular molecule although it does not totally relax the DNA.  $\omega$  protein binds strongly to single-stranded DNA, will not relax positively supercoiled DNA and does not require ATP. Wang (1971) suggested a model for  $\omega$  activity : the protein binds to a single-stranded region of DNA in a negatively supercoiled molecule, nicks one or both strands and remains bound to one end of a free strand. The free energy in the molecule allows rotation around the helical axis (to increase  $\alpha$ ) and the energy stored in the  $\omega$ -DNA bond is used to reseal the break. Depew et al. (1978) found that a complex was formed between  $\omega$  protein and single-stranded circular fd phage DNA. Moreover, when

treated with alkali the DNA was found to be cleaved and the protein bound, presumably covalently, to the 5' end of the DNA. Examination by electron microscopy revealed that at low protein to DNA ratios the DNA was cleaved once, but at higher ratios the DNA was cleaved at several sites. No sequence dependence was evident and the protein could not be visualised by this procedure. However, no rigorous proof of nicked intermediates in double-stranded circular molecules has been reported. No mutants have been reported with a defective  $\omega$  protein and  $\omega$  is not required in any in vitro DNA replication system so far described.

(ii) Eukaryotic DNA Topoisomerases

A number of eukaryotic enzymes have been isolated which will relax supercoiled DNA. These enzymes are found in nuclei associated with chromatin and appear to be single polypeptides of molecular weight 60-70,000 (see Champoux, 1978). Some sources of these enzymes are rat liver, mouse embryo, calf thymus, HeLa cells and chicken erythrocytes. The eukaryotic DNA topoisomerases share some properties which distinguish them from the E.coli  $\omega$  protein. (1) The eukaryotic enzymes are maximally active in 0.15 - 0.2 M salt and in the absence of magnesium, whereas maximum  $\omega$  activity is achieved in 1 mM magnesium in the absence of salt (see Champoux, 1978). (2) The eukaryotic enzymes will completely relax both positively and negatively supercoiled DNA (Champoux and Dulbecco, 1972; Vosberg et al., 1975).

Eukaryotic topoisomerases, like  $\omega$ , do not require ATP and a mechanism of action similar to that proposed for the E.coli enzyme has been independently suggested by Champoux and Dulbecco (1972). Some

support for this model involving the formation of a protein-DNA bond which is maintained during the reaction has been obtained by Champoux (1976). When rat liver enzyme was incubated with negatively supercoiled SV40 DNA in a buffer containing a low salt concentration to slow down the reaction, a nicked DNA intermediate was detected on alkaline sucrose gradients. This intermediate disappeared if the reaction was allowed to proceed to completion at high salt concentrations. Champoux (1977) went on to demonstrate that this intermediate contained a protein, presumed to be the untwisting enzyme, bound to the 3'-phosphoryl termini of the DNA molecules.

In all the DNA topoisomerases studied, the reaction with closed circular DNA was shown to be both catalytic and step-wise. The latter can be demonstrated by analysing the products of the reaction by agarose gel electrophoresis. Keller (1975) has shown that supercoiled DNA has a greater mobility than the relaxed (closed) form but that molecules with intermediate superhelicity run as discrete bands between these two forms. Thus the DNA topoisomerases must undergo cycles of nicking and closing, perhaps at different sites on the DNA molecule, to achieve relaxation.

### (iii) Bacteriophage DNA Topoisomerases

DNA relaxing enzymes are also produced by the bacteriophages  $\lambda$  and T4. Liu et al. (1979) and Stetler et al. (1979) found that the T4 genes 39, 52 and 60 coded for a multi-subunit enzyme which would relax negatively or positively supercoiled PM2 DNA molecules. Amber mutants in any of these genes results in the delayed initiation of T4 DNA replication upon infection of non-suppressor strains; such mutants are

termed "DNA-delay" mutants (McCarthy et al., 1976). McCarthy (1979) found that although normal T4 DNA replication was insensitive to inhibitors of DNA gyrase, DNA replication in the DNA-delay mutants was sensitive, suggesting that under these conditions gyrase can substitute for the defective phage enzyme complex. This suggests that DNA gyrase may carry out a relaxation reaction in vivo; this will be discussed later when the activities of gyrase are reviewed.

Kikuchi and Nash (1979) have shown that there is a nicking-closing activity associated with the  $\lambda$  int gene product. This protein is required for both the integration and excision reactions between  $\lambda$  DNA and the host chromosome (Echols, 1970). Integrative recombination can be carried out in vitro using one  $\lambda$  DNA molecule containing both the bacterial and phage DNA sequences that are necessary for recombination (Mizuuchi and Nash, 1976). Kikuchi and Nash (1978) found this reaction required only the  $\lambda$  int protein, supercoiled circular substrate and some unidentified bacterial proteins. When purified  $\lambda$  int protein was incubated in the reaction mixture without the bacterial extract, it catalysed the removal of negative supercoils from the substrate DNA without promoting recombination (Kikuchi and Nash, 1979). The int protein was shown to relax both positively and negatively supercoiled DNA and, unlike the T4 enzyme, did not require ATP.

The int protein may nick and reseal the DNA substrate(s) in the course of integration and excision reactions, allowing rapid exchange of  $\lambda$  and chromosomal DNA. Since integrative recombination does not require ATP in vitro, it is possible that the  $\lambda$  int protein may catalyse strand breakage and reunion in a manner similar to that proposed

for the E.coli  $\omega$  protein and the eukaryotic enzymes; i.e. an int protein-DNA bond could conserve the energy necessary for resealing the DNA scission whilst DNA strands rotate about their helical axis.

It should be emphasised here that a DNA relaxing enzyme need not function in vivo to remove supertwists. Rather the DNA topoisomerase activity of these enzymes in vitro could simply reflect a nicking and resealing activity in vivo. This may or may not be accompanied by rotation of the helix in vivo to alter the superhelicity of the DNA.

### III.(d) DNA Gyrase

#### (i) Characterisation of the Enzyme

As outlined previously, Mizuuchi, Gellert and Nash demonstrated that supercoiled  $\lambda$  DNA was required for in vitro integrative recombination; relaxed closed circular  $\lambda$  DNA was inactive as a substrate unless first incubated with ATP plus an E.coli cell extract (Gellert et al., 1976a; Mizuuchi et al., 1979a). This cell fraction was subsequently shown to contain an enzymic activity, termed DNA gyrase, which introduced negative supercoils into relaxed closed circular DNA e.g.  $\lambda$ , ColE1 or SV40 DNAs (Gellert et al., 1976a). DNA gyrase is conveniently assayed by the method of Keller (1975) using agarose gel electrophoresis as described in the previous section. Because gyrase is removed prior to examination of the supercoiled products, it must act by altering the linking number ( $\alpha$ ) of the DNA, and thus it is termed a DNA topoisomerase (Liu and Wang, 1978a). Gellert et al. (1976a) found that gyrase activity required ATP and magnesium, and that the super-

coiling reaction proceeded in a series of steps as judged by the presence of many molecules of intermediate superhelical density amongst the reaction products.

The supercoiling activity catalysed by DNA gyrase is blocked by two groups of antibiotics represented by novobiocin and coumermycin A<sub>1</sub> (Gellert *et al.*, 1976b; see part II), and nalidixic acid and oxolinic acid (Sugino *et al.*, 1977; Gellert *et al.*, 1977). These compounds inhibit DNA synthesis in *E.coli* (see part II and Chapter 3) and resistance to their activities map at separate loci; nalA at 48 minutes (resistance to nalidixic acid and oxolinic acid (Hane and Wood, 1969)) and cou at 82 minutes (resistance to novobiocin and coumermycin (Ryan, 1976)).

Exhaustive purification of DNA gyrase by Mizuuchi *et al.* (1978a) and of its individual subunits by Higgins *et al.* (1978) has shown that the enzyme is composed of two different subunits of molecular weights 100-110,000 and 90-95,000, coded by the nalA and cou genes respectively. Gyrase purified from a nalidixic acid resistant strain is insensitive to nalidixic acid and oxolinic acid, but is sensitive to novobiocin and coumermycin and vice versa (Sugino *et al.*, 1977; Gellert *et al.*, 1977). Cloning of the nalA gene on a lambda transducing phage has confirmed the nalA protein to be a 110,000 dalton polypeptide (Kreuzer *et al.*, 1978). Similarly, the cou gene has been cloned using  $\lambda$ , and has been shown to code for a 92-95,000 dalton protein (Orr *et al.*, 1979; Hansen and von Meyenburg, 1979; this thesis). The purification and reconstitution studies indicated above showed that the native gyrase enzyme is most likely a tetramer consisting of two molecules of each subunit. However,



it should be noted that the existence of other subunits, although unlikely, has not yet been ruled out.

## (ii) Activities of DNA Gyrase

In addition to the introduction of supercoils, DNA gyrase has other activities which may be divided into three reactions. All four reactions are now known to require equimolar amounts of both subunits (Higgins et al., 1978) in contrast to earlier studies with partially purified nalA protein (Sugino et al., 1977). Gellert et al. (1977) and Sugino et al. (1977) found that in the absence of ATP, gyrase would relax both positively or negatively supercoiled DNA. This activity is inhibited by nalidixic acid and oxolinic acid, but not by novobiocin or coumermycin. This suggested that the nicking closing activity resided in the nalA subunit, but that the presence, although not the activity, of the cou subunit was required for its functioning. In this context, it is interesting to note that the nalA protein and the E.coli  $\omega$  protein have apparently identical molecular weights on SDS-polyacrylamide gel electrophoresis. However, Sugino et al. and Gellert et al. found that  $\omega$  activity was not inhibited by nalidixic acid and that gyrase catalysed relaxation was unaffected by antiserum to the  $\omega$  protein.

The addition of nalidixic acid or oxolinic acid to a gyrase-catalysed relaxation reaction does not simply inhibit gyrase activity. If the reaction products are subsequently treated with 1% SDS followed by proteinase K, the circular DNA molecule is cleaved and co-migrates with the linear form of the DNA (Sugino et al., 1977; Gellert et al., 1977). Peebles et al. (1979) have shown that the omission of proteinase K allows cleavage, although the DNA product has a reduced mobility due

to the presence of protein attached to the DNA. This study also showed that circular DNA was not the only substrate for gyrase-induced cleavage; linear ColE1 DNA could be cleaved in the reaction resulting in two main fragments, both of which had protein bound to them as judged by their reduced mobility in the absence of proteinase K digestion. This gyrase-induced cleavage of double-stranded DNA is insensitive to novobiocin and coumermycin (Gellert et al., 1977; Sugino et al., 1977) and may reflect the cleavage of DNA by the nalA subunit during the course of relaxation of supercoiling.

Peebles et al. (1979) found that each linear DNA species was not always cleaved at one unique site, but that each DNA contained a few primary sites and several secondary sites. Morrison and Cozzarelli (1979) examined the cleavage reaction in detail using  $\phi$ X174 RFI DNA and found that the resulting linear fragments were not substrates for T4 polynucleotide kinase, even after proteinase K treatment. This implies that the 5' ends of polynucleotide chains generated by gyrase cleavage are blocked. However, this DNA did provide a template-primer for E.coli DNA polymerase I, showing that gyrase creates a staggered break with a recessed 3' hydroxyl group. Morrison and Cozzarelli mapped the primary gyrase cleavage sites in  $\phi$ X174 and determined their nucleotide sequence. The sequences are not unique as in the case of restriction endonucleases, however considerable similarities between different sites do exist. This will not be discussed further.

The DNA-protein complex which is seen after SDS induced cleavage of oxolinic acid inhibited gyrase reactions is clearly analogous to the relaxation complexes of many plasmids (see Helinski et al., 1975).

When ColE1 or R6-K is isolated in the superhelical form, between 20 and 80% of the molecules are complexed with protein. In the case of ColE1 three proteins are attached to the DNA and after treatment with protein denaturing agents the DNA is cleaved in one strand and one of these proteins (molecular weight 60,000) is covalently attached to the 5' end of the free DNA (Lovett and Helinski, 1975). This cleavage site is site-specific (see Tomizawa et al., 1977) and occurs very close to, and perhaps at, the origin of transfer of this plasmid (Warren et al., 1978).

The principle difference between the gyrase-DNA complex and a plasmid relaxation complex or the  $\phi$ X174 RFII - CisA protein complex (see part I) is that the former involves double-stranded cleavage of the DNA. This need not imply a totally different mechanism of formation and cleavage; rather the similarities suggest that covalent attachment of a protein to a free end of a DNA molecule after nicking may be an important structural feature of a nicking-closing reaction.

The last activity of DNA gyrase I will discuss is the DNA-dependent hydrolysis of ATP yielding ADP and inorganic phosphate (Pi).

Sugino et al. (1978) observed that when ATP was added to the standard gyrase cleavage reaction with linear duplex ColE1 DNA, the two major bands diminished in intensity and two new bands appeared, corresponding to a change in position of the principle gyrase cleavage site. This alteration was also seen with App(NH)p, a non-hydrolyzable analogue of ATP, but in both cases the effect was blocked by novobiocin or coumermycin. This implies that novobiocin inhibits the binding of ATP to the gyrase-DNA complex, and it is this binding, and not the

hydrolysis of ATP, that effects the alteration in the cleavage site. In addition, novobiocin and coumermycin were shown to be competitive inhibitors of the ATPase activity of DNA gyrase using the standard supercoiling assay. Mizuuchi et al. (1978a) found that ATP bound specifically to the cou subunit of gyrase in the absence of novobiocin and that the novobiocin sensitive ATPase activity of gyrase was only effective on double-stranded relaxed or linear DNA. Finally, Sugino et al. (1978) noted that DNA gyrase did not turn-over, i.e. act catalytically in the supercoiling reaction when incubated with App(NH)p instead of ATP; the number of supercoils introduced into each DNA molecule increased only with increasing enzyme concentration.

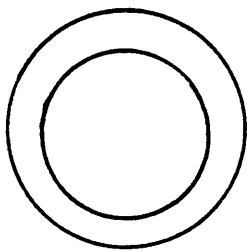
### (iii) A Model for DNA Gyrase Activity

The above reactions may now be compiled to form the basis of a model for the introduction of supercoils into DNA by DNA gyrase. The model presented is based upon that of Sugino et al. (1978) and is shown diagrammatically in Figure 1.8. In step 1, the two subunits of gyrase (E) bind to covalently closed, relaxed circular duplex DNA. A gyrase-DNA complex has in fact been demonstrated by Peebles et al. (1979); it is stable to dilution but is broken down by SDS or heating to 70°C. In step 2, ATP binds to the cou subunit of gyrase. This causes a conformational change in the enzyme (E) to form E and leads to a movement of the DNA relative to the enzyme. It is this movement which is reflected in the alteration of gyrase cleavage sites as discussed above. Various models have been proposed to explain how this movement results in the introduction of supercoils (Sugino et al., 1978; Liu and Wang, 1978a,b; Mizuuchi et al., 1978a; Peebles et al., 1979). These models

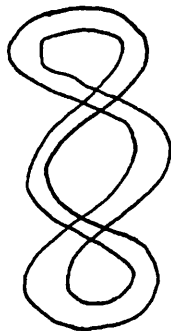
Figure 1.8

A Model for DNA Gyrase Activity

E and E = DNA Gyrase



Relaxed Double Stranded  
Covalently Closed DNA



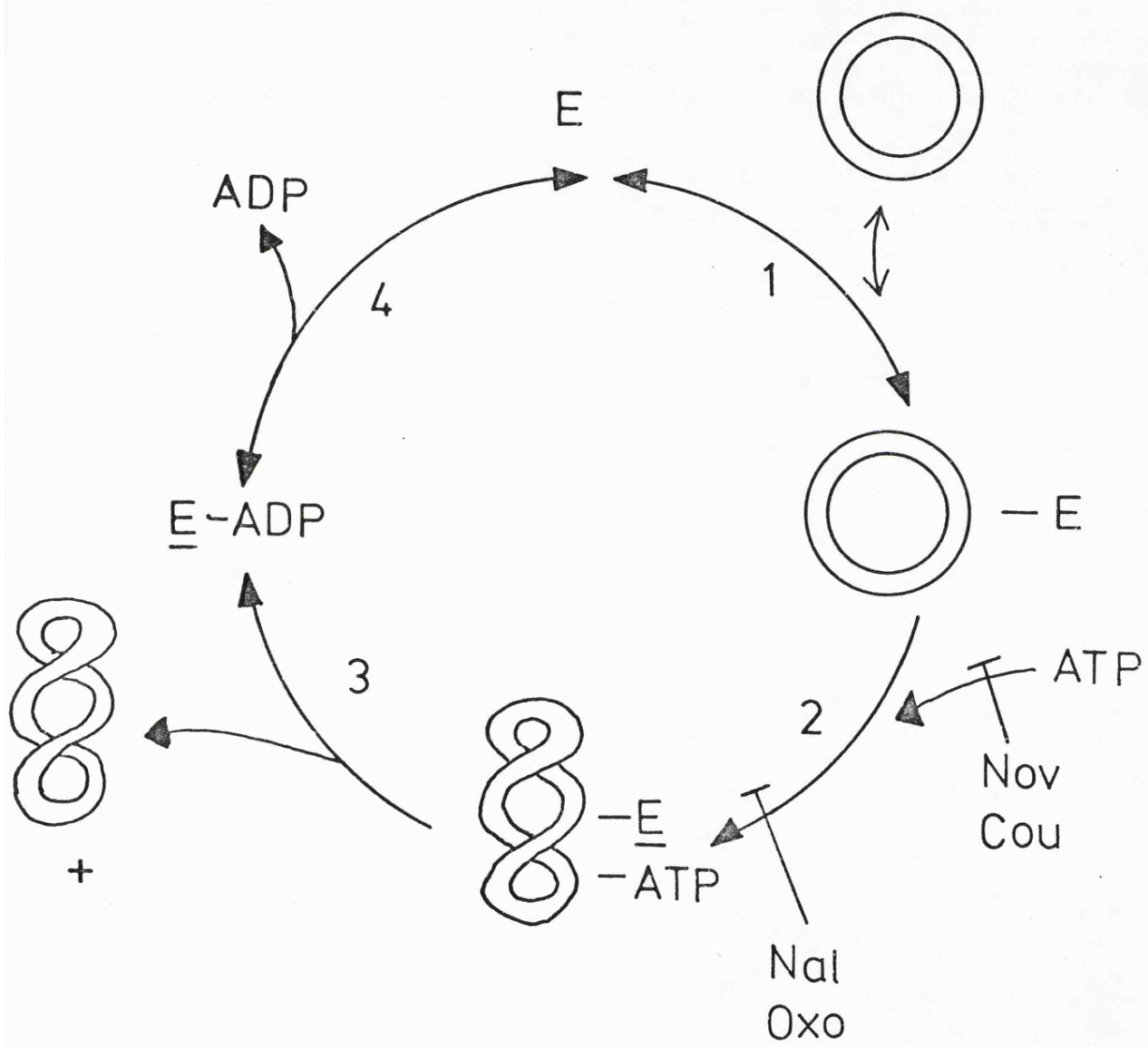
Supercoiled Double Stranded  
Covalently Closed DNA

Nov = Novobiocin

Oxo = Oxolinic Acid

Cou = Coumermycin

For explanation see text



will not be discussed in detail, but all include the common feature of the generation of both positively and negatively supercoiled domains in the DNA, and the subsequent relaxation of the positive supercoils by the nalA protein activity. In the third step, the DNA is dissociated from the complex accompanied by hydrolysis of ATP to yield E-ADP and Pi. This step is irreversible and gives direction to the reaction. In the final step, E-ADP is dissociated to ADP and E.

The relaxation of positive and negative supercoils can be explained by gyrase (E) binding to the supercoiled DNA. In the absence of ATP, no domains of supercoiling are created and the nalA protein nicks and reseals both negative and positive supercoils. The supercoiling and relaxation reactions are catalytic because the enzyme is released after one (or many) cycles and can therefore catalyse an identical reaction on other DNA molecules. The reactions are progressive because one cycle of events as outlined in Figure 1.8 only produces a limited number of supercoils per DNA molecule.

### III.(d) The Function of DNA Gyrase In Vivo

We have seen that DNA gyrase catalyses the supercoiling or relaxation of closed circular duplex DNA in vitro. Does gyrase have a similar role in vivo? To answer this question it is necessary to examine the tertiary structure of DNA in vivo.

#### (i) Eukaryotic DNA

The chromosome of simian virus 40 (SV40), whether isolated from virions or intact cells, is a circular DNA molecule. At intervals around

the molecule the DNA is condensed with histones to form nucleosomes (Huang et al., 1972; Griffith, 1975), discrete particles containing DNA apparently coiled around the histones. These nucleosomes are connected by naked DNA, and so the chromosome takes on the appearance of a "string of beads". The DNA in this form is under a constraint as it is compacted 5-7 fold in length, and when isolated and deproteinised exhibits a superhelical density of -0.05.

Germond et al. (1975, 1979) reconstituted SV40 chromatin from purified relaxed SV40 DNA (containing no superhelical turns) and a mixture of histones. The resulting complexes, which took on the appearance of normal nucleosomes as judged by electron microscopy, were treated with an untwisting enzyme to remove any supertwists in the DNA, but left unaltered any structural changes imposed upon the DNA by the association with histones. After deproteinisation, the DNA was found to contain negative supercoils in proportion to the amount of histones initially added. When DNA and histones were added in the proportions seen in nucleosomes isolated from virions, the superhelicity of the DNA after treatment was the same as that of the DNA in virions. From these studies it can be concluded that the DNA in the nucleosomes is not under winding strain (i.e. it cannot be relaxed by untwisting enzymes) and that the superhelical turns observed after deproteinisation are all due to the previous winding of the DNA around the histones.

It would be reasonable then to conclude that a eukaryotic cell does not contain a supercoiling enzyme e.g. DNA gyrase, since free negative supercoils do not appear to exist in the cell. However, Mattern and Painter (1979) measured DNA synthesis in both intact and permeabilised



Chinese hamster ovary cells in the presence of novobiocin, and found that DNA synthesis was reduced by 50% by 200  $\mu\text{g/ml}$  in intact cells. In contrast the same level of inhibition required 200  $\text{mg/ml}$  in permeabilised cells. Since initiation of DNA replication does not occur in permeabilised cells, Mattern and Painter concluded that novobiocin primarily inhibited initiation. In addition, centrifugation of chromatin from nucleoids of novobiocin treated cells in ethidium bromide-caesium chloride gradients revealed that the antibiotic reduced the superhelical density of the DNA. This suggests that a supercoiling enzyme may exist within the cell, and that it may act in a similar way to bacterial DNA gyrase. Further work is necessary to resolve this apparent contradiction to the findings with the SV40 studies.

(ii) Prokaryotic DNA

It is now generally accepted that the DNA within bacteria is negatively supercoiled and is not condensed with significant amounts of histone-like proteins. In this section I will discuss some of the evidence for this conclusion and in support of a role for DNA gyrase in introducing and maintaining the DNA in this underwound state.

Plasmid DNA can be isolated from bacteria without deproteinisation, and is found to be in a negatively supercoiled form. Timmis et al. (1976) studied the replication of the small plasmid pSC101 by pulse labelling cells with  $[^3\text{H}]$ -thymidine followed by varying chase periods. The buoyant density of the plasmid population was then determined by centrifugation to equilibrium in caesium chloride and ethidium bromide. After a short chase, a band corresponding to the closed circular relaxed form was seen; this band appeared concomitantly with the

negatively supercoiled form and disappeared at the same time as the peaks of intermediate density. This fully relaxed form of the DNA was concluded to be the end product of plasmid replication, which was only subsequently converted to the supercoiled form. A similar conclusion was reached by Crosa et al. (1976) using the plasmid RSF1040.

A direct demonstration of the role of DNA gyrase in vivo was obtained by Gellert et al. (1976b) who determined the form of  $\lambda$  DNA after superinfection of an E.coli  $\lambda$  lysogen (there is no replication of the infecting  $\lambda$  under these conditions). In the absence of coumermycin, the  $\lambda$  DNA, which immediately circularises upon infection, was recovered in a negatively supercoiled form. If coumermycin was present during infection, the  $\lambda$  DNA was isolated in a relaxed form containing only 15% of the normal superhelical density. Nalidixic acid also reduced the superhelical density of  $\lambda$  DNA under similar conditions (Gellert et al., 1977). These experiments demonstrate that extrachromosomal DNA in E.coli is actively supercoiled, most probably by DNA gyrase.

As previously stated, E.coli cells can be gently lysed to yield a "folded chromosome" containing DNA, RNA and protein. Worcel and Burgi (1972) found that this chromosomal DNA behaved as a single covalently closed circular molecule when titrated with ethidium bromide, with a superhelical density very similar to other cyclic DNAs from E.coli. However, Worcel and Burgi found that the introduction of between 12 and 80 single-strand nicks was required to fully relax this DNA, and concluded that the chromosome was organised into approximately 50 independent supercoiled loops or domains. Pettijohn and Hecht (1973)

found that when cells were pretreated with rifampicin, which inhibits RNA synthesis, the sedimentation coefficient of the folded chromosomes was greatly reduced while the superhelical density remained unchanged. However, the number of domains of supercoiling appeared to decrease under these conditions. The folded chromosome of E.coli may then be visualised as a single circular DNA duplex divided into many superhelical domains which are somehow maintained by PNA molecules to form a condensed or folded structure.

Drlica and Snyder (1978) showed that the addition of coumermycin (50 µg/ml) to cells 35 minutes prior to isolation of the folded chromosomes resulted in a 70-75% decrease in the superhelical density of the DNA. The DNA was still covalently closed and, interestingly, the sedimentation coefficient both in the presence and absence of ethidium bromide was reduced. Thus inhibition of DNA gyrase not only inhibited supercoiling of the DNA, but also caused the chromosome to partially unwind, reminiscent of the effect of rifampicin.

#### IV. AIMS OF THE PRESENT STUDY

The work reviewed in this chapter has shown that DNA replication is a complex process, dependent upon many factors including the superhelicity of the DNA.

When this study was commenced the mode of action of novobiocin and coumermycin was not fully understood although it appeared probable that they inhibited DNA synthesis. I set out therefore to study this inhibition using the closely related antibiotic clorobiocin. This goal was made simpler by the subsequent discovery of DNA gyrase and its inhibition by novobiocin and coumermycin. I have therefore characterised the activity of clorobiocin and have used this antibiotic to study the effects of changes in superhelicity of the DNA on the physiology of the E.coli cell.

## Chapter 2

### MATERIALS AND METHODS

#### 1. BACTERIAL STRAINS

The strains of E.coli used in many of the experiments were E.coli K12 LE234 and its derivatives (see Table 2.1). LE234 was constructed by Dr P. Meacock by mating AB2147 F<sup>-</sup> with KL25 Hfr supE and selecting strA his<sup>+</sup> recombinants. LE234 is a putative supE clone picked from such a cross. All other strains are listed in Table 2.1. Strains were maintained at 4°C on nutrient or M9 minimal agar plates or at -20°C in liquid medium containing 20% glycerol.

#### 2. CHEMICALS

All chemicals with the exception of those listed below were analytical grade, obtained from Fisons Ltd, Loughborough. Amino acids, thiamine, thymidine, uridine, dimethylaminobenzaldehyde, Trisma base, 2-mercaptoethanol, bovine serum albumin, chymotrypsinogen, lactoglobulin, D-galactonate and lysozyme were from Sigma Laboratories, London. Acrylamide and bis-acrylamide were from Eastman Kodak and were purified by extraction with activated charcoal before use. Electrophoresis grade ammonium persulphate, sodium dodecyl sulphate and Coomassie brilliant blue were obtained from Bio-Rad Laboratories Ltd. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) was from Nuclear Enterprises Ltd. Aquasol aqueous scintillation fluid was obtained from New England Nuclear. NCS tissue solubiliser was obtained from the Amersham/Searle Corporation. Radiochemicals were from the Radiochemical Centre, Amersham.

Table 2.1

Bacterial Strains

All strains are derivatives of E.coli K-12 except LEB18 and LEB18 rRM98 which are E.coli B/r substrain F.

The spontaneous chlorobiocin-resistant mutants of LE234 - LE316 and LE701 - are described in the text.

The Met<sup>+</sup> derivatives of LE234, LE316 and LE701 were prepared by transduction using P1 grown on C600.

The recA56 derivatives of LE234, LE316 and LE701 were prepared by conjugation of thyA derivatives with Hfr JC5088 as described in the text.

The points of origin of the Hfr strains are given in Figure 5.1 or in Low (1972).

Genetic nomenclature is that given by Bachmann et al. (1976).

| <u>Strain</u>        | <u>Genotype</u>  | <u>Reference or Source</u>       |
|----------------------|--|----------------------------------|
| A3                   | F <sup>-</sup> <u>thr</u> <u>leu</u> <u>thi</u> <u>lacY</u> <u>supE44</u> <u>dnaA46</u>  | Tresguerres <u>et al.</u> (1975) |
| AB2147               | F <sup>-</sup> <u>argH</u> <u>his</u> <u>metB</u> <u>ilv</u> <u>rpsL</u> <u>tsx</u> <u>tna</u><br><u>thi</u> <u>rpsL</u>                               | E. Orr                           |
| AB312                | Hfr <u>thr</u> <u>leu</u> <u>thi</u> <u>lacZ</u> <u>rpsL</u>   | Bachmann (1972)                  |
| C600                 | F <sup>-</sup> <u>thr</u> <u>leu</u> <u>thi</u> <u>supE44</u> <u>lacY</u> <u>tonA</u>  | Bachmann (1972)                  |
| C0101                | HfrC <u>thi</u> (?) <u>dgoD</u>  | Cooper (1978)                    |
| C0509( $\lambda^+$ ) | F <sup>-</sup> <u>metB</u> <u>dgoD</u> ( $\lambda^+$ )   | See Orr <u>et al.</u> (1979)     |
| D21                  | F <sup>-</sup> <u>trp</u> <u>proA</u> <u>his</u> <u>lac</u> <u>tsx</u> <u>rpsL</u><br><u>ampA</u> ( $\lambda^+$ )                                      | Boman and Monner<br>(1975)       |
| D21e7                | <u>lpsA</u> mutant of D21 with galactose-<br>deficient lipopolysaccharide (LPS)  | Boman and Monner<br>(1975)       |
| D21f1                | mutant of D21e7 with glucose-<br>deficient LPS   | Boman and Monner<br>(1975)       |
| D21f2                | mutant of D21e7 with heptose-<br>deficient LPS   | Boman and Monner<br>(1975)       |
| JE1011               | F <sup>-</sup> <u>thr</u> <u>leu</u> <u>trp</u> <u>his</u> <u>thy</u> <u>thi</u> <u>ara</u><br><u>lac</u> <u>gal</u> <u>xyl</u> <u>mtl</u> <u>rpsL</u> | Tamaki <u>et al.</u> (1971)      |
| NS2                  | novobiocin - supersensitive mutant<br>of JE1011  | Tamaki <u>et al.</u> (1971)      |

| <u>Strain</u> | <u>Genotype</u>  | <u>Reference or Source</u>                        |
|---------------|--|---|
| F'111         | JC1553/F'111 <u>metB</u> <u>argG</u> <u>leuB</u> <u>his</u><br><u>rpsL</u> <u>recA1</u> <u>tonA</u> <u>lacY</u> <u>xyl</u> <u>malA</u><br><u>supE44</u> <u>mtl</u> <sup>R</sup> F'111 carries<br><u>met</u> <sup>+</sup> <u>arg</u> <sup>+</sup> <u>ilv</u> <sup>+</sup> | Bachmann <u>et al.</u><br>(1976)<br>also see text |
| F'133         | JC1553/F'133 as JC1553/F'111 for<br>chromosomal markers. F'133 is<br>shorter than F'111 but carries<br><u>met</u> <sup>+</sup> <u>arg</u> <sup>+</sup> <u>ilv</u> <sup>+</sup>   | As above  |
| KL25          | F <sup>-</sup> <u>supE42</u>   | Bachmann (1972)                                   |
| LE234         | F <sup>-</sup> <u>ilv</u> <u>argH</u> <u>metB</u> <u>rpsL</u> <u>tna</u> <u>thi</u><br><u>xyl</u> <u>supE44</u> ?  | See text  |
| LEB18         | <u>E.coli</u> B/r F <sup>-</sup> <u>lacZ</u> <u>rpsL</u>   | Boyd (1979)                                       |
| LEB18         | LEB18 harbouring the plasmid   | Iyer <u>et al.</u> (1978)                         |
| rRM98         | RM98 Ap Sm Tc  |   |
| P10           | Hfr <u>thr</u> <u>leu</u> <u>thi</u> <u>supE44</u> <u>tonA</u> <u>malB</u>   | Bachmann (1972)                                   |
| W1895         | HfrC <u>metB</u> <u>rel</u>  | Ennis (1971)                                      |
| H101          | Synergistin A - sensitive mutant<br>of W1895   | Ennis (1971)                                      |



| Strain | Genotype   | Reference or Source           |
|--------|--|-------------------------------|
| LE705  | <u>recA56</u> derivative of LE234  | this study                    |
| LE706  | <u>recA56</u> derivative of LE701  | this study                    |
| LE707  | <u>recA56</u> derivative of LE316  | this study                    |
| LE747  | <u>met</u> <sup>+</sup> derivative of LE234  | this study                    |
| LE748  | <u>met</u> <sup>+</sup> derivative of LE701  | this study                    |
| LE746  | <u>met</u> <sup>+</sup> derivative of LE316  | this study                    |
| 833    | <u>lacZ</u> <u>his</u> <u>rpsL</u> <u>tonA</u> <u>tsx</u><br>∇( <u>trpED</u> <u>attλ</u> <u>gal</u> )  | W.J. Brammer                  |
| 159(λ) | <u>gal</u> <u>uvrA6</u> (λ <u>ind</u> <sup>-</sup> )   | W.J. Brammer                  |
| P400   | F <sup>-</sup> <u>argE</u> <u>proA</u> <u>thr</u> <u>leu</u> <u>lacY</u> <u>mtl</u> <u>xyl</u><br><u>galK</u> <u>supE</u> <u>rpsL</u> <u>thi</u> | Manning and Reeves<br>(1978)  |
| P407   | <u>tsx</u> mutant of P400  | Manning and Reeves<br>(1978)  |
| JC5088 | Hfr <u>thi</u> <u>rel</u> <u>thr</u> <u>ilv</u> <u>spc</u> <u>recA56</u>   | Bachmann <u>et al.</u> (1976) |
| D22    | <u>envA</u> mutant of D21  | Normark <u>et al.</u> (1969)  |
| Yme1   | <u>mel</u> <u>supE</u> <u>supF</u> <u>tonA</u> λ <sup>-</sup>  | Bachmann (1972)               |

### 3. ANTIBIOTICS

Clorobiocin (18, 631 RP) was a gift from May and Baker Ltd, Dagenham, Essex. The majority of the experiments were performed using Batch No. CZS 605, prepared by Rhone-Poulenc, S.A., Vitry, France. Clorobiocin was used as a 10 mg/ml stock solution in absolute ethanol (or occasionally in methanol) and incubated at 37°C until completely dissolved. Fresh stocks were prepared on the day of use.

Coumermycin A1 (A447) was manufactured by Bristol Laboratories, Syracuse, New York, USA and was a gift from May and Baker Ltd. Coumermycin was dissolved in dimethyl-sulphoxide to 10 mg/ml and was stored at -20°C.

Nalidixic acid was obtained from Boehringer and was made up fresh as a 10 mg/ml stock in 25 mM NaOH. Rifampicin was from Sigma, and was freshly prepared as 20 mg/ml in 50<sup>v</sup>/v ethanol, 0.1M K<sub>2</sub>CO<sub>3</sub>. Chloramphenicol was obtained from Sigma, and Trimethoprim was a gift from Burroughs Wellcome. Streptomycin was obtained from Glaxo.

### 4. MEDIA

The following liquid media were used: Nutrient Broth (25g Oxoid No.2 Nutrient Broth Powder per litre of distilled water); Luria Broth (10g Difco Bacto Tryptone, 5g Difco Bacto yeast extract, 5g NaCl per litre of distilled water); Tryptone Soy Broth (17g Oxoid Tryptone No. L42, 3g Oxoid Soya Peptone No. L44, 2.5g Dextrose, 5g NaCl, 2.5g K<sub>2</sub>HPO<sub>4</sub> per litre of distilled water and adjusted to pH 7.3).

M9 minimal salts medium contained per litre of distilled water : 1g NH<sub>4</sub>Cl, 6g Na<sub>2</sub>HPO<sub>4</sub>, 3g KH<sub>2</sub>PO<sub>4</sub>, 5g NaCl (all added from a sterile

x10 stock solution) and 1 mM  $\text{CaCl}_2$  and 10 mM  $\text{MgSO}_4$  (added from a X100 sterile stock). Glucose (0.4% w/v) was the usual carbon source employed. Amino acids (40  $\mu\text{g}/\text{ml}$  final conc.) and thiamine (2  $\mu\text{g}/\text{ml}$  final conc. - filtered sterilised) were added when required.

Agar plates were prepared by solidifying liquid media with 15g Davis New Zealand Agar per litre; 6g agar per litre was used to prepare soft agar overlays.

BBL agar was used to grow  $\lambda$  phages and contained 10g Trypticase (Baltimore Biological Laboratories) and 5g NaCl per litre of distilled water and was solidified with 25g or 6g agar as above.

Bacterial buffer : 3g  $\text{KH}_2\text{PO}_4$ , 7g  $\text{Na}_2\text{HPO}_4$ , 4g NaCl, 0.1g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per litre of distilled water.

Lambda buffer : 6 ml 1M Tris-HCl pH 7.2, 2.46g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 50mg gelatin per litre of distilled water.

## 5. GROWTH OF BACTERIA IN LIQUID CULTURE

For many experiments cultures in balanced exponential growth were used. This was achieved by taking a fresh overnight culture grown in M9 minimal medium and diluting this culture in the same medium to  $A_{450} = 0.1$ . The culture was again diluted in the same medium 1 in 100 to the desired volume. This culture was grown in a conical flask (of capacity of at least five times the culture volume) in a New Brunswick gyrotory shaking water bath. This procedure allowed at least 6 generations of growth before sampling.

The absorbance,  $A_{450}$ , of cultures was determined by withdrawing a 2 ml sample and measuring in a Gilford Microsample spectrophotometer at 450 nm.

## 6. BACTERIAL CELL NUMBERS

These were determined using a Model B Coulter Counter fitted with a 30  $\mu\text{m}$  orifice. 0.2 ml samples of cultures were mixed with 1.8 ml of filtered 0.9% w/v saline containing 0.8% w/v formaldehyde to fix the cells, and diluted further in 0.9% w/v saline (usually 1 in 25) to an appropriate density for counting. The instrument settings used were as follows: lower threshold = 8.0, upper threshold between 100 and  $\infty$ ,  $1/\text{aperture current} = 1$ ,  $1/\text{amplification} = \frac{1}{8}$ .

For determination of relative cell volume, a Coulter Counter Model ZB1 fitted with a Channelyzer C/1000 was used. This machine was used while on loan from Coulter Electronics Ltd. A constant number of cells was counted and the readouts were recorded on an X-Y plotter. The instrument settings were the same as above except that  $1/\text{aperture current} = 0.354$ .

## 7. MEASUREMENT OF DNA SYNTHESIS

To measure the accumulation of DNA in a growing culture, the medium included uridine (1.5 mM) and either  $[^3\text{H}]$ -thymidine (1  $\mu\text{Ci}/\mu\text{g}$ , 1  $\mu\text{g}/\text{ml}$ ) or  $[^{14}\text{C}]$ -thymidine (0.1-0.16  $\mu\text{Ci}/\mu\text{g}$ , 2  $\mu\text{g}/\text{ml}$ ). In both cases non-radioactive thymidine was added to give the desired specific activity. The isotope, cold thymidine and uridine were added to the medium prior to the addition of diluted culture (see section 5). 1 ml samples were taken with either a glass pipette or an automatic adjustable Finpipette into 2 ml ice cold 10% w/v trichloroacetic acid (TCA) containing 100  $\mu\text{g}$  thymidine per ml. These samples were left on ice for at least one hour and were collected by suction on 27 mm membrane filters (Sartorius,

0.45  $\mu\text{m}$  pore size) presoaked in 2 mg/ml thymidine. The tubes containing the samples were each washed out five times with distilled water heated to 95°C, and the filters a further ten times. Filters were dried under an infra red lamp and transferred to plastic scintillation vials which were subsequently fitted with enough non-aqueous scintillation fluid to cover the filters. This scintillation fluid contained, per litre of toluene : 33 mg dimethyl POPOP and 5g of 2,5-diphenyloxazole (PPO). The vials were stoppered and placed in glass scintillation vials and counted in a Packard Liquid Scintillation Spectrophotometer.

#### 8. THE RATES OF DNA AND RNA SYNTHESIS

The rates of nucleic acid synthesis were measured by withdrawing 0.5 ml aliquots of culture into 50 or 100  $\mu\text{l}$  of prewarmed medium containing either [ $^3\text{H}$ ]-thymidine (1  $\mu\text{Ci}$ , 85 Ci/mmol) or [ $^{14}\text{C}$ ]-uridine (0.5  $\mu\text{Ci}$ , 57.4 mCi/mmol). The pulses were terminated after 2 minutes by the addition of 2 ml 10% w/v TCA containing 100  $\mu\text{g}$  thymidine or uridine per ml. TCA precipitable counts were determined as described above.

#### 9. ESTIMATION OF THE DURATION OF A ROUND OF DNA REPLICATION

The C time of a culture was determined by measuring the amount of DNA synthesis ( $\Delta G$ ) which occurred after inhibition of initiation of replication by the addition of rifampicin (200  $\mu\text{g}/\text{ml}$ ) in a culture grown in the presence of [ $^3\text{H}$ ]-thymidine as described above. The C time was calculated from  $\Delta G$  and  $\gamma$ , the generation time, as described in Chapter 6. The equation quoted there is complex and was not solved for

every combination of  $\Delta G$  and  $\gamma$  obtained. Instead a computer programme readout was consulted which contains many values of  $\Delta G$  calculated from probable combinations of  $C$  and  $\gamma$ . Chloramphenicol (100  $\mu\text{g/ml}$ ) was occasionally substituted for rifampicin.

#### 10. DETERMINATION OF DNA BREAKDOWN

A culture was grown to  $A_{450} = 0.4$  at  $37^{\circ}\text{C}$  in M9 minimal medium containing 1.5 mM uridine and  $[^3\text{H}]$ -thymidine (2  $\mu\text{Ci/ml}$ , 0.5  $\mu\text{g/ml}$ ). The cells were centrifuged at  $4^{\circ}\text{C}$  in the Sorvall SS34 rotor, washed twice with non-radioactive medium containing 100  $\mu\text{g/ml}$  thymidine, and resuspended in this medium to the original absorbance. The culture was then divided into 8 ml aliquots and treated as required. Duplicate 0.5 ml samples were taken into 0.5 ml 10% w/v TCA containing 100  $\mu\text{g/ml}$  thymidine. The samples were left on ice for at least one hour when 10  $\mu\text{l}$  bovine serum albumin (10  $\text{mg/ml}$ ) was added as coprecipitate and the samples centrifuged in the Sorvall SM24 rotor (20 min, 12,000 rpm). Duplicate 50  $\mu\text{l}$  samples were taken from the supernatant to assay for total acid soluble radioactivity in both the cells and medium. The pellet was then broken up by vigorous vortexing and the samples treated for 30 minutes at  $90^{\circ}\text{C}$  to extract the hot acid soluble material. Duplicate 50  $\mu\text{l}$  samples were taken from the supernatant. 1 ml non aqueous scintillation fluid (Aquasol) was added to each sample which was then counted in a Packard Liquid Scintillation Spectrophotometer. The percentage solubilisation was then calculated as the proportion of the total hot acid soluble counts present as cold soluble counts.

## 11. MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF ANTIBIOTICS

### (i) Tube Dilution Method

A series of tubes were constructed containing two-fold dilutions of the compound of interest in 4.5 ml of either TS broth or M9 minimal medium. 0.1 ml of an overnight culture diluted 1 in  $10^3$  was added to each tube. After incubation at 30°C or 37°C for 16 hours, the MIC was read as the lowest concentration of compound which completely inhibited overnight growth as judged from the turbidity of the cultures.

### (ii) Agar Spot Test

Minimal agar plates containing various concentrations of clorobiocin were prepared as follows. The agar was autoclaved, essential supplements added and then cooled to 50°C in a water bath. Measured quantities were poured into prewarmed sterile flasks and clorobiocin was added to the desired concentration from a 10 mg/ml stock in ethanol. After thorough mixing the agar was poured into plastic petri dishes. Care was taken to avoid air bubbles. The plates were used the same day or within 2 days if kept at 4°C. Addition of clorobiocin to very hot agar, or the use of plates more than 4-5 days old led to an apparent decrease in the activity of clorobiocin.

The sensitivity of a strain was determined by spotting 10 µl of a  $10^{-2}$  dilution of an overnight culture on to a series of plates containing varying concentrations of clorobiocin. The MIC was taken as the lowest concentration of clorobiocin which effectively prevented the formation of a confluent growth of bacteria. Similar plates were used to determine the concentration of clorobiocin required to prevent formation of single colonies.

## 12. ISOLATION OF CLOROBIOICIN RESISTANT MUTANTS

10 ml overnight cultures of LE234 in minimal medium were centrifuged and resuspended in 0.2 - 0.4 ml bacterial buffer. 0.1 ml ( $10^{10}$  cells) was spread on minimal agar plates containing various concentrations of clorobiocin and the plates were incubated at 30°C or 37°C for several days until growth appeared. Single colonies or areas of strong growth were purified to single colonies on similar plates and tested for resistance to clorobiocin over a range of concentrations of the antibiotic. Further details are given in Chapter 5.

## 13. GENETIC TECHNIQUES

These were carried out basically as described by Miller (1972).

### (a) Hfr x F<sup>-</sup> matings

10 ml cultures of the Hfr and F<sup>-</sup> strain were grown overnight in nutrient broth at 37°C, diluted 1 in 50 into fresh prewarmed broth and grown to  $A_{450} = \sim 0.5$ . Hfr and F<sup>-</sup> were mixed in the ratio 1 to 10 and incubated with very slow shaking. After an appropriate time, 0.5 ml samples were withdrawn, vortexed thoroughly, diluted in bacterial buffer, and plated on to selective plates. Parental strains were always plated separately as controls.

### (b) F-prime x F<sup>-</sup> matings

Essentially as above, except that the F' strain was grown overnight in minimal medium to maintain selection for the episome before dilution into broth. Equal numbers of F-prime and F<sup>-</sup> strains were mixed and incubated for 1-2 hours before dilution and plating for progeny.



(c) P1 transduction

Recipient strains were grown in nutrient broth at 37°C (or 30°C for temperature-sensitive strains) to  $A_{450} = \sim 0.7$ , centrifuged in a MSE bench centrifuge, resuspended in 4 ml 0.01M  $\text{CaCl}_2$ , 0.1M  $\text{MgSO}_4$  and aerated for 20 minutes. 0.1 ml aliquots of cells were added to 0.1 ml of  $10^0 - 10^{-4}$  dilutions of donor P1vir and incubated for 20 minutes at 30°C or 37°C. 0.2 ml 1M sodium citrate was added to each tube, the contents mixed with 3 ml 0.6% water agar, and poured on to selective plates. Controls without phage or cells were always included. Transduction frequencies were usually between 1 and 20 transductants per  $10^5$  p.f.u. of P1.

(d) Tests for various phenotypes

A loopful of cells from a plate was resuspended in 0.1 ml bacterial buffer. Approximately 10  $\mu\text{l}$  was spotted on to selective plates either with a 0.1 ml pipette or a toothpick. DgoD<sup>+</sup> strains were identified by their ability to grow on 10 mM D-galactonate when this replaced glucose. For identification of tna<sup>+</sup> (tryptophase) strains, the remainder of the cell suspension described above, or a single colony, was grown overnight in 1.0 ml of either Luria broth + 500  $\mu\text{g/ml}$  tryptophan or M9 minimal medium containing 0.4% glycerol (no glucose) and 500  $\mu\text{g/ml}$  tryptophan. 0.1 ml of a culture was mixed with 0.1 ml Ehrlich's reagent (100 mg dimethylaminobenzaldehyde in 5 ml absolute ethanol, + 12 ml (92% ethanol, 8% conc.  $\text{H}_2\text{SO}_4$ )). Tna<sup>+</sup> cultures rapidly turned pink; Tna<sup>-</sup> cultures remained yellow. Temperature-sensitivity was determined by incubating strains on pre-warmed plates.

(e) Growth of bacteriophage

(i) P1. 0.1 ml of various dilutions of phage ( $10^0 - 10^{-5}$ ) was mixed with 1 ml of a bacterial culture grown to late exponential phase nutrient broth containing 5 mM  $\text{CaCl}_2$ . After 20 minutes at  $37^\circ\text{C}$  (or  $30^\circ\text{C}$ ) to allow the phage to adsorb, 3 ml soft nutrient agar + 5 mM  $\text{CaCl}_2$  was added and the mixture poured on to a nutrient agar plate. The plates were incubated at  $37^\circ\text{C}$  (or  $30^\circ\text{C}$ ) until the resulting plaques were just touching each other on one plate (about 8-10 hours). 3 ml nutrient broth was added and the top layer was collected with this broth, chloroformed and centrifuged in a MSE bench centrifuge for 20 minutes. The supernatant was collected and stored over chloroform. Phage was titred by mixing 0.1 ml of various dilutions of phage with 0.1 ml of a late exponential bacterial culture resuspended in bacterial buffer + 5 mM  $\text{CaCl}_2$ . 3 ml soft nutrient agar + 5 mM  $\text{CaCl}_2$  was added and the contents were poured on to nutrient agar plates. Titres of P1 were normally  $5 \times 10^9 - 10^{11}$  p.f.u. per ml.

(ii)  $\lambda$ . Stocks were prepared as described above except that the bacteria used was 0.1 ml of an overnight culture grown in Luria broth and resuspended in Lambda buffer. The bacteria and cells were plated on Luria agar using BBL soft agar overlays. After lysis, the top layer was removed with 3 ml lambda buffer and treated as above. Titres were normally between  $10^9$  and  $5 \cdot 10^{10}$  p.f.u. per ml.

(f) Isolation of  $\lambda$  transducing phages

Based on the method of Schrenck and Weis berg (1975). 0.1 ml of an overnight culture of bacterial strain 833 att $\lambda$  $\nabla$  was mixed with

$\lambda_{\text{cI857Sam7}}$  at a multiplicity of infection of 5, and incubated at  $32^{\circ}\text{C}$  for 20 minutes. 0.1 ml of  $10^0$ ,  $10^{-1}$  and  $10^{-2}$  dilutions was spread on Luria agar plates together with  $\sim 10^9 \lambda_{\text{cIh}}^{82}$  to kill non-lysogenic cells. Plates were incubated at  $32^{\circ}\text{C}$  until colonies (lysogens) appeared. Plates containing 500 colonies were taken and all the bacteria on the plate suspended in several hundred ml Luria broth to an  $A_{650} \approx 0.05$ . This culture was grown at  $32^{\circ}\text{C}$  until the  $A_{650} = 0.2$ , shifted to  $42^{\circ}\text{C}$  for 30 minutes to induce the phage (which produce a temperature-sensitive repressor) and grown for a further 2-3 hours at  $37^{\circ}\text{C}$  to allow phage DNA replication and packaging. The cells were centrifuged, resuspended in

5 ml lambda buffer and lysed by the addition of 0.1 ml chloroform. Cell debris was removed by centrifugation and the supernatant titred for  $\lambda_{\text{cI857Sam7}}$  phages by using supE and supF (Ymel) strains. The  $S^{-}$  defect cannot be overcome by the use of a supE mutant.

(g) Identification of bacterial proteins specified by  $\lambda$  transducing phages

This was based on the method of Ptashne (1967) as modified by G.S. Plastow (personal communication). Bacterial strain 159 (uvrA,  $\lambda_{\text{ind}}^{-}$ ) was grown in M9 minimal medium containing maltose (0.4%) and glycerol (0.4%) to  $A_{450} = 0.64$  ( $2 \times 10^8$  cells/ml). For each sample, 5 ml culture was irradiated with UV light ( $12,000 \text{ ergs} \cdot \text{mm}^{-2}$  (12 Joules)). The cells were centrifuged in a SS34 rotor (10,000 rpm, 10 min) and resuspended in 1 ml growth medium containing 10 mM  $\text{MgSO}_4$ . Purified transducing phage, or that mixed with helper phage, was added at an  $\text{moi} = 5$ . This phage was dialysed against Lambda buffer in order to remove methionine and so increase the amount of  $[^{35}\text{S}]$ -methionine

incorporated. After adsorption at 37°C for 15 minutes the mixture was diluted with growth medium (no addition  $\text{MgSO}_4$  was added) to 5 ml final volume. After aeration at 37°C for 10 minutes, 100  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]-methionine ( $\sim 800$  Ci/mmol) was added and incubation continued for 30 minutes before addition of an equal volume of ice-cold "stop" solution containing 3.75 mg/ml methionine and 600  $\mu\text{g/ml}$  chloramphenicol. The cells were harvested and treated for SDS-PAGE as described in section 16.

(h) Isolation of *recA* mutants

Strains were first made thymine-requiring by the procedure of Stacey and Simson (1965). Cells were grown overnight at 37°C (30°C for temperature-sensitives) in M9 minimal medium without thymine, and diluted 100 times into fresh medium containing thymine (200  $\mu\text{g/ml}$ ) and trimethoprim (20  $\mu\text{g/ml}$ ). If significant growth occurred after 1 day the culture was diluted into fresh medium containing thymine and trimethoprim. After 2 days, cultures were plated out on minimal medium + thymine (200  $\mu\text{g/ml}$ ) to give single colonies which were then tested for growth without thymine.  $\text{Thy}^-$  (*thyA*) colonies were picked and checked.

*RecA* derivatives were obtained by mating *thyA* recipients with the Hfr JC5088 *thy*<sup>+</sup> *recA56* (*Str*<sup>S</sup>). *Thy*<sup>+</sup> *Str*<sup>R</sup> recombinants were selected and tested for the presence of the *recA56* allele by sensitivity to U.V. light. *RecA* strains did not grow after irradiation of 300  $\text{ergs.mm}^{-2}$ .

#### 14. PREPARATION OF CELL LYSATES FOR SDS-PAGE

Radioactively labelled cells ( $10^7$ - $10^8$ ) were transferred to centrifuge tubes and, for most experiments, unlabelled cells ( $\sim 2 \times 10^9$  per sample) were added to provide carrier material. In some experiments, additional [ $^3\text{H}$ ]-leucine labelled cells were added to provide an internal standard (see section 17 below). The cells were harvested and washed twice in ice-cold 10 mM sodium phosphate buffer, pH 7.2, by successive centrifugations in the Sorvall SS34 rotor (10,000 rpm, 10 minutes,  $4^\circ\text{C}$ ). The washed pellet was either resuspended directly in 100  $\mu\text{l}$  of SDS sample buffer or firstly in 50  $\mu\text{l}$  phosphate buffer followed by the addition of 50  $\mu\text{l}$  sample buffer. The samples were transferred to Eppendorf 1.5 ml plastic vials and immediately boiled for 5 minutes to complete cell lysis and solubilise protein, and then vortexed to shear the DNA. Cell lysates were stored at  $-20^\circ\text{C}$  and were always reboiled before electrophoresis. Typically 5-20  $\mu\text{l}$ , containing 50-200  $\times 10^3$  counts per minute, was loaded into each gel slot.

#### 15. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

The basic procedure and buffer system was that of Laemmli (1970). The constitution of buffers and solutions is given in Table 2.2. Only one acrylamide solution was used in any one gel. Gels were composed of a 7.2% stacking gel and an 11% or 15% separating gel. After mixing, gel solutions were deaerated on ice for several minutes before pouring. Freshly prepared ammonium persulphate was always used. Electrophoresis was carried out using a Bio-Rad slab gel system (Model 220). Samples

Table 2.2

Solutions and Buffers used in Electrophoresis

A. Separating Gel Buffer

0.75M Tris-HCl pH 8.8; 0.2% w/v SDS

B. Stacking Gel Buffer

0.25M Tris-HCl pH 6.8; 0.2% w/v SDS

C. Acrylamide Solutions

(i) 44% w/v acrylamide; 0.8% w/v N,N'-methylene-bis-acrylamide (bis)

(ii) 44% w/v acrylamide; 0.4% w/v bis

D. Electrophoresis Buffer

0.125M Tris; 0.192M glycine; 0.1% w/v SDS

(pH ~8.3 with no adjustment)

E. Sample Buffer

0.0625M Tris-HCl pH 6.8; 10% w/v glycerol;

2% w/v SDS; 5% v/v mercaptoethanol

11% Separating Gel

20 ml Buffer A

10 ml Acrylamide Solution (i) or (ii)

9 ml Distilled Water

1.0 ml Ammonium persulphate (10 mg/ml)

0.08 ml TEMED

were prepared as described elsewhere and were always boiled immediately prior to electrophoresis. Electrophoresis was carried out at a constant current of 25 mA per gel, until the tracking dye was approximately 1 cm from the bottom of the gel. Gels were stained overnight in 300 ml 10% v/v acetic acid, 25% v/v isopropanol, 0.05% w/v Coomassie brilliant blue. Diffusion destaining was carried out by shaking the gel in 300 ml of 10% v/v acetic acid, 10% v/v isopropanol for two three hour periods. Gels were stored in 10% v/v acetic acid and were photographed on 4" x 5" Ilford FP4 film plates using background illumination and a deep orange filter.

#### Autoradiography and Fluorography

Autoradiographs were prepared by drying the gels on to a sheet of Whatman No.17 Chromatography Paper in a Bio-Rad gel drying unit. The dried gels were then placed in a Kodak X-ray cassette with a Kodak X-Omat XRP5 X-ray film plate and exposed at room temperature. Fluorographs were prepared by dehydrating the gels by two successive washes in dimethylsulphoxide, followed by impregnation with PPO exactly as described by Bonner and Laskey (1974). The gels were dried and exposed to film as above, except that the latter was at  $-80^{\circ}\text{C}$ .

#### 16. PREPARATION OF BACTERIAL CELL ENVELOPES

The basic procedure was that described by Boyd (1979). Cells ( $10^7$ - $10^8$ ) labelled with  $[^{35}\text{S}]$ -methionine were transferred to 25 ml beakers and approximately  $3 \times 10^9$   $[^3\text{H}]$ -leucine labelled cells were added to provide an internal standard (see section 17). Unlabelled carrier cells of the same strain from an exponentially growing culture at  $A_{450}$

= 0.5 were added to each sample to give approximately  $2 \times 10^{10}$  cells per sample. The combined sample (10 ml) was sonicated for three 30 second intervals, with 30 second cooling periods, using a  $\frac{3}{4}$  inch diameter probe in a MSE ultrasonic disintegrator. This and all subsequent steps were carried out at 4°C.

Sonicated samples were centrifuged in a Sorvall SM24 rotor (7,500 rpm, 5 min) to remove unbroken cells. The supernatant was transferred to a second tube and centrifuged at 18,000 rpm for 30 minutes. The pellet was resuspended in 1 ml sodium phosphate buffer, pH 7.2 and transferred to a polycarbonate centrifuge tube and centrifuged in a Beckman ultracentrifuge using the Type 40 rotor (35,000 rpm, 30 minutes). The pellet of cell envelopes was resuspended in 100  $\mu$ l 0.5% w/v Sarkosyl NL97 in phosphate buffer and incubated for 30 minutes at room temperature. The outer membrane, which is insoluble in this detergent (Filip et al., 1973) was recovered by centrifugation of the samples at 39,000 rpm for 2 hours in the Type 40 rotor. Both the supernatants (inner membranes) and the pellets (outer membranes) were prepared for SDS-PAGE by the addition of 100  $\mu$ l SDS sample buffer and boiling for 5 minutes. Samples were stored at -20°C. Radioactivity in the fractions was measured by taking 5 or 10  $\mu$ l aliquots into aqueous scintillation fluid and counting in a Packard Liquid Scintillation Spectrophotometer.



## 17. USE OF AN INTERNAL STANDARD OF [ $^3\text{H}$ ]-LEUCINE LABELLED CELLS

In some experiments described in Chapter 7, incorporation of [ $^{35}\text{S}$ ]-methionine into cell envelope fractions and individual gel bands was measured. In order to obviate the need for reproducible recovery from sample to sample, an internal standard was used. The method used was that devised by A. Boyd (1979). A 20 ml exponentially growing culture ( $A_{450} = 0.1 - 0.2$ ) was labelled by the addition of 100 - 200  $\mu\text{Ci}$  [ $^3\text{H}$ ]-leucine (100 Ci/mmol). After about two generations growth, unlabelled leucine (final concentration 20  $\mu\text{g/ml}$ ) was added. The culture was concentrated by centrifugation and a constant volume was added to each [ $^{35}\text{S}$ ] labelled sample before processing. The  $^{35}\text{S}/^3\text{H}$  ratio in any cell fraction or gel band derived from these samples was thus a measure of the relative amount of  $^{35}\text{S}$  radioactivity in the whole of the original sample.

Counting was performed using the  $^{14}\text{C}/^3\text{H}$  setting in a Packard Model 3255 Liquid Scintillation Spectrophotometer. Samples labelled with each isotope alone were always included to measure cross-channel spillover. Spillover was always  $<1\%$  from the  $^3\text{H}$  channel to the  $^{14}\text{C}$  and was disregarded. Spillover in the opposite direction was 60 - 100%, and  $^3\text{H}$  cpm were always corrected for spillover before calculation of isotope ratios. The ratio of  $^3\text{H}$  cpm /  $^{14}\text{C}$  cpm was usually  $>10:1$ , so that spillover correction was  $<10\%$  of total  $^3\text{H}$  radioactivity.

#### 18. DIRECT MEASUREMENT OF RADIOACTIVITY IN INDIVIDUAL GEL BANDS

The method used was that of Ames (1974). Stained radioactive gels were dried down onto a sheet of Whatman No.4 Chromatography Paper. The bands of interest were carefully cut out using fine scissors, and placed in plastic scintillation vials. 50  $\mu$ l of distilled water was added to each vial, with care being taken to wet thoroughly the gel slices. After 10 minutes, 5 ml of scintillation fluid was added containing per litre; 923 ml toluene, 77 ml NCS solubiliser, 3.75 g PPO and 56 mg dimethyl POPOP. The vials were stoppered, vortexed and incubated at 37°C overnight. The vials were cooled and counted as described elsewhere.

#### 19. STANDARD MOLECULAR WEIGHT MARKERS FOR SDS-PAGE

Concentrated stocks (10 mg/ml) of the following proteins were prepared : lysozyme (14,300 MW), lactoglobulin (17,000 MW), chymotrypsinogen (25,000 MW), lactate dehydrogenase (36,000 MW), ovalbumin (43,000 MW), pyruvate kinase (57,000 MW) and phosphorylase A (94,000 MW). The proteins were mixed and diluted in SDS sample buffer to a final concentration of 50  $\mu$ g of each protein per ml. The mixture was boiled and 20  $\mu$ l was applied to a gel slot.

## 20. PHOTOGRAPHY OF BACTERIA

Bacteria were grown in liquid medium to  $A_{450} \approx 0.4$ . 5 ml of culture was centrifuged at 4°C in a Sorvall SS34 rotor and resuspended in approximately 0.1 ml bacterial buffer. 10-20  $\mu$ l was placed on a clean microscope slide previously coated with a thin layer of 0.7% agarose (Sigma). The bacteria were left to dry into the agarose which was then covered with a microscope slide. The bacteria were viewed under phase-contrast using a Wild microscope (x1000 magnification) and photographed using a Nikon camera and Kodak Pan X film. A calibrated graticule was also photographed and was used to calculate the magnification factor.

## Chapter 3

### CHARACTERISATION OF THE ANTIBIOTIC CLOROBIOCIN

#### I. Introduction

Clorobiocin is an antibiotic structurally similar to novobiocin and coumermycin A<sub>1</sub> (see Figure 1.4). In order to use clorobiocin as an inhibitor of DNA gyrase it was necessary, and of interest, to study the activities of clorobiocin and to compare these with published results with novobiocin and coumermycin.

Since clorobiocin, like novobiocin, is a hydrophobic compound (May and Baker Ltd., personal communication) it was expected that strains lacking portions of the LPS from the outer membrane would be particularly sensitive to clorobiocin. Further, since novobiocin and coumermycin preferentially inhibit DNA synthesis in E.coli (see Chapter 1) it was likewise expected that clorobiocin would also primarily inhibit DNA synthesis. The results obtained will be compared with those found using nalidixic acid, a potent inhibitor of DNA synthesis in E.coli (Goss et al., 1965) and also an inhibitor of DNA gyrase.

#### II. Measurement of the Sensitivity of E.coli to Clorobiocin

The minimum inhibitory concentration (MIC) of an antibiotic is, by definition, the lowest concentration of that compound which will inhibit overnight growth of a bacterial culture. The most common method of determination of an MIC value is the tube dilution method. The MIC

values of clorobiocin and novobiocin determined in this way are given in Table 3.1. All strains tested were more sensitive to clorobiocin than to novobiocin. As expected, strains NS-2 and D21f2 were very sensitive to clorobiocin; NS-2 was isolated as a novobiocin-sensitive mutant (Tamaki *et al.*, 1971) and has a reduced LPS content of the outer membrane. D21f2 lacks heptose in the LPS (see Figure 1.3) whereas D21f1 and D21e7 have longer LPS chains and therefore would not be expected to be as sensitive to clorobiocin as D21f2. Strain D22, the *envA* chain-forming mutant of D21 (Normark *et al.*, 1969), was not unusually sensitive to clorobiocin; the same result was obtained with H101, a mutant which has been reported to be unusually sensitive to many antibiotics and detergents (Ennis, 1971). The strains LE316 and LE701, clorobiocin resistant mutants derived from LE234 (see Chapters 2 and 5), had increased MIC values although these were not outside those values obtained for some "wild-type" strains of *E.coli* K-12 e.g. D21 and W1895.

A more sensitive test was devised which could detect smaller but consistent differences between the sensitivity of different strains to clorobiocin. This was the "agar spot test" as described in Chapter 2 and the results are shown in Table 3.2. The strains D21e7, D21f1 and D21f2 were all more sensitive to clorobiocin than their parent strain D21. In this test the *envA* mutant (D22) was also clearly more sensitive than D21. The introduction of the plasmid rRM98 into the *E.coli* B/r strain LEB18 also resulted in increased sensitivity to clorobiocin, presumably due to the absence of the major outer membrane protein (36.5K) which results from the presence of this plasmid in this strain (Iyer *et al.*, 1978). In this context it is interesting to note that the strains

Table 3.1

Minimum Inhibitory Concentration (MIC) of Novobiocin and Clorobiocin  
Determined by the Tube Dilution Method

(a) For a full description of strains see Chapter 2.

(b), (c) For method see Chapter 2. Unless otherwise stated all  
determinations were performed in tubes standing at 37°C.

T.S. = tryptone soy broth

M9 = M9 minimal medium plus essential amino acids

N.D = not determined

| Strain (a) | MIC (b) (µg/ml) |             | Conditions (c)    |
|------------|-----------------|-------------|-------------------|
|            | Novobiocin      | Clorobiocin |                   |
| JE1011     | > 500           | 31          | T.S               |
| NS-2       | 1.6             | 0.4         | T.S               |
| W1895      | N.D             | 62          | M9; shaking       |
| H101       | N.D             | 31          | M9; shaking       |
| D21        | N.D             | 62          | M9                |
| D22        | N.D             | 31          | M9                |
| LE234      | > 500           | 16          | M9                |
| LE234      | > 500           | 16          | T.S               |
| LE316      | > 500           | 62          | T.S 30°C and 37°C |
| LE701      | > 500           | 32-64       | T.S               |
| D21e7      | N.D             | 8           | T.S               |
| D21f1      | N.D             | 8           | T.S               |
| D21f2      | N.D             | 0.4         | T.S               |

Table 3.2

Minimum Inhibitory Concentration (MIC) of Clorobiocin Determined by  
the Agar Spot Test

- (a) For a full description of strains see Chapter 2.
- (b) MIC determined as the lowest concentration of clorobiocin  
in agar that inhibited overnight colony formation. For full  
details of method see Chapter 2.



| Strain (a)    | MIC (b)( $\mu$ g/ml) | Phenotype of Strain                   |
|---------------|----------------------|---------------------------------------|
| D21           | 60                   | wild-type                             |
| D22           | 10                   | <u>envA</u> ; chain forming           |
| D21e7         | 10                   | galactose deficient LPS               |
| D21f1         | 10                   | glucose deficient LPS                 |
| D21f2         | 1                    | heptose deficient LPS                 |
| LE234         | 30                   | wild-type                             |
| LE316         | 60                   | clorobiocin resistant; from LE234     |
| LE701         | 60                   | clorobiocin resistant; from LE234     |
| LEB18         | 60                   | <u>E.coli</u> B/r wild-type           |
| LEB18 (rRM98) | 30                   | outer membrane protein b<br>deficient |

D21e7, D21f1 and D21f2 all have decreased levels of the major outer membrane proteins (Lugtenberg et al., 1976).

An alternative method used to determine the sensitivity of strains to clorobiocin was the addition of clorobiocin to a growing culture and measurement of the subsequent increase in mass as indicated by the optical density ( $A_{450}$ ). Figure 3.1 shows the effect of clorobiocin on the mass increase of four strains; in all cases the accumulation of mass was reduced within one generation of the addition of clorobiocin. In the two particularly sensitive strains, as defined by the previous tests, mass increase was more severely affected and the envA mutant essentially stopped growing after only 100 minutes.

The effect of clorobiocin on the viability of LE234 was determined and the results shown in Figure 3.2. In both growing and non-growing cultures viability immediately decreased upon addition of clorobiocin. However this effect was modest, particularly in non-growing cultures where less than 50% of the cells were killed after two hours. This is consistent with results which have shown a recovery from inhibition of both mass and DNA synthesis by removal of clorobiocin from a treated culture (data not shown).

### III. The Effect of Clorobiocin on Mass and DNA Synthesis

Strain LE234 was selected for further study in view of the presence of genetic markers which were suitable for the subsequent analysis of clorobiocin resistant mutants (see Chapter 5). The growth rate of LE234 was first determined in the presence of sub-MIC concentrations of

Figure 3.1

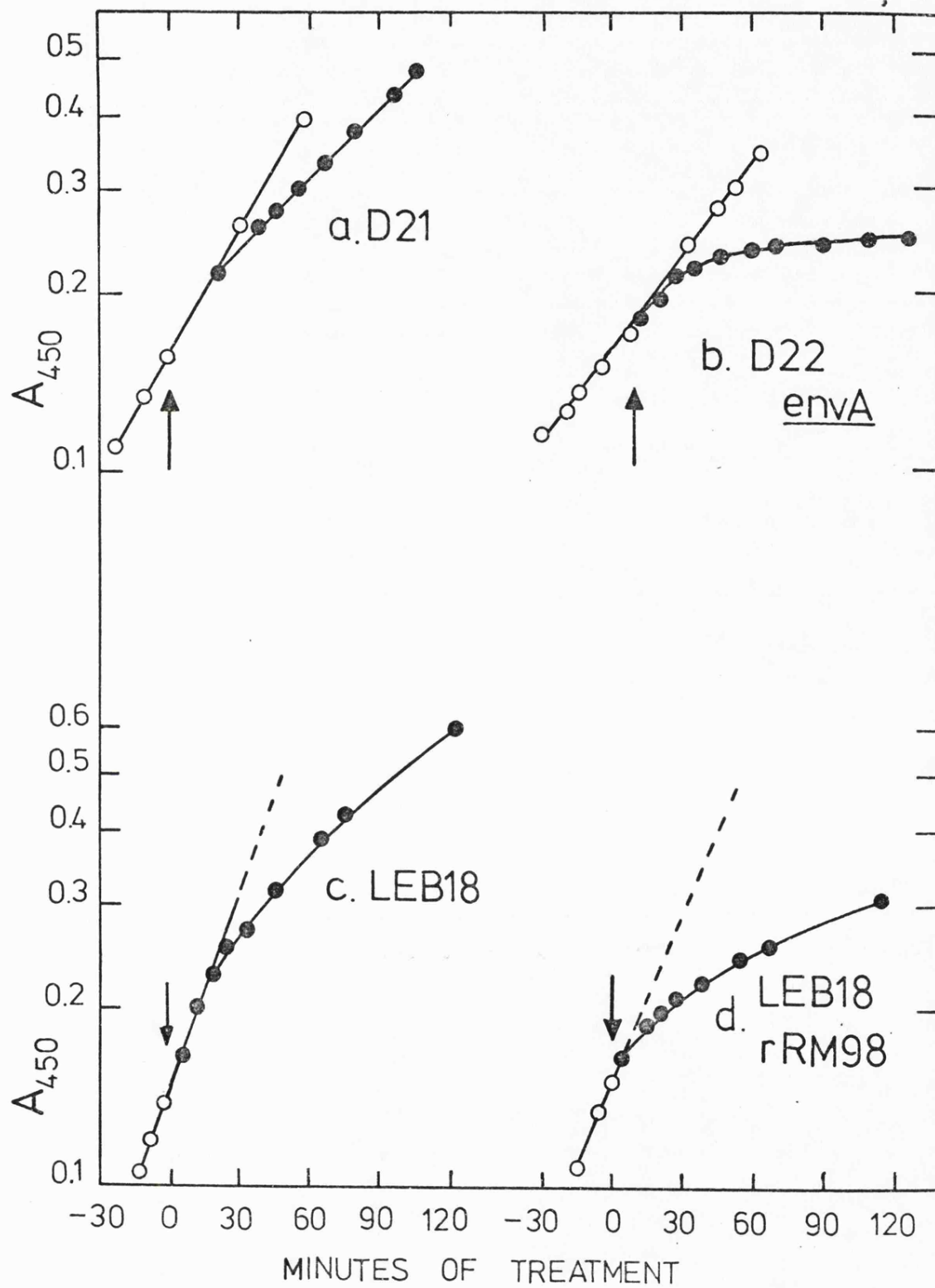
The Effect of Clorobiocin on the Growth of Four *E.coli* K-12 Strains

a, b. D21 and D22 (envA) were grown at 37°C in M9 minimal medium.

c, d. LEB18 and LEB18 (rRM98) were grown at 37°C in nutrient broth.

At zero time, as indicated by the arrow, clorobiocin was added to a final concentration of 10 µg/ml.

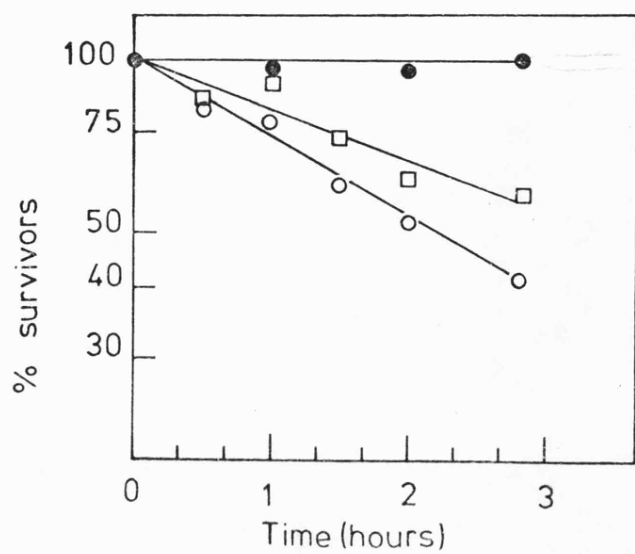
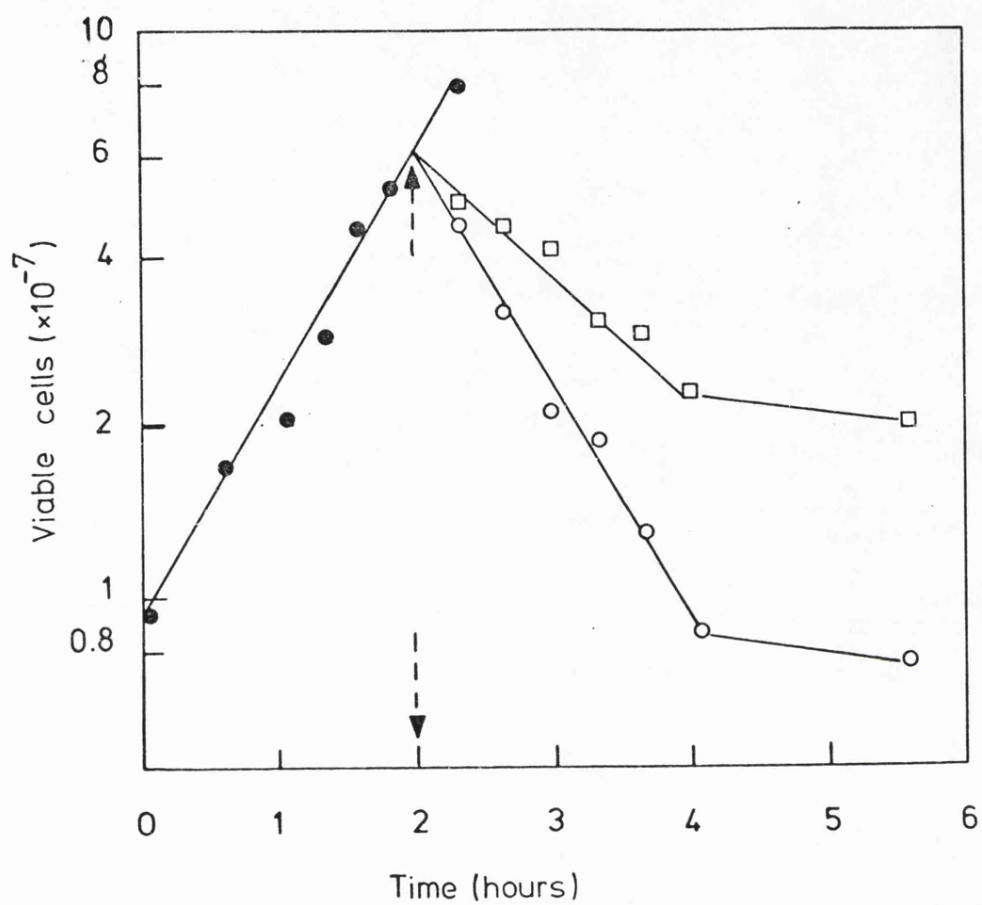
(○) untreated culture; (●) clorobiocin (10 µg/ml) treated culture.



### Figure 3.2

#### The Effect of Clorobiocin on the Viability of *E.coli* K-12 LE234

- a. A culture of LE234 was grown at 37°C in M9 minimal medium with essential amino acids to an  $A_{450} = 0.17$ . The culture was distributed into three flasks : two flasks received clorobiocin to final concentrations of 50 µg/ml (□) and 100 µg/ml (○) respectively; the third flask received ethyl alcohol to 1% v/v (●). Samples were taken and viable counts determined on nutrient agar without clorobiocin.
- b. A culture of LE234 was grown at 37°C in M9 minimal medium with essential amino acids to an  $A_{450} = 0.4$ . The cells were centrifuged, washed once in bacterial buffer and resuspended in this buffer in the original volume. The culture was divided and treated as in a.(●), control culture; (□) 50 µg/ml clorobiocin; (○), 100 µg/ml clorobiocin.



clorobiocin and the results are shown in Table 3.3. Although these concentrations did not result in total inhibition of growth, the growth rate was reduced in approximate proportion to the concentration of clorobiocin present. This indicates that the cou subunit of DNA gyrase may be essential for normal growth of E.coli. The effects on cell division and septation will be discussed in Chapter 4.

The effect of clorobiocin on DNA synthesis was measured in LE234 and the results shown in Figure 3.3. At both 50 and 100 µg/ml clorobiocin (compare with the MIC value of 16 µg/ml in this medium), DNA synthesis was severely inhibited although even at the higher concentration the block was not complete and synthesis continued at about 14% of the control rate. Cell mass was also immediately affected but to a lesser extent than DNA synthesis. The increase in mass during clorobiocin treatment appeared to be linear as opposed to logarithmic and this was confirmed by replotting the data as shown in Figure 3.4.

To ensure that the continued accumulation of DNA observed during clorobiocin treatment was not due to an artifact of labelling with [ $^3\text{H}$ ]-thymidine, the experiment was repeated over a wide range of concentrations using [ $^{14}\text{C}$ ]-thymidine. These two isotopes differ by the position of the radioactive label in the thymidine molecule. The results, as shown in Figure 3.5, were qualitatively similar to those in Figure 3.3. DNA synthesis was not completely blocked at 100 µg/ml clorobiocin and, interestingly, at low concentrations some recovery was apparent after about 1.5 hours. At all concentrations the effect of clorobiocin upon DNA synthesis was greater than that upon mass.

Table 3.3

The Effect of Clorobiocin on the Growth Rate and Morphology of  
LE234

An overnight culture of LE234, grown in M9 minimal medium with essential amino acids, was diluted 200-fold and distributed to 8 flasks to which clorobiocin was added to the final concentrations indicated. The alcohol content was adjusted to 0.1% v/v and the cultures grown through approximately 7 generations to  $A_{450} = 0.1$ . The growth rate was determined over the next 2 generations of exponential growth. The cells were then examined by phase contrast microscopy.



| Sample | Clorobiocin<br>conc. ( $\mu\text{g/ml}$ ) | $\tau$<br>(mins) | Morphology of Cells   |
|--------|---|------------------|---|
| A      | 0   | 49               | Normal short rods   |
| B      | 0.05                                      | 49               | Occasional chain of 3 cells. No long cells  |
| C      | 0.1                                       | 55               | As B; also a few long fat cells   |
| D      | 0.5                                       | 59               | As C; also some chains of 3-4 cells   |
| E      | 1.0                                       | 59               | As D; although increased frequency of both chains and long, fat cells   |
| F      | 2   | 62               | As E; frequency of chains higher. Filaments of 2-3 x cell length  |
| G      | 5   | 77               | Decreased frequency of chains and increased frequency of filaments (x 4-6 cell length); an occasional very small cell |
| H      | 10  | 100              | Nearly all cells longer than those in A and many perhaps wider. Very few chains; an occasional very small cell        |

### Figure 3.3

#### Mass and DNA Synthesis in LE234 during Clorobiocin Treatment

A culture of LE234 was grown in M9 medium with essential amino acids supplemented with  $[^3\text{H}]$ -thymidine ( $1\text{ }\mu\text{g/ml}$ ,  $1\text{ }\mu\text{Ci}/\mu\text{g}$ ) to an  $A_{450} = 0.08$ . At intervals  $1.0\text{ ml}$  samples were removed into ice-cold TCA for determination of total acid precipitable counts. At  $A_{450} = 0.1$  the culture was distributed into 3 flasks. Two cultures received clorobiocin and the third an equal volume of ethyl alcohol. Samples were then removed as before.

Upper : Cell Mass                      Lower : DNA

(O) control; (●)  $50\text{ }\mu\text{g/ml}$  clorobiocin; (□)  $100\text{ }\mu\text{g/ml}$  clorobiocin.

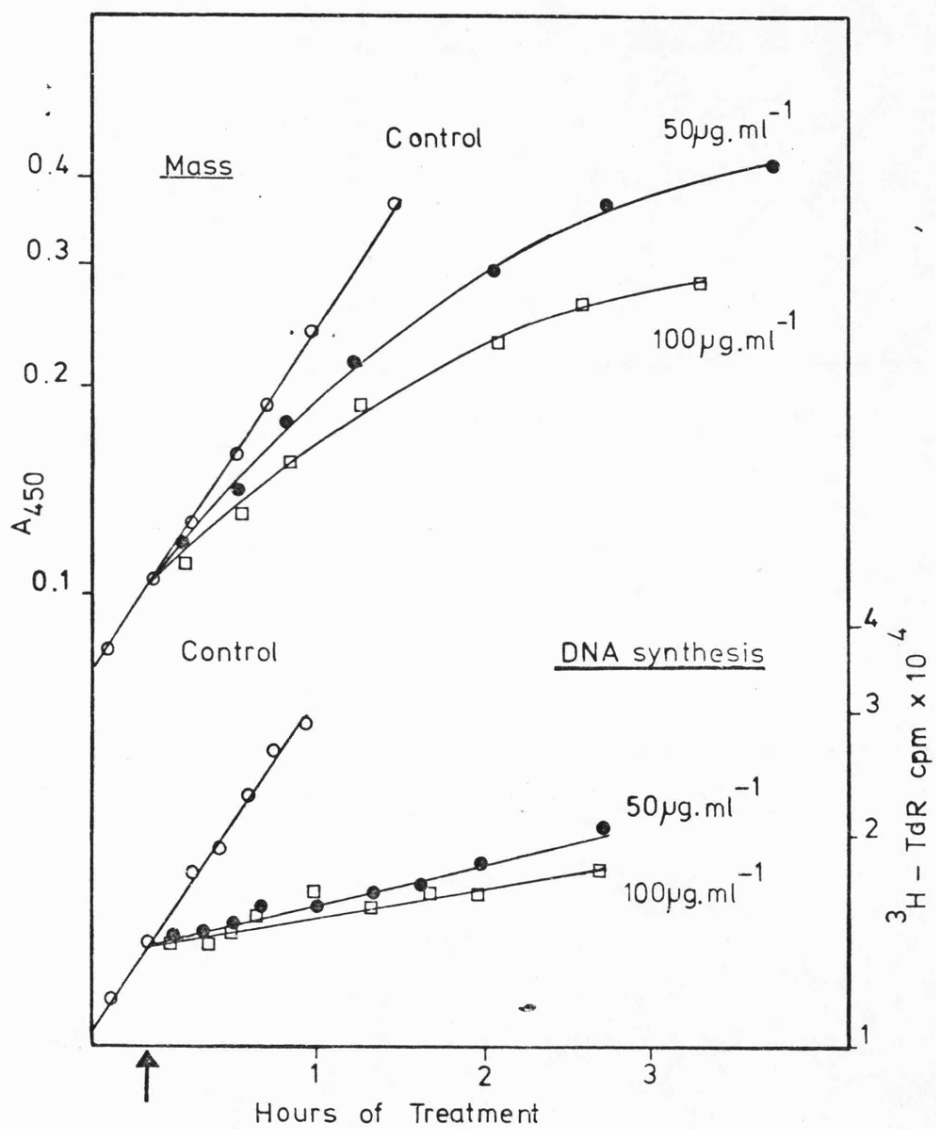


Figure 3.4

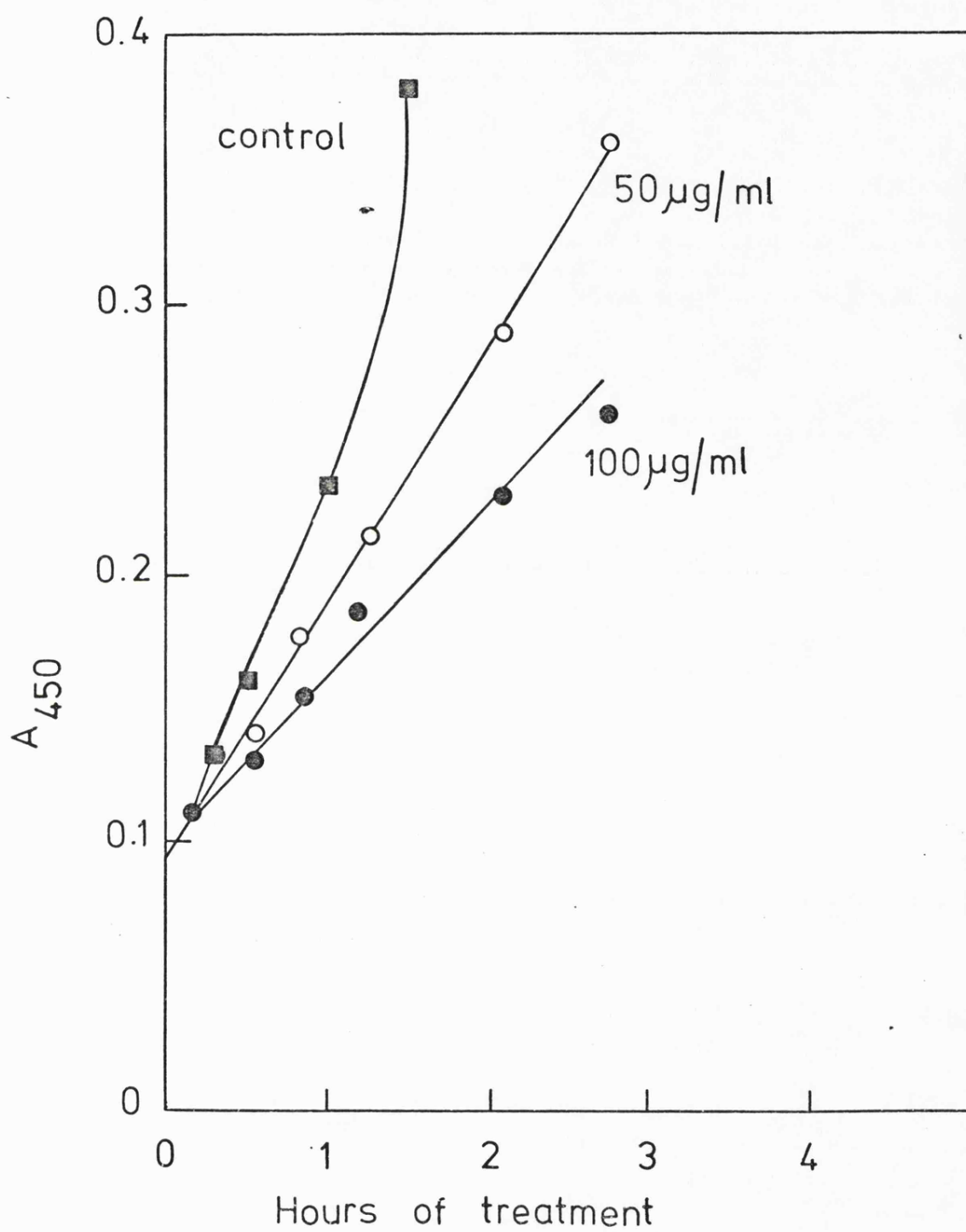
Mass Synthesis in LE234 during Clorobiocin Treatment

The  $A_{450}$  data in Figure 3.3 are replotted on a linear scale.

(■) control

(○) 50  $\mu\text{g/ml}$  clorobiocin

(●) 100  $\mu\text{g/ml}$  clorobiocin



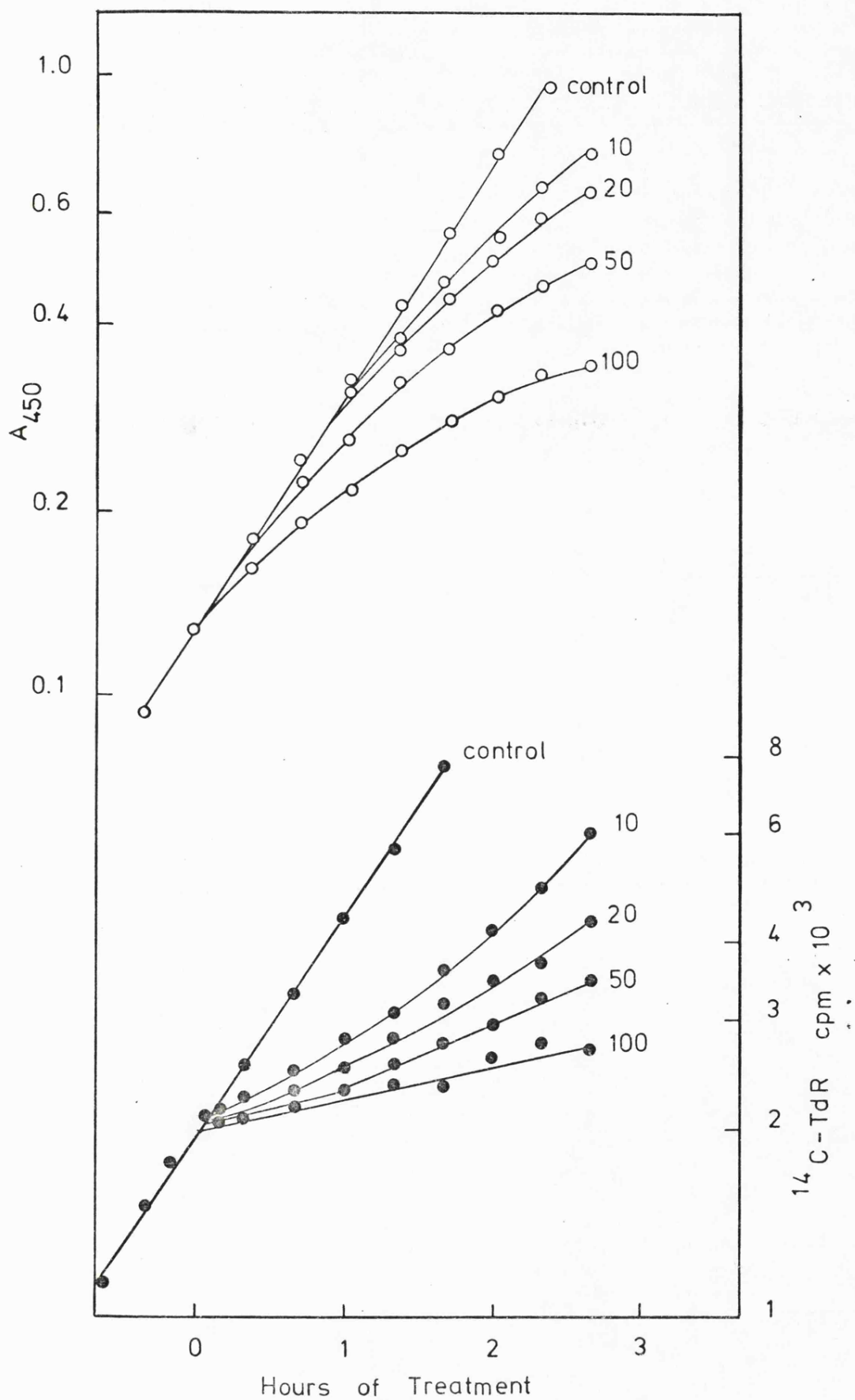
### Figure 3.5

#### Mass and DNA Synthesis in LE234 during Clorobiocin Treatment

A culture of LE234 was grown in M9 medium with essential amino acids supplemented with  $[^{14}\text{C}]$ -thymidine (2  $\mu\text{g}/\text{ml}$ , 0.05  $\mu\text{Ci}/\text{ml}$ ) to an  $A_{450} = 0.08$ . At intervals 1.0 ml samples were removed into ice-cold TCA for determination of total acid precipitable counts. At  $A_{450} = 0.13$  the culture was distributed into five flasks which received clorobiocin to the final concentrations (in  $\mu\text{g}/\text{ml}$ ) indicated on the right of each curve. The control culture received an equivalent volume of ethyl alcohol.

Upper : Cell Mass

Lower : DNA



The effect of clorobiocin on these two parameters was compared to that of nalidixic acid (Figure 3.6). As expected (see Goss *et al.*, 1965), a high concentration of nalidixic acid (50 µg/ml) caused an immediate cessation of DNA synthesis. Mass, however, continued to accumulate exponentially for about 45 minutes (slightly less than one generation time) before inhibition occurred. At a lower concentration of nalidixic acid (20 µg/ml) DNA synthesis continued at a reduced rate for about 100 minutes before ceasing abruptly. The significance of this continued synthesis is not clear; it may reflect continued movement of some or all replication forks before DNA synthesis finally ceases. When clorobiocin (50 µg/ml) and nalidixic acid (20 µg/ml) were added together, the effect of nalidixic acid upon DNA synthesis appeared to be dominant. However a synergistic effect on mass synthesis was observed consistent with a similar additive effect of the two drugs on protein synthesis (see section IV).

The low levels of DNA synthesis observed in the presence of clorobiocin and nalidixic acid were studied further by pulse labelling a culture of LE234 with [ $^3\text{H}$ ]-thymidine to measure the instantaneous rate of DNA synthesis during antibiotic treatment. As shown in Figure 3.7, the rate of DNA synthesis fell by almost 90% within 8 minutes of the addition of clorobiocin (50 µg/ml) and continued at about this level for 2.75 hours. By comparison, nalidixic acid (20 µg/ml) caused an immediate 80% reduction in the rate of DNA synthesis. The residual synthesis, however, was only observed for 80 minutes before the rate fell to less than 5% of the initial value. This confirms the accumulation data and shows that DNA synthesis continued at a reduced rate in the presence of these concentrations of clorobiocin and nalidixic acid.



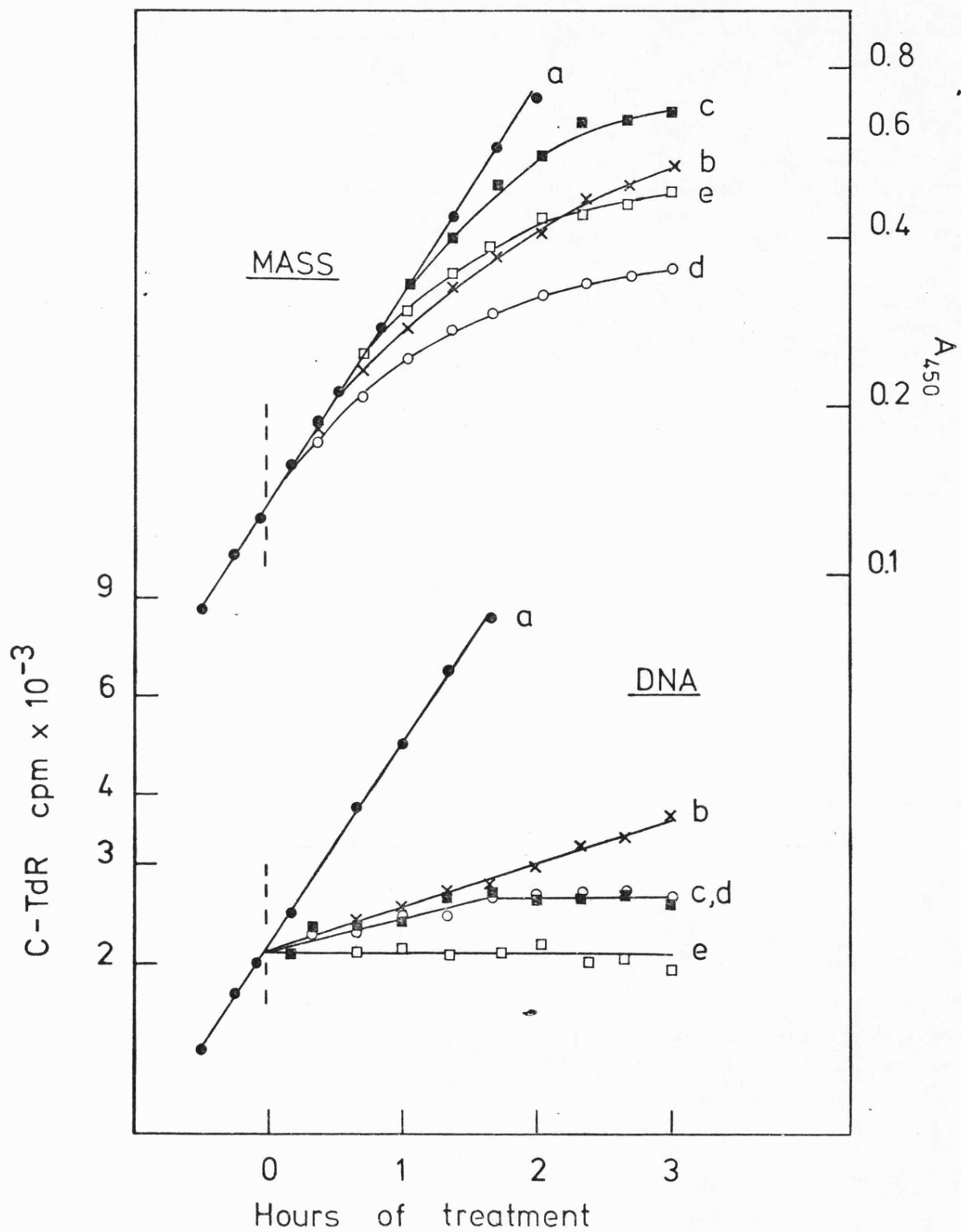
### Figure 3.6

#### Mass and DNA Synthesis in LE234 during Nalidixic Acid and Clorobiocin Treatments

A culture of LE234 was grown in M9 medium with essential amino acids supplemented with  $[^{14}\text{C}]$ -thymidine (2  $\mu\text{g}/\text{ml}$ , 0.05  $\mu\text{Ci}/\text{ml}$ ) to an  $A_{450} = 0.08$ . At intervals 1.0 ml samples were removed into ice-cold TCA for determination of total acid precipitable counts. At  $A_{450} = 0.13$  the culture was distributed into 5 flasks which received nalidixic acid or clorobiocin to the final concentrations indicated below. Samples were then removed as before.

Upper : Cell Mass                      Lower : DNA

- a. (●) control
- b. (X) 50  $\mu\text{g}/\text{ml}$  clorobiocin
- c. (■) 20  $\mu\text{g}/\text{ml}$  nalidixic acid
- d. (O) 20  $\mu\text{g}/\text{ml}$  nalidixic acid plus 50  $\mu\text{g}/\text{ml}$  clorobiocin
- e. (□) 50  $\mu\text{g}/\text{ml}$  nalidixic acid



### Figure 3.7

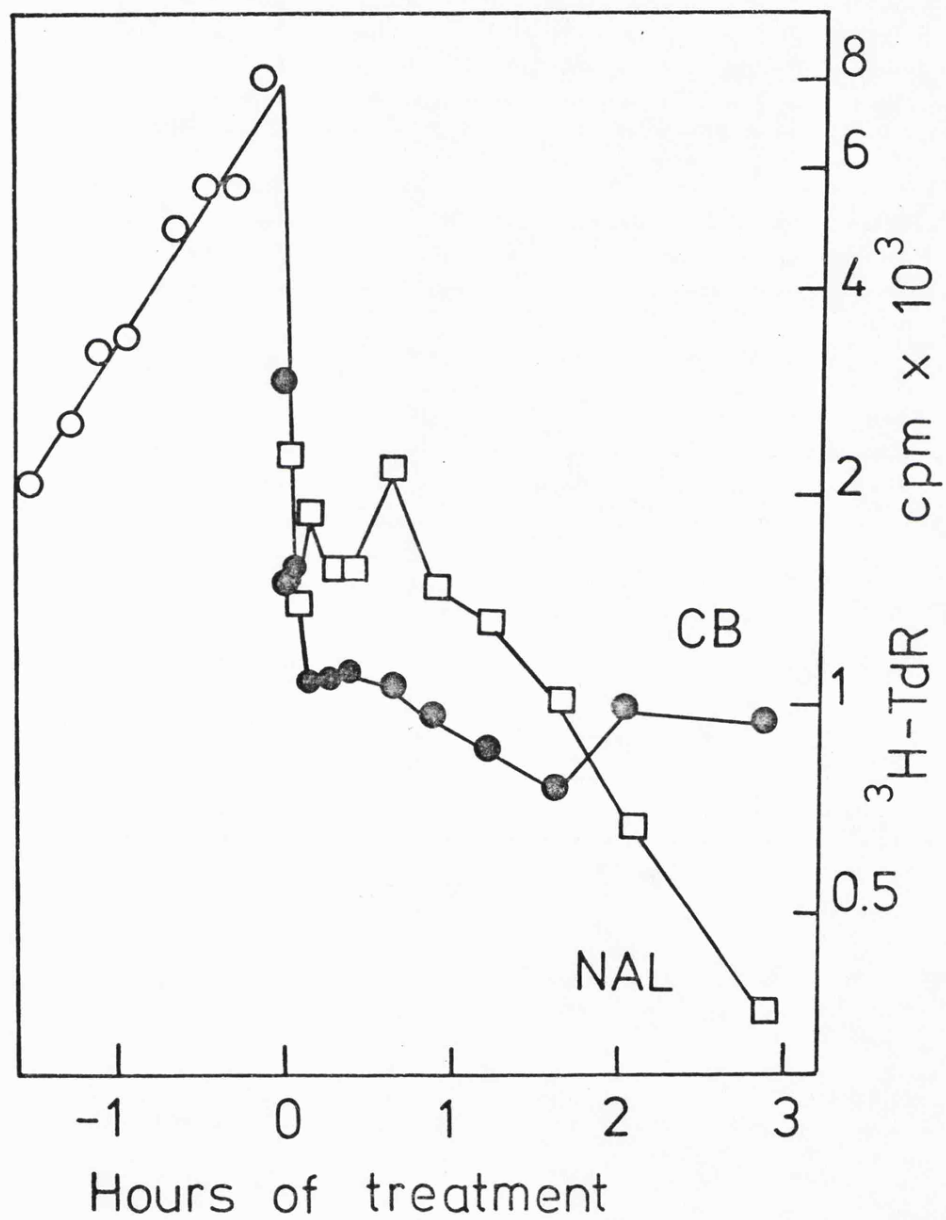
#### Rate of DNA Synthesis in LE234 during Clorobiocin and Nalidixic Acid Treatments

A culture of LE234 was grown in M9 medium with essential amino acids to an  $A_{450} = 0.03$ . 0.5 ml samples were withdrawn and pulse-labelled for 2 minutes with 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]-thymidine (85 Ci/mmol). At an  $A_{450}$  0.12 the culture was distributed into two flasks which immediately received clorobiocin or nalidixic acid. Samples were then removed as before.

(O) control

(●) 50  $\mu\text{g/ml}$  clorobiocin

(□) 20  $\mu\text{g/ml}$  nalidixic acid



#### IV. An Attempt to Characterise the Reduced Rate of DNA Synthesis

The reduced rate of DNA synthesis observed in the presence of low concentrations, e.g. 10 µg/ml of clorobiocin, could be due to; (1) a reduced frequency of initiation of replication, (2) a reduced rate of movement of some or all of the replication forks in the culture, or (3) some combination of these two factors. In order to test the second explanation the pattern of DNA synthesis was examined in the presence of rifampicin. Rifampicin blocks the initiation of DNA replication and the time taken for DNA synthesis to completely cease, or runout, is a measure of the C-time of that culture i.e. the velocity of the replication forks (Pritchard and Zaritsky, 1970). A more accurate determination of the C-time can be computed from the increment of DNA synthesis ( $\Delta G$ ) which is a measure of the number of replication forks per chromosome immediately before blockage of further initiation of replication (Pritchard and Zaritsky, 1970). The formula used is:

$$\Delta G = \frac{2^n n \cdot \ln 2}{2^n - 1} - 1$$

where  $n = \frac{C}{\tau}$

add  $C =$  time taken for one round of replication

and  $\tau =$  generation time (minutes)

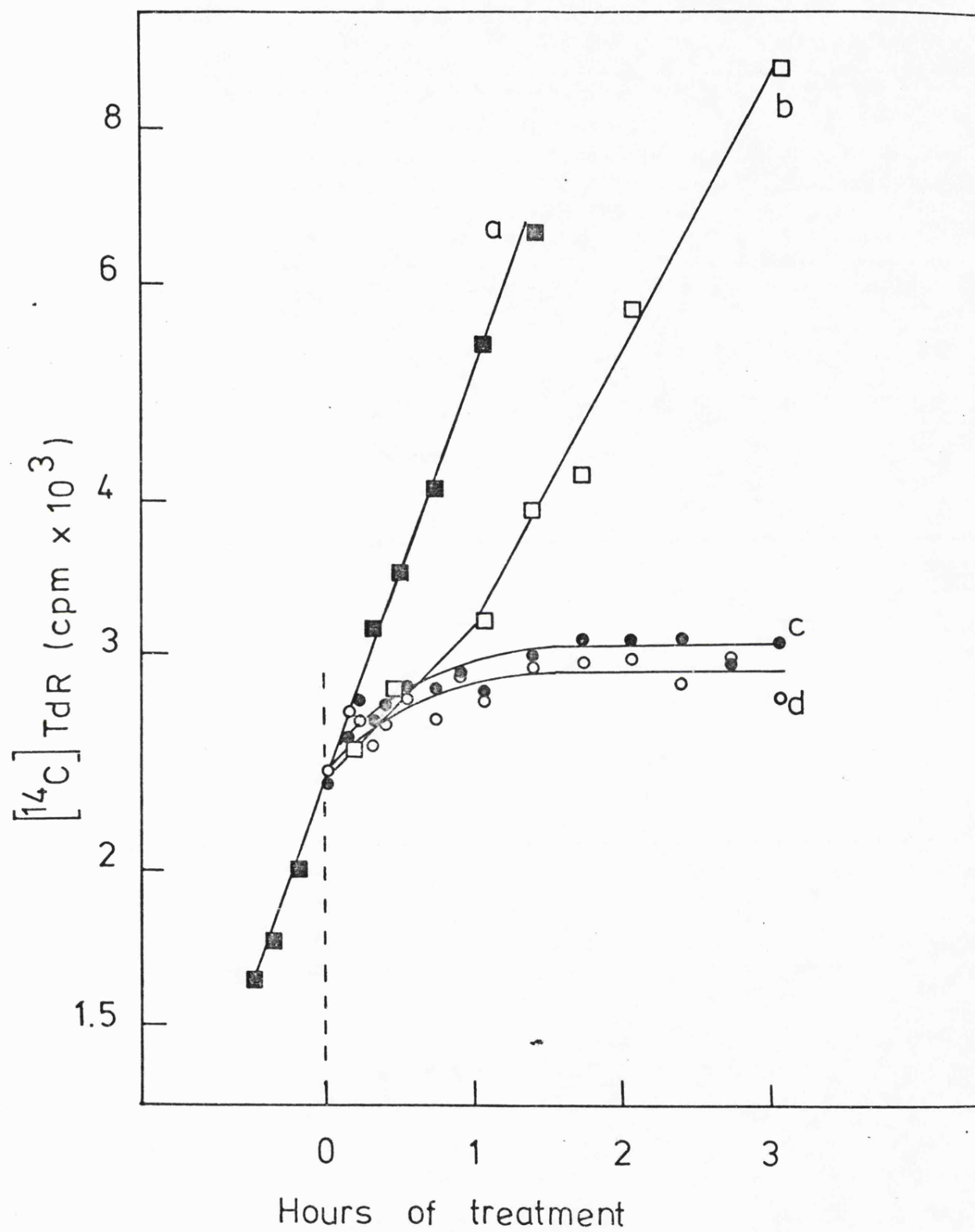
In the experiment shown in Figure 3.8 clorobiocin and rifampicin were added either separately or together to LE234 and the runout times compared. Although there is a certain amount of scatter in the points, it is clear that the clorobiocin and rifampicin treated culture synthesised

### Figure 3.8

#### DNA Synthesis in LE234 in the Presence of Clorobiocin and Rifampicin

A culture of LE234 was grown in M9 medium with essential amino acids supplemented with  $[^{14}\text{C}]$ -thymidine (0.05  $\mu\text{Ci/ml}$ , 2  $\mu\text{g/ml}$ ) to an  $A_{450} = 0.12$ . Samples were then withdrawn into ice-cold TCA for determination of total acid precipitable counts. At an  $A_{450} = 0.18$  the culture was distributed into 4 flasks which received either clorobiocin or rifampicin as indicated below. Samples were then taken as before.

- a. (■) control
- b. (□) 10  $\mu\text{g/ml}$  clorobiocin
- c. (●) 200  $\mu\text{g/ml}$  rifampicin
- d. (○) 10  $\mu\text{g/ml}$  clorobiocin + 200  $\mu\text{g/ml}$  rifampicin



less (i.e. 80%) DNA than did the culture treated with rifampicin alone. The time taken to runout was the same for both cultures, about 85 minutes. This value is more than twice the computed C-time of the rifampicin treated culture (41 min.) as calculated from the  $\Delta G$  value. However, estimations of C-times from the runout times can yield abnormally high values (see Chapter 6; also Orr et al., 1979). The important point to note is that because the cultures did not synthesise the same amount of DNA, no comparison can be made concerning the respective velocities of the replication forks. However this point does allow the conclusion that some replication forks are effectively stalled during clorobiocin treatment.

#### V. The Effect of Clorobiocin on RNA and Protein Synthesis

Both novobiocin and coumermycin have been reported to inhibit RNA synthesis in E.coli (Ryan, 1976; Smith and Davis, 1967) and the effect of clorobiocin on mass described in section III indicated that this drug acted similarly. The effect on RNA synthesis was specifically examined by pulse labelling a culture with  $[^{14}\text{C}]$ -uridine in the presence of clorobiocin or nalidixic acid. The results are shown in Figure 3.9. Upon addition of clorobiocin (50  $\mu\text{g/ml}$ ) the rate of RNA synthesis fell sharply to less than 30% of the pretreatment level. However this reduction was transient and was followed by a rapid recovery to an approximately constant rate at about 75% of the pretreatment value. After 2.5 hours the rate of RNA synthesis declined.



Figure 3.9

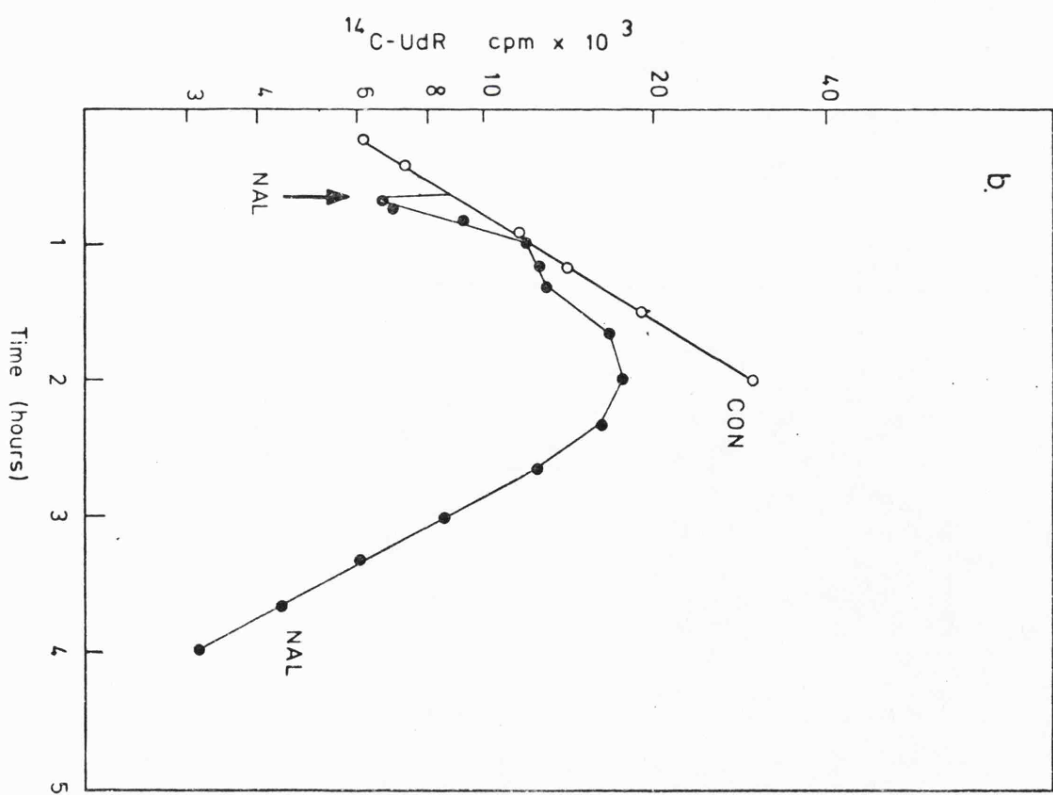
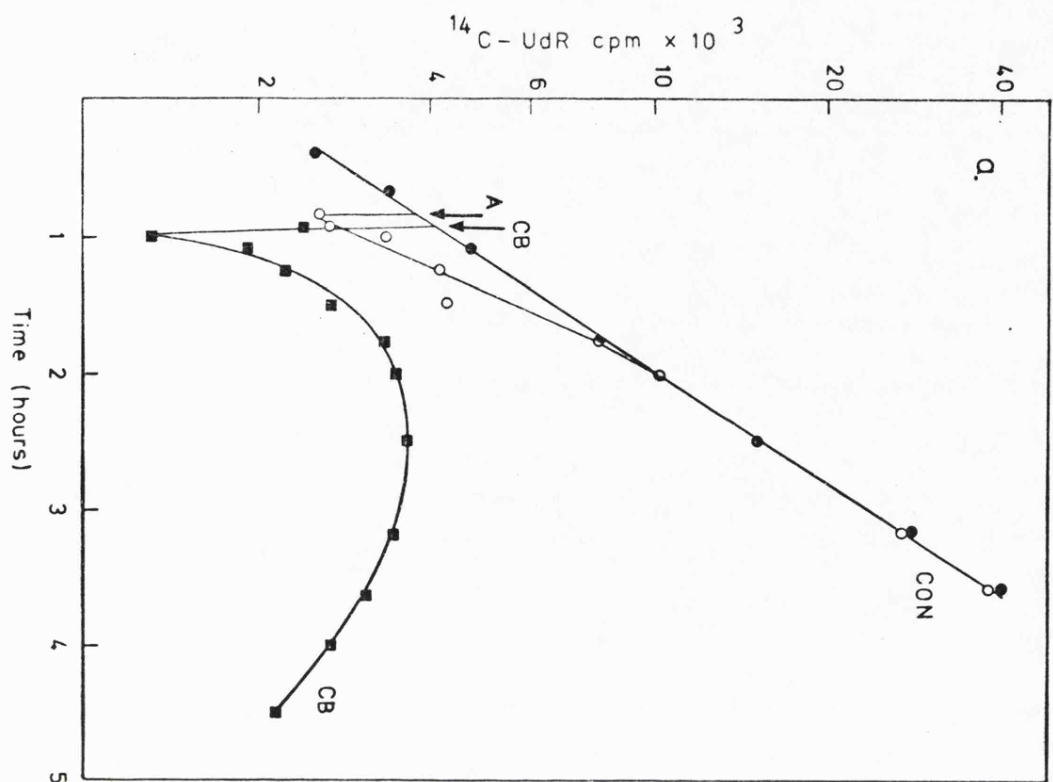
The Rate of RNA Synthesis in LE234 during Clorobiocin and  
Nalidixic Acid Treatments

- (a) A culture of LE234 grown in M9 minimal medium with essential amino acids was split 20 minutes prior to the addition of ethyl alcohol (final concentration 0.5% v/v) or clorobiocin (final concentration 50 µg/ml). The arrows beneath A and CB indicate the times of addition of alcohol and clorobiocin respectively. 0.5 ml samples were taken and pulse-labelled for 2 minutes with [ $^{14}\text{C}$ ]-uridine (0.5 µCi; 57.4 mCi/mmol).

CON (●) control culture  
A (○) alcohol treated  
CB (■) clorobiocin treated culture

- (b) A culture of LE234, grown as in (a), was treated with nalidixic acid (20 µg/ml) and samples were taken for determination of the rate of RNA synthesis as before.

CON (○) control culture  
NAL (●) nalidixic acid treated culture



The interpretation of the transient fall in rate of RNA synthesis is complicated by a similar effect which was observed after addition of ethyl alcohol (the solvent for clorobiocin). As shown in Figure 3.9a, the transient fall in this case was smaller and was followed by a recovery to the control exponential rate. A similar effect was seen upon addition of clorobiocin to the resistant strain LE701 (data not shown). Most probably the substantial fall in the rate of RNA synthesis, which was observed in several experiments including some in which  $[^3\text{H}]$ -uridine was used instead of  $[^{14}\text{C}]$ -uridine, is a specific effect of the antibiotic. In any case the subsequent period of an essentially constant rate of RNA synthesis which continued for about two hours before declining was clearly a direct effect of the clorobiocin.

The addition of nalidixic acid (20  $\mu\text{g/ml}$ ) also caused a transient fall in the rate of RNA synthesis as shown in Figure 3.9b. However this reduction in rate was even less than that with ethyl alcohol (the nalidixic acid was dissolved in 0.02M NaOH) and may be entirely attributable to an artifact of the experimental procedure. The important point to note is that subsequently the rate of RNA synthesis was approximately exponential and close to that of the untreated control culture over the first hour; thereafter the rate declined rapidly.

It is concluded from this data that at these concentrations clorobiocin is the more effective inhibitor of RNA synthesis, at least during the first hour of treatment.

The rate of protein synthesis was also measured during antibiotic treatment. Figure 3.10 shows that clorobiocin was the more effective inhibitor preventing any significant increase in rate above the

Figure 3.10

Rate of Protein Synthesis in LE745 during Clorobiocin and  
Nalidixic Acid Treatments

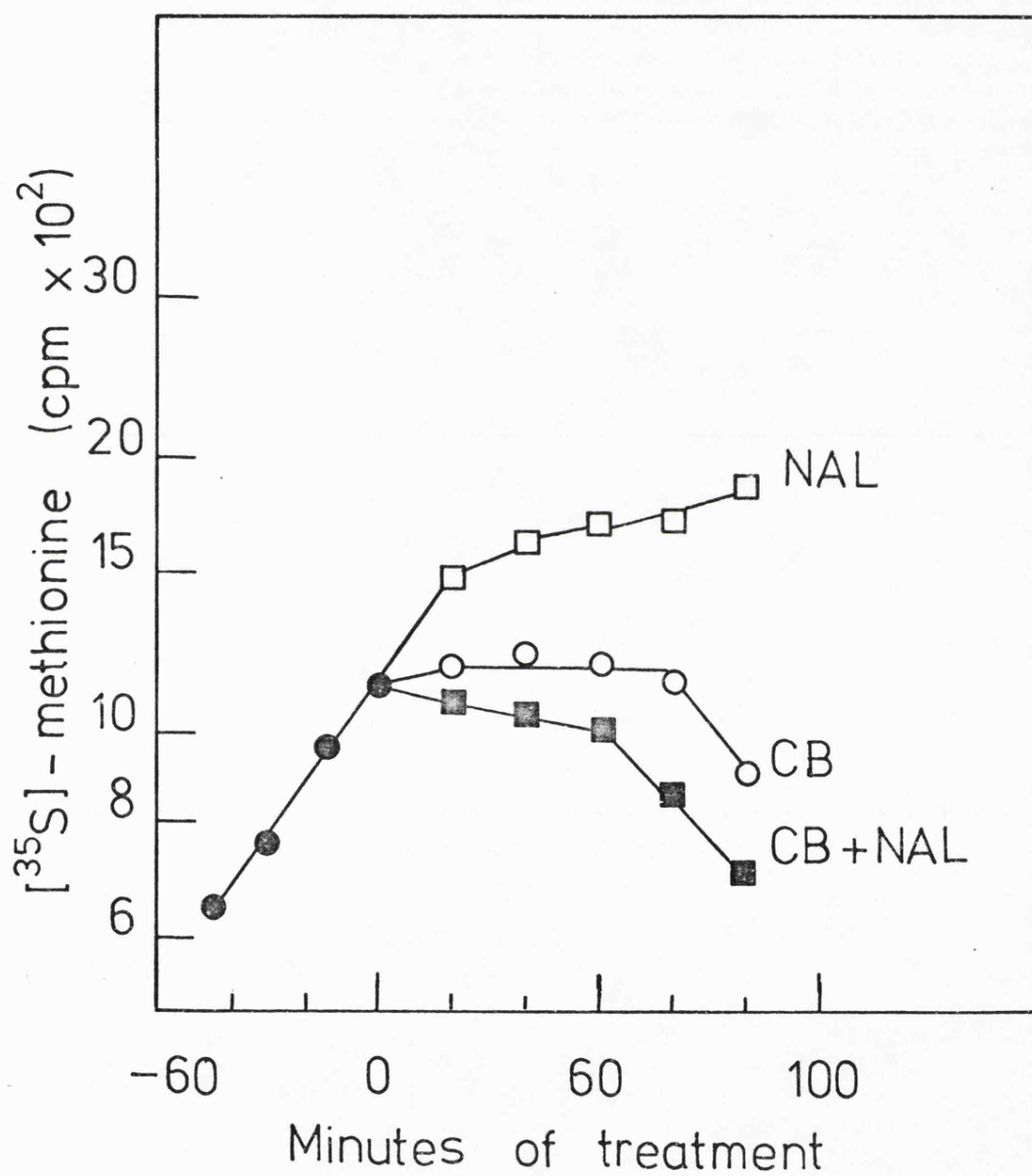
A culture of LE745 (LE234 Met<sup>+</sup>) was grown in M9 minimal medium with essential amino acids to an A<sub>450</sub> = 0.1. 1 ml samples were withdrawn, pulse-labelled with 1 ml medium containing 3  $\mu$ Ci [<sup>35</sup>S]-methionine and acid precipitable counts determined. At an A<sub>450</sub> = 0.19 the culture was split and clorobiocin and nalidixic acid added as indicated.

(○) control

(□) nalidixic acid (50  $\mu$ g/ml)

(○) clorobiocin (50  $\mu$ g/ml)

(■) clorobiocin and nalidixic acid (50  $\mu$ g/ml each)



pretreatment level for 80 minutes. With nalidixic acid (50 µg/ml) there was an exponential increase in the rate of protein synthesis for 20 minutes before inhibition was observed. Once again the two antibiotics were synergistic and caused an immediate reduction in rate of protein synthesis when added together.

## VI. Discussion

Clorobiocin is preferentially active against certain mutants of E.coli which lack LPS and/or protein components in the outer membrane. As discussed in Chapter 1, this phenomenon has been observed with many other hydrophobic compounds and has been attributed to a reduction in the barrier properties of the outer membrane. Clorobiocin was also more active against two mutants, the LPS compositions of which have not yet been reported; strains LEB18 (rRM98) and D22 (envA). The presence of the plasmid rRM98 in LEB18 blocks the synthesis of the major outer membrane protein (Iyer et al., 1978), and the increased permeability towards clorobiocin may be due either directly to the absence of this protein or to any increase in phospholipid which may accompany this loss. The envA mutant is defective in cell division, forming chains of 3-10 cells and is therefore apparently defective in the final stages of division, a process which involves the ingrowth of the outer membrane prior to septation (Normark et al., 1971). It would be of interest to determine the outer membrane protein composition of this mutant, and if this was abnormally low or showed a lack of any specific proteins, this might

account for both the defect in cell division and the abnormal sensitivity towards hydrophobic compounds.

The data in Figures 3.3 and 3.5 show clearly that DNA synthesis is inhibited more than mass synthesis during clorobiocin treatment. The pattern of inhibition of these processes is similar to that seen with novobiocin and coumermycin (Smith and Davis, 1967; Ryan, 1976) and this, together with the similarity of the structures of these compounds and the genetic evidence (see Chapter 5), makes it almost certain that clorobiocin is an inhibitor of DNA gyrase.

The continued low level of DNA synthesis even at high concentrations of clorobiocin suggests that a slow rate of polymerisation continues at the replication fork. If DNA synthesis was completely dependent upon active DNA gyrase, this continued synthesis would probably reflect incomplete inhibition of the enzyme by the drug. Alternatively, DNA gyrase may only be required for maximal DNA synthesis and the residual synthesis seen in the presence of clorobiocin would then reflect gyrase-independent replication. This alternative is consistent with results from clorobiocin resistant mutants which were found to be defective in initiation, but not elongation, of replication forks (see Chapter 6; also Orr *et al.*, 1979). The rifampicin runout experiment could not confirm the slow fork movement in the presence of clorobiocin, but did indicate that some replication forks were prematurely halted by clorobiocin. The recovery of DNA synthesis at low concentrations of clorobiocin must also be explained. Although clorobiocin is sensitive to high temperatures (see Chapter 2) it is unlikely that the drug was

inactivated at 37°C during the experiments. This is reinforced by the reduced growth rate of LE234 which persists even in cultures grown for eight generations in very low concentrations of clorobiocin. Rather it appears that the treated cultures, or at least the synthesis of DNA in such cultures, was adapted to the presence of clorobiocin. Since coumermycin and novobiocin, and therefore presumably clorobiocin, competitively inhibit the binding of ATP to the cou subunit of DNA gyrase, this adaptation could perhaps be achieved by raising the intracellular concentration of ATP. The recovery of DNA synthesis could also be due to extra initiation events which would increase the number of replication forks per cell. This would only be possible if the progress of these replication forks was not substantially affected by the presence of clorobiocin. It is therefore difficult to imagine this second alternative accounting entirely for the observed effect. The cultures in question were not followed long enough to determine whether the synthesis of cell mass also recovered.

Clorobiocin appeared to inhibit transcription in a complex manner. It has been shown that transcription from closed circular DNA in vitro is dependent upon the superhelical density of the DNA; this is apparently due to the energetically favourable "melting" of supercoiled promoter regions by RNA polymerase (see Chapter 1). The transient fall in the rate of transcription observed with clorobiocin could be due to a decrease in the rate of synthesis of one specific class of RNA, and the recovery due to increased synthesis of a second class of RNA. These two classes of RNA would then differ in the sensitivity of their promoters to a decrease in superhelical density of the DNA.



Recently Smith et al. (1978) found that nalidixic acid, and to a lesser extent coumermycin, inhibited transcription of the E.coli trp operon from a phage ( $\phi$ 80) promoter in preference to that from the trp promoter. Further indication of the selective inhibition of transcription by these inhibitors of DNA gyrase comes from a study by Sanzey (1979) who has shown that both novobiocin and nalidixic acid preferentially inhibit transcription of catabolite-sensitive operons via an effect on the promoters of these operons. Interestingly, Sanzey found that both nalidixic acid and novobiocin increased the synthesis of three separate gene <sup>products</sup> when these were under the control of a mutated, catabolite-insensitive form of the lac promoter (lacPUV5).

The effects of clorobiocin and nalidixic acid on transcription are emphasised by the synergistic effects of these two antibiotics on RNA and protein synthesis, but not on DNA synthesis. This may indicate that the cou subunit of DNA gyrase functions independently of the nalA subunit in transcription, and perhaps that the roles of the subunits in transcription differ from that in replication.

## Chapter 4

### THE EFFECT OF CLOROBIOCIN ON CELL DIVISION AND INDUCTION OF THE SOS FUNCTIONS

#### I. Introduction

Treatments which inhibit DNA synthesis in E.coli generally cause the induction of the SOS functions (Gudas, 1976; Gudas and Pardee, 1976; Little and Hanawalt, 1977; see Chapter 1). This induction is most probably due to the presence of acid-soluble products of DNA degradation. As discussed in Chapter 1, the induction of the SOS functions has complicated the interpretation of some experiments designed to increase our understanding of the regulation of the cell cycle of E.coli; in particular the hypothesis that termination of a round of chromosomal replication is a prerequisite for subsequent cell division (Helmstetter and Pierucci, 1968; Jones and Donachie, 1973). Some data do in fact contradict this hypothesis, namely that continued cell division resulting in the production of DNA-less cells is seen both during continued growth of a dnaA mutant at 42°C, and after treatment of a recA mutant with nalidixic acid (Hirota et al., 1968; Inouyi, 1971). However it is precisely under these conditions that the SOS functions are not induced. Thus, if termination of replication is a prerequisite for a subsequent division, it would be necessary to postulate that this control system must be defective in these mutants. Of course it is possible that the recA and dnaA genes are normally active in such a pathway.

Alternatively, it is equally possible that cell division is not timed from any point in the replication process, and that the cell relies upon the induction of the SOS system to halt division when DNA synthesis is disturbed. It was of interest, therefore, to study the effects of clorobiocin on cell division and to determine its ability to induce the SOS functions.

## II. The Effect of Clorobiocin on the SOS Functions

Induction of the SOS system is accompanied by increased mutagenesis, induction of phage  $\lambda$ , inhibition of cell division and the synthesis of large amounts of a protein, termed X, now known to be the recA gene product (see Chapter 1).

### (a) DNA Degradation

The ability of clorobiocin to induce DNA degradation was determined by measuring the solubilisation of DNA prelabelled with [ $^3\text{H}$ ]-thymidine. The results, as shown in Figure 4.1, show that no significant DNA breakdown occurred after 4 hours incubation with clorobiocin (50  $\mu\text{g/ml}$ ). In contrast, nalidixic acid (50  $\mu\text{g/ml}$ ) and UV light (100  $\text{J.m}^{-2}$ ) caused 21% and 31% solubilisation above the control level respectively. The high value after 4 hours clorobiocin treatment is probably not significant as it was not seen on repetition of the experiment (data not shown). The high control value of around 20% of the thymidine label in a TCA soluble form appears to be a property of the strain LE234 and was always observed in such experiments.

Figure 4.1

DNA Degradation Caused by Clorobiocin, Nalidixic Acid and  
UV Light

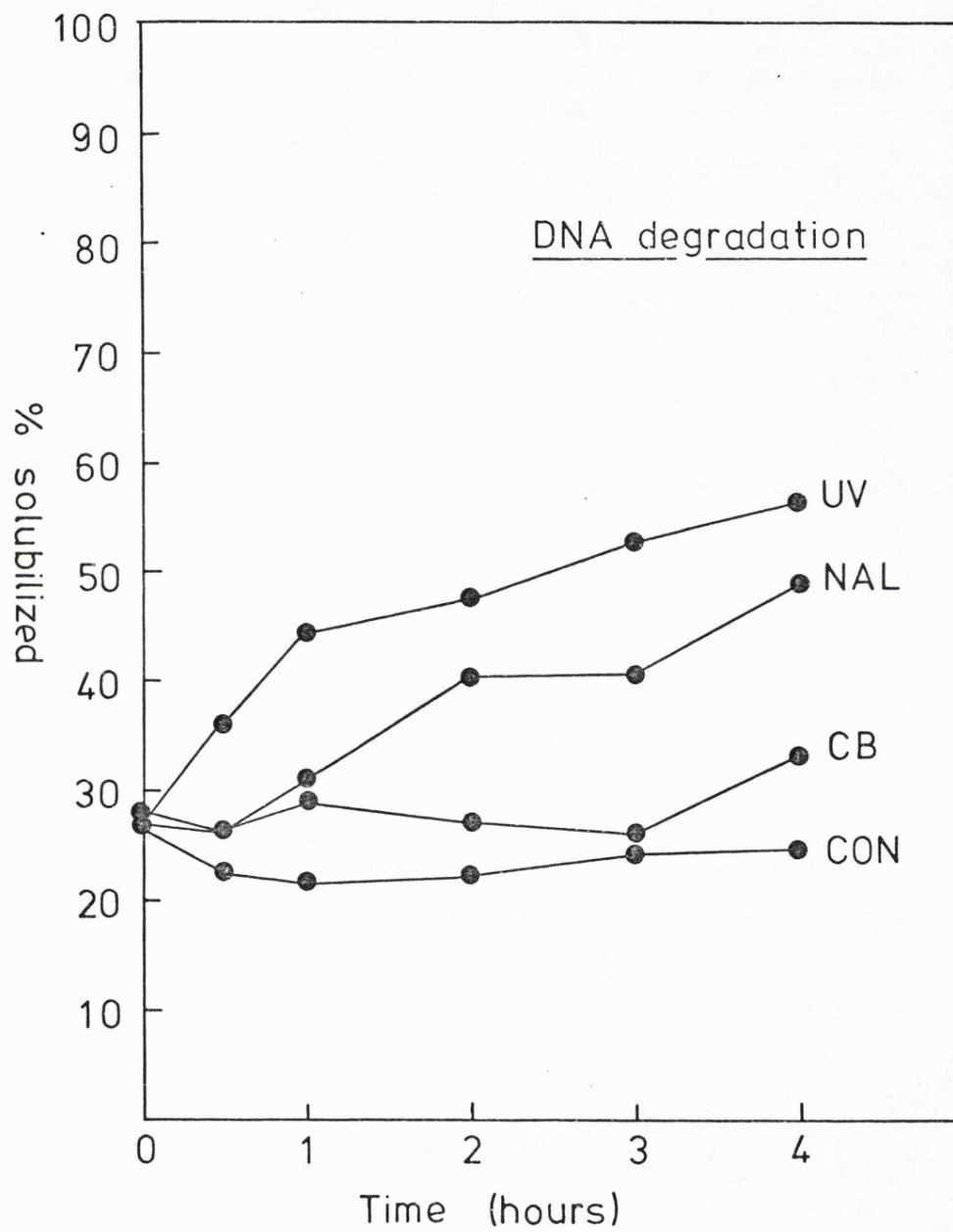
A culture of LE234 was prelabelled with  $^3\text{H}$  -thymidine, washed and aliquots were treated as indicated below. Samples were removed and acid-insoluble and -soluble counts determined. DNA solubilisation is expressed as the percentage acid-soluble counts present.

NAL      50  $\mu\text{g/ml}$  nalidixic acid

CB        50  $\mu\text{g/ml}$  clorobiocin

UV        100  $\text{J.m}^{-2}$

CON      control (no treatment)



(b) Induction of Protein X by Clorobiocin and Nalidixic Acid

A simpler and more sensitive assay for the induction of the SOS functions is the increased synthesis of protein X, the recA gene product of molecular weight 40K. Figure 4.2 shows the analysis, by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), of total cellular proteins synthesised by LE747 during nalidixic acid and clorobiocin treatments. Nalidixic acid clearly induced protein X while no detectable induction was apparent with clorobiocin. Clorobiocin did, however, alter the pattern of protein synthesis. The rate of synthesis of a 24K polypeptide was markedly increased during clorobiocin treatment and to a lesser extent during nalidixic acid treatment. This will be considered further in Chapter 7. The rate of synthesis of a 42K protein, which migrated between Eftu (44K) and protein X (40K), was also increased by clorobiocin but not by nalidixic acid (see also Figure 4.3). This latter change was only observed when the monomer to bis ratio in the acrylamide gel was 44:0.8. When this ratio was increased to 44:0.3 (see Chapter 6) the 40K and 42K bands comigrated and were not easily resolved (data not shown).

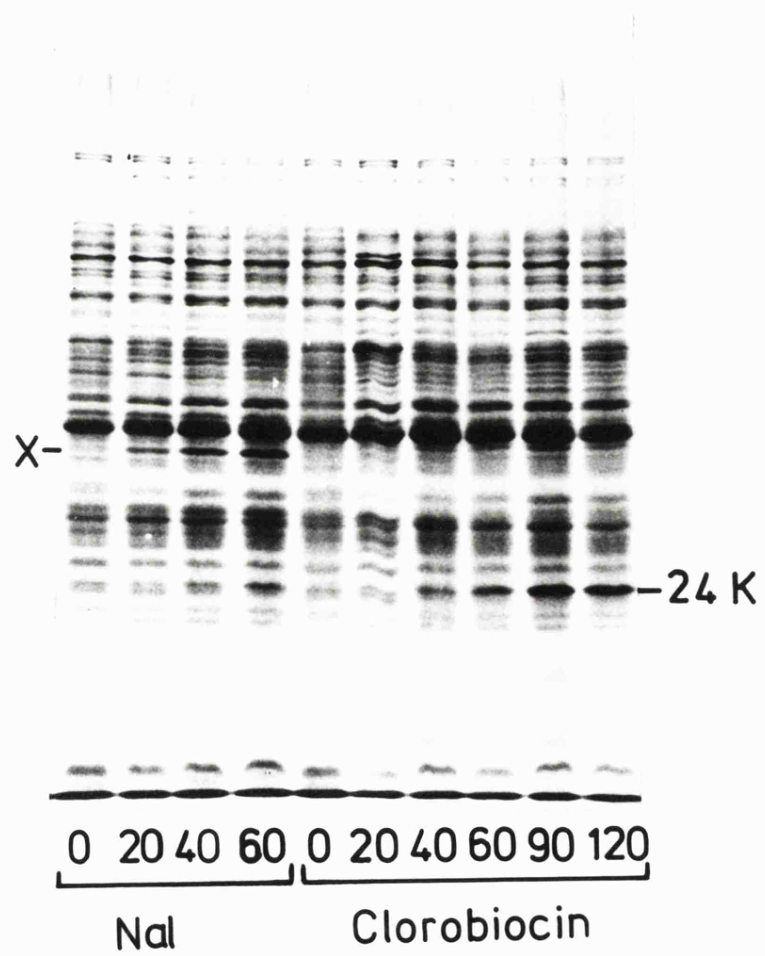
In order to rule out the possible repression of protein X synthesis by clorobiocin (as opposed to a failure to induce), the two antibiotics were added together and the proteins synthesis were again visualised by SDS-PAGE. Figure 4.3 shows that clorobiocin did not dramatically inhibit the induction of protein X by nalidixic acid although a slight decrease cannot be ruled out (compare lanes 8 and 9 with lanes 12 and 13).

## Figure 4.2

### Failure of Clorobiocin to Induce Protein X

A culture of LE747 was grown in M9 minimal medium without methionine to an  $A_{450} = 0.2$ . The culture was split; one portion received clorobiocin (50  $\mu\text{g/ml}$ ), the other nalidixic acid (50  $\mu\text{g/ml}$ ). 1.0 ml samples of the cultures were pulse-labelled for 5 min with 5  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]-methionine ( $> 800 \text{ Ci/m.mol}$ ) and total cell proteins were analysed by SDS-PAGE.

The numbers under the slots refer to the time of the pulse, in min., after addition of the antibiotic.



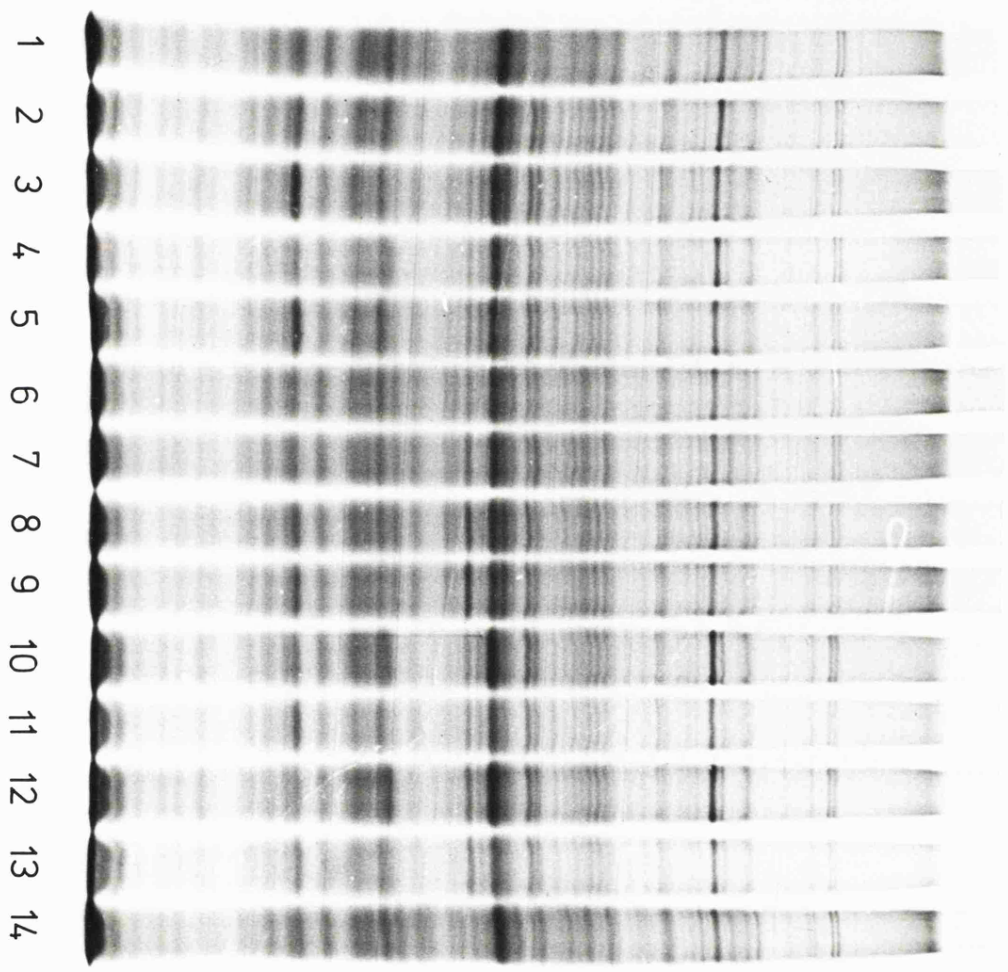


### Figure 4.3

#### Induction of Proteins by Clorobiocin and Nalidixic Acid

A culture of LE747 was grown in M9 minimal medium without methionine to an  $A_{450} = 0.2$ . The culture was split and portions received either clorobiocin (50 or 100  $\mu\text{g/ml}$ ), nalidixic acid (20 or 50  $\mu\text{g/ml}$ ), both clorobiocin (50  $\mu\text{g/ml}$ ) and nalidixic acid (20  $\mu\text{g/ml}$ ) or both clorobiocin (50  $\mu\text{g/ml}$ ) and nalidixic acid (50  $\mu\text{g/ml}$ ). 1.0 ml samples were pulse-labelled with 5  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]-methionine (50  $\mu\text{Ci}/\mu\text{g}$ ) after either 30 or 60 min. treatment and total cell proteins analysed by SDS-PAGE.

|      |       |   |            |  |
|------|-------|---|------------|--|
| Lane | 1     | Control No treatment                                |            |  |
| "    | 2,3   | CB 50 $\mu\text{g/ml}$                              | 30, 60 min |  |
| "    | 4,5   | CB 100 $\mu\text{g/ml}$                             | 30, 60 "   |  |
| "    | 6,7   | NAL 20 $\mu\text{g/ml}$                             | 30, 60 "   |  |
| "    | 8,9   | NAL 50 $\mu\text{g/ml}$                             | 30, 60 "   |  |
| "    | 10,11 | CB 50 $\mu\text{g/ml}$ + NAL (20 $\mu\text{g/ml}$ ) | 30, 60 min |  |
| "    | 12,13 | CB 50 $\mu\text{g/ml}$ + NAL (50 $\mu\text{g/ml}$ ) | 30, 60 min |  |
| "    | 14    | Control No treatment                                | 30, 60 min |  |



EfTu  
X

—24K

### III. The Effect of Clorobiocin on Cell Division

In Chapter 3, Table 3.3 it was shown that low concentrations of clorobiocin reduced the growth rate of LE234 and this was accompanied by some chain formation and/or filamentation of the bacteria. The effect of clorobiocin on cell division was studied further in LE234 and the results are shown in Figure 4.4. Division was immediately inhibited upon addition of clorobiocin. However this inhibition was not absolute and was concentration dependent. At 20 µg/ml the culture partially recovered from the inhibition after two hours and the cells divided more frequently.

The inhibition of division by 50 µg/ml clorobiocin was studied further by phase contrast microscopy. Figures 4.5 and 4.6 show a marked heterogeneity of cell sizes in the clorobiocin treated cultures, including a class of cells smaller than the untreated control cells. These appear to be the products of abnormal divisions from longer cells. In contrast, nalidixic acid caused only filamentation with very few abnormal divisions (Figure 4.5b). The heterogeneity of cell sizes was confirmed by a size distribution analysis using a Coulter Counter fitted with a Channelyzer (see Figure 4.7). This showed a general increase in cell volume during the course of the  $2\frac{1}{2}$  hour experiment. The small cells observed by phase contrast microscopy could account for the small peak at the very low cell volume in the 150 minute sample.

As mentioned previously, the presence of a recA mutation does not allow the expression of the SOS functions. In order to test more directly whether the inhibition of cell division by clorobiocin was due

Figure 4.4

Inhibition of Cell Division by Clorobiocin

A culture of LE234 was grown in M9 minimal medium with essential amino acids. At an  $A_{450} = 0.13$  the culture was split and portions received clorobiocin as indicated.

Upper : Cell Mass

Lower : Cell Number

(○) : Control

(□) : 20  $\mu\text{g/ml}$  clorobiocin

(■) : 50  $\mu\text{g/ml}$  clorobiocin

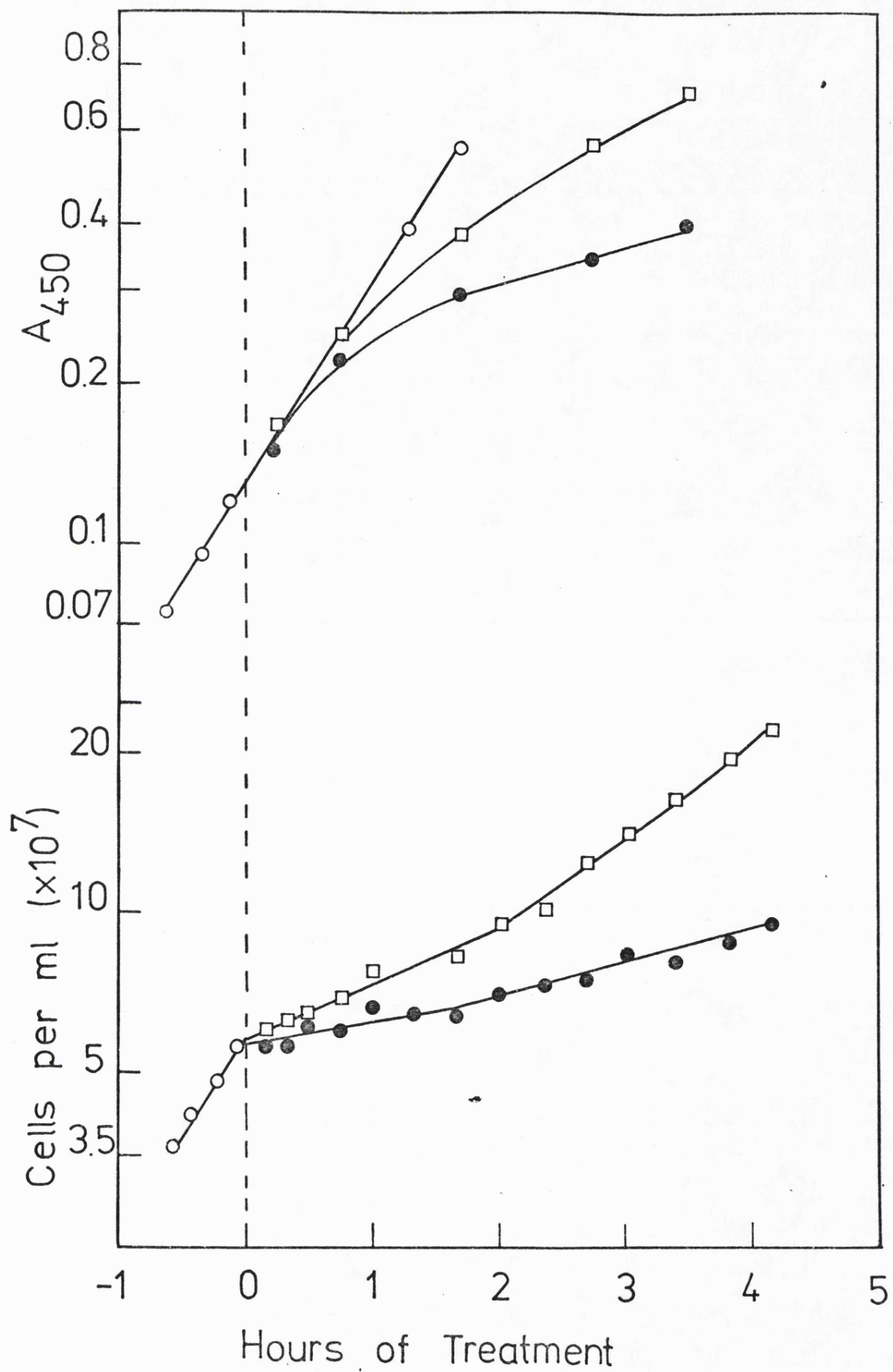


Figure 4.5

Morphology of Cultures Treated with Clorobiocin or Nalidixic Acid

A culture of LE234 was grown in M9 minimal medium with essential amino acids to an  $A_{450} = 0.2$ . The culture was distributed into 3 flasks; one received nalidixic acid (20  $\mu\text{g/ml}$ ), one clorobiocin (50  $\mu\text{g/ml}$ ) and the third alcohol (1% v/v). After 2.5 hours, 5.0 ml samples were taken and the bacteria photographed using phase contrast microscopy.

- a. control
- b. nalidixic acid (20  $\mu\text{g/ml}$ )
- c. clorobiocin (50  $\mu\text{g/ml}$ )

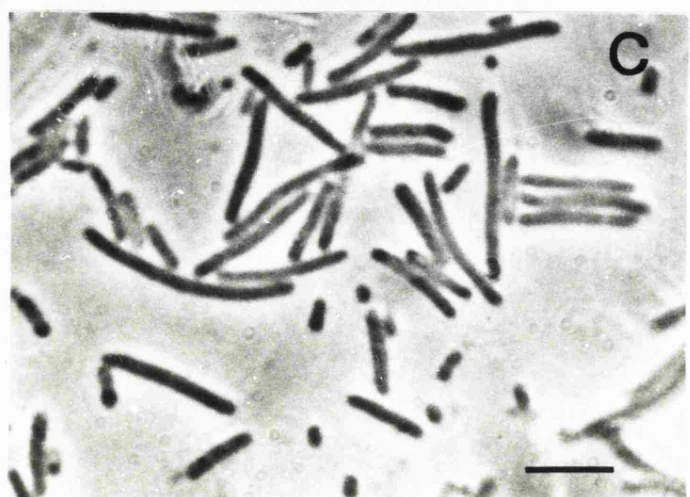
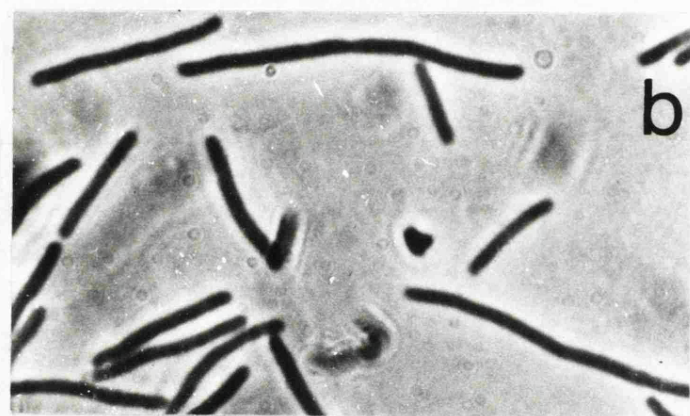
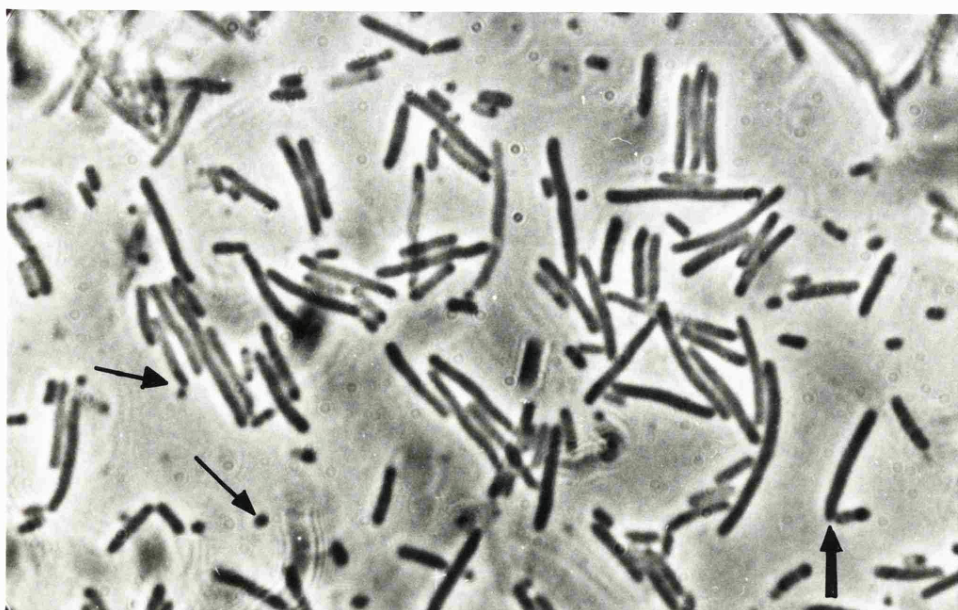
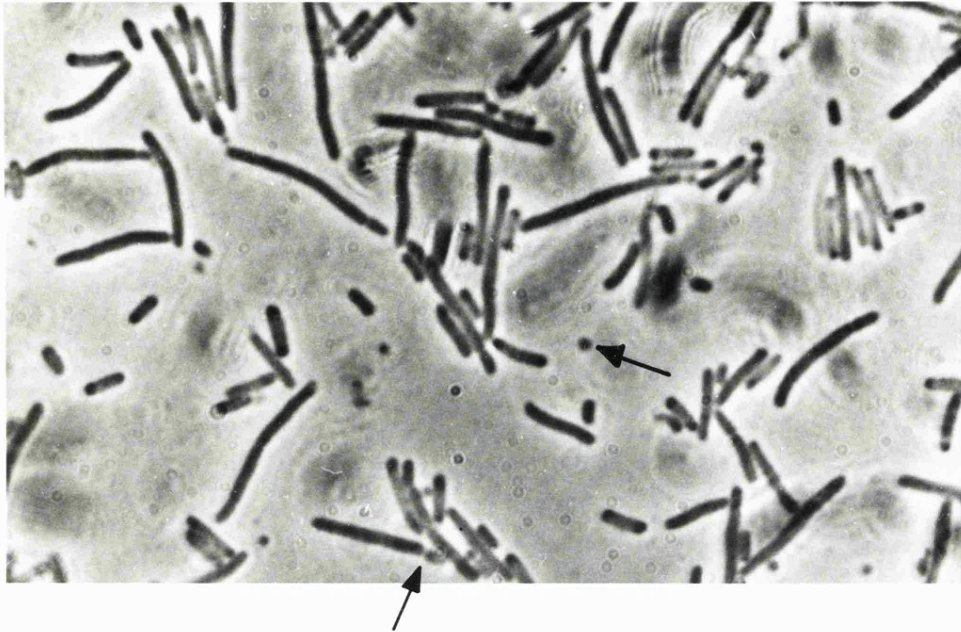


Figure 4.6

Morphology of Cultures During Clorobiocin Treatment

The clorobiocin treated culture of LE234 described in Figure 4.5 was photographed by phase contrast microscopy. The arrows point to abnormal division sites and small cells.





### Figure 4.7

#### Size Distribution of Cultures Treated with Clorobiocin

LE234 was grown in M9 minimal medium with essential amino acids to an  $A_{450} = 0.15$ . Clorobiocin was added to a final concentration of 50  $\mu\text{g/ml}$  and the distribution of cell volumes, and hence cell sizes, measured using a Coulter Counter fitted with a Channelyzer.

The numbers above the curves indicate the times, in minutes, of the samples taken after addition of clorobiocin. A constant number of cells from each sample was analysed.

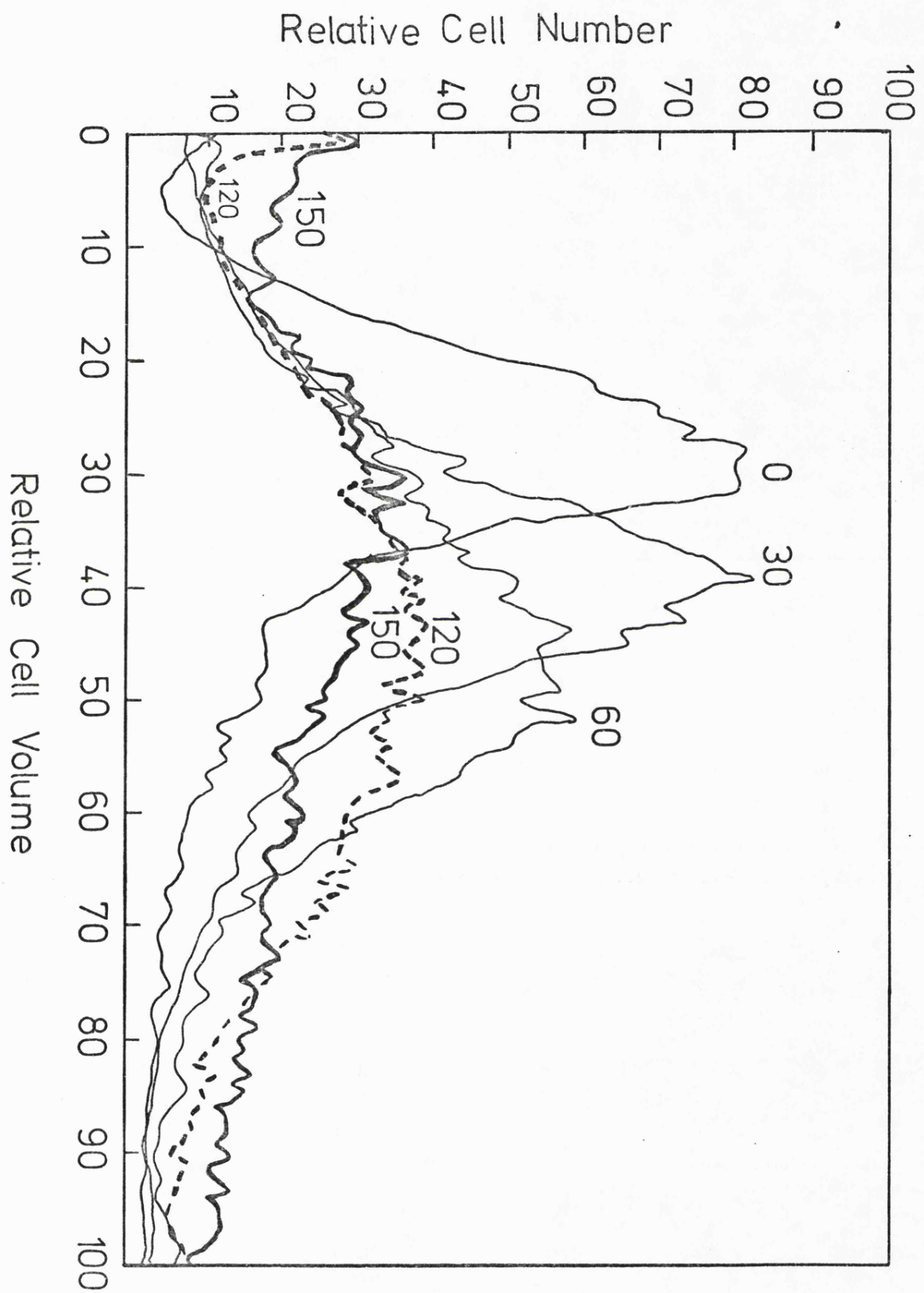


Figure 4.8

Inhibition of Cell Division in  $\text{recA}^+$  and  $\text{recA}^-$  Strains of LE234  
During Treatment with Clorobiocin or Nalidixic Acid

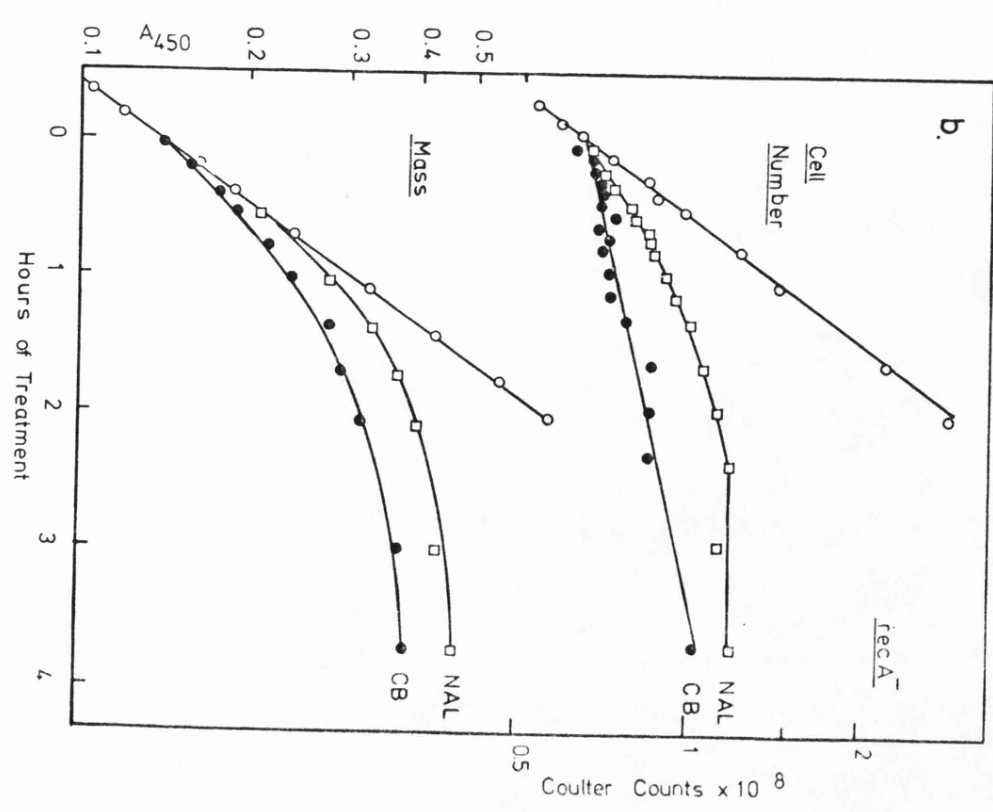
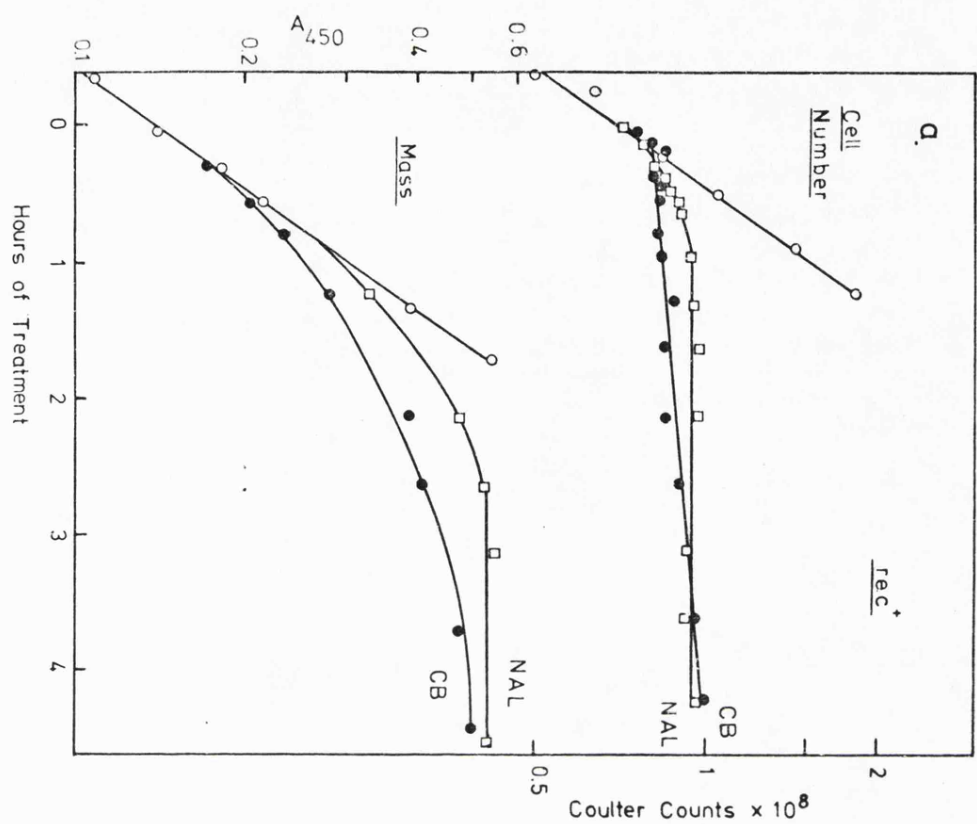
Cultures of LE234 and LE705 were grown in M9 minimal medium with essential amino acids to an  $A_{450} = 0.13$ . The cultures were split and clorobiocin or nalidixic acid added as indicated.

a.  $\text{recA}^+$       b.  $\text{recA}^-$

(○) control

(□) clorobiocin (50  $\mu\text{g/ml}$ )

(●) nalidixic acid (20  $\mu\text{g/ml}$ )



to the induction of the SOS functions, clorobiocin and nalidixic acid were added to LE234 and a recA derivative, LE705. The results (Figure 4.8a) show that nalidixic acid treatment resulted in a run-out of divisions. Calculation showed that the increment of divisions obtained in this experiment would exactly represent the number of bacteria in the D period if the D-time were 20.2 minutes. This calculation derives from the equation:

$$\text{no. of cells in } D = 2^{D/\tau} - 1$$

where  $\tau$  = generation time in minutes

In contrast, clorobiocin did not allow such a runout and therefore inhibited division in those cells already in the D period. In the recA mutant (Figure 4.8b), increment of divisions before growth ceased with nalidixic acid was much greater than in the isogenic rec<sup>+</sup> strain. In contrast clorobiocin still drastically reduced the rate of divisions but not to the same extent as in the rec<sup>+</sup> strain.

#### IV. Discussion

The major conclusions of this chapter are; (1) clorobiocin does not induce the SOS repair system and (2), clorobiocin is an effective inhibitor of cell division. The failure to induce the SOS repair system (as judged by the synthesis of protein X) presumably reflects the absence of DNA degradation products and so provides further circumstantial evidence that such products may be the inducer of the error prone repair system. The inhibition of cell division by clorobiocin is not then due

to the induction of this system. The inhibition of division is also not due to a failure to terminate rounds of chromosome replication, since clorobiocin inhibited division in those cells which had apparently completed this event. Of course this does not mean that termination is not also a prerequisite for division, and could imply that clorobiocin acts to block a later step in the division process.

What then is the reason for the inhibition of division by clorobiocin, and how can this be reconciled with the inhibition of DNA gyrase? Before speculating on this point, it is of interest to recall an alternative to the two models cited above which attempts to explain the inhibition of division following the cessation of DNA synthesis. Pritchard (1974) proposed that inhibition of cell division due to inhibition of DNA synthesis could result from a disruption of the relative rates of accumulation of cell envelope and cell mass. This followed from the construction of a model which invoked the exponential increase in cell mass (which is known to occur) and the linear accumulation of some component of the cell envelope, the rate of synthesis doubling at a discrete time during the cell cycle corresponding to the replication of an unregulated gene or genes. This model therefore predicted that cell mass and cell volume never increase at the same rate. A doubling of the linear rate of cell envelope synthesis results in a relative increase in cell envelope material which then leads to division. It is of interest that Boyd and Holland (1979) found that outer membrane protein synthesis, but not that of the inner membrane, did increase linearly with an abrupt doubling in rate occurring about the time of termination of a round of DNA replication.

Thus it is possible that clorobiocin selectively induces or represses, via conformational changes in promoters as discussed in Chapter 3, the transcription of one or more genes to alter the differential rate of synthesis of crucial envelope components sufficiently to block division.

Finally, as an alternative to "transcriptional change" model cited above, clorobiocin could inhibit cell division and promote abnormal divisions by reducing the overall degree of supercoiling of the chromosome leading to the disruption of a DNA-membrane complex which might be essential for a late step in septum formation. These hypotheses are not mutually exclusive; an effect upon transcription could in turn result in the formation of a defective DNA-membrane complex (by for example altering the ratios of structural RNA or protein components) thereby leading to abnormal division events. A study of the effect of clorobiocin on membrane protein synthesis is presented in Chapter 7.



## Chapter 5

### A GENETIC ANALYSIS OF CLOROBIOCIN RESISTANT MUTANTS

#### I. Introduction

The isolation and genetic analysis of mutants resistant to coumermycin was first reported by Ryan (1976) who found that such strains carried a mutation, designated cou, which mapped very close to the dnaA locus at 82 minutes on the E.coli genetic map. Ryan concluded that the gene order was cou, dnaA, ilv. Since the results in Chapter 3 and the similar results of Ryan (1976) suggested that clorobiocin and coumermycin were preferential inhibitors of DNA synthesis, at the commencement of this work the interesting possibility existed that cou and dnaA were in fact one and the same gene. At that time the many different dnaA mutants (which were not proved to be allelic) were known to map to the left of ilv, but their exact position with respect to the many nearby markers, including oriC, was uncertain (see Bachmann et al., 1976).

I decided to isolate mutants resistant to clorobiocin and to map the mutations with respect to four markers whose presumptive order was dgoD dnaA tna ilv (see Figure 5.1). The method of isolation of these mutants is given in Chapters 2 and 6.

## Figure 5.1

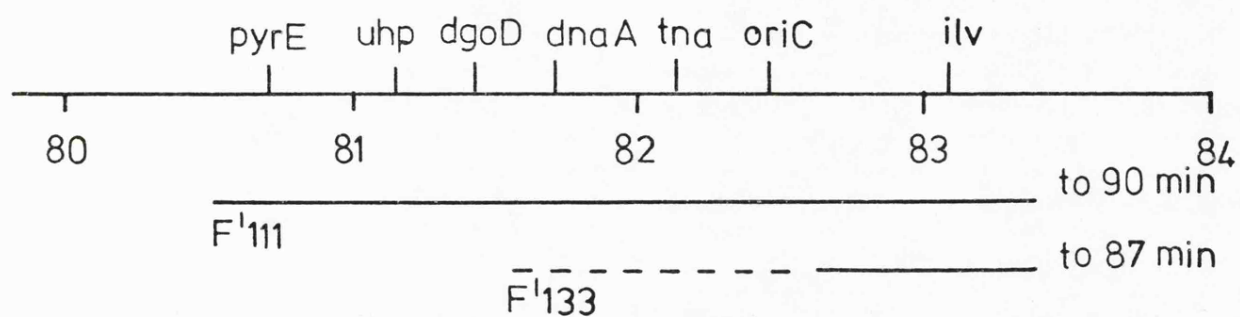
### The Location of Genetic Markers on the E.coli Chromosome

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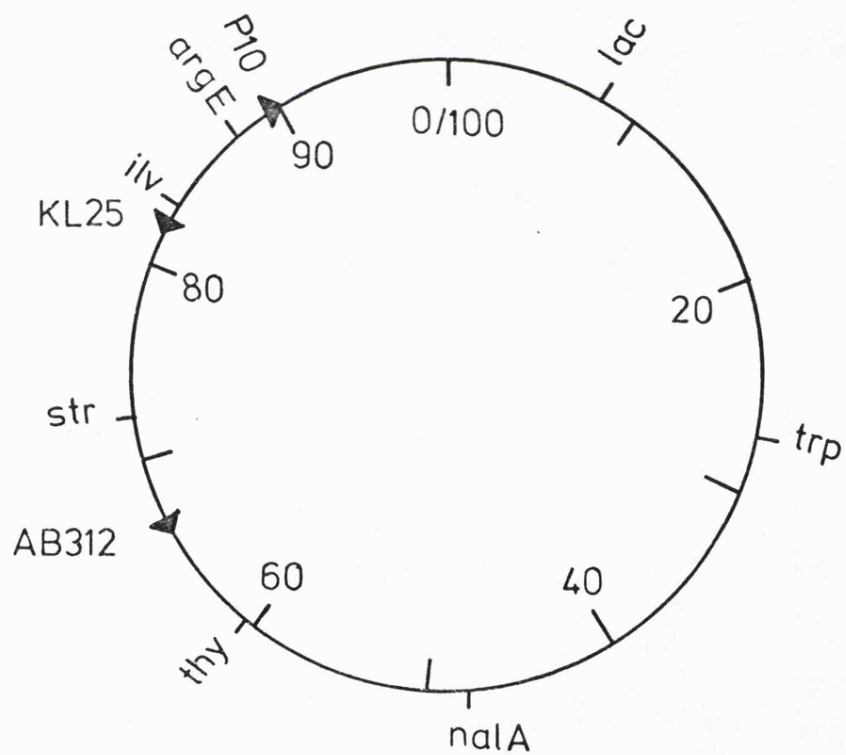
- a. The entire chromosome showing the position of a few markers of interest. The points of origin and direction of transfer of 3 Hfr strains are shown.
- b. The 80-83 minute region showing the order of markers of interest and the estimated portions of the genome carried by F-primes 111 and 133. The dotted line indicates uncertainty (see text).

Based upon the data of Bachmann et al. (1976) and Cooper (1978).

b.



a.



## II. Mapping of the Mutation in Strain LE316 by Bacteriophage P1

### Transduction

Strain LE316 carries the genetic markers ilv, tna and ts16 in addition to other markers (see Chapter 2). The ts16 symbol designates the genetic defect responsible for both the chlorobiocin resistance (at 30°C) and the temperature sensitivity for growth, which will be shown to be most probably due to a single mutation.

A preliminary transduction was carried out using P1 grown on C600 (P1.C600) with selection in LE316 for ilv<sup>+</sup> (see Table 5.1). After scoring for unselected markers the least frequent class was found to be (ilv<sup>+</sup>) tna ts16<sup>+</sup>, and if we assume this was generated by a double crossover then the order ilv tna ts16 is suggested while that of ilv ts16 tna is not. All temperature resistant recombinants were found to be chlorobiocin sensitive.

Similar transductions were carried out using P1 grown on strains C0101 (dgoD) and A3 (dnaA46). The results with P1.C0101 are shown in Table 5.2. The transduction frequencies suggest the order dgoD ts16 tna ilv assuming all three unselected markers lie to the same side of ilv. The four classes containing the fewest transductants could all be generated by 4 crossovers only if the order were that suggested above. If ts16 lay between tna and ilv, the class tna<sup>+</sup> dgoD<sup>+</sup> ts16 would be expected to contain less transductants than the class tna dgoD<sup>+</sup> ts16.

When P1 grown on A3 (dnaA46) was introduced into LE316 and ilv<sup>+</sup> transductants selected, the transduction frequencies suggested the order (dnaA ts16) tna ilv (see Table 5.3). The order of dnaA and ts16 was not

Table 5.1

Transduction of  $ilv^+$  from C600 into LE316

Donor: P1 grown on C600 ts16<sup>+</sup> tna<sup>+</sup> ilv<sup>+</sup>  
 Recipient: LE316 ts16 tna ilv  
 Selection:  $Ilv^+$  30°C

| Recombinants |     | Number in class |
|--------------|-----|-----------------|
| ts16         | tna | (total = 199)   |
| +            | +   | 45              |
| -            | +   | 13              |
| +            | -   | 0               |
| -            | -   | 141             |

Transduction frequencies

ilv - tna 29%

ilv - ts16 22%

Concluded order: ts16 tna ilv

Table 5.2

Transduction of  $ilv^+$  from C0101 into LE316

Donor: P1 grown on C0101 dgoD ts16<sup>+</sup> tna<sup>+</sup> ilv<sup>+</sup>  
 Recipient: LE316 dgoD<sup>+</sup> ts16 tna ilv  
 Selection: Ilv<sup>+</sup> 30°C

| Recombinants |             |            | Number in class |
|--------------|-------------|------------|-----------------|
|              |             |            | (total = 100)   |
| <u>dgoD</u>  | <u>ts16</u> | <u>tna</u> |                 |
| +            | +           | +          | 6               |
| +            | -           | +          | 6               |
| -            | +           | +          | 3               |
| -            | -           | +          | 0               |
| +            | +           | -          | 0               |
| +            | -           | -          | 84              |
| -            | +           | -          | 1               |
| -            | -           | -          | 0               |

Transduction frequencies

Ilv - tna 15%  
Ilv - ts16 10%  
Ilv - dgoD 4%

Concluded order: dgoD ts16 tna ilv

Table 5.3

Transduction of  $ilv^+$  from A3 into LE316

Donor: P1 grown on A3 dnaA ts16<sup>+</sup> tna<sup>+</sup> ilv<sup>+</sup>  
 Recipient: LE316 dnaA<sup>+</sup> ts16 tna ilv  
 Selection: Ilv<sup>+</sup> 30°C

| Recombinants (a) |      |     |  | Number in class |
|------------------|------|-----|--|-----------------|
| ts16 (Clb)       | dnaA | tna |  | (total = 400)   |
| + (S)            | +    | +   |  | 0               |
| + (S)            | -    | +   |  | 6               |
| - (R)            | +    | +   |  | 31              |
| - (R)            | -    | +   |  | 0               |
| + (S)            | +    | -   |  | 0               |
| + (S)            | -    | -   |  | 4               |
| - (R)            | +    | -   |  | 359             |
| - (R)            | -    | -   |  | 0               |

Transduction frequencies

ilv - tna 9.2%

ilv - ts16 2.5%

ilv - dnaA 2.5%

(a) dnaA<sup>-</sup> was scored by its cold sensitive phenotype, i.e. failure to grow at 26°C on nutrient agar (Orr et al., 1978).

ts16<sup>-</sup> was identified as resistant to chlorobiocin.

Concluded order: (ts16 dnaA) tna ilv

determinable since no crossovers occurred between them, presumably because they are very closely linked. Analysis of the number of crossovers required to generate each observed class also failed to order these two markers but otherwise the data again suggested the order (dnaA ts16) tna ilv. The alternative order tna (dnaA ts16) ilv would require that the second most frequent class of recombinants observed (tna<sup>+</sup> ts16 dnaA<sup>+</sup>) be generated by four crossovers which is unlikely.

The interpretation of the data in Tables 5.1, 5.2 and 5.3 is only valid if ts16 lies to the same side of ilv as dgoD, dnaA and tna. This was confirmed by the crosses described in Tables 5.4 and 5.5. These show that ts16 is highly cotransducible with both tna and dgoD and therefore ts16 must lie to the left of ilv. Do these crosses help us to order the markers independently of the crosses described previously? By analysing the number of crossovers required to generate each class of recombinant, it can be concluded that for the order dgoD tna ts16 ilv, double crossovers would be required to generate the classes tna dgoD ilv<sup>+</sup> and tna dgoD<sup>+</sup> ilv<sup>+</sup>. If however the order were dgoD tna ts16 ilv, double crossovers would generate the classes tna dgoD ilv<sup>+</sup> and tna dgoD ilv. In both experiments, recombinants in this latter class were recovered whereas tna ilv<sup>+</sup> recombinants were not. This again suggests the order dgoD ts16 tna ilv. However it must be noted that due to the very close linkage of ts16 to tna, the low numbers of tna<sup>-</sup> recombinants could reduce the significance of this latter result. In the cross described in Table 5.4, all ts<sup>+</sup> transductants were chlorobiocin sensitive suggesting further that the temperature sensitivity for growth and resistance to chlorobiocin are due to a single mutation.



Table 5.4

Transduction of  $ts16^+$  from C0101 into LE316

Donor: P1 grown on C0101 dgoD ts16<sup>+</sup> tna<sup>+</sup> ilv<sup>+</sup>

Recipient: LE316 dgoD<sup>+</sup> ts16 tna ilv

Selection: Growth at 42°C on nutrient agar

| Recombinants (a) |            |            | Number in class |
|------------------|------------|------------|-----------------|
| <u>dgoD</u>      | <u>tna</u> | <u>ilv</u> | (total = 171)   |
| -                | +          | +          | 16              |
| -                | +          | -          | 116             |
| +                | +          | +          | 28              |
| +                | +          | -          | 6               |
| -                | -          | +          | 0               |
| -                | -          | -          | 2               |
| +                | -          | +          | 0               |
| +                | -          | -          | 3               |

Transduction frequencies

ts16 - tna 97%

ts16 - dgoD 78%

ts16 - ilv 26%

(a) All ts<sup>+</sup> transductants were sensitive to chlorobiocin (50 µg/ml).

Concluded order: dgoD ts16 tna ilv

Table 5.5

Transduction of  $ts16^+$  from C0101 into LE316

Donor: P1 grown on C0101 dgoD ts16<sup>+</sup> tna<sup>+</sup> ilv<sup>+</sup>

Recipient: LE316 dgoD<sup>+</sup> ts16 tna ilv

Selection: Growth at 42°C on nutrient agar

| Recombinants |            |            | Number in class |
|--------------|------------|------------|-----------------|
| <u>dgoD</u>  | <u>tna</u> | <u>ilv</u> | (Total = 150)   |
| -            | +          | +          | 7               |
| -            | +          | -          | 97              |
| +            | +          | +          | 13              |
| +            | +          | -          | 6               |
| -            | -          | +          | 0               |
| -            | -          | -          | 5               |
| +            | -          | +          | 0               |
| +            | -          | -          | 4               |

Transduction frequencies

ts16 - tna 94%

ts16 - dgoD 72%

ts16 - ilv 13%

Concluded order: dgoD ts16 tna ilv

Thus the ts16 mutation lies to the left of tna, very close to dnaA. The four markers dgoD, ts16, dnaA and tna were also ordered by a further cross as shown in Table 5.6. In this case the selection for temperature resistance forced a crossover to occur between dnaA and ts16 since both mutations confer temperature sensitivity. By analysing the distribution of flanking markers, it was hoped to order these two mutations with respect to dgoD and tna. Figure 5.2 shows that if the order were dgoD ts16 dnaA tna, the class dgoD tna would be expected to constitute the major category of transductants. Conversely, if the order were dgoD dnaA ts16 tna the major category would be expected to be dgoD<sup>+</sup> tna<sup>+</sup> (not illustrated). As Table 5.6 shows, the first of these alternatives is true and therefore the order illustrated in Figure 5.2 is inferred to be correct.

In order to extend the analysis of the mutation in LE316 and to study the effects of this mutation in combination with other mutations, an attempt was made to transfer ts16 by P1 transduction. However it proved impossible to grow P1 on LE316 whereas high titres were readily obtained on LE234, the parent strain.

### III. Analysis of the Mutation in LE701

A second mutant, LE701, was also chosen for further study. LE701 was isolated as resistant to chlorobiocin, but unlike LE316 it is not temperature sensitive for growth (see Chapter 6). The mutation in LE701 will be designated clb701, although evidence will be presented suggesting that clb701 does in fact map close to, or within, cou.

Table 5.6

Transduction of  $ts16^+$  from LE312 into LE316

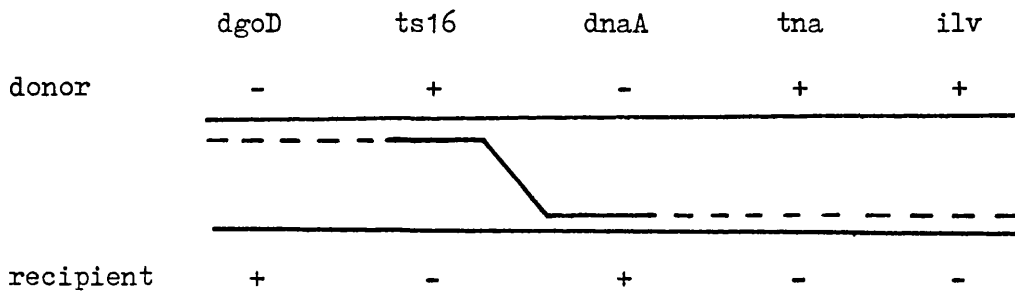
Donor: P1 grown on LE312 dgoD ts16<sup>+</sup> dnaA tna<sup>+</sup> ilv<sup>+</sup>  
 Recipient: LE316 dgoD<sup>+</sup> ts16 dnaA<sup>+</sup> tna ilv  
 Selection: Growth at 42°C on nutrient agar

| Recombinants |            |            | Number in each class |
|--------------|------------|------------|----------------------|
| <u>dgoD</u>  | <u>tna</u> | <u>ilv</u> | (Total = 147)        |
| +            | +          | +          | 2                    |
| +            | +          | -          | 7                    |
| -            | +          | +          | 1                    |
| -            | +          | -          | 13                   |
| -            | -          | +          | 11                   |
| -            | -          | -          | 66                   |
| +            | -          | +          | 12                   |
| +            | -          | -          | 35                   |

Figure 5.2

Transduction of  $ts16^+$  from LE312 into LE316

This figure shows diagrammatically the cross described in Table 5.6 (see opposite).



The order of genes shown above is the only one which is consistent with the results in Table 5.6 (see text). The bold line between the two "chromosomes" represents the selection imposed, and the dotted line represents the most frequent class of recombinant.

(a) P1 Transduction

P1 grown on strain C0101 was introduced into LE701 and ilv<sup>+</sup> recombinants selected and scored for chlorobiocin sensitivity. As shown in Table 5.7, in two separate experiments no chlorobiocin sensitive transductants were recovered although 5 dgoD and 48 tna<sup>+</sup> recombinants were found. This result was unexpected given the cotransduction frequency of 10% between ilv and ts16 in a similar cross (Table 5.2). Since the transduction frequencies obtained in such experiments varied according to the host upon which the P1 was grown, the cross was repeated using P1 grown on C600 since this same phage preparation had previously given a cotransduction frequency of 22% between ilv and ts16. However this also gave no chlorobiocin sensitive recombinants amongst 400 ilv<sup>+</sup> transductants tested (data not shown). Thus it appeared that clb701 did not map at cou.

(b) Hfr Matings

LE701 was used as the recipient in matings with three Hfr strains whose points of origin and direction of transfer are given in Figure 5.1. Ilv<sup>+</sup> Str<sup>R</sup> transconjugants were selected and tested for the presence of various markers. The results are given in Table 5.8. With KL25 no chlorobiocin sensitive (Clb<sup>+</sup>) transconjugants were obtained while 33 out of 36 of those from the cross with AB312 were Clb<sup>+</sup>. This indicated that the clb701 mutation lay between 65 and 82 minutes. This is consistent with the low numbers of Clb<sup>+</sup> transconjugants obtained with Hfr P10. It should be noted here that technical problems were encountered in scoring sensitivity and resistance to chlorobiocin in these crosses. While it

Table 5.7

Transduction of  $\text{ilv}^+$  from C0101 into LE701

Donor: P1 grown on C0101  $\text{dgoD}$   $\text{clb}^+$   $\text{tna}^+$   $\text{ilv}^+$   
 Recipient: LE701  $\text{dgoD}^+$   $\text{clb}$   $\text{tna}$   $\text{ilv}$   
 Selection:  $\text{ilv}^+$

| Recombinants (a) |            | Number in class (b) |
|------------------|------------|---------------------|
| <u>dgoD</u>      | <u>tna</u> | (total = 100, 99)   |
| +                | +          | 21, 34              |
| -                | +          | 1, 2                |
| -                | -          | 0, 0                |
| +                | -          | 78, 63              |

(a) All recombinants were clb i.e. resistant to chlorobiocin

(b) The data are the results of two separate experiments

Table 5.8

Conjugation of LE701 with Hfr Strains P10, KL25 and AB312

Matings were performed for 30 minutes as described in Chapter 2. Selection in all cases was for  $Ilv^+ Str^R$ . The points of origin of the Hfr strains are shown in Figure 5.1.

| Hfr   | Recombinants |                                   |                      |
|-------|--------------|-----------------------------------|----------------------|
|       | Total        | Met <sup>+</sup> Arg <sup>+</sup> | Clb <sup>S</sup> (a) |
| P10   | 35           | 27                                | 5                    |
| KL25  | 35           | 11                                | 35                   |
| AB312 | 36           | 0                                 | 33                   |

(a) Clb<sup>S</sup> designates sensitivity to 50 µg/ml chlorobiocin



was possible to determine the sensitivity of a few strains after several attempts using the agar spot test (see Chapter 2), or better still by growth to single colonies on agar containing chlorobiocin, it proved difficult to score large numbers of recombinants by these procedures. The ilv<sup>+</sup> transductants of LE701 (see (a)) were scored by three separate tests on plates containing different concentrations of chlorobiocin, and the growth compared to that of LE234 and LE701. With LE316 this problem was minimised because of the very clean temperature sensitivity of this strain at 42°C.

#### IV. Are Mutations to Chlorobiocin Resistance Recessive?

There is a degree of controversy as to whether dnaA mutations are dominant or recessive (see Chapter 1). Since at the commencement of this study it was by no means certain that the cou and dnaA loci were distinct, I decided to determine whether the mutations in LE316 and LE701 were dominant or recessive. Such information can sometimes throw light on the nature mutations as well as providing a convenient method for rapid mapping of recessive mutations (Low, 1973).

##### (a) The F-prime Factor F'133

In their experiments with dnaA mutants in E.coli B/r, Messer and co-workers found that all dnaA mutations tested were dominant upon introduction of the episome F'133 (Beyersmann et al., 1974; Zahn et al., 1977) which carries chromosomal genes from ilv to argH (82 to 87 min.). Messer and co-workers have concluded that F'133 carries dnaA<sup>+</sup> because when they isolated F'133 carrying ilv<sup>+</sup> dnaA204<sup>-</sup>, this conferred temperature sensitivity upon a dnaA<sup>+</sup> host.

Table 5.9

Phenotypes of Clorobiocin Resistant Strains Carrying F-prime Factors

(a) + indicates complementation of the genetic defect by the F-prime. Ts<sup>+</sup> indicates temperature-resistance and clorobiocin sensitivity. Clb<sup>+</sup> indicates clorobiocin sensitivity.

| <u>Strain</u>        | <u>F-prime</u> | <u>Phenotype of Diploid</u> (a) |     |     |     |    |     |
|----------------------|----------------|---------------------------------|-----|-----|-----|----|-----|
|                      |                | Ilv                             | Met | Arg | Tna | Ts | Clb |
| LE234                | 133            | +                               | +   | +   | -   |    |     |
| "                    | 111            | +                               | +   | +   | +   |    |     |
| LE316                | 133            | +                               | +   | +   | -   | -  | -   |
| "                    | 111            | +                               | +   | +   | +   | +  | +   |
| LE701                | 133            | +                               | +   | +   | -   |    | -   |
| "                    | 111            | +                               | +   | +   | +   |    | +   |
| LE705                | 133            | +                               | +   | +   | -   |    |     |
| (LE234 <u>recA</u> ) | 111            | +                               | +   | +   | +   |    |     |
| LE707                | 133            | +                               | +   | +   | -   | -  | -   |
| (LE316 <u>recA</u> ) | 111            | +                               | +   | +   | +   | +  | +   |
| LE706                | 133            | +                               | +   | +   | -   |    | -   |
| (LE701 <u>recA</u> ) | 111            | +                               | +   | +   | +   |    | +   |

I therefore transferred this episome into LE316 and LE701 but found no return to either temperature resistance or chlorobiocin sensitivity (Table 5.9). In addition, these partial diploids were all  $\text{Tna}^-$  when grown with selection for the episome in either a  $\text{rec}^+$  or a  $\text{recA}$  background. These results indicate that F'133 does not carry tna or any of the genes to the left of this marker including dnaA.

These results are in conflict with those of Messer but there are several possible explanations for this. Firstly, Messer's first experiments with F'133 were reported in 1974 (Beyersmann *et al.*, 1974) and so it is likely that the work was carried out at least five years before this study. During this time F'133 could have lost a portion of its DNA containing the dnaA region. Indeed as Zahn *et al.* (1977) point out, F'133 may have lost a central fragment around oriC and, in the isolate we obtained from B. Bachmann for this study, such a deletion could have extended to all genes carried on this episome anticlockwise of oriC. Secondly, Messer's experiments were carried out using E.coli B/r (carrying the host specificity of E.coli K-12) and it is possible that the order of markers in this strain differs from that in E.coli K-12. However this second point does not explain how complementation of dnaA<sup>+</sup> was achieved using F'133 derived from E.coli K-12.

(b) The F-prime Factor F'111

As an alternative to F'133, the episome F'111 was used. This carries genes from pyrE to malK (see Figure 5.1) and was therefore expected to carry cou. F'111 was introduced into LE316 and LE701 and this resulted in

a return to chlorobiocin sensitivity in both these strains and temperature resistance in LE316 (Table 5.9). These diploids were not stable in rec<sup>+</sup> backgrounds even with continual selection for Ilv<sup>+</sup>. In LE316 F'111, 50% of the Ilv<sup>+</sup> colonies picked at 30°C were not found to be Met<sup>+</sup> Arg<sup>+</sup> Tna<sup>+</sup> Temp<sup>R</sup> Clb<sup>S</sup>. Amongst these partial diploids were some isolates in which the F'111 had apparently lost some genes to one side or the other of ilv, and even some which retained only ilv. When temperature resistance was lost in such partial diploids of LE316, this was always accompanied by a return to chlorobiocin resistance. In the recA background, all Ilv<sup>+</sup> isolates retained the full phenotype of the episome, i.e. Met<sup>+</sup> Arg<sup>+</sup> Tna<sup>+</sup> Temp<sup>R</sup> Clb<sup>S</sup>. However upon removal of selection for the episome by growth in medium containing methionine, arginine, valine and isoleucine at 30°C, these strains rapidly lost the episome as judged by their subsequent failure to grow on minimal medium at 42°C with amino acids or at 30°C without one or all of the amino acids. With LE701 recA, as indicated above, introduction of F'111 also resulted in a return to chlorobiocin sensitivity. Like LE316 recA/F'111, the LE701 recA partial diploids rapidly lost F'111 in the absence of selection as judged by a failure to grow without isoleucine and valine and a return to chlorobiocin resistance. These results demonstrated that chlorobiocin resistance is recessive to chlorobiocin sensitivity. In addition it can be concluded that clb701 maps in a region of the chromosome covered by F'111 but not by F'133. This, taken with the Hfr mating results, suggests that clb701 lies between the point of origin of KL25 and the end point of F'111, i.e. close to the cou locus (see Figure 5.1).

Partial diploids with F'111 were also used to test the dnaA46 mutation for its alleged dominance to dnaA<sup>+</sup>. This experiment was performed by Dr E. Orr who found that this particular allele was recessive to dnaA<sup>+</sup>.

## V. λ Transducing Phages Carrying dgoD and cou

### (a) Transductional Analysis

A powerful method for the analysis of short segments of chromosomal DNA of E.coli is to clone such fragments on to bacteriophage λ. By utilising the abundance of secondary attachment sites for λ on the E.coli chromosome (Schmida et al., 1972), it is possible to isolate λ transducing phages which carry genes from many sites on the chromosome (Schrenck and Weisberg, 1975). This section describes the initial characterisation of λddgoD and λdcou transducing phages. These phages were isolated by Dr E. Orr by selection for growth on D-galactonate using the strain C0509(λ<sup>+</sup>) infected with a generalised transducing lysate prepared essentially as described in Chapter 2. The lysate used for the isolation of the transducing phages was prepared and kindly donated by Dr B.G. Spratt.

Many λddgoD phages were isolated and were screened for the presence of the cou gene by transducing LE316(λ<sup>+</sup>) and selecting for growth at 42°C. Two phages, λddgoD12 and λddgoD25, when present as prophages completely abolished the temperature sensitivity and chlorobiocin resistance of LE316 and are concluded to carry the cou gene. Another phage, λddgoD28, only complemented dgoD. These phages were tested for

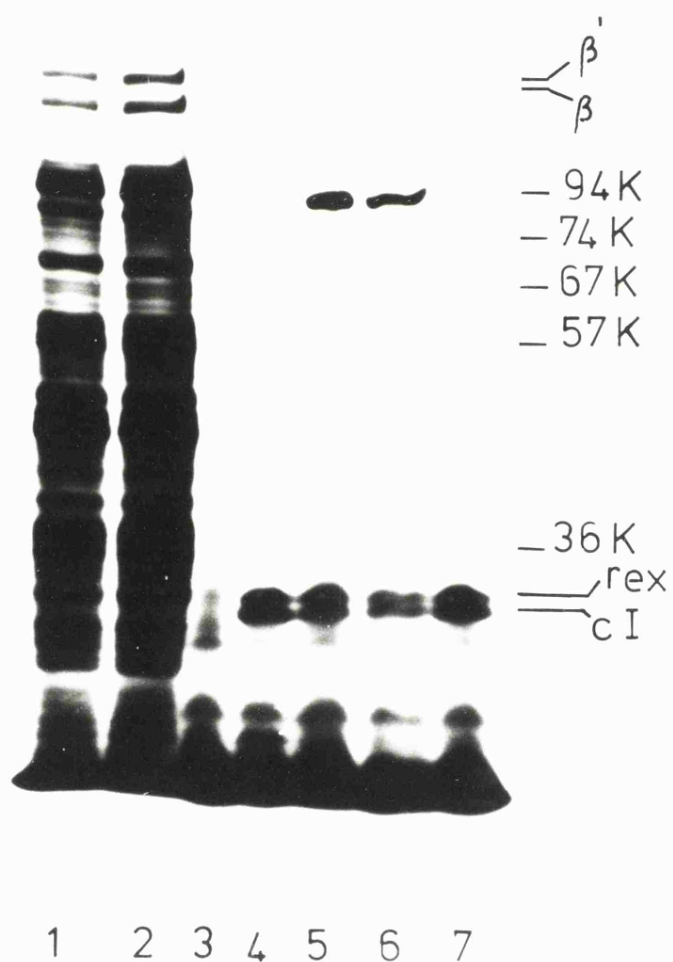
### Figure 5.3

#### Analysis of Proteins Specified by $\lambda_{dgoD}$ Transducing Phages

Strain 159( $\lambda^+$ ) was UV-irradiated, infected with  $\lambda$  transducing phages and pulse labelled with [ $^{35}\text{S}$ ]-methionine as described in Materials and Methods. The proteins were visualised by fluorography after polyacrylamide gel electrophoresis on an 11% gel.

The positions of RNA polymerase subunits  $\beta$  and  $\beta'$  (155K; 160K respectively) and of standard molecular weight proteins were determined on the stained profile.

|       |   |     |   |   |                            |
|-------|---|-----|---|---|----------------------------|
| Lanes | 1 | and | 2 | : | Unirradiated cells         |
| "     |   |     | 3 | : | Irradiated cells, no phage |
| "     |   |     | 4 | : | " " $\lambda^+$            |
| "     |   |     | 5 | : | " " $\lambda_{ddgoD12}$    |
| "     |   |     | 6 | : | " " $\lambda_{ddgoD25}$    |
| "     |   |     | 7 | : | " " $\lambda_{ddgoD28}$    |





the presence of the dnaA<sup>+</sup> gene by selecting for temperature resistance using LE315( $\lambda^+$ ), an ilv<sup>+</sup> dnaA46 transductant of LE234. None of these phages complemented the dnaA46 mutant; this is true for every other  $\lambda$ ddgoD isolate so far examined (E. Orr, personal communication). It is concluded that the dnaA gene does not lie between dgoD and cou, and this supports the earlier conclusion that cou maps to the left of dnaA. This conclusion is of course dependent upon the recessive nature of the dnaA46 mutation as reported in the previous section.

(b) Analysis of the Proteins Encoded by  $\lambda$ ddgoD Transducing Phages

It is possible to investigate the proteins encoded by genes on transducing phages by analysing the proteins synthesised after infection of a heavily UV-irradiated strain (Ptashne, 1967). The chromosomal DNA is damaged beyond repair and if such a strain is lysogenic for  $\lambda$ ind<sup>-</sup>, this ensures repression of transcription from the infecting phage promoters P<sub>L</sub> and P<sub>R</sub>. The only proteins synthesised after infection in this system are the cI and rex gene products of  $\lambda$  and those encoded by bacterial DNA contained within the transducing fragment.

Figure 5.3 shows the analysis, by SDS-PAGE, of proteins synthesised after infection of strain 159( $\lambda$ ind<sup>-</sup>) with  $\lambda$ ddgoD transducing phages.  $\lambda$ ddgoD12 and 25, which complemented ts16, synthesised a protein of molecular weight 92K;  $\lambda$ ddgoD28, which complemented only dgoD, did not. Since the molecular weight of the cou gene product is estimated to be between 90K and 95K (Higgins *et al.*, 1978; Muzuuchi *et al.*, 1978a), it is most probable that the 92K protein visualised is in fact the cou gene

product. The dgo proteins were not visualised in this system, presumably because the cells were not grown with D-galactonate as a carbon source.

## V. Discussion

The genetic locus cou which determines resistance to coumermycin (Ryan, 1976) has been identified as the structural gene for the B subunit of DNA gyrase (Gellert et al., 1976b; Mizuuchi et al., 1978a). The results presented in this Chapter indicate that the conditional lethal mutation in LE316 maps within the cou locus. This is consistent with the observed resistance of LE316 and LE701 to coumermycin (see Chapter 6). The gene order deduced from P1 transduction was:

dgoD ts16 (= cou) dnaA tna ilv.

The results obtained with the  $\lambda$  transducing phages are also consistent with dnaA and cou as distinct genes. This interpretation is also consistent with the results of another study conducted at about the same time as this work (Hansen and von Meyenburg, 1979). These authors used  $\lambda$ tna transducing phages and found that the dnaA gene mapped to the right of cou, and coded for a 54K protein distinct from the cou product.

The mapping data in LE701 is incomplete. F-prime complementation placed the clb701 mutation in a region of the chromosome covered by F'111 but not by F'133. Thus clb701 lies either between pyrE and ilv, or between argE and malK. The Hfr matings appear to rule out the latter but the complete lack of co-transduction with ilv remains a mystery, unless clb701 lies at the extreme left hand end of the chromosome covered by

F'111. LE701, unlike other chlorobiocin resistant mutants, is resistant to low concentrations of nalidixic acid (see Chapter 6). It has not been ruled out that LE701 has mutations at both nalA and cou, however the complementation of chlorobiocin resistance by F'111 (which does not cover nalA) makes this unlikely. It is possible that, because the gyrase subunits interact in vivo, an alteration in the cou gene product is sufficient to cause an alteration in the conformation of the nalA product thereby resulting in nalidixic acid resistance. This mutant deserves further study as this phenomenon may help to elucidate the role and interactions of the DNA gyrase subunits in vivo.

In conclusion, LE316 is mutant at the cou locus and therefore may produce a temperature-sensitive DNA gyrase. The mutant LE701 may be considered to be a cou mutant, although rigorous proof was not obtained. These mutants provided the basis for a study of the role of DNA gyrase in cell growth including DNA replication as presented in Chapter 6.

Finally, in connection with the close linkage of dnaA and cou, it is of interest that one strain (E517) originally classified as a dnaA mutant (although it was noted that this strain, unlike all other dnaA mutants, could not be integratively suppressed (Wechsler and Gross, 1971)), now appears to be mutant at the cou locus. Projan and Wechsler (1979) examined the form of the <sup>pMB9</sup>/ plasmid DNA isolated from this strain before and after a shift to 42°C, and found that the negative supercoils present at the permissive temperature were lost at the non-permissive temperature. Interestingly, strain E517 is not itself resistant to coumermycin, but between 20 and 80% of temperature resistant revertants were found to be coumermycin resistant. This

strain therefore may be mutant in a different region of the cou locus than ts16, and the temperature resistant revertants may map at a second site, perhaps in the same region as ts16.

## Chapter 6

### STUDIES ON THE GROWTH OF CLOROBIOCIN RESISTANT MUTANTS

#### I. Introduction

In Chapter 5 it was established that, in at least one mutant, clorobiocin resistance was due to mutation at the cou locus, now known to be the structural gene for subunit B of DNA gyrase. The precise physiological role of DNA gyrase however is not clear. While the experiments in Chapters 3 and 4 indicate an involvement of DNA gyrase in DNA, RNA and protein synthesis and in cell division, other workers have consistently considered an activity in the elongation step of DNA replication to be a major role of this enzyme (Gellert et al., 1976b, 1977; Itoh and Tomizawa, 1977; Sugino et al., 1977).

One approach to an understanding of the role of a gene product is to study mutants in which the gene product is either absent or altered by mutation. This chapter describes the isolation of clorobiocin resistant mutants (including conditional lethal mutants) and studies on these strains designed to probe the role of DNA gyrase in cell growth and particularly in DNA replication.

## II. Isolation of Clorobiocin Resistant Mutants

### (a) Clorobiocin Resistant Mutants

In one experiment, 9 spontaneous clorobiocin resistant mutants were isolated at 37°C on plates containing 100 µg/ml clorobiocin as described in Chapter 2. Upon purification these mutants (designated with CLB prefixes) did not grow well on plates containing 100 µg/ml clorobiocin, but did grow significantly better than LE234 on 50 and 75 µg/ml clorobiocin.

Overnight cultures of these mutants were prepared in M9 minimal medium containing 0 or 50 µg/ml clorobiocin and the cells were examined by phase contrast microscopy. As shown in Table 6.1, all 9 mutants appeared longer than LE234 when grown in the absence of clorobiocin. However most mutants appeared to elongate further, or develop abnormal shapes upon growth in the presence of clorobiocin. CLB30 was judged to be slightly larger than LE234 and did not significantly alter in shape when grown in the presence of clorobiocin. This strain was renamed LE701 and was chosen for further study.

From a different experiment, another mutant of LE234 was isolated by growth on plates containing 50 µg/ml clorobiocin. This mutant, designated CLB8 (LE739) also appeared larger than LE234. However CLB8 grew poorly in liquid medium containing 50 µg/ml clorobiocin, forming chains and filaments.

Table 6.1

Morphology of Clorobiocin Resistant Mutants

Cultures of LE234, and 9 spontaneous clorobiocin resistant mutants were grown overnight in M9 minimal medium (with essential supplements) at 37°C in the presence of either clorobiocin (50 µg/ml) or ethyl alcohol (0.5% v/v).

All cultures grew well in the presence of clorobiocin except LE234 which grew poorly. The cultures were examined under phase contrast microscopy.

| Strain  | Clorobiocin Absent                                   | Clorobiocin Present  |
|---------|--|--|
|         |  | (50 µg/ml)   |
| LE234   | Short rods, i.e. 1 cell length                       | Very little growth X2-3 cell lengths. Many abnormal shapes |
| CLB21   | X2-3 cell lengths                                    | Abnormal shapes including dumpy "cigar shapes"             |
| CLB22   | X2-3 cell lengths                                    | Abnormal shapes including "minicells"                      |
| CLB23   | As CLB22. Also normal sized cells and long filaments | As without clorobiocin                                     |
| CLB25   | X2-3 cell lengths. Some chains                       | Abnormal shapes including some long, fat cells             |
| CLB26   | X2-3 cell lengths                                    | Many abnormal shapes                                       |
| CLB27   | As CLB26   | As CLB26   |
| CLB28   | X2-3 cell lengths. Some very small cells             | As without clorobiocin; also some abnormal shapes          |
| CLB29   | X1-2 cell lengths. Some very small cells             | Many abnormal cells including some fat cells               |
| CLB30   | Homogeneous size perhaps larger than                 | As without clorobiocin                                     |
| = LE701 | LE234  |  |



(b) Temperature-sensitive Clorobiocin Resistant Mutants

Mutants hopefully producing a temperature-sensitive DNA gyrase were isolated by obtaining clorobiocin resistant mutants at 30°C, and picking those that were unable to grow on plates at 42°C. Such mutants would of course only be obtained if DNA gyrase were an enzyme essential for cell growth.

In one experiment a total of 107 clorobiocin resistant mutants were isolated at 30°C on 75 µg/ml clorobiocin. Of these, 4 were fully temperature-sensitive and 6 were partially temperature-sensitive as judged by their ability to form colonies on minimal agar (without clorobiocin) at the restrictive temperature. Like the mutants described in II (a), these temperature-sensitive mutants did not grow well on plates containing the same concentration of clorobiocin on which they were isolated, but they did grow significantly better than LE234 on 50 µg/ml clorobiocin. As with other clorobiocin resistant mutants, these temperature-sensitive mutants appeared larger (longer) than LE234 at 30°C when examined by phase contrast microscopy.

In addition, the clorobiocin resistant mutant LE316 (isolated from LE234) which is temperature-sensitive for growth was obtained from Dr E. Orr (Orr et al., 1979). This mutant was isolated by selection for growth on 30 µg/ml clorobiocin at 30°C. The mutation in this strain was mapped as described in Chapter 5.

### III. Sensitivity of Clorobiocin Resistant Mutants to Coumermycin and Nalidixic Acid

All clorobiocin resistant mutants studied were found to be resistant to 15 µg/ml coumermycin, a concentration which totally inhibited growth of the parent, LE234 (see Table 6.2). These tests were carried out on minimal agar plates containing various concentrations of coumermycin; resistance was defined as the ability to grow to single colonies after 48 hours. On the basis of other results (May and Baker Ltd; see also Godfrey and Price, 1972), one might expect the MICs of clorobiocin and coumermycin to be approximately equal on a weight to weight basis. In fact, with the strains used in this study, the MIC for coumermycin was less than one half of that for clorobiocin. This was not due to the presence of the dimethyl sulphoxide used to dissolve coumermycin since this solvent did not inhibit the growth of LE234. Due to the limited supply of coumermycin available, the lower MIC value for this compound was not investigated further.

The mutants were also tested for sensitivity to nalidixic acid. Surprisingly one mutant, LE701, was resistant when tested on agar containing 20 µg/ml nalidixic acid. Furthermore, the sensitivity of LE316 to nalidixic acid in the plate tests recorded here may not be straight forward; DNA synthesis in LE316 appears to be partially resistant to nalidixic acid, at least at the non-permissive temperature (E. Orr, personal communication).

Table 6.2

Properties of some Clorobiocin Resistant Mutants

The strains LE316, LE701 and LE739 are all spontaneous clorobiocin resistant mutants of LE234 as described in the text.

- (a) All measurements were at 37°C, and the strains grown in M9 minimal medium, except the generation time, DNA/mass ratio and C times of LE316 which were measured at 36°C in the presence of 0.5% casamino acids, and are taken from Orr et al. (1979).
- (b) DNA/mass and mass/cell are expressed in arbitrary units relative to the value of LE234.
- (c) C times (the length of time taken for one round of replication) were calculated from either the values of  $\Delta G$  and  $\gamma$  obtained from runout experiments in the presence of rifampicin or chloramphenicol ( $C_1$ ); or directly from the runout times in such experiments ( $C_2$ ).
- (d) Sensitivity to antibiotics was determined by growth to single colonies on minimal plates containing the appropriate compound and incubated at 34°C for 2 days. Similar results were obtained for the recA derivatives of LE234, LE316 and LE701.
- (e) (-) indicates not determined.

| <u>Measurement</u> (a)           |                        | <u>Strain</u> |       |       |       |
|----------------------------------|------------------------|---------------|-------|-------|-------|
|                                  |                        | LE234         | LE701 | LE739 | LE316 |
| Generation time (min)            |                        | 49            | 62    | 55    | 41    |
| DNA/mass (b)                     |                        | 1.0           | 0.7   | 0.75  | 0.58  |
| Mass/cell (b)                    |                        | 1.0           | 2.48  | (-)   | (-)   |
| <u>C-times</u> (c)               |                        |               |       |       |       |
| Rifampicin                       |                        |               |       |       |       |
| $\Delta G$ (%)                   |                        | 31.6          | 38.6  | (-)   | 61    |
| $C_1$ (min)                      |                        | 41            | 62    | (-)   | 60    |
| $C_2$ (min)                      |                        | 45            | 66    | (-)   | 50    |
| Chloramphenicol                  |                        |               |       |       |       |
| G (%)                            |                        | 40.0          | 41.8  | (-)   | (-)   |
| $C_1$ (min)                      |                        | 51            | 67    | (-)   | (-)   |
| $C_2$ (min)                      |                        | 55            | 70    | (-)   | (-)   |
| <u>Antibiotic Resistance</u> (d) |                        |               |       |       |       |
| Clorobiocin                      | (50 $\mu\text{g/ml}$ ) | Sens          | Rest  | Rest  | Rest  |
| Coumermycin                      | (15 $\mu\text{g/ml}$ ) | Sens          | Rest  | Rest  | Rest  |
| Nalidixic acid                   | (20 $\mu\text{g/ml}$ ) | Sens          | Rest  | Sens  | Sens  |

#### IV. Physiology of Clorobiocin Resistant Mutants

##### (a) Effect of Clorobiocin on the Growth of LE701

The two processes in LE234 most markedly inhibited by clorobiocin were DNA synthesis and cell division (Chapters 3 and 4). A fully resistant mutant would be expected to be insensitive to such inhibition, and this was tested with the mutant LE701. Figure 6.1 shows that upon addition of clorobiocin, DNA synthesis and cell division continued essentially undisturbed. This of course does not show that this mutant contains a resistant target enzyme, but does suggest that inhibition of cell division is not a non-specific effect of the drug.

##### (b) LE701 is Defective in Initiation of DNA Replication

The growth of LE701 was compared to that of LE234 by measuring the accumulation of DNA, mass and cell number as shown in Figure 6.2. Two points are evident; (1) LE701 has a reduced DNA concentration (DNA to mass ratio) compared to LE234, and (2) LE701 has an increased mass per cell compared to the wild type strain. The values are given in Table 6.2.

DNA concentration has been shown to be determined by three parameters : the average cell mass per chromosome origin at the time of initiation of a round of chromosome replication; (2) the replication time (C) of the chromosome; and (3) the growth rate (Pritchard and Zaritsky, 1970). An increase in the initiation mass, an increase in C, or an increase in the growth rate all reduce the DNA concentration. This reduction may be calculated from the equation:

Figure 6.1

Effect of Clorobiocin on Mass, Cell Number and DNA Synthesis in LE701

LE701 was grown at 37°C in M9 minimal medium containing [ $^3\text{H}$ ]-thymidine (1  $\mu\text{Ci/ml}$ , 1  $\mu\text{g/ml}$ ) and uridine (1.5 mM). At  $A_{450} = 0.12$  the culture was split; one portion (closed symbols) received no treatment, the other (open symbols) received clorobiocin to a final concentration of 50  $\mu\text{g/ml}$ . Samples were taken for optical density, cell number and DNA.

- (●) Optical density - no treatment
- (○) Optical density - clorobiocin treated
- (■) Cell number - no treatment
- (□) Cell number - clorobiocin treated
- (▲) DNA - no treatment
- (△) DNA - clorobiocin treated

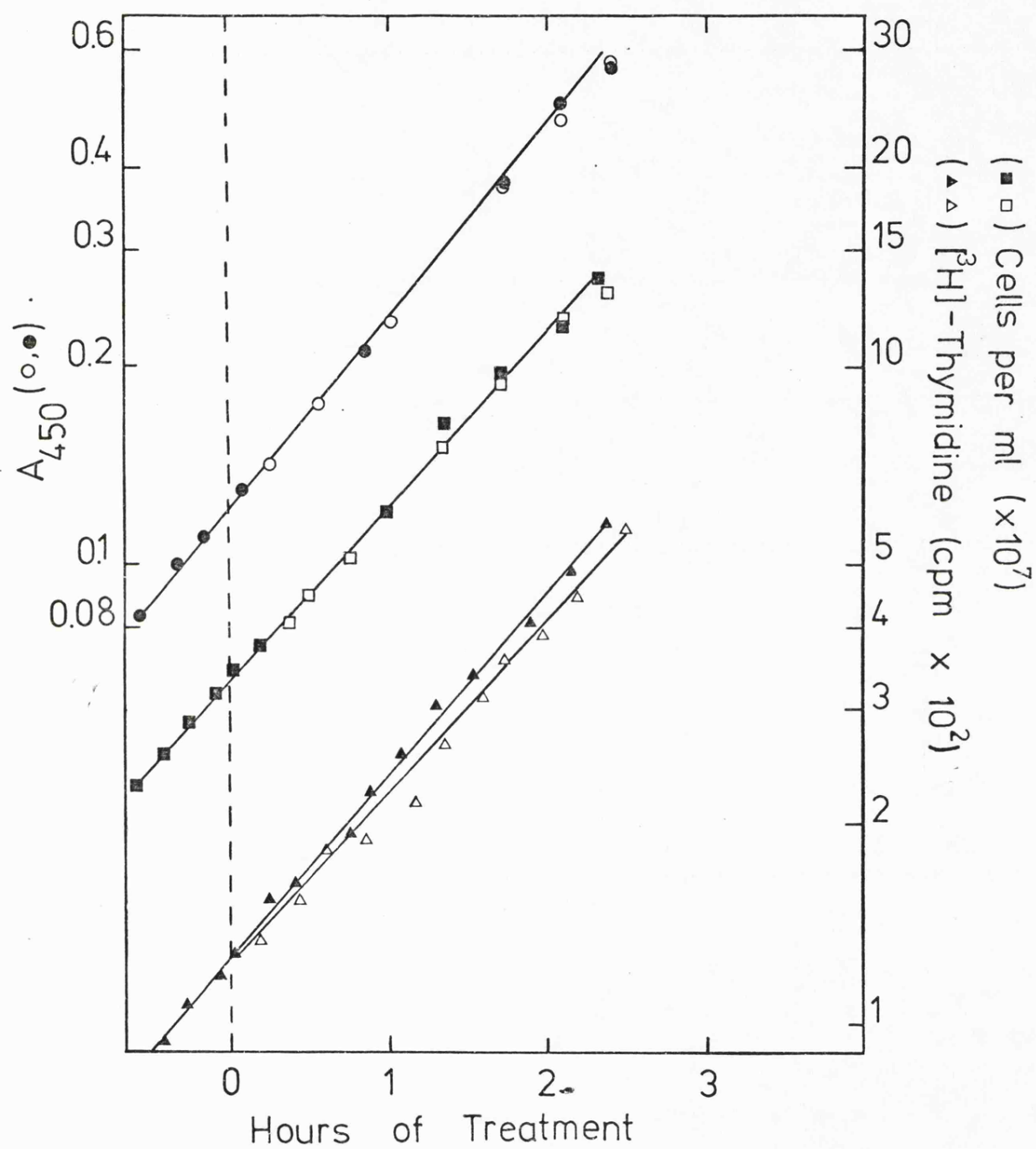


Figure 6.2

DNA, Mass and Cell Number of LE701 Relative to LE234

Cultures of LE234 and LE701 were grown at 37°C in aliquots of the same M9 minimal medium containing essential amino acids, [<sup>3</sup>H]-thymidine (1 µCi/ml, 1 µg/ml) and uridine (1.5 mM) at 37°C. Samples were taken for determination of mass, DNA and cell number.

(a) LE701

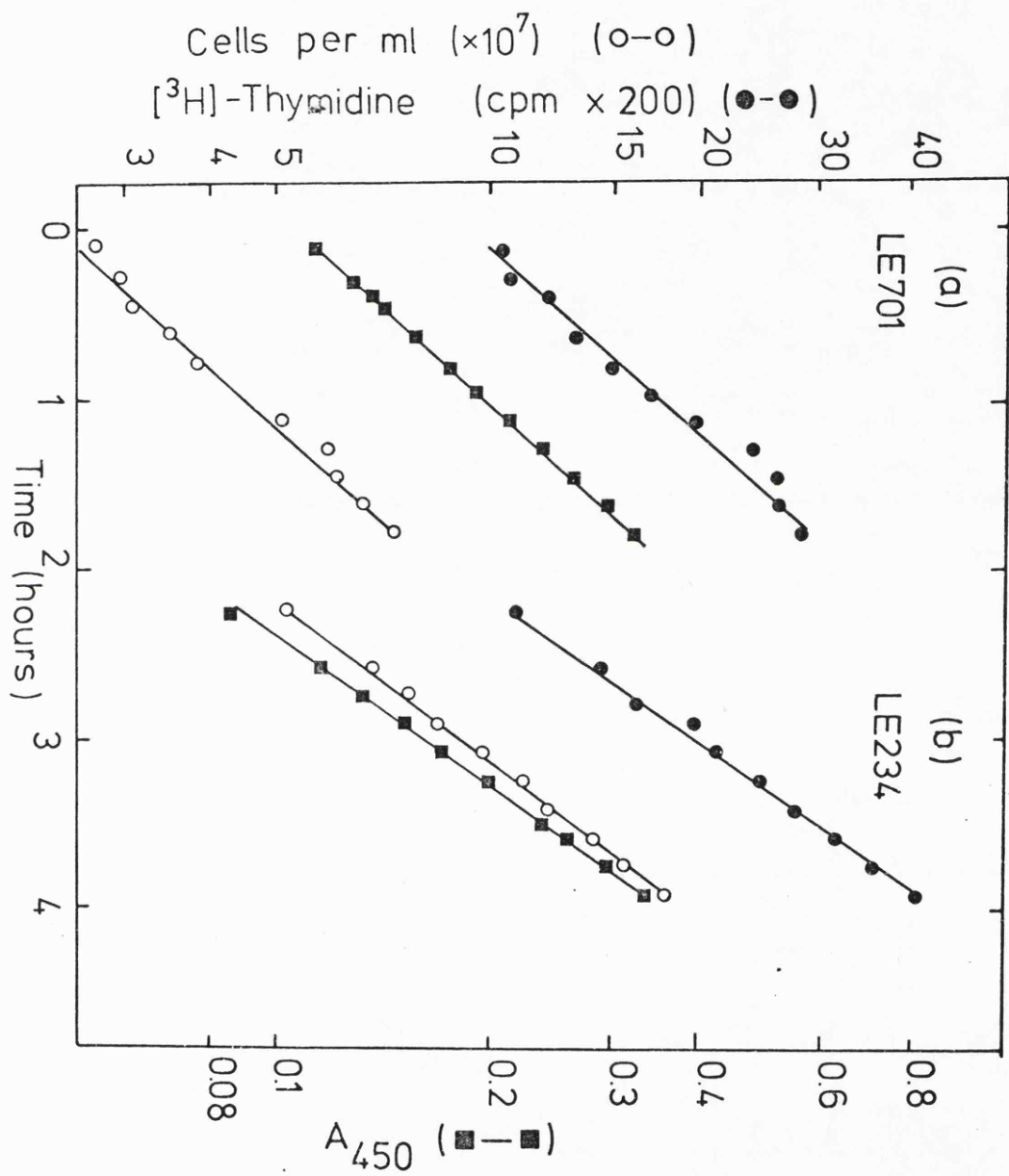
(b) LE234

(●) DNA

(○) Cell Number

(■) Mass (A<sub>450</sub>)





$$\frac{\bar{G}}{\bar{M}} = \frac{\tau}{k \cdot C \cdot \ln 2} \quad (1 - 2^{-C/\tau})$$

where  $\bar{G}$  is the average amount of DNA per cell

$\bar{M}$  is the average cell mass

$\tau$  is the doubling time

and  $k$  is the initiation mass.

Since the growth rate of LE701 was less than that of LE234, this could not account for the observed decreased DNA concentration in LE701. The reduced DNA concentration must therefore be due to an increased  $C$  time, i.e. slower movement of the replication forks, or to an increased cell mass per chromosome origin at the time of initiation of replication.

The  $C$  time of a strain may be calculated by measuring the increment of DNA synthesis ( $\Delta G$ ) after the addition of rifampicin (Pritchard and Zaritsky, 1970) as described in Chapter 3 using the expression (Sueoka and Yoshikawa, 1965).

$$\Delta G = \frac{2^n \cdot n \cdot \ln 2}{2^n - 1} - 1$$

where  $n = C/\tau$ .

The  $C$  times of LE234 and LE701 were calculated from such an experiment (Figure 6.3) and the results are given in Table 6.2. If the reduction in DNA concentration in LE701 were due solely to an increase

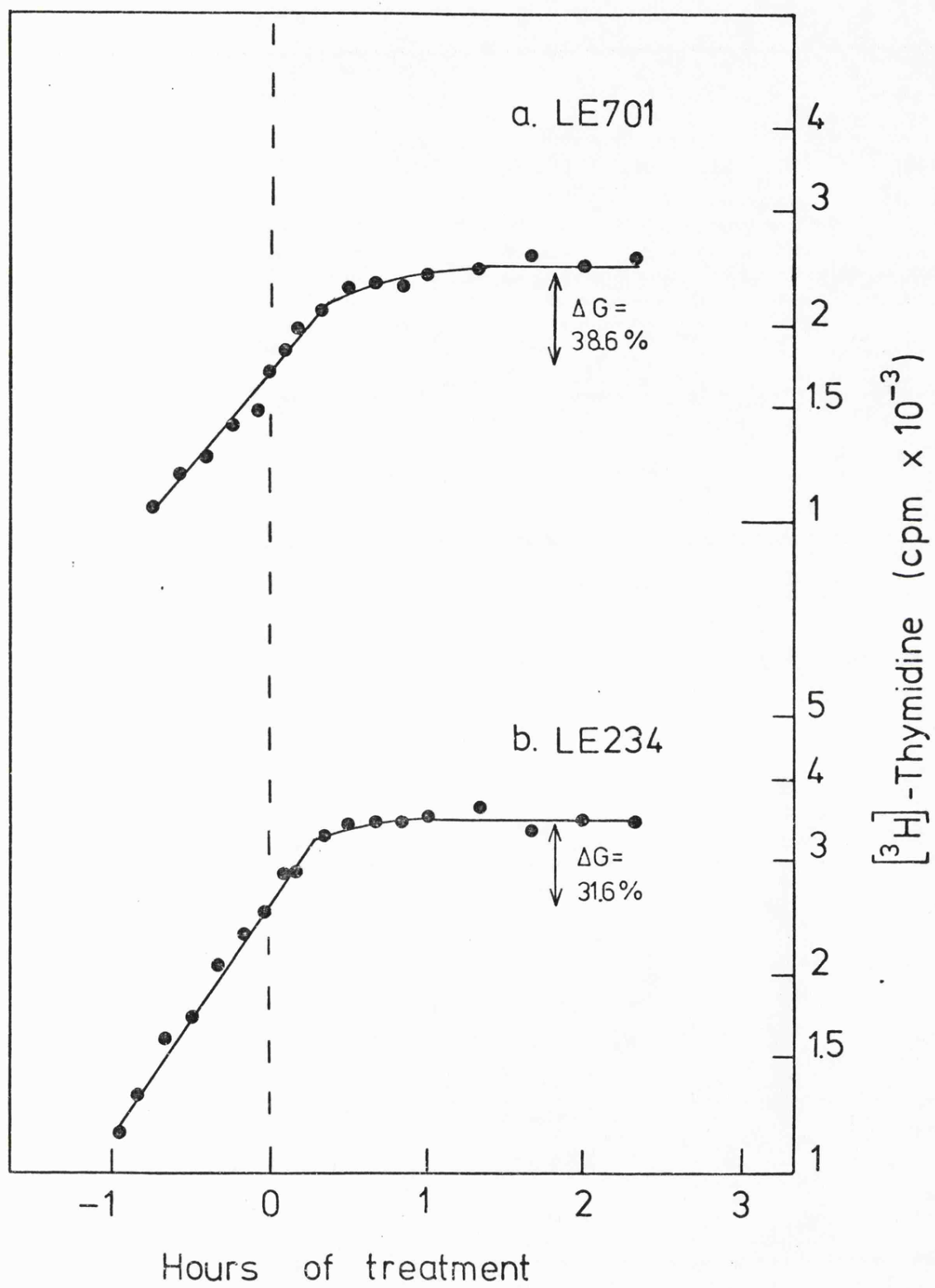
Figure 6.3

Effect of Rifampicin on DNA Synthesis in Strains LE234 and LE701

Cultures of LE234 and LE701 were grown at 37°C in aliquots of the same M9 minimal medium containing essential amino acids, [<sup>3</sup>H]-thymidine (1 µCi/ml, 1 µg/ml) and uridine (1.5 mM) to A<sub>450</sub> = 0.2 when rifampicin (200 µg/ml) was added to each culture. 1.0 ml samples were withdrawn into ice-cold TCA for determination of acid insoluble counts. The ΔG value was then calculated as the increment of DNA synthesis after addition of rifampicin.

a. LE701

b. LE234



in C, the resulting C time would be approximately three times that of LE234. The values of C obtained for LE234 and LE701 were 41 and 62 minutes respectively. Thus although the replication fork velocity may be slightly slower in LE701, the difference observed does not account for the reduced DNA concentration and I therefore conclude that LE701 has an increased initiation mass. The values of C may also be determined directly from the time taken for all DNA synthesis to cease. However these values are often larger than those calculated directly from  $\Delta G$ . This may be due to non-specific binding of rifampicin to components of the replication apparatus causing the forks to slow down. The values calculated from  $\tau$  and  $\Delta G$  should not be subject to this possible artifact since they depend entirely on the rate of fork movement before addition of rifampicin providing all rounds of replication do eventually terminate. In confirmation of the rifampicin run-out data, similar values of C were obtained when rifampicin was replaced with chloramphenicol, an antibiotic which blocks protein synthesis and therefore does not allow further initiation of chromosome replication (Table 6.2).

As indicated above, LE701 was found to have an increased mass per cell (Figure 6.2 and Table 6.2). If cell division is timed from some stage in the DNA replication cycle, then delayed initiation of replication will result in delayed cell division and a consequent increase in cell mass. The size difference between cells of LE234 and LE701 was confirmed by phase contrast microscopy (Figure 6.4); the cells of LE701 appeared both wider and longer than those of LE234. This difference in cell size was substantiated by size analysis using a Coulter Counter fitted with a channelyzer (Figure 6.5).

Figure 6.4

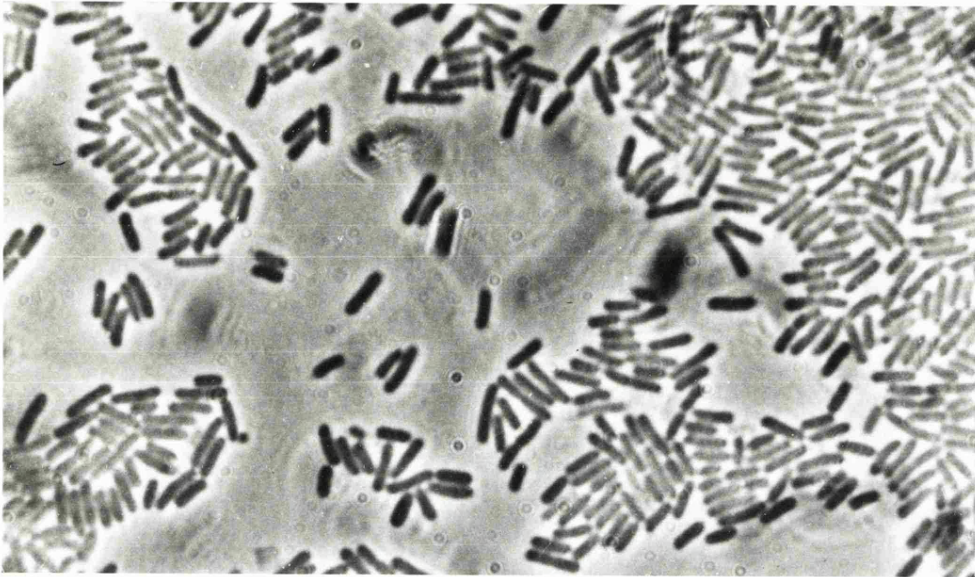
Photographs of Strains LE234 and LE701

Strains LE234 and LE701 were grown at 37°C in M9 minimal medium to  $A_{450} = 0.4$ . The bacteria were photographed as described in Chapter 2.

a. LE234

b. LE701

[a] LE234



[b] LE701

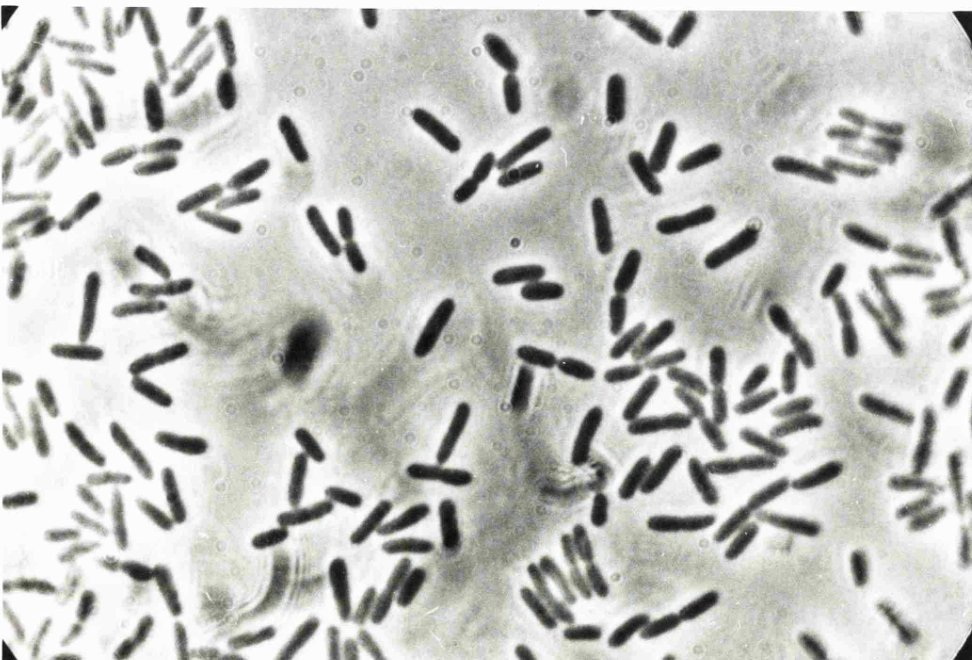


Figure 6.5

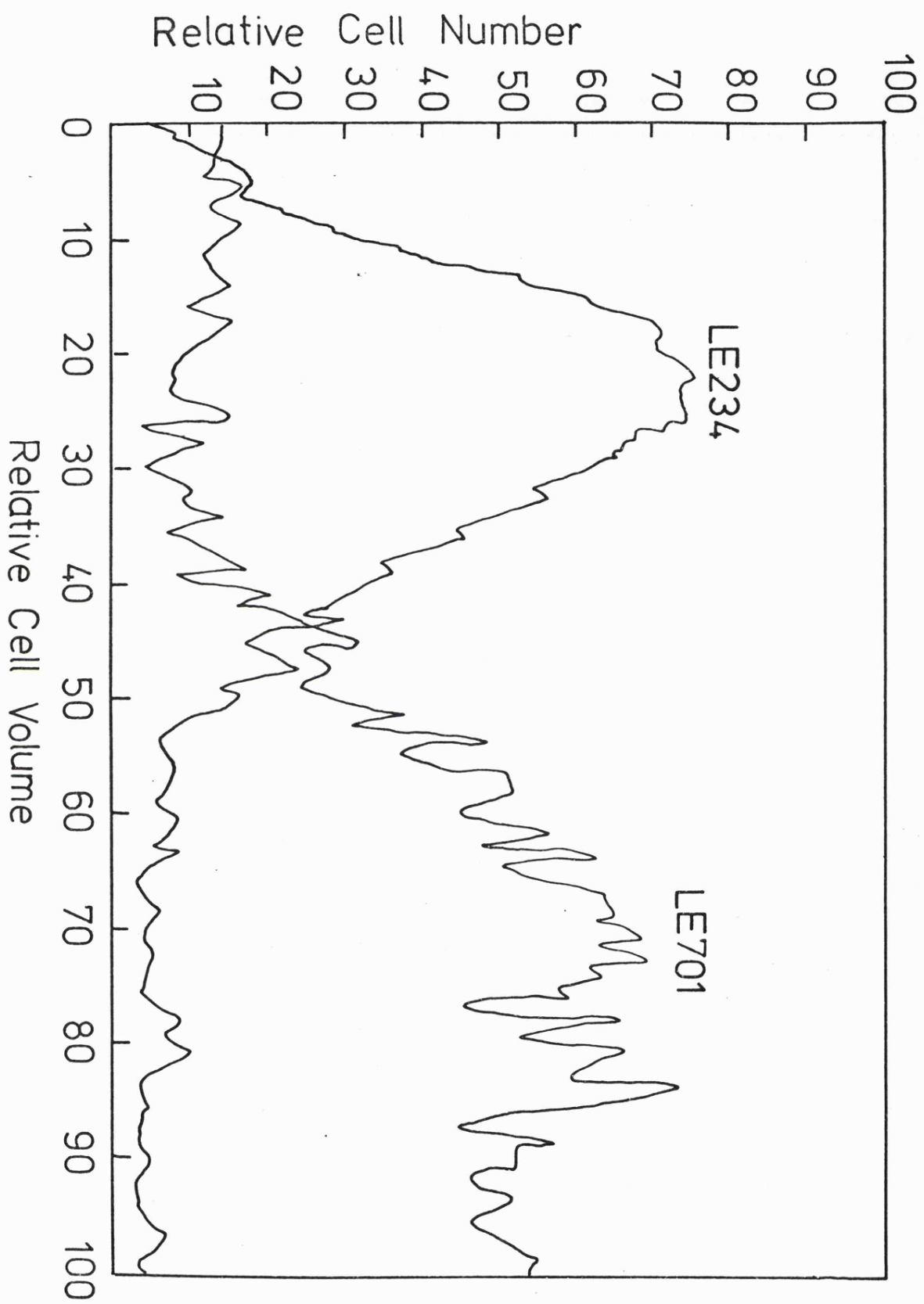
Distribution of Relative Cell Volumes of LE234 and LE701

Strains LE234 and LE701 were grown in M9 minimal medium at 37°C. Samples were taken for determination of relative cell volume by analysis using a Coulter Counter fitted with a Channelyzer.

(a) LE234

(b) LE701





(d) Growth of Temperature-Sensitive Mutants

If DNA gyrase is essential for "ongoing" DNA replication, one might expect to isolate temperature-sensitive mutants which immediately cease to synthesise DNA upon a shift to the non-permissive temperature. Such mutants would be expected to filament (or produce DNA-less cells) and eventually stop growing.

Four temperature sensitive mutants were grown at 30°C to a low cell density and shifted to 42°C (see Figure 6.6). Unexpectedly, these strains continued to grow normally through at least five generations at 42°C finally to enter stationary phase. Thus these mutants appeared to be temperature sensitive on agar but not in the same medium when not solidified. For this reason they were not studied further.

The conditional lethal mutant LE316 was also studied (see Figure 6.7). Upon a shift from 30°C to 42°C synthesis of cell mass appeared initially to accelerate and then continued at a constant, i.e. linear, rate before ceasing after two mass doublings. The rate of DNA synthesis did not significantly change from that at 30°C for about one hour before declining as cell growth ceased. If DNA gyrase is completely defective in this mutant at 42°C, this result shows that gyrase is not essential for "ongoing" DNA replication. Rather the reduced DNA concentration of LE316 (see Figure 6.8, also Table 6.2) suggests that perhaps the defect lies in initiation of replication. The pattern of DNA synthesis in LE316 both at 30°C and 42°C was studied further by Dr E. Orr (Orr et al., 1979) who showed that the increment of DNA synthesised after the temperature shift was reduced to less than one half if rifampicin was

## Figure 6.6

### Growth of Temperature-Sensitive Chlorobiocin Resistant Mutants

Four temperature-sensitive chlorobiocin resistant mutants were isolated from LE234 (see text) and designated CLB3, CLB6, CLB7 and CLB10. They were grown in M9 minimal medium at 30°C to  $A_{450} = 0.2$  and were diluted into fresh prewarmed medium and incubation was continued at 42°C. Samples were taken for determination of cell mass ( $A_{450}$ ).

These results may be compared to the growth of LE234 and LE316 (see Figures 6.8 and 6.7).

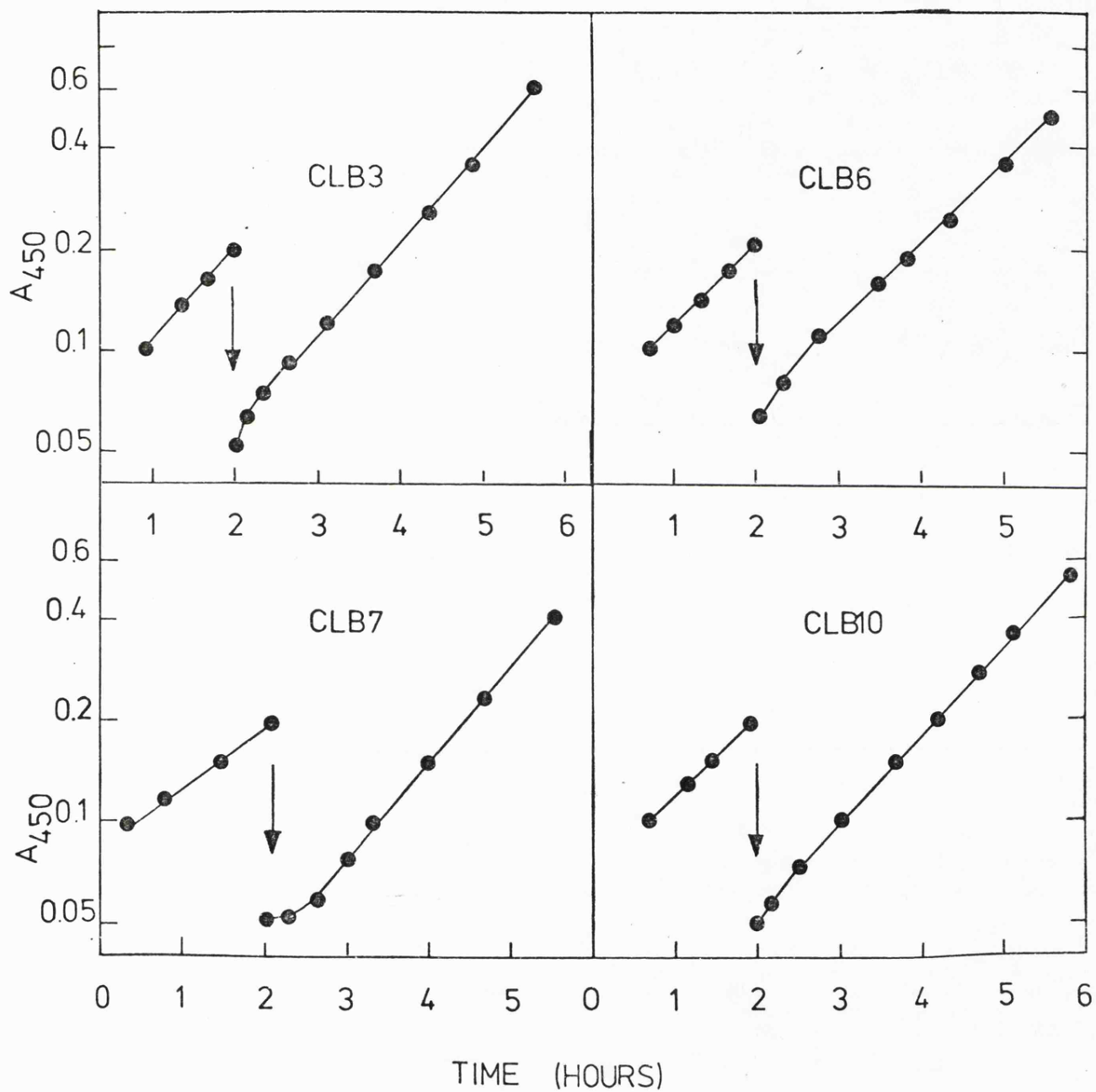


Figure 6.7

DNA and Mass Synthesis in LE316 at 30°C and 42°C

LE316 was grown at 30°C in M9 minimal medium with essential amino acids, [<sup>3</sup>H]-thymidine (1 µCi/ml, 1 µg/ml) and uridine (1.5 mM). The culture was shifted to 42°C by dilution with prewarmed (42°C) medium. Samples were withdrawn for determination of mass and DNA as described in Chapter 2.

(●) Optical density

(■) DNA

(○) and (□) Optical density and DNA respectively  
after dilution to 30°C

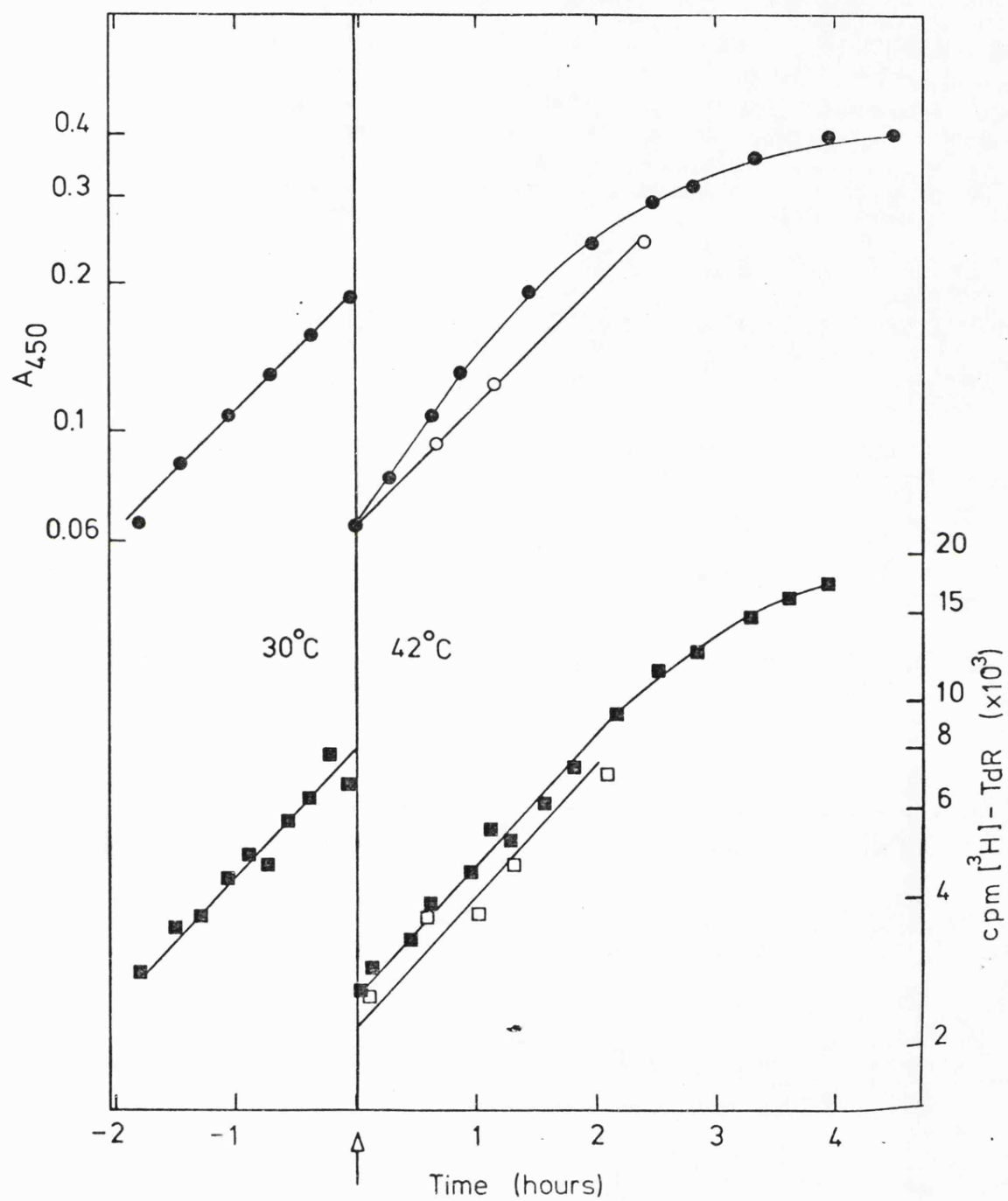


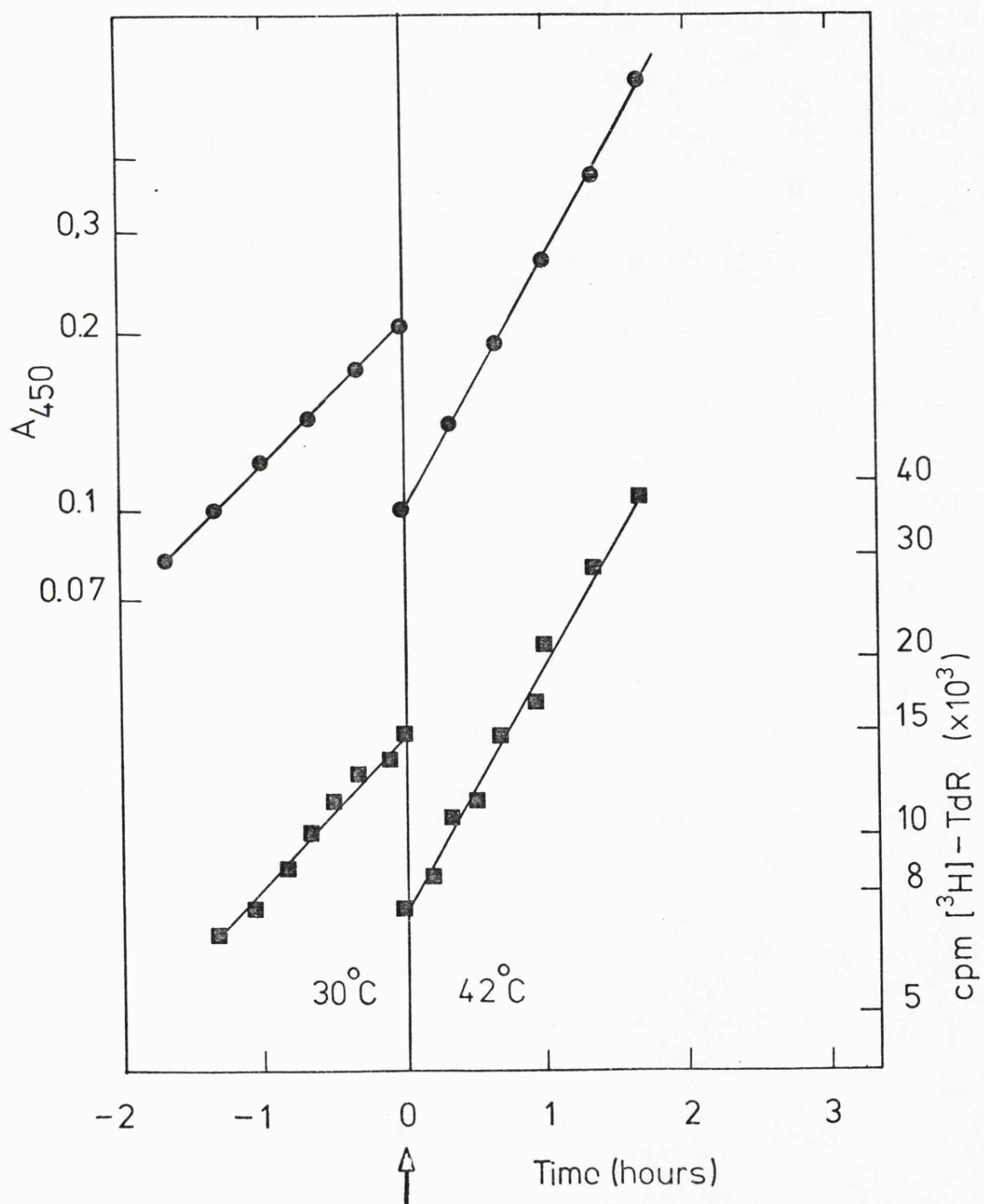
Figure 6.8

DNA and Mass Synthesis in LE234 at 30°C and 42°C

LE234 was grown at 30°C under identical conditions to those described in Figure 6.7. The culture was shifted to 42°C by dilution with prewarmed medium and samples were withdrawn for determination of mass and DNA as described previously.

(●) Optical density

(■) DNA





added immediately upon the shift to 42°C. Thus LE316 is not completely defective in initiation of replication (like for example a dnaA mutant). Furthermore, the C times of LE234 and LE316 were not substantially different at any temperature tested, including 42°C, indicating that elongation of replication forks is not impeded in LE316. Thus the reduced DNA concentration of LE316 (see Figures 6.7 and 6.8) and the reduction in DNA synthesis at 42°C are most probably due to defective or delayed initiation events.

## VI. Discussion

The data presented in this Chapter indicates that the subunit B of DNA gyrase is involved in the initiation of chromosome replication in E.coli. In LE701 the near normal velocity of the replication forks and the reduced DNA concentration indicate that initiation is delayed.

The ability to isolate conditional lethal mutants by mutating the cou gene indicates that some activity of this subunit of DNA gyrase is essential for cell growth. However, LE316 was not completely defective in initiation of replication at 42°C suggesting that perhaps some initiations can occur, albeit inefficiently, in the absence of an active DNA gyrase. RNA synthesis and cell division were also inhibited in LE316 at 42°C and Geimsa staining of LE316 revealed a gross disorganisation of the nucleus at the non-permissive temperature. This latter result suggest a loss of supercoiling in the folded chromosome and it would obviously be informative to assay DNA gyrase activity from LE316 at 42°C in vitro to verify that this activity is absent or very much reduced at this temperature.

In view of the probable involvement of DNA gyrase in initiation of chromosomal replication, it was interesting to record that the dnaA46 mutant was more sensitive to chlorobiocin than its isogenic dna<sup>+</sup> derivative (data not shown). The dnaA46 mutant is not abnormally sensitive to other antibiotics (Orr et al., 1979). Increased sensitivity of dnaA46 mutants to coumermycin was also found by Filutowicz (1980) although in this case, and in contrast to the findings of Orr et al. (1978), abnormal sensitivity to nalidixic acid was observed. Thus it is possible that the cou, dnaA and nalA products interact during initiation of replication, or that the dnaA product requires a supercoiled template in order to implement its action.

## Chapter 7

### THE EFFECTS OF CLOROBIOCIN ON THE SYNTHESIS OF CELL ENVELOPE PROTEINS

#### I. Introduction

In Chapter 4 it was shown that the addition of clorobiocin to a growing culture of LE234 caused an immediate but incomplete block to cell division. The processes of septation and cell division must be tightly coupled to the synthesis of cell envelope material, and previous studies have shown that while total cellular protein is synthesised at an exponentially increasing rate throughout the cell cycle of E.coli B/r (Ecker and Kokaisl, 1969; Churchward and Holland, 1976), bulk outer membrane protein is synthesised at a constant rate which abruptly doubles 10-15 minutes before cell division (Boyd and Holland, 1979). This doubling in rate could be one prerequisite for division, and since clorobiocin inhibits division and reduces the rate of total protein synthesis, it was of interest to study the effect of clorobiocin on cell envelope protein synthesis.

#### II. Effects of Clorobiocin on Envelope Protein Synthesis in LE234

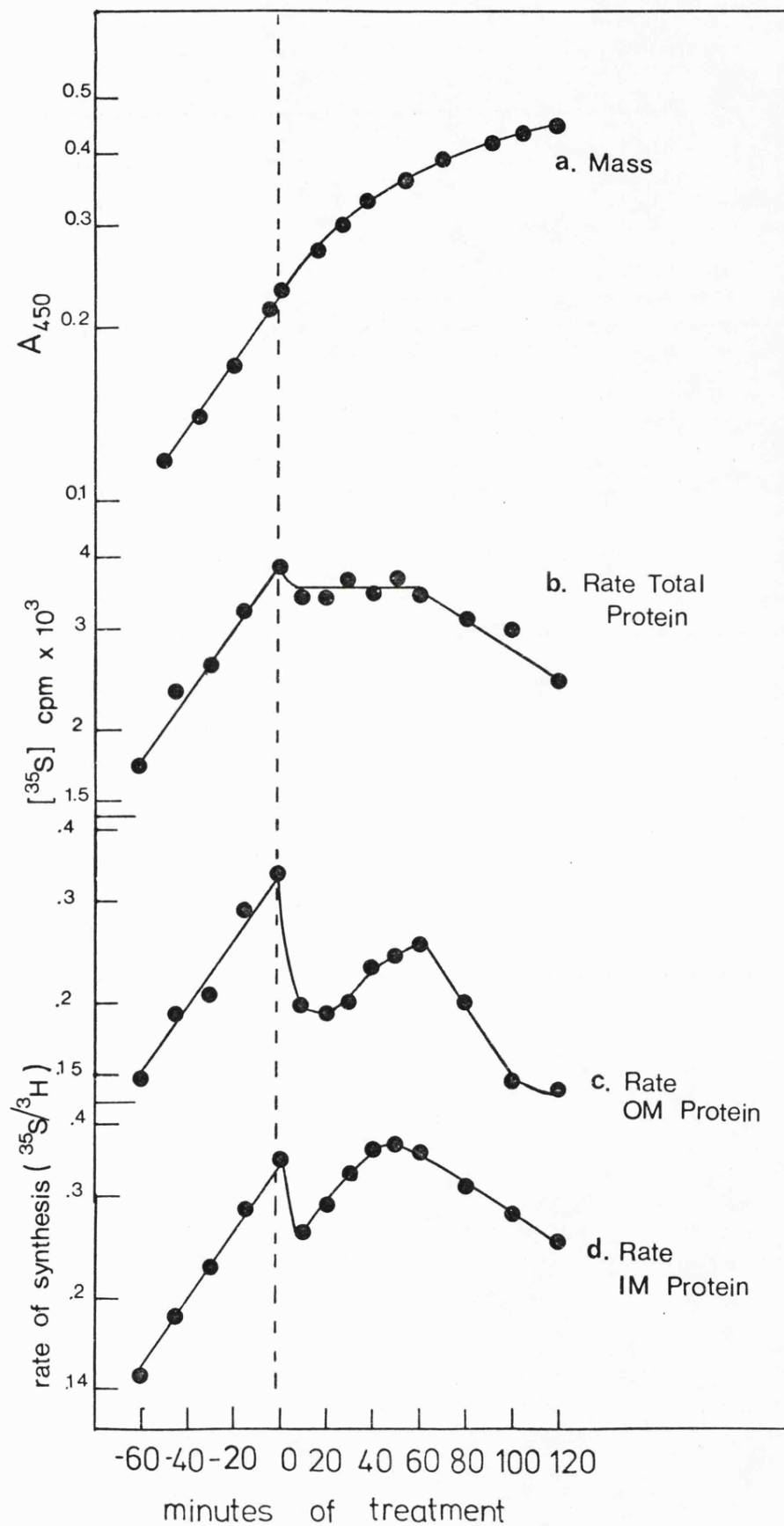
The rates of synthesis of cell protein fractions of LE234 were determined before and during clorobiocin treatment as shown in Figure 7.1. The rate of synthesis of total cell protein increased exponentially up to the time of addition of clorobiocin; thereafter the rate of synthesis remained constant for about one hour before declining.

## Figure 7.1

### Rates of Synthesis of Membrane Protein Fractions of LE234 During Clorobiocin Treatment

A culture of LE234 ( $\text{Met}^+$ ) was grown in M9 minimal medium with essential amino acids to  $A_{450} = 0.23$  when clorobiocin was added to a final concentration of 50  $\mu\text{g/ml}$ . 1 ml samples were withdrawn and were pulse-labelled by incubation with 1 ml prewarmed medium containing 20  $\mu\text{Ci}$   $[^{35}\text{S}]$ -methionine (200  $\mu\text{Ci}/\mu\text{g}$ ). After 2 minutes the pulses were terminated by the addition of 2 ml ice-cold "stop" solution containing methionine (3.75  $\text{mg/ml}$ ) and chloramphenicol (600  $\mu\text{g/ml}$ ). 50  $\mu\text{l}$  aliquots were withdrawn for determination of TCA insoluble material prior to the addition to each sample of a constant volume of  $[^3\text{H}]$ -leucine labelled cells as described in Chapter 2. Additional unlabelled carrier cells were added and cell envelopes were prepared. Incorporation of  $[^{35}\text{S}]$ -methionine into envelope fractions is therefore expressed as a  $^3\text{H}/^{35}\text{S}$  ratio and represents the rate of synthesis of the protein in these fractions.

- a. Optical density.
- b. Rate of total cellular protein synthesis.
- c. Rate of outer membrane (OM) protein synthesis.
- d. Rate of inner membrane (IM) protein synthesis.



The cell envelope fraction was separated into inner (cytoplasmic) and outer membranes on the basis of differential solubility in the detergent Sarkosyl (Filip et al., 1973). The rate of synthesis of both the inner and outer membrane proteins fractions fell markedly upon addition of clorobiocin but recovered to some extent before finally declining further. This pattern is reminiscent of the rate of bulk RNA synthesis seen during clorobiocin treatment (see Chapter 3). That outer membrane protein synthesis was inhibited to a greater extent than that of the inner membrane can be seen clearly in Figure 7.2. During the second hour of treatment, the differential rate of synthesis of outer membrane protein fell below the pretreatment level while that of the inner membrane rose above this level. Consequently the rate of synthesis of outer membrane protein relative to that of the inner membrane showed a substantial reduction upon addition of clorobiocin (Figure 7.2(c)). This reduction was always seen when the experiment was repeated and was therefore not a consequence of loss of incorporation of isotope into the outer membrane in this one experiment.

It should be noted here that some of the data presented in Figure 7.1 are expressed as a  $^{35}\text{S}/^3\text{H}$  ratio. This is a result of the inclusion of a [ $^3\text{H}$ ]-leucine internal standard of uniformly labelled (untreated) cells which minimises errors in the measurement of radioactivity in cell fractions and isolated gel bands (see Boyd, 1979; also see Chapter 2).

## Figure 7.2

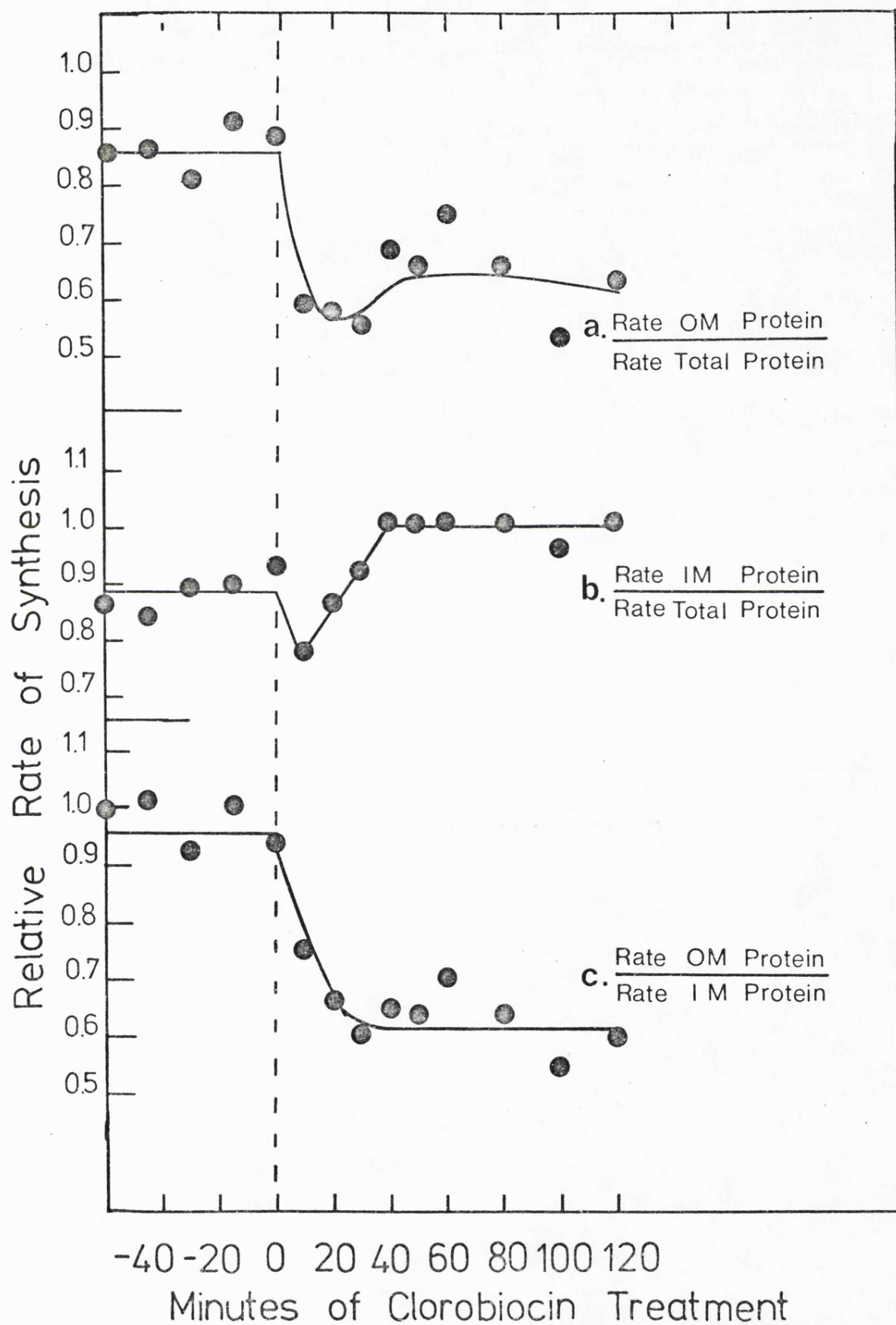
### The Differential Rate of Synthesis of Membrane Protein Fractions During Clorobiocin Treatment

The data in Figure 7.1 are plotted as rate of synthesis of envelope protein fractions relative to that of total cell protein.

(a) 
$$\frac{\text{Rate of synthesis of outer membrane protein}}{\text{Rate of synthesis of total cell protein}}$$

(b) 
$$\frac{\text{Rate of synthesis of inner membrane protein}}{\text{Rate of synthesis of total cell protein}}$$

(c) 
$$\frac{\text{Rate of synthesis of outer membrane protein}}{\text{Rate of synthesis of inner membrane protein}}$$





### III. Rates of Synthesis of Outer Membrane Proteins during Clorobiocin Treatment

The fall in the relative rate of outer membrane protein synthesis described above was studied further by the analysis of the rates of synthesis of individual outer membrane proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The outer membrane of E.coli was chosen for analysis in preference to the inner membrane for several reasons. The proteins of the inner membrane of E.coli are numerous (over 50 can be seen on SDS-PAGE) and they are not well characterised. In contrast, the outer membrane proteins are currently receiving much attention in this and many other laboratories. This is primarily due to the relatively few outer membrane proteins which can be detected on SDS-PAGE, and the existence of 3 or 4 of these as "major" proteins. The importance of several outer membrane proteins is just being realised, following the elucidation of their role in the diffusion of small hydrophilic compounds across the outer membrane (see Chapter 1).

The analysis by SDS-PAGE of outer membrane proteins synthesised during clorobiocin treatment is shown in Figure 7.3. The most prominent change is the increase in the rate of synthesis of a 24K protein. This protein migrated at exactly the same position as the clorobiocin induced 24K protein which can be seen in gel analysis of total cell protein lysates as shown in Chapter 4. Three other major changes are apparent : the disappearance of the 38K protein (protein a); the transient increase in the rate of synthesis of the 34K protein (protein

### Figure 7.3

#### Outer Membrane Protein Synthesis during Clorobiocin Treatment

A culture of LE234 (Met<sup>+</sup>) was grown to an  $A_{450} = 0.22$  when clorobiocin was added to a final concentration of 50 µg/ml. Samples of the culture were pulse-labelled as described in Figure 7.1. Outer membranes were prepared and analysed by SDS-PAGE (15% w/v acrylamide; 0.01% bisacrylamide). The gel was dried down and autoradiographed. Whole cell lysates of cultures which had been treated with clorobiocin or nalidixic acid were also pulse-labelled with [<sup>35</sup>S]-methionine and electrophoresed.

#### Outer membranes

Lanes 1-4 : 55, 40, 20 and 0 minutes prior to the addition of clorobiocin.

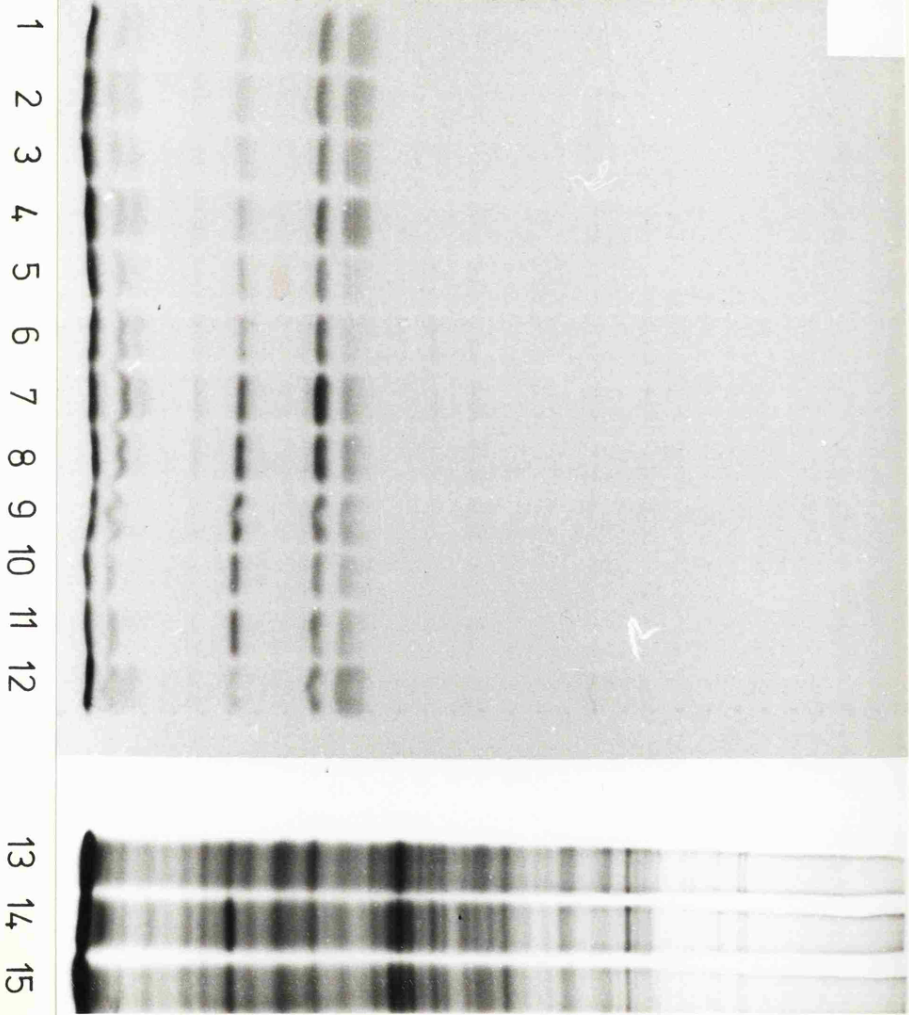
Lanes 5-11 : 10, 20, 40, 60, 80, 100, 120 minutes after the addition of clorobiocin.

Lane 12 : 20 minutes prior to the addition of clorobiocin.

#### Whole cell lysates

Lanes 13-15 : LE234 total cellular proteins after one hour of;  
(13) no treatment, (14) clorobiocin treatment (50 µg/ml),  
(15) nalidixic acid treatment (20 µg/ml).

$\begin{array}{c} a \\ \diagdown \\ b+c \\ \diagup \\ d \end{array}$   
 24K-



- 24K

d); and the transient derepression of synthesis of the 36.5K and 35K proteins. These two latter proteins (designated b and c respectively) often migrate together in SDS-PAGE but may be separated under appropriate conditions of electrophoresis (Lugtenberg et al., 1975).

The changes in the rates of synthesis of proteins (b+c), d, and the 24K protein were measured quantitatively by cutting out the appropriate stained gel band and determining its radioactive content. Figure 7.4 shows the result of an experiment similar to that of Figure 7.3 where the rates of synthesis have been determined in this way. The rate of synthesis of the 24K protein was increased to over four times its uninduced level. Likewise proteins (b+c) and d were affected in a similar way to that implied by the autoradiographic analysis shown in Figure 7.3.

#### IV. Analysis of the 24K Protein

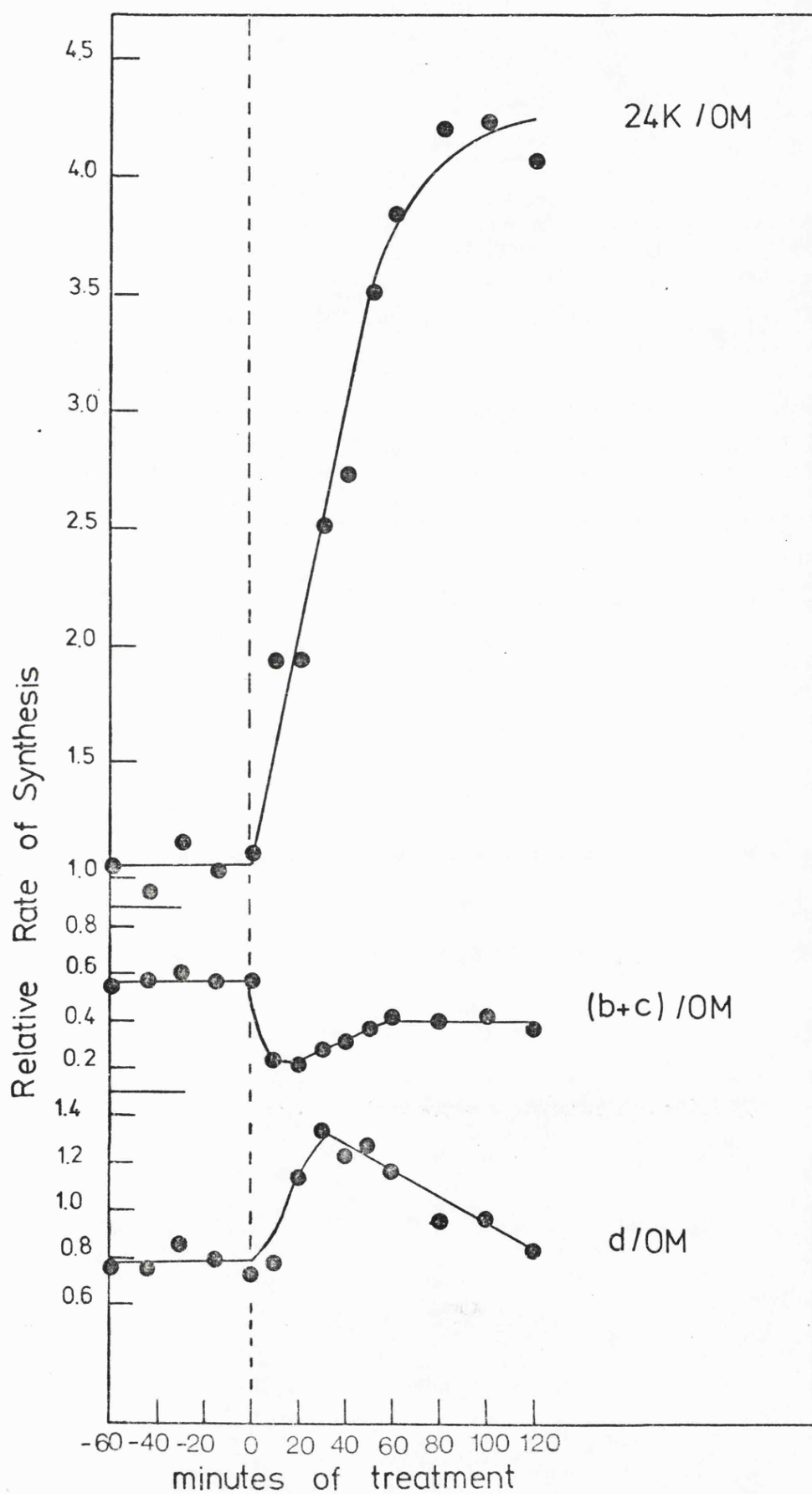
Because of the substantial increase in the rate of synthesis of the 24K protein upon addition of chlorobiocin it was of interest to identify this protein, particularly as two other outer membrane proteins of E.coli K12 have been reported to have similar molecular weights. These are the phage T6 receptor protein (tsx protein) which has recently been shown to act as a porin for the uptake of nucleosides and colicin K (Hantke, 1976), and the undenatured form of protein d (Manning and Reeves, 1977; 1978). That the 24K protein was either of these two proteins seemed unlikely on two accounts. Firstly, strain LE234 is resistant to phage T6 and would therefore be expected to lack the tsx protein. Secondly, samples were always boiled immediately prior to

#### Figure 7.4

#### Effects of Chlorobiocin on Outer Membrane Protein Synthesis in LE234

The outer membranes prepared from the culture described in Figure 7.1 were analysed by SDS-PAGE (15% acrylamide; 44:0.3 acrylamide : bis-acrylamide). The stained bands were cut out and their radioactive content determined (see Chapter 2). The rates of synthesis of each protein is expressed relative to that of total outer membrane protein.

- (a) 24K protein
- (b) proteins b+c (36.5K and 35K)
- (c) protein d (34K)



electrophoresis and therefore no undenatured proteins should be present.

The outer membranes of two isogenic strains, P400 (tsx<sup>+</sup>) and P407 (tsx) were prepared and electrophoresed together with the outer membrane proteins of LE234. Figure 7.5 shows the relative positions of the tsx protein and the 24K protein and demonstrated clearly that they are distinct. It can also be seen from Figure 7.5 that in the stained profile of LE234 outer membrane proteins there appeared to be two proteins present in the 24K region, although only one band was distinguishable upon autoradiography. In fact these two bands were cut out as one band for determination of the rate of synthesis of the 24K protein (Figure 7.4). A diagrammatic representation of the proteins present in this region is given in Figure 7.5 lanes 12 and 13. This representation is very similar to the pattern found by Hantke (1976) for other E.coli K12 strains.

Figure 7.5 also compares the inner and outer membrane proteins of LE234 both before and after chlorobiocin treatment (lanes 1-6). In the inner membrane the ratio of 24K protein to protein d (or proteins b+c) appeared to be higher than that of the outer membrane (compare lanes 1 and 4). Since any proteins (b+c) or d present in the inner membrane fraction is probably due to outer membrane contamination, it is probable that some of the 24K protein seen in inner membrane preparations actually represents its presence there in vivo. This conclusion is strengthened by the appearance of the 24K protein in large amounts in the inner membrane 60 and 120 minutes after the addition of chlorobiocin.

## Figure 7.5

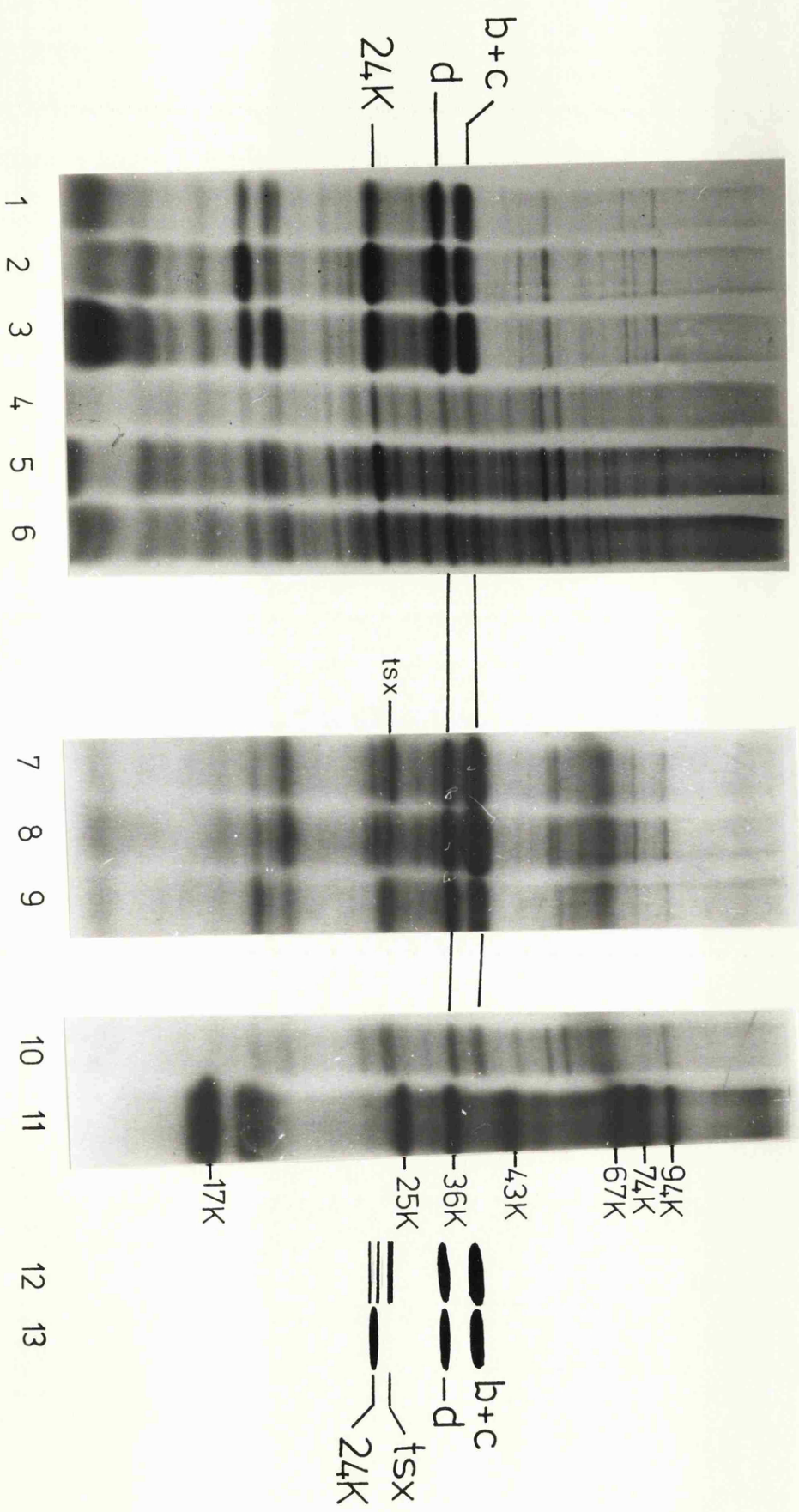
### Analysis of the 24K Protein Induced by Clorobiocin

A culture of LE234 (Met<sup>+</sup>) was grown in M9 minimal medium and treated with clorobiocin (CB) (50 µg/ml). Samples were pulse-labelled with 10 µCi [<sup>35</sup>S]-methionine (100 µCi/µg), unlabelled carrier cells added and inner and outer membrane proteins prepared. A constant amount of inner or outer membrane protein was applied to a 15% SDS-PAGE and the gel was subsequently autoradiographed.

Outer membranes of strains P400 (tsx<sup>+</sup>) and P407 (tsx) were also electrophoresed.

- Lane 1. Outer membrane of LE234. No CB treatment
2. " " " " 60 min CB treatment
3. " " " " 120 min CB treatment
4. Inner " " " No CB treatment
5. " " " " 60 min CB treatment
6. " " " " 120 min CB treatment
7. P400 (tsx<sup>+</sup>) Outer membrane. Stained profile
8. P407 (tsx) " " " "
9. LE234 " " " "
10. LE234 Inner membrane. " "
11. Protein standards
12. Diagrammatic representation of the outer membrane
13. proteins of strains P400 (12) and LE234 (13)





The question of the 24K being the undenatured form of protein d was not resolved in this study. However the kinetics of synthesis of protein d and of the 24K protein are so different that they are unlikely to be related. Furthermore, E. Herrero (personal communication) has identified the undenatured form of protein d as a band running well above the 24K protein.

## V. Discussion

The data presented in this Chapter shows that the pattern of membrane protein synthesis in E.coli is greatly influenced by the addition of clorobiocin. These effects are presumably due to a reduction in the level of supercoiling of the chromosomal DNA which in turn affects transcription of genes determining the synthesis or assembly of membrane proteins.

The pattern of outer membrane protein synthesis after clorobiocin treatment was complex. In particular the rate of synthesis of protein d was transiently increased while that of proteins (b+c) was reduced. A similar effect was observed by Boyd and Holland (1979) who found that induction of synthesis of the 81K (feuB) protein in E.coli B/r was accompanied by a decrease in the rate of synthesis of the major outer membrane protein b. Thus it is probable that not all the observed changes in outer membrane protein synthesis are due to altered DNA gyrase activity; it is possible that the rate of synthesis of only one protein is altered by clorobiocin (e.g. the 24K protein) and that the other changes are a consequence of this initial alteration. Boyd and Holland proposed the existence of a factor which limited overall outer

membrane protein synthesis and that the regulation of this factor might also reflect the observed doubling in rate of bulk outer membrane protein synthesis at a specific point in the cell cycle.

The observed reduction in the differential rate of outer membrane protein synthesis may be directly related to the inhibition of cell division. If, as suggested in section I, a doubling in the linear rate of outer membrane protein synthesis during the cell cycle is one prerequisite for division, then prevention of this by inhibition of DNA gyrase might result in the observed inhibition of cell division. Inhibition of DNA gyrase may lead to a reduction in the differential rate of outer membrane protein synthesis by direct inhibition of transcription of outer membrane protein genes. Alternatively, a reduction in the rate of synthesis of enzymes which determine peptidoglycan synthesis could inhibit outer membrane protein synthesis by reducing the rate of expansion of available surface for their assembly as suggested by Boyd and Holland (1979).

The correlation between the differential reduction in rate of synthesis of outer membrane protein and inhibition of cell division is strengthened by a very similar reduction which is seen in the temperature-sensitive cou (gyrB) mutant LE316 upon a shift to 42°C when division is also blocked (E. Herrero, personal communication). Interestingly, induction of the 24K protein is also seen under these conditions.

## Chapter 8

### DISCUSSION

E.coli DNA gyrase (DNA topoisomerase II) is an enzyme which introduces negative supercoils into covalently closed circular duplex DNA in vitro (Gellert et al., 1976a). This enzyme activity is inhibited by novobiocin and coumermycin (Gellert et al., 1976b), and by nalidixic acid and oxolinic acid (Gellert et al., 1977; Sugino et al., 1977). DNA gyrase is presumed to have supertwisting activity in vivo. This is based upon the observations that when E.coli ( $\lambda$ ) is superinfected with  $\lambda$  in the presence of coumermycin or oxolinic acid, the superhelical density of the superinfecting  $\lambda$  DNA after circularisation is reduced when compared to a similar infection carried out in the absence of the drugs (Gellert et al., 1976b, 1977). In addition, the DNA in folded chromosomes isolated from E.coli after treatment with coumermycin was found to have a reduced superhelical density (Drlica and Snyder, 1978). Interestingly, low concentrations of oxolinic acid (which inhibit DNA replication in vivo) did not reduce the superhelical density of DNA in folded chromosomes (Snyder and Drlica, 1979).

In this study I have used clorobiocin as an inhibitor of subunit B of DNA gyrase. The suggestion that clorobiocin, like coumermycin and novobiocin, would inhibit DNA gyrase came initially from the structural similarity of these three compounds (see Figure 1.1). The other lines of evidence which were obtained in this study are : (1) clorobiocin resistant mutants are also resistant to coumermycin, and in the mutant LE316 the mutation responsible was mapped between dnaA and dgoD,

presumably at cou (gyrB); (2) DNA synthesis is inhibited by clorobiocin. In addition, very recent evidence indicates that clorobiocin inhibits the supercoiling of ColE1 DNA in vitro by DNA gyrase, and extracts of the gyrB mutant LE316 do not contain an active DNA gyrase (E. Orr, personal communication).

It has been suggested that a major role of DNA gyrase in vivo could be to introduce and maintain negative supercoils in the DNA immediately ahead of the replication fork, and thus compensate for positive supercoils which may result from a moving replication fork (Gellert et al., 1976a; Alberts and Sternglanz, 1977; Champoux, 1978).

This study has provided evidence that may contradict this hypothesis. Firstly, DNA synthesis is not completely inhibited by high concentrations of clorobiocin, indicating that some gyrase-independent replication may occur at the replication fork. Secondly, a reduction in the frequency of initiation of chromosome replication, but not a reduced fork velocity appears to be responsible for the low DNA to mass ratio of some clorobiocin resistant mutants. In addition, Orr et al. (1979) found that when the conditional lethal gyrB mutant LE316 was grown at 42°C, the rate of fork movement was not reduced significantly. This last result indicates that "ongoing" replication does not require the activity of the B subunit of DNA gyrase. However, the appreciable inhibition of DNA synthesis by clorobiocin cannot be accounted for solely by a block to the initiation of replication, and if we accept that the activity of DNA gyrase subunit B is not required for elongation of replication forks, we must look to another explanation for the sensitivity of replication to clorobiocin. Replication forks might be

impeded by the presence of gyrase-DNA-chlorobiocin complexes which may form at specific sites all around the chromosome, and which may prevent the separation of parental strands prior to the synthesis of daughter strands. Support for this idea comes from the work of Drlica and Snyder (1978, 1979) who have suggested that DNA gyrase is not confined to the replication forks but is in fact situated at about 50 sites on the chromosome, each site perhaps corresponding to one supercoiled domain as described by Worcel and Burgi (1972).

The complete inhibition of DNA synthesis by high concentrations of nalidixic acid (Chapter 3), and the almost complete cessation of replication in temperature-sensitive gyrA mutants at the non-permissive temperature (Kreuzer and Cozzarelli, 1980) suggest that, in contrast to the B subunit, the activity of the A subunit is essential for "ongoing" replication. This implies that the subunit A of gyrase, again in contrast to the B subunit, is intimately associated with the replication fork. To explain this, one could postulate that some A and B subunits of gyrase exist independently of each other in vivo, or that the B subunit when present in the replication fork complex is inactive. However, measurements of the in vitro activities of DNA gyrase have shown that neither subunit has any activity by itself, and that maximum activity of DNA gyrase is obtained when the A and B subunits are present in equimolar amounts (Higgins et al., 1978; Peebles et al., 1979).

One explanation for this apparent paradox is based on the discovery of a third DNA topoisomerase (DNA topoisomerase II') which is very closely related to DNA gyrase (Brown et al., 1979; Gellert et al., 1979). This enzyme is composed of the gyrA coded A subunit of DNA

gyrase, and a 50,000 dalton polypeptide ( $\gamma$ ) which is probably a proteolytic cleavage product of the 92,000 gyrase B subunit. The A and  $\gamma$  subunits, when present in equimolar amounts (but not separately) will catalyse the relaxation, but not the introduction, of negative or positive supercoils in an ATP-independent reaction. The relaxation of negative supercoils is inhibited by oxolinic acid but not by novobiocin (Brown *et al.*, 1979; Gellert *et al.*, 1979). Inhibition of the relaxation of positive supercoils was not studied. Thus this newly identified DNA topoisomerase could exist *in vivo* as an enzyme in its own right, and may be the activity which relaxes positive supercoils introduced by the replication fork. This would explain the dependence of replication on the A subunit, but not on the B subunit of DNA gyrase. It is possible that the *gyrB* mutations in strains LE316 and LE701 lie outside the DNA encoding  $\gamma$ , and that these strains produce an active  $\gamma$ . Initiation of replication probably requires the activity of the entire subunit B, and this would explain the delay in initiation in these strains (Orr *et al.*, 1979; see Chapter 6; and discussed below). Alternatively, subunit A could relax positive supercoils *in vivo* either by itself (in contrast to *in vitro*), or in combination with other yet unidentified components of the replication apparatus.

The inability of clorobiocin to induce synthesis of the *recA* protein, protein X, (Chapter 4) is not surprising if DNA degradation products are the signal for the induction of this protein and other constituents of the SOS pathway. Clorobiocin did not induce detectable DNA breakdown, as found previously with novobiocin (Smith and Oishi, 1978). It may be significant in this respect that clorobiocin did not

completely block DNA synthesis. A similar result has now been found for coumermycin (E. Herrero, personal communication). Perhaps the complete cessation of fork movement is somehow required to initiate DNA breakdown from the replication forks. If the replication complexes are sensitive to disruption, then a total halt of DNA synthesis could expose DNA to an endonuclease, perhaps the recBC enzyme or even the gyrase A subunit itself (see also discussion below).

The apparent delay in the initiation of DNA replication in gyrB mutants could be due to a reduced rate of formation, or a reduced stability, of a suitably supercoiled DNA structure at or near the origin of replication (oriC). This structure, which could be located in one particular domain of the folded chromosome, may be required at some critical cell mass as a substrate for cleavage by an endonuclease similar to the  $\phi$ X174 cisA protein which is only active on supercoiled DNA (Marians et al., 1977). The nucleotide sequence of the E.coli K12 origin of replication contains many direct and inverted repeats (Meijer et al., 1979; Sugimoto et al., 1979). Thus single stranded regions of DNA could form local complex secondary structures within the supercoiled domain, and consequently provide recognition sites for endonucleolytic attack.

Alternatively, DNA gyrase activity may be necessary to modify the tertiary structure of DNA in the oriC region in order to promote specific transcription which is considered essential for initiation in both E.coli (Lark, 1972; Messer et al., 1975; Orr et al., 1978) and phage  $\lambda$  (Dove et al., 1979). From sequence analysis Sugimoto et al. (1979) noted that the oriC region contains four sequences which are homologous



to the binding site for DNA primase on the phage G4 complementary strand. The synthesis of the origin RNA may then be initiated by the dnaG protein (DNA primase) from one or more of these sites. Clearly negative supercoiling of this region could "open up" these sites for interaction with DNA primase. More direct evidence for the role of RNA in initiation has been reported recently by Conrad and Cambell (1979). They found that an RNA transcript of about 100 nucleotides in length was synthesised during replication of ColE1 DNA in vitro. A small insert into the DNA coding for this transcript resulted in an increased copy number of the plasmid, and the authors proposed that this RNA is involved in the regulation of initiation of replication.

The isolation of conditional lethal gyrB mutants (Chapter 5) has shown that the activity of DNA gyrase subunit B is essential for cell growth. A similar conclusion for the A subunit can be made on the basis of the isolation of conditional lethal gyrA mutants (Krenzer and Cozzarelli, 1980). It is of interest that the gyrB locus is situated very close to dnaA, a gene involved in the initiation of DNA replication, and to oriC. The presence of a third locus in this region involved in DNA replication is suggested by the isolation of temperature-sensitive amber mutants which are defective in DNA synthesis at the high temperature and which apparently map very close to, but to the left of, dnaA (Kimura et al., 1979). These mutants are complemented by  $\lambda$  transducing phage specifying a 43K protein. Hansen and von Meyenburg (1979) found that the gyrB locus (product = 92K) and the dnaA locus (product = 54K) were separated by a segment of DNA coding for a 45K protein. It is

probable therefore that the 43 and 45K proteins are the same, and are the product of a dna gene lying between dnaA and gyrB. It is also possible that these three genes are coordinately regulated and that their products interact to form a complex necessary for the initiation of chromosome replication. Evidence that at least the dnaA and gyrB products interact comes from the abnormal sensitivity of dnaA mutants to clorobiocin (Chapter 6) and coumermycin (Filutowicz, 1980).

The mutation to clorobiocin resistance in strain LE701, although not precisely mapped, is in all probability located in gyrB (Chapter 5). This is supported by the finding that LE701 is also resistant to coumermycin and nalidixic acid. This cross resistance to nalidixic acid has been reported for the coumermycin resistant strain NI741 (Yang *et al.*, 1979). In addition Inoue *et al.* (1979) isolated novel nalidixic acid resistant mutants (nalC and nalD) which were mapped at about 82 minutes on the E.coli K12 genetic map. Although these mutations do not confer resistance to novobiocin, they may well be alleles of the gyrB locus. If so they provide further circumstantial evidence for an association *in vivo* between the gyrA and gyrB gene products.

Sensitivity of transcription to nalidixic acid and coumermycin has been demonstrated both *in vivo* (Sanzey, 1979; Smith *et al.*, 1978; Ryan, 1976) and *in vitro* (Ryan and Wells, 1976; Yang *et al.*, 1979). This study has shown that clorobiocin is also an effective inhibitor of transcription. The effects of all these drugs on transcription is almost certainly due to their effect on the degree of supercoiling of the DNA. Indeed supercoiled DNA has been shown to enhance transcription *in vitro* (Botchan *et al.*, 1973; Wang, 1974; Richardson, 1975), and an interaction between DNA

gyrase, or DNA which has been acted upon by gyrase, and RNA polymerase is suggested by the finding that mutations in rpoB and rpoC can increase or decrease the sensitivity of strains to coumermycin (Mirkin et al., 1979).

The overall rate of RNA synthesis, and more particularly protein synthesis, was essentially constant over the first 90 minutes of clorobiocin treatment. At present it is not clear what a linear, as opposed to an exponential, rate of protein synthesis might be due to. It is possible that clorobiocin specifically represses transcription of one or more genes whose products may be rate limiting for transcription, and therefore for protein synthesis.

A detailed analysis of the synthesis of one particular class of proteins, the outer membrane proteins, revealed that although the overall rate of synthesis of these proteins was reduced, the various individual proteins were affected to different extents by clorobiocin (Chapter 7). This indicates that the degree of supercoiling of the DNA affects different promoters in different ways, and that supercoiling plays an important role in the regulation of transcription in the cell.

Two other results remain to be discussed. These are the differential reduction in the rate of synthesis of outer membrane proteins and the inhibition of cell division, both of which occur upon treatment of cells with clorobiocin (Chapters 4 and 7) and upon a temperature shift of the conditional lethal gyrB mutant, LE316 (Orr et al., 1979, but see below). It is possible that this inhibition of cell division is either due to an alteration in the rate of transcription of a gene or genes involved in the regulation of cell division, or to an overall reduction in the

degree of supercoiling of DNA which may subsequently disrupt the formation of a DNA - membrane complex necessary in some way for septum formation.

Alternatively, it is possible that the inhibition of cell division is a direct consequence of the reduction in rate of outer membrane protein synthesis. Some support for this explanation comes from the correlation between these two events upon a temperature shift of the conditional lethal gyrB mutant LE316. Under conditions of slow growth, which result in an almost complete block to cell division, the rate of synthesis of outer membrane proteins is reduced. At fast growth rates when the block to division is incomplete and many DNA-less cells are produced (Orr et al., 1979), the differential rate of the outer membrane proteins is not reduced, but actually increases (E. Herrero, personal communication). This data is therefore consistent with a model in which one of the prerequisites for cell division is an increase in the rate of synthesis of outer membrane proteins. Such an increase is in fact observed in E.coli B/r, and occurs about 10-15 minutes before cell division (Boyd and Holland, 1979).

With regard to the inhibition of cell division by nalidixic acid in rec<sup>+</sup> strains, but to a much lesser extent in recA strains (see Chapter 4, also Inouyi, 1971), it is probable that in the former this is due to the induction of the SOS repair system, and not to any alteration in the degree of supercoiling of the DNA. Indeed loss of supercoiling in the DNA was not found after the addition of oxolinic acid to a growing culture of E.coli (Snyder and Drlica, 1979), and the DNA was only shown to be nicked after treatment of the extracted DNA with SDS to induce the

gyrase dependent cleavage of DNA (see Chapter 1). Thus the fragmentation of DNA necessary for induction of the SOS system may be very specific, and qualitative rather than quantitative. The study of cell division in temperature-sensitive gyrA mutants and the effect of nalidixic acid on outer membrane protein synthesis may throw light on the role, if any, of the A subunit of DNA gyrase in cell division.

As discussed in Chapter 1, DNA gyrase is not the only enzyme involved in forming, regulating and maintaining the tertiary structure of the DNA in E.coli. The  $\omega$  protein (Wang, 1971), perhaps like DNA topoisomerase II<sup>1</sup> described above, may relax negative supercoils in vivo. However, unlike DNA gyrase, the lack of any antibiotics which inhibit  $\omega$  activity, and the consequential lack of resistant mutants producing an altered  $\omega$  activity have so far prevented the elucidation of the role of this protein in vivo. The rep protein and the DNA unwinding protein (Eisenberg et al., 1976; Abdel-Monem et al., 1976) probably unwind DNA prior to replication by invading the duplex from the exposed single-stranded regions of the parental DNA (see Figure 1.6). Various DNA binding proteins, including the E.coli HD-protein (ssb) involved in DNA replication, and other proteins not yet identified, and perhaps also the RNA which is found in the folded chromosome, may perform essential functions in the formation and stabilisation of the tertiary structure of the chromosome.

One other protein which may be involved in determining the tertiary structure of the DNA in vivo is the E.coli HU protein. This protein, which is present in about 30,000 copies per cell, is apparently composed of at least two subunits each of molecular weight  $\sim 10,000$  (Rouvière-Yaniv

and Gros, 1975). The HU protein introduces negative supercoils into SV40 DNA in the presence of a chromatin extract or a purified eukaryotic nicking-closing enzyme. The resulting molecules take on the appearance of beaded structures in the electron microscope, similar to chromatin formed by reassociation of SV40 DNA with histones (Rouvière-Yaniv et al., 1979; see also Chapter 1). This then is an indication that prokaryotic DNA may form condensed histone-like structures in vivo. Another similarity between the organisation of DNA in prokaryotes and eukaryotes is that a novel DNA gyrase activity, that is formation and resolution of DNA catenanes (knotted circles), has been identified recently in many eukaryotes (see Kreuzer and Cozzarelli, 1980; Liu et al., 1980). Moreover, the initiation of DNA replication in Chinese hamster ovary cells is sensitive to novobiocin (Mattern and Painter, 1979; see Chapter 1). Thus the tertiary structure of DNA in all organisms may have a common basis, determined by a balance of enzymic supercoiling and unwinding, and stabilisation of supercoiled structures by DNA binding proteins.

In summary, this thesis describes some important activities of the antibiotic clorobiocin, an inhibitor of DNA gyrase. The results presented here, and those subsequently obtained in this and other laboratories, show that transcription is sensitive to the degree of negative supercoiling of the DNA. Supercoiling of promoter regions may therefore be one important factor in the regulation of the initiation of transcription. One particular class of transcription which appears to be very sensitive to the degree of supercoiling of the DNA is that involved in the initiation of chromosomal replication in E.coli, which is delayed in mutants containing an altered DNA gyrase.

## Chapter 9

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## ABSTRACT

Title            GENETICAL AND PHYSIOLOGICAL STUDIES IN  
                  ESCHERICHIA COLI K-12 USING CLOROBIOCIN,  
                  AN INHIBITOR OF DNA GYRASE

Neil F. Fairweather

Escherichia coli, in common with other bacteria studied, contains an enzyme termed DNA gyrase which introduces negative supercoils into closed circular DNA. DNA gyrase is inhibited by novobiocin and coumermycin, two structurally related antibiotics.

In this study I have used clorobiocin, an antibiotic structurally similar to novobiocin, as an inhibitor of DNA gyrase. Mutants of E.coli K-12, including those exhibiting a conditional lethal phenotype, were isolated as resistant to clorobiocin and the mutation responsible was mapped at gyrB (cou), the gene coding for the B subunit of DNA gyrase. The gene order was concluded to be : dgoD, gyrB (cou), dnaA, tna, ilv.

One resistant mutant was studied in detail, and was shown to have a reduced DNA concentration. This defect could not be accounted for by a reduced velocity of replication, and it was concluded that the initiation of DNA replication must be delayed in this strain.

An examination of the mode of action of clorobiocin demonstrated that this antibiotic caused an immediate inhibition of DNA synthesis and cell division in exponential cultures of E.coli; RNA and protein synthesis were affected to a lesser extent. The rate of synthesis of total outer membrane protein relative to that of the inner membrane fell immediately upon clorobiocin treatment, although the rates of synthesis of individual outer membrane proteins varied widely during the course of antibiotic treatment.

The role of DNA gyrase in the control of DNA replication, transcription and cell division is discussed.