THE INTERACTION OF QUINOLONE ANTIBACTERIALS WITH DNA GYRASE

by

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March 1993
To Ann and Peter Willmott
   (my parents)
and
Anne Willmott
   (my wife)
ACKNOWLEDGMENTS

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My wife Anne has patiently endured the encroachment of this thesis into our first nine months of married life.

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ABBREVIATIONS AND CONVENTIONS

A<sub>260</sub>  Absorbance at 260 nM
Amp  Ampicillin
AMPS  Ammonium persulphate
ADP  Adenosine 5'-diphosphate
ADPNP  5'-adenylyl-β,γ-imidodiphosphate
ATP  Adenosine 5'-triphosphate
ATPase  Adenosine 5'-triphosphatase
BSA  Bovine serum albumin
bp  Base pairs
CAT  Chloramphenicol acetyltransferase
CFX  Ciprofloxacin
CIP  Calf intestinal phosphatase
DFX  Difloxacin
DHFR  Dihydrofolate reductase
DMSO  Dimethylsulphoxide
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
EDTA  Ethylene diaminetetra-acetic acid
ENX  Enoxacin
FPLC  Fast protein liquid chromatography
gyrA  gene for the A subunit of DNA gyrase
GyrA  the A subunit of DNA gyrase
gyrB  gene for the B subunit of DNA gyrase
GyrB  the B subunit of DNA gyrase
HPLC  High pressure liquid chromatography
IPTG  Isopropyl-β-D-thiogalactopyranoside
kb  Kilobase pairs
kD  KiloDaltons
LB  Luria-Bertani broth
Mar  Multiple-antibiotic-resistant
MDR  Multiple drug resistance
MIC  Minimum inhibitory concentration
MOPS  3-[N-morpholino]propanesulphonic acid
MRSA  Methicillin-resistant <i>Staphylococcus aureus</i>
NAL  Nalidixic acid
NFX  Norfloxacin
OD<sub>595</sub>  Optical density at 595 nM
<table>
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<td>OFX</td>
<td>Ofloxacin</td>
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<td>OXO</td>
<td>Oxolinic acid</td>
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<td>Polyacrylamide gel electrophoresis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PEG</td>
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<td>PFX</td>
<td>Pefloxacin</td>
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<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<td>RNA</td>
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<td>RNAP</td>
<td>RNA polymerase</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SFX</td>
<td>Sparfloxacin</td>
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<tr>
<td>TEMED</td>
<td>N, N', N'-tetramethylethylenediamine</td>
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<tr>
<td>TRIS</td>
<td>Tris (hydroxymethyl) amino methane</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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<td>UV</td>
<td>Ultra Violet</td>
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Quinolone drugs are a clinically significant family of antibacterial compounds that are known to affect the activity of DNA gyrase, an essential bacterial enzyme involved in controlling the topological state of DNA. Gyrase holoenzyme, a complex of two A and two B subunits, can introduce negative supercoils into DNA using energy derived from the hydrolysis of ATP.

Although addition of quinolones rapidly inhibits the supercoiling activity of gyrase, it was found that quinolone-dependent DNA cleavage was a slow process, leading to the suggestion that there may be two levels of interaction between quinolones and the gyrase-DNA complex.

Rapid gel-filtration experiments have shown that stable quinolone binding requires the presence of both gyrase and DNA; no significant binding was found to either gyrase or DNA alone. Enzyme containing gyrase A protein with the mutation SerG to Trp (which is known to confer quinolone resistance) showed greatly reduced drug binding. It is concluded that efficiency of binding is primarily determined by the gyrase A subunits.

Investigation of transcription by T7 and Escherichia coli RNA polymerases has found that quinolone-mediated stabilisation of a gyrase-DNA complex prevents passage of polymerase along the template. Inhibition of transcription required the presence of gyrase and quinolone together; RNA polymerase was unaffected by either quinolone or gyrase alone, implying that polymerase can normally pass or displace gyrase. In the presence of ciprofloxacin, gyrase was found to shield a region of about 26 bp of DNA from transcription by T7 RNA polymerase, with especially strong protection of a 20 bp core. Preliminary experiments performed using an in vitro DNA replication system suggest that DNA polymerases may be similarly interrupted by a gyrase-quinolone-DNA complex.

**ABSTRACT**

Quinolone drugs are a clinically significant family of antibacterial compounds that are known to affect the activity of DNA gyrase, an essential bacterial enzyme involved in controlling the topological state of DNA. Gyrase holoenzyme, a complex of two A and two B subunits, can introduce negative supercoils into DNA using energy derived from the hydrolysis of ATP.

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CHAPTER 1

Introduction
1.1 The place of antibacterials in modern medicine

The annual UK expenditure of 270 million pounds on 47 million "antibiotic" prescriptions must surely be indicative of the significant role played by antibacterial agents in modern medicine (Price, 1992). The chance observation that a culture of *Penicillium notatum* produced a substance that inhibited the growth of *Staphylococcus aureus*, and the subsequent introduction, in 1941, of penicillin G as a treatment for streptococcal and staphylococcal infections was a major milestone in chemotherapy. Since then, a plethora of diverse antimicrobial compounds have been developed, of which the quinolones are amongst the most recent and important families. The search continues for new compounds that are either potent against a broader spectrum of bacteria or exhibit enhanced activity against a particular sub-population of clinically significant microbes. The financial rewards for a pharmaceutical company that develops an efficacious new agent can be considerable.

1.1.1 Resistance to antibacterials

In Alice's adventures through the looking glass, the Red Queen attempts to explain the nature of life in her country, noting that "it takes all the running you can do, to keep in the same place" (Carroll, 1871). Some analogy can be drawn to the resistance development that has accompanied the use of antimicrobial agents. A period of initial success in combating infection with a new compound is often followed by bacterial counter-attack that returns the battleline to the original, or potentially a worse, position and requires an alternative strategy to be pursued.

Three general routes to antibacterial resistance have been identified (Dever and Dermody, 1991). Firstly, bacteria may have or may acquire a means of excluding sufficient drug from the cell, either as a result of permeability barriers against entry or, less frequently, active efflux mechanisms. Secondly, the antibacterial agent may be subject to enzymic degradation before it reaches its target. Thirdly, the target itself may be altered to reduce its affinity for the drug.

Given the vast range of antimicrobial agents that are now available to the medical world, it would be neither possible nor appropriate to give here a full description of all the compounds. Nevertheless, an overview of some clinically significant categories of
antibacterial agents, the current understanding of their mode of action and the mechanism(s) by which resistance can arise, summarised in Table 1.1, will enable us to see the context in which quinolones have been developed.

1.2 A survey of antibacterial agents
1.2.1 β-lactams

1.2.1.1 Background information Penicillins, cephalosporins and other agents of this group are united by the possession of a central β-lactam nucleus, see Figure 1.1 (Dever and Dermody, 1991). Cephalosporins and penicillins are distinguished by the ring structure adjoining the β-lactam ring; penicillins have a thiazolidine ring, cephalosporins have a dihydrothiazine ring. The nature of the acyl sidechain (R₁) and, in the case of cephalosporins, the R₂ group contribute to the spectrum of activity and the efficacy of these compounds. Despite extensive resistance development (see below) and a significant level of allergic response, β-lactams remain the most widely used antibacterials. According to the British National Formulary (BNF, the “prescriber’s bible”), penicillin G (benzylpenicillin) remains the drug of choice in infections with susceptible staphylococci, streptococci, gonococci and meningococci. Ampicillin is a broad spectrum penicillin and as such is a commonly prescribed agent, notably for infections of the urinary and respiratory tracts and salmonellosis (Lambert and O’Grady, 1992). There are few absolute indications for the use of cephalosporins, although they are often provided in a prophylactic capacity during surgery.

1.2.1.2 Modes of action β-lactams bring about bacterial cell death via the interruption of cell wall synthesis. Under normal circumstances, alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) are built up into peptidoglycan chains and assembled into cell walls by transpeptidase-catalysed cross-linking. The β-lactam ring represents an alternative substrate for hydrolysis by transpeptidase, and the antibiotic molecule becomes covalently linked to the enzyme, rendering it incapable of performing subsequent transpeptidation. Peptidase enzymes that serve as β-lactam targets have been termed penicillin-binding proteins (PBPs).
FIGURE 1.1: Penicillins and cephalosporins

(a) Generic Structures

Penicillins

\[ R\text{-}\mathrm{C\text{-}\text{NH}}\text{-}N\text{-O COOH} \]

Cephalosporins

\[ R_1\text{-}\mathrm{C\text{-}\text{NH}}\text{-}N\text{-O COOH} \]

(b) Action of \(\beta\)-lactamases (shown for penicillin)

\[ R\text{-}\mathrm{C\text{-}\text{NH}}\text{-}N\text{-O COOH} \]

\[ \Downarrow \text{\(\beta\)-lactamase} \]

\[ R\text{-}\mathrm{C\text{-}\text{NH}}\text{-}N\text{-OH H} \text{-}O\text{-OH H} \text{-COOH} \]
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1.2.1.3 Resistance mechanisms  Resistance to β-lactams occurs most frequently through enzyme-mediated antibiotic degradation. β-lactamases catalyse hydrolysis of the β-lactam bond, preventing the antibiotic from interacting with the peptidases. They can either be chromosome or plasmid-encoded, with the latter being of greater significance in a clinical context because of the ease with which resistance can be passed on to other bacterial species that may only be distantly related. Degradation under the action of acylases and esterases (on cephalosporins) has also been reported but is insubstantial compared to β-lactamases (Dever and Dermody, 1991).

β-lactamases have been independently classified on the basis of structural features or substrate specificity, with some differences in the resultant groupings. TEM-1, for example, is paired with SHV-1 on the basis of their common ability to hydrolyse penicillins and early cephalosporins, without efficacy against more recent cephalosporins, such as ceftazidime and cefotaxime. TEM-1 shares only 65% amino acid homology with SHV-1, compared to 99% homology with TEM-3, an enzyme capable of efficiently hydrolysing the newer cephalosporins. For the clinician, detection of β-lactamase production is an important step in the selection of an appropriate therapeutic regime. This decision is aided by testing for hydrolysis of nitrocefin, a chromogenic cephalosporin that changes colour when its β-lactam ring is opened (Rosenblatt, 1991).

In England and Wales, as many as 85% of *Staphylococcus aureus* may now be resistant to penicillin, and over 50% of *Escherichia coli* resistant to ampicillin, a penicillin derivative (Amyes and Gemmell, 1992). The continued use of this class of compounds has therefore required further strategies to be employed, notably the design of compounds with improved stability against β-lactamases (eg. methicillin) or the co-administration of β-lactamase inhibitors such as sulbactam or clavulanic acid. In other words, a convoluted situation exists with sulbactam or clavulanic acid provided as an alternative hydrolysis substrate for the β-lactamase which cannot then act against the antibacterial compound.

Methicillin has an altered β-lactam nucleus with increased stability against Gram-positive β-lactamases. This does not, however, provide methicillin with bactericidal carte blanche as resistance has developed through a β-lactamase-independent route, namely the
mutation of the target enzyme PBP2 to a form with a lower binding affinity for the drug. Methicillin-resistant *Staph. aureus* (MRSA) are increasingly common and outbreaks have been reported in the UK, Europe, the USA and Australia. They are now considered to be a clinical problem world-wide (Marples and Cooke, 1988), not least as a result of their ready ability to acquire resistance to other unrelated agents.

1.2.2 Antifolates

1.2.2.1 Background information Trimethoprim and sulphonamides are classified, and often administered together, on the basis of their similar mode of action (see below). They are frequently used for selected infections of the genitourinary, respiratory and gastrointestinal tracts, particularly with ampicillin-resistant *Shigella* or *Salmonella* species. Concerns about toxicity and increased resistance (see below) has, however, decreased the importance of trimethoprim and sulphonamides as chemotherapeutic agents. Figure 1.2 shows the structure of trimethoprim and an example compound from each of the classes surveyed here.

1.2.2.2 Mode of action Tetrahydrofolic acid is an essential intermediate in the biosynthesis of amino acids and nucleotides. The inability of bacteria to take up preformed folic acid derivatives make the folate synthesis pathway an ideal target for antibacterial action. Dihydropteroate synthetase (DPS), which is not required by man, is inhibited by sulphonamides and dihydrofolate reductase (DHFR) is targeted by trimethoprim. The affinity of the human DHFR for trimethoprim is at least 3000-fold lower than its bacterial equivalent (Baccanari et al., 1982).

1.2.2.3 Resistance mechanisms Careless use of trimethoprim in developing countries has been blamed for the widespread resistance reported. The main cause of resistance appears to be the production of reduced affinity DHFR mutants. At least seven different groups of plasmid-encoded insusceptible DHFRs have so far been identified in Gram-negative bacteria and one in staphylococci, which apparently bears no relationship to the Gram-negative mutants (Amyes and Towner, 1990). Overproduction of wild-type DHFR and impermeability mutations have also been cited as resistance mechanisms.
FIGURE 1.2: Structure of some significant antibacterials

Trimethoprim

Tetracycline

Streptomycin

Chloramphenicol

Erythromycin
FIGURE 1.2 (continued):

Vancomycin
Two distinct plasmid-encoded DPS mutants with reduced affinity for sulphonamides have been characterised in Gram-negative bacteria (Swedberg and Sköld, 1980). Additionally, hyperproduction of \( p \)-aminobenzoic acid, a substrate for DPS, may provide a means of overcoming the metabolic block.

1.2.3 Aminoglycosides

1.2.3.1 Background information Streptomycin and gentamicin are perhaps the most well-known agents of the aminoglycoside family. Gentamicin is routinely used in burn creams and streptomycin has been valuable against tuberculosis. Widespread prophylactic use of aminoglycosides over the last 20 years has, however, led to increasing resistance, particularly in Southern Europe.

1.2.3.2 Mode of action Aminoglycosides disrupt protein synthesis by binding to the prokaryotic ribosome. Binding to both the 50S and 30S subunits has been observed, and chemical footprinting experiments may imply that the primary interaction is with 16S rRNA (Moazed and Noller, 1987a). Protection of bases A1408 and G1494 from modification by dimethyl sulphate (DMS) was noted with neomycin, paromomycin, gentamicin and (to a lesser extent) kanamycin. Interestingly, these bases are weakly protected by aminoacyl tRNA (AA-tRNA) occupying the A-site, and are adjacent in the ternary structure to A1492 and G1493 which are strongly protected (Noller, 1991). The suggestion has therefore been made that binding of an aminoglycoside antibiotic to the 16S rRNA in a position adjacent to the A-site can stabilise the binding to that site of an incorrect AA-tRNA, i.e. carrying a residue that is not determined by the mRNA codon. This would then lead to translational mis-incorporation and/or termination.

1.2.3.3 Resistance mechanisms The action of aminoglycoside modifying enzymes (AMEs) is the most common, and clinically significant, route to acquired resistance. A number of AMEs have been identified and categorised on the basis of their modes of action, principally N-acetylation, O-nucleotidylation or O-phosphorylation. Most AMEs are plasmid encoded. Impermeability accounts for less than 10% of resistance, though this is increasing, particularly in \textit{Pseudomonas aeruginosa} (Gerding et
Single-step mutation at position 1491 of the 16S rRNA results in decreased binding to the 30S subunit (De Stasio et al., 1989) but has not, to date, been reported to have clinical significance. The gentamicin producing organism, *Micromonospora purpurea* is self-protected by methylation at G-1405 (Beauclerk and Cundliffe, 1987).

### 1.2.4 Glycopeptides

**1.2.4.1 Background Information** Vancomycin and teicoplanin are glycopeptide antibiotics that are active against staphylococci, streptococci and other Gram-positive bacteria, indeed, vancomycin is the drug of choice for the treatment of infections due to MRSA (Wilhelm, 1991). The resistance of Gram-negatives to these compounds is thought to result from the outer membrane structure preventing entry of glycoproteins, which are large, rigid molecules.

**1.2.4.2 Mode of action** Like β-lactams, glycopeptides elicit antibacterial activity by blocking the polymerisation of peptidoglycan in cell wall biosynthesis. Unlike β-lactams, glycopeptides bind to the acyl-D-alanyl-D-alanine arrangement of nascent amino acid chains, rather than to an enzyme involved in the catalysis.

**1.2.4.2 Resistance mechanisms** Given that vancomycin has been in use for over 30 years, occurrence of bacterial resistance has been remarkably low and has yet to become a major clinical problem. Plasmid-mediated resistance to vancomycin and teicoplanin has been detected in some enterococci and a gene, *vanA*, from *Enterococcus faecium* has been isolated (Brisson-Noel et al., 1990). The VanA protein encoded by the resistance gene has been identified as a D-alanyl-D-alanine ligase with altered substrate specificity (Dukta-Malen et al., 1990; Bugg et al., 1991). VanA strains have inducible resistance to all glycopeptides tested associated with the production of a 39 kD cytoplasmic membrane protein. In conjunction with the α-keto acid dehydrogenase VanH, VanA synthesised a pentapeptide with an altered C-terminal structure, to which vancomycin has low affinity (Bugg et al., 1991). A second resistant phenotype, VanB, has been reported in which bacteria exhibit inducible production of a 39.5 kD membrane protein and are resistant to vancomycin but remain susceptible to teicoplanin. A third
phenotype, VanC, has recently been described in *Enterococcus gallinarum*, with low-level constitutive resistance to vancomycin linked to a putative D,D-carboxypeptidase activity (Vincent et al., 1992).

1.2.5 Tetracyclines

1.2.5.1 Background Information A family of compounds produced by *Streptomyces* species and their semi-synthetic derivatives, the tetracyclines are broad-spectrum antibacterials recommended for the treatment of simple UTIs, infections caused by *Chlamydia*, acne vulgaris and Lyme disease (*Borrelia burgdorferi*).

1.2.5.2 Mode of action Tetracyclines are understood to prevent binding of AA-tRNA via attachment to the 30S subunit; chemical footprinting has revealed interaction with 16S rRNA (Moazed and Noller, 1987a). Binding is reversible and tetracyclines are considered bacteriostatic, not bactericidal. Recent reports suggest an alternative mode of action for "atypical" tetracyclines, eg. chelocardin and 6-thiatetracycline, with evidence that their primary target is in fact the bacterial cell wall, and that interference with membrane permeability leads to cell lysis and death (Oliva et al., 1992).

1.2.5.3 Resistance mechanisms Increased occurrence of resistance in Gram-negative bacilli has impinged upon the usefulness of tetracyclines. Active efflux and ribosomal protection have been recognised as two distinct routes to tetracycline resistance (Levy, 1988; Salyers et al., 1990). Tetracycline efflux, mediated by a 43 kD cytoplasmic membrane protein (Tet) is the major mechanism of tetracycline resistance among Gram-negative bacilli. Hydropathy analysis of Tet proteins reveals 12 hydrophobic regions, and it is suggested that each of these is membrane-spanning in a manner that forms a channel through the membrane. Similarly, an active efflux system involving a 50 kD membrane protein has been identified in Gram-positive cocci and *Bacillus* species. In general, however, ribosomal protection mediated by a 72 kD cytoplasmic protein is now seen to be the principle resistance mechanism among Gram-positives. First noted in *Streptococcus* (Burdett, 1986), ribosomes of tetracycline-resistant cells were shown to be less sensitive to the antibacterial than ribosomes of wild-type cells. Treatment with high salt concentration
reverses the resistance, and has been interpreted to show that the product of the
tetracycline resistance gene either binds directly to the ribosome, or else activates a third
cytoplasmic component to do so (Salyers et al., 1990).

1.2.6 Chloramphenicol

1.2.6.1 Background information Used in chemotherapy for over 40 years,
concern over toxicity, particularly suppression of bone marrow, has significantly reduced
the role of chloramphenicol. Apart from routine use against conjunctivitis,
chloramphenicol is reserved for the treatment of life-threatening infections, particularly
those caused by Haemophilus influenzae, and also for typhoid fever.

1.2.6.2 Mode of action Chloramphenicol disrupts protein synthesis by inhibition
of peptidyl transferase that links amino acids to the growing peptide chain (Cundliffe and
McQuillen, 1967). Although the exact nature of the interaction has not been elucidated,
chloramphenicol binds to the 50S ribosomal subunit and is thought to interfere with the
correct binding of AA-tRNA to the peptidyltransferase site (Cooperman et al., 1990).
Protection of a number of bases in the central loop of domain V of 23S rRNA has been
reported (Moazed and Noller, 1987b). As with tetracyclines, inhibition by
chloramphenicol is bacteriostatic.

1.2.6.3 Resistance mechanisms Resistance to chloramphenicol arises through
reduced permeability or by O-acetylation of the antibiotic under the action of
chloramphenicol acetyltransferase (CAT) with acetyl-coenzyme A as the acyl donor. The
modified drug no longer binds to the bacterial ribosome (Shaw and Unowsky, 1968). In
addition, resistance mutations at positions 2057, 2447, 2451, 2452, 2503 and 2504 have
been noted (Noller, 1991).

1.2.7 Erythromycin

1.2.7.1 Background information Erythromycin is produced by Streptomyces
erythraeus and is the most clinically significant macrolide antibiotic, consisting of a 14-
membered lactone ring that is glycosylated in two positions by different monosaccharides.
Considered one of the safest antibiotics, erythromycin is the drug of choice for the treatment of *Mycoplasma pneumoniae* and *Legionella* infections (Wilkowske, 1991). With a similar, though not identical, antibacterial spectrum to that of penicillin, it is a suitable alternative in instances of penicillin resistance or allergy.

1.2.7.2 Mode of action  Macrolide antibiotics compete with the binding of chloramphenicol to the 50S ribosomal subunit and are therefore thought to have similar, though not necessarily identical sites of interaction (Cundliffe, 1990).

1.2.7.3 Resistance mechanisms  *S. erythraeus* has an erythromycin resistance methylase (erm) that dimethylates residue A-2058 of the 23S rRNA (Skinner et al., 1983), and serves to protect the bacterium from the agent it produces. Mutation of a number of residues in 23S rRNA gives rise to erythromycin resistance and the limited cross resistance to chloramphenicol, only one of four reported modifications gives resistance to chloramphenicol, is taken as evidence that the mode of action of the two classes of inhibitor is not identical.

1.3 Quinolones

1.3.1 Structures and structure-activity relationships  An entirely synthetic family, the quinolones are rapidly rising to prominence in chemotherapy. Since nalidixic acid (Lesher et al., 1962), some 5000 analogues have been reported. Comparison of compounds differing in only one substituent group has allowed limited assignment of functionality. An exhaustive review of the relationship between quinolone structure and antibacterial activity has recently been published (Chu and Fernandes, 1991).

The general structure of quinolones consists of a bicyclic ring; a 1-substituted-1,4-dihydro-4-oxopyridine-3-carboxylic moiety combined with an aromatic or heteroaromatic ring, see Figure 1.3. Due to the complexity of the systematic names, the 4-quinolones has been suggested as a generic name for this class of agents (Smith, 1984). A subdivision into quinolones and fluoroquinolones is useful, since the presence of a C-6 fluorine atom dramatically enhances antibacterial activity (Koga et al., 1980) and distinguishes first generation compounds, which are now largely redundant in clinical practice, from the
FIGURE 1.3: Examples of quinolone structures

Basic Quinolone Structure

Nalidixic Acid

Oxolinic Acid

Norfloxacin

Ciprofloxacin

Difloxacin

Ofloxacin
second (and third) generation agents that have superseded them. The fluoro group at position 6 may improve both the inhibitory activity against purified gyrase (2- to 17-fold) and cell penetration (1- to 70- fold; Domagala et al., 1986). A C-6 fluorine atom has therefore been a feature of nearly all of the recently synthesised quinolones (Chu and Fernandes, 1991).

Although some modification at the C-3 position has been accommodated, it is generally considered that the C-3 carboxyl and C-4 keto groups are necessary for antibacterial activity; indeed, suggestion has been made that the the planarity of the C-3 carboxyl and C-4 keto groups is vital (Chu et al., 1990). Some interest has been shown in C-3 variations which have improved water solubility and pharmacokinetic properties. These compounds are not themselves antibacterial, but can be subsequently metabolised in vivo to the C-3 carboxyl derivative in a manner that yields a higher concentration of the active form than was achievable directly. One example of this scenario is the reported conversion of 3-formyl norfloxacin to norfloxacin (NFX) in mice, with resultant serum concentrations twice as high as those achieved with NFX alone (Kondo et al., 1988).

Conservation of the N-1 nitrogen atom is also considered vital for quinolone potency. Some significance is attached to the possession of a side-group at this position, frequently an ethyl group, as for nalidixic acid (NAL) and NFX, or a cyclopropyl group, as in ciprofloxacin (CFX) and many compounds currently undergoing clinical trials. Little is known about the structure-activity relationship of C-2 modifications though they are generally deemed unfavourable since 2-methyl and 2-hydroxyl substitutions result in a loss of biological activity (Chu et al., 1990). Inclusion of a 5-amino group is thought to increase potency against Gram-positives when presented in conjunction with a C-8 fluoro and an N-1 cyclopropyl group, eg. sparfloxacin (SFX), but not when the N-1 substituent is ethyl (Chu and Fernandes, 1991). In other circumstances, the C-5 position is best left unsubstituted.

Modification of C-7 and C-8 (N-8 for naphthyridine derivatives, such as NAL and enoxacin) have been comprehensively studied. Only fluoro, chloro, methyl and methoxyl substituents at C-8 have proved more efficacious than the hydrogenated form. N-8
analogues are, in general, less active \textit{in vitro} than the corresponding C-8 counterparts, but this is in some measure counteracted \textit{in vivo} by superior absorption. A C-8 methoxyl group is reported to improve the stability of fluoroquinolones against UV light (Matsumoto \textit{et al.}, 1992). The nature of the C-7 substituent group is considered to be the predominant factor affecting cell permeability (Chu and Fernandes, 1991). This view is based partly upon computer-automated structure evaluation (CASE) analysis (Klopman \textit{et al.}, 1987). A C-7 piperazinyl group, as for NFX (see Figure 1.3), enhances potency against Gram-positives.

\subsection*{1.3.2 Clinical applications} Nalidixic acid showed activity against Gram-negative bacteria and found use in the treatment of urinary tract infections (UTIs). However, the inability to achieve adequate blood concentrations, and the rapid development of bacterial resistance, severely limited the applications of the first generation (non-fluorinated) quinolones.

The inclusion of a fluorine atom at the C-6 position has given the 4-quinolone family a new lease of life. Six conditions have been identified where the use of fluoroquinolones may be preferable to the use of other agents currently available (Hooper and Wolfson, 1991). Firstly, for the treatment of complicated UTIs, where patients have structural or functional abnormalities of the urinary tract. Secondly, as a quick response to patients who are seriously ill with bacterial gastroenteritis and require treatment before the results of culture analyses are known. Thirdly, in cases of typhoid fever resulting from carriage of \textit{Salmonella typhi}. Fourthly, in patients with cystic fibrosis and mild respiratory exacerbations associated with \textit{P. aeruginosa} in the sputum. Fifthly, for the treatment of Gram-negative bacillary osteomyelitis and sixthly for the therapy of invasive external otitis caused by \textit{P. aeruginosa}. In a further range of UTI, respiratory tract and skin infections, treatment with fluoroquinolones is a valid alternative should complications have arisen with chemotherapy using the first choice agents. Recent reports have suggested the possible use of fluoroquinolones against \textit{Legionella} ("Legionaire's disease") and malaria (Meyer, 1991; Krishna \textit{et al.}, 1988). The world health organisation (WHO) is investigating the efficacy of ofloxacin (OFX) in combating \textit{Mycobacterium leprae} that
causes leprosy (Lienhardt and Fine, 1992). Prophylactic use against traveller's diarrhoea has also been mooted (eg. Johnson et al., 1986) but remains controversial since, in addition to the potential for significant increase in and distribution of resistant bacteria, there has been description of opportunistic infection by *Clostridium difficile* associated with quinolone therapy (Dan and Samra, 1989).

Double-blind clinical trials comparing fluoroquinolones with non-quinolone treatments and placebos have generally concluded that fluoroquinolones are safe and well-tolerated drugs, exhibiting a safety profile that equals or surpasses commonly used non-quinolone agents (Wolfson and Hooper, 1991). Where adverse results have been reported, they have principally been gastrointestinal disturbances (1.8 - 5%), reactions of the central nervous system (0.9 - 1.6%) or skin reactions (0.6 - 1.4%). Observation of abnormal cartilage development in young animal models means that quinolones are not prescribed to children, adolescents and pregnant or breast-feeding women (Stahlmann, 1990). The image of quinolones has recently been tarnished by the worldwide withdrawal of temafloxacin in June 1992. Advertised in the clinical press as being "designed to exceed the reach of other quinolones" it may have been the attempt to combine the good solubility of OFX with the potent antimicrobial activity of CFX and the prolonged serum half-life of pefloxacin (PFX) in one optimised compound that led to its downfall (Percival, 1991).

Ciprofloxacin is, to date, the most widely used fluoroquinolone, available in over 60 countries (Grohe, 1992), with a worldwide expenditure of more than 500 million pounds a year (Emsley, 1991). It is by no means implicit that such usage was always appropriate; the ease of administration, the broad spectrum of activity, excellent pharmacokinetics and low level of toxicity have led to inappropriate use of the new quinolones (Frieden and Mangi, 1990; Van Landuyt et al., 1990). Overuse and misuse of 4-quinolones is undoubtedly contributing to the increasing observation of resistance, most notably in strains of *S. aureus* and *P. aeruginosa*.

1.3.3 Resistance mechanisms Resistance to 4-quinolones has been shown to result from either reduction of the intracellular drug concentration or by mutation of the
target enzyme, DNA gyrase. As yet, there have been no reports of resistance stemming from enzymic modification of the drugs and, given that quinolones are entirely synthetic agents, it might fairly be assumed that such a route to resistance is unlikely to develop.

Reduction of the intracellular quinolone concentration can arise through lower uptake (e.g. Hirai et al., 1986; Hooper et al., 1989) or active efflux (e.g. Cohen et al., 1988; Yoshida et al., 1990b). The 4-quinolone family of antibacterials includes hydrophobic (e.g. NAL, OXO, SFX) and hydrophilic compounds (e.g. NFX, CFX, OFX). This diversity may well contribute to the difficulties that have been apparent in determining the way quinolones enter bacterial cells, and it is almost certain that uptake occurs by more than one mechanism.

1.3.3.1 Reduced uptake Many studies have reported involvement of the outer membrane porin protein OmpF in the entry of quinolones into Gram-negatives (Bedard et al., 1987; Cohen et al., 1989; Hirai et al., 1986; Hooper et al., 1989). The expression of ompF, which encodes OmpF protein, is subject to both transcriptional and translational regulation (Misra and Reeves, 1987; Slauch and Silhavy, 1989). Quinolone-resistant cells (nfxB, nfxC, cfxB) can exhibit reduced OmpF expression as a result of overexpression of micF, which encodes an antisense RNA complementary to the 5' end of the ompF mRNA (Hooper et al., 1992). Findings from study of multiple-antibiotic-resistant (Mar) E. coli reveal that reduction of OmpF expression is only responsible in part for quinolone resistance (Cohen et al., 1989). Transposon insertion at the marA locus restored complete susceptibility to all antimicrobial agents (tetracycline, chloramphenicol, NAL, NFX and CFX) to which the recipient strain had formerly been resistant (this does not indicate that all quinolone resistance need involve marA). Transposon inactivation of ompF did not account for all the resistance observed in the Mar mutants, which were still significantly less susceptible to fluoroquinolones and had lower steady-state accumulation of NFX than the ompF::Tn5 mutant. An interesting aside to this work was the observation that cross-resistance to fluoroquinolones arose some 1000-fold more frequently in E. coli selected on tetracycline or chloramphenicol than in cells selected directly on NFX (Cohen et al., 1989). This could have important repercussions in any clinical context where
fluoroquinolone treatment was sought as a replacement for an agent that had failed to overcome infection by a Mar-like mutant.

Bedard and coworkers conclude from their experiments that uptake of enoxacin (ENX) by E. coli and B. subtilis occurs by simple diffusion (Bedard et al., 1987; Chapman and Georgopapadakou, 1988). This view was supported by the lack of saturability and the energy independence of ENX uptake. A slight intracellular accumulation of fluoroquinolone, perhaps 2-4 fold above the extracellular concentration, has been suggested (Bedard et al., 1987; Hooper et al., 1989; D.C. Hooper, personal communication) but this would be insufficient to explain the "MIC paradox", see Section 1.5.

1.3.3.2 Active efflux The occurrence of endogenous active efflux of quinolones was first noted in a susceptible strain of E. coli (Cohen et al., 1988) but has since been described in other Gram-negative and Gram-positive bacteria (Yoshida et al., 1990b; Celesk and Robillard, 1989). Efflux has been most comprehensively studied in Staphylococcus aureus, where the norA gene has been cloned (Ubukata et al., 1989) and characterised (Yoshida et al., 1990b). The NorA polypeptide confers relatively high resistance to hydrophilic quinolones but low or no resistance to hydrophobic agents. It has a predicted mass of 42 kD, features 12 hydrophobic membrane-spanning regions and shares significant homology with the tetracycline efflux proteins described in Section 1.2.5.3 and the multidrug efflux transporter of B. subtilis (Neyfakh, 1992). Evidence suggests that an endogenous efflux system, perhaps with lower activity, is present in susceptible as well as resistant cells (Yoshida et al., 1990b). Southern hybridization revealed that the norA gene is present in sensitive strains of S. aureus and drug accumulation differences in the presence and absence of the inhibitor carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which blocks energy production by destroying the proton motive force (PMF), point to the existence of an energy-dependent efflux system in susceptible cells. The addition of CCCP led to uptake by formerly-resistant cells of wild-type concentrations of fluoroquinolone (Cohen et al., 1988; Yoshida et al., 1990b; Yoshida S. et al., 1991). Ohshita and coworkers report an Asp to Ala point mutation towards the C-
terminus of the NorA protein in resistant cells, though it remains unclear how this could lead to the resistance phenotype (Ohshita et al., 1990). It is possible that mutation could alter the substrate specificity of a protein that is normally expressed.

1.3.3 Resistance via DNA gyrase Discussion of the manner in which quinolone resistance may arise through mutation of DNA gyrase will be greatly facilitated by prior introduction to DNA topoisomerases and gyrase in particular. This subject will, therefore, be considered at some length in Section 1.6.

1.4 DNA gyrase and other topoisomerases

1.4.1 DNA topoisomerases

The structure of DNA poses many topological problems during, for example, its replication and subsequent partitioning between daughter cells. The difficulties are most obvious when considering the replication of closed-circular DNAs, such as the bacterial chromosome. The two strands of the double-helix are coiled around each other (Watson and Crick, 1953). With a little thought, it is apparent that for both backbones to be sealed in a way that leaves no free ends, the two strands are necessarily interlinked. Segregation after semi-conservative replication requires that at least one strand is broken and a strand (or duplex) passed through the gap in a manner that facilitates the separation of the two molecules with the break being subsequently resealed. The search for an agent that could carry out such a reaction in a controlled manner was the impetus for much of the early work in this field (Wang, 1985). Manoeuvring of the replication (or for that matter transcriptional) machinery along the template, or vice versa (Cook and Gove, 1992), presents gross topological difficulties. The role of proteins capable of manipulating DNA in this manner is all the more significant when it is remembered that a considerable length of DNA, from approximately 1.5 mm (for E. coli) up to several metres (for Homo sapiens) must be packaged into a cell/nucleus that is little more than 1 μm in diameter.

Enzymes capable of altering the topological state of DNA are termed DNA topoisomerases. The family can be sub-divided into type I and type II enzymes on the
basis of whether they nick and reseal one or both strands, respectively. As a consequence of this difference, type I topoisomerases alter the number of supercoils in DNA in increments of one while type II topoisomerases remove or, in the case of gyrase, introduce supercoils in steps of two; the mechanism by which gyrase achieves this transition will be discussed in further detail in Section 1.4.2.2. The formation of phosphotyrosine covalent interaction(s) between enzyme and DNA is a feature common to all topoisomerases.

1.4.1 Type I topoisomerases The first topoisomerase discovered was *E. coli* topoisomerase I, then termed c o protein (Wang, 1971). *E. coli* topoisomerase I was shown to relax negatively supercoiled DNA without the need for a high energy cofactor such as ATP or NAD. A year later, a similar activity was detected in an extract from mouse cells, although it apparently differed from *E. coli* topoisomerase I in its capacity to relax both negatively and positively supercoiled DNA (Champoux and Dulbecco, 1972). During DNA cleavage *E.coli* topoisomerase I (in common with all type II topoisomerases) is covalently linked to the 5' end of the broken strand (Tse et al., 1980). Eukaryotic topoisomerase I, however, forms a 3' phosphotyrosine bond (Champoux, 1977). This mechanistic difference reflects structural dissimilarity; there is little amino acid sequence homology between topoisomerase I from *E. coli* and *S. cerevisiae* (Tse-Ding and Wang, 1986). Despite these differences, expression of yeast topoisomerase I can complement a conditional lethal topoisomerase I mutation in *E. coli* (Bjornsti and Wang, 1987). An N-terminal sequence motif found in *S. cerevisiae, Schizosaccharomyces pombe* and human topoisomerase I is thought to determine nuclear localisation for the protein (Alsner et al., 1992).

Further type I topoisomerases have now been identified. In *E.coli*, a second enzyme of this class, termed topoisomerase III, was first detected as a weak relaxation activity in a strain carrying a deletion of *topA*, the gene that encodes topoisomerase I (Dean et al., 1982) and was subsequently shown to act as a decatenase (Digate and Marians, 1988). It has been confirmed that this protein is the product of a distinct gene, *topB*, and not a fragment of gyrase or topoisomerase I (Digate and Marians, 1989). A hyper-recombination mutation in *S. cerevisiae* led to the cloning of TOP3, the gene for yeast
topoisomerase III (Wallis et al., 1989). Unlike yeast topoisomerase I, this novel protein has significant sequence homology (39% identical or conserved residues) with the bacterial topoisomerase I. Furthermore, the E. coli protein was shown to complement the slow-growth phenotype that results from mutation in TOP3. There has been speculation, on the basis of sequence homology, about whether the HPR1 gene of S. cerevisiae encodes a third type I topoisomerase in this organism (Aguilera and Klein, 1990). Mutation of this gene has also been associated with a hyper-recombination phenotype, yet the authors reported that it lacks the characteristic active-site tyrosine that is a hallmark of topoisomerases. Reassessment of the amino acid sequence of HPR1 (Wang et al., 1990) has located a putative active-site tyrosine and homology has been noted to RAG-1, a mammalian protein implicated in sequence-specific recombination in the immunoglobulin genes. It is therefore possible that the type I topoisomerase family contains further members that have yet to be identified.

Thermophilic archaeabacteria have been shown to possess an ATP-dependent type I topoisomerase, termed reverse gyrase, which is capable of introducing positive supercoils (Kikuchi and Asai, 1984). The manner in which this supercoiling is achieved has yet to be elucidated (Boutier de la Tour et al., 1990).

1.4.1.2 Type II topoisomerases Enzymes capable of altering DNA topology in increments of two are as ubiquitous as those that break one strand only. DNA gyrase, originally isolated from E.coli (Gellert et al., 1976a), was the first, and to this date remains the only, example of a topoisomerase that can introduce negative supercoils into DNA (Gellert et al., 1976a; see Section 1.4.2.2). The introduction of supercoils is energetically unfavourable and is performed by gyrase with energy derived from ATP hydrolysis (structural and mechanistic details of gyrase follow in Section 1.4.2). The gyrase genes from several bacteria have now been cloned (reviewed by Reece and Maxwell, 1991a), and without exception the enzyme is composed of two nonidentical subunits, the products of the gyrA and gyrB genes.
A second *E. coli* type II topoisomerase, referred to as topoisomerase IV, was recently described (Kato *et al.*, 1990). Despite the new enzyme, the product of the parC and parE genes, sharing with gyrase both considerable structural homology and the need for ATP as a cofactor, topoisomerase IV is unable to supercoil DNA. It has been shown to have relaxation activity, and mutants are deficient in chromosome segregation. Simultaneous overexpression of the ParC and ParE proteins can compensate for deficiency in topoisomerase I activity.

Like bacterial topoisomerase IV, eukaryotic topoisomerase II requires ATP to relax DNA and is structurally homologous to gyrase; for example, 30 residues are absolutely conserved between *E.coli* GyrA, *B. subtilis* GyrA, *E. coli* ParC and type II topoisomerases from T4 phage, *S. cerevisiae*, *Sch. pombe* and *Drosophila melanogaster* (Kato *et al.*, 1990). Unlike their bacterial counterparts, eukaryotic topoisomerase IIIs are homodimers. There is good evidence that eukaryotic topoisomerase II is a component of the nuclear scaffold (eg. Klein *et al.*, 1992). In yeast, the gene encoding topoisomerase II is essential for growth, and study of temperature-sensitive mutants has shown a requirement for the enzyme in the decatenation of sister chromatids, and for condensation of chromatin before mitotic segregation (DiNardo *et al.*, 1984; Uemara *et al.*, 1987).

### 1.4.2 DNA gyrase

#### 1.4.2.1 Subunit and domain structure of gyrase

The gyrase holoenzyme consists of two A and two B subunits (Mizuuchi *et al.*, 1978). In *E. coli*, these proteins have molecular weights of $97 \text{ kD}$ and $90 \text{ kD}$ respectively and though the $A_2B_2$ structure is common to all gyrase Investigated, the molecular weight of each subunit may vary between bacterial species; a range of $90-115 \text{ kD}$ has been noted for GyrA, but GyrB is generally smaller than the *E. coli* equivalent, being often only $71-72 \text{ kD}$ (Reece and Maxwell, 1991a). Comparison of *E. coli* GyrB with the B subunit from *Bacillus subtilis* reveals an additional 169 amino acid sequence, residues 564-732, in *E. coli* (Yamagishi *et al.*, 1986).
There is now good evidence that both the gyrase A and B proteins are organised into functional domains, see Figure 1.4. Limited digestion of *E. coli* GyrA with trypsin yields an N-terminal fragment of 64 kD and a C-terminal fragment of 33 kD (Reece and Maxwell, 1989). The 64 kD portion and smaller derivatives (down to 58 kD) have been shown to catalyse the breakage and reunion of DNA (Reece and Maxwell, 1989 and 1991b). The N-terminal 64 kD fragment has been crystallised in two forms (Reece et al., 1990), one of which diffracts x-rays to a resolution of 4.5 Å, but this is insufficient for high resolution structure determination. The C-terminal 33 kD polypeptide has been found to be a DNA-binding protein and by so doing to induce positive supercoils, which suggests it may be involved in wrapping of DNA in the gyrase-DNA complex (Reece and Maxwell, 1991c).

The observation of an approximately 50 kD protein (termed B') in preparations of GyrB was an early indicator of the existence of domain structure within the gyrase subunits (Brown et al., 1979; Gellert et al., 1979). In conjunction with GyrA, B' forms a complex (topoisomerase II) that has the capacity to relax both positively and negatively supercoiled DNA but could not introduce supercoils into DNA. When the full sequence of the *gyrB* gene became known (Adachi et al., 1987) it was clear that B' is the C-terminal 47 kD of GyrB, and represents the portion of GyrB that interacts with GyrA. ATP hydrolysis (or a means of coupling the energy derived from ATP hydrolysis to the supercoiling reaction) must be a feature of the remaining 43 kD, the N-terminal fragment of GyrB. Production of the 43 kD fragment as a direct gene product (Jackson et al., 1991) confirmed that this domain can bind and hydrolyse ATP (Ali et al., 1993). The 43 kD fragment has been crystallised in the presence of the non-hydrolysable ATP analogue ADPNP (Jackson et al., 1991) and the structure solved to 2.5 Å resolution (Wigley et al., 1991). The 43 kD protein dimerises and a sub-domain structure is apparent; ADPNP is bound to the N-terminal region which also contributes most of the interaction between the monomer units. Each C-terminal domain forms one side of a ~20 Å hole in the protein dimer, through which DNA could pass during the supercoiling reaction (see Section 1.4.2.2).
FIGURE 1.4: Domains structure of E. coli gyrase subunits.
1.4.2.2 Mechanism of action As befits its categorisation as a type II topoisomerase, gyrase acts to introduce or remove supercoils by catalysing the transient cleavage of DNA in both strands, the passing of duplex DNA through the gap and the subsequent resealing of the breaks. Several lines of evidence (reviewed by Reece and Maxwell, 1991a) have contributed to the mechanistic model depicted in Figure 1.5. A segment of approximately 120 bp is wrapped with a positive writhe around the enzyme (Liu and Wang, 1978; Morrison and Cozzarelli, 1981; Fisher et al., 1981; Kirkegaard and Wang, 1981; Kirchhausen et al., 1985; Rau et al., 1987; Krueger et al., 1990). Cleavage occurs in both strands of the DNA and involves the covalent attachment of each GyrA to a 5' phosphate group via residue Tyr122 (Horowitz and Wang, 1987). There is a 4 bp stagger between the broken bond in each DNA backbone (Morrison and Cozzarelli, 1979). A region of DNA bound elsewhere by gyrase is passed through the break and almost certainly through at least part of the protein complex; the existence of cavities or channels within the protein has been suggested by electric dichroism and small-angle neutron scattering (Rau et al., 1987; Krueger et al., 1990) and a 20Å central hole is a feature in the crystal of the 43 kD N-terminal fragment of GyrB (Wigley et al., 1991).

The introduction of negative supercoils is energetically unfavourable and somewhere within this process there is the requirement for ATP hydrolysis. This energy-providing reaction is performed by the GyrB subunits, specifically within the N-terminal 43 kD domain of GyrB (Wigley et al., 1991; Ali et al., 1993). Given that each gyrase holoenzyme contains two GyrB subunits and each has one ATP-binding pocket, it is probable that one round of supercoiling necessitates the hydrolysis of two ATP molecules and there are limited data to support such a proposal (Maxwell and Gellert, 1986). The observation of limited supercoiling in the presence of the non-hydrolysable ATP analogue ADPNP has led to the proposal that nucleotide binding is sufficient to promote the reactions up to and including the strand passage step, but ATP hydrolysis is required to allow dissociation of products from the active site of the enzyme, permitting further reactions to occur (Peebles et al., 1978).
FIGURE 1.5: Proposed mechanism for the supercoiling of DNA by *E. coli* DNA gyrase

- **Relaxed closed-circular DNA**
- **DNA gyrase**
- **Positive writhe**
- **Negative writhe**
- **Double-stranded break in DNA**
- **Negatively supercoiled DNA**
- **DNA strand-passage through DNA gap then protein**
- **Break resealed**
- **2 ATP**
- **2 ADP**
- **2 Pi**
1.4.2.3 Control of bacterial DNA supercoiling. There is now strong evidence that the extent of intracellular DNA supercoiling affects the expression of some genes (reviewed by Drlica, 1992). The growth environment, for example nutrient availability (Balke and Gralla, 1987), levels of oxygenation (Dorman et al., 1988) and osmotic pressure (Higgins et al., 1988) can all influence DNA supercoiling. It is suggested, though as yet unsubstantiated, that each of these effects may be exerted via alteration of the [ATP]/[ADP] ratio (Drlica, 1992). That levels of DNA supercoiling result primarily from the interplay of gyrase and topoisomerase I is, however, well established.

A cell functioning normally will tend to maintain its DNA superhelicity within tightly defined boundaries. There will inevitably need to be a means of controlling fluctuations, not only as a result of environmental factors of the kind outlined above, but also because any process that requires the separation of the strands of the double-helix, such as DNA replication and RNA transcription will upset the local status quo. The effect of RNA transcription serves most clearly to outline the roles played by gyrase and topoisomerase I in vivo (Liu and Wang, 1987). According to the twin transcriptional-loop model, the DNA ahead of the advancing RNA polymerase becomes overwound, leading to a build-up of positive supercoils, with concomitant production of negative supercoils behind the enzyme. In the situation where a plasmid carries one transcriptional unit, the negative and positive supercoils can cancel out. If the DNA was tethered in some way, or if a plasmid carried two genes arranged in opposite directions (such as the tet and bla genes of pBR322), the regions behind and in front of the transcribed regions are in effect isolated domains that will have either a build-up of positive or of negative supercoils. It is proposed that gyrase acts to relax the positive supercoils ahead of the transcription complex and topoisomerase I relaxes the negative supercoils behind it. Plasmid pBR322 serves as a good indicator of this phenomenon; which explains both the observation of positively supercoiled plasmid in E. coli cells treated with OXO (Lockshon and Morris, 1983) and the especially high degree of negative supercoiling of pBR322 in a strain in which a deletion mutation in the topA gene has eliminated topoisomerase I activity (Pruss and Drlica, 1986).
The antagonistic effects of gyrase and topoisomerase I also explain why strains carrying a topA deletion mutation, which would normally be lethal, can survive if a gyrase gene, either gyrA or gyrB, carries a compensatory mutation (DiNardo et al., 1982: Pruss et al., 1982). The molecular basis of the compensatory mutations has recently been established; *E. coli* strain DM800 has a mutation of gyrB that leads to insertion of alanine and arginine between residues 382 and 383 of GyrB (McEachern and Fisher, 1989). DM750 has two point mutations from wild-type GyrA, Ala569 to Thr and Thr586 to Ala (Oram and Fisher, 1992). It is interesting that both the GyrB mutation of DM800 and the GyrA mutations of DM750 are located close to the domain boundaries identified by proteolysis of the respective subunits (Adachi et al., 1987; Reece and Maxwell, 1989).

It is also important in this section to stress that the gyrA and gyrB genes are themselves responsive to changes in the degree of intracellular supercoiling (Menzel and Gellert, 1983 and 1987). Increased supercoiling of the DNA template down regulates the expression of the gyrase genes and a decrease in the level of supercoiling leads to the expression of the genes. In this way the level of gyrase activity within any cell can be homeostatically regulated. If gyrase is inactivated by the addition of inhibitors (e.g., OXO or coumermycin A1, see Section 1.7.1), a corresponding increase in gyrA expression has been observed (Franco and Drlica, 1989).

Recent work has identified two proteins encoded by the F plasmid that serve to regulate the supercoiling activity of DNA gyrase (Maki et al., 1992; Miki et al., 1992). It was known that the product of the letD gene (11.7 kD) has a lethal effect on the host cell and that the letA gene product (8.3 kD) serves to suppress this effect. The manner in which this result was achieved had been unclear but evidence now points to the formation of a stable LetD-GyrA complex that inactivates the topoisomerase. In the presence of LetD, gel filtration showed that the inactivated GyrA was somewhat larger than the active form (Miki et al., 1992). The identification of a point mutation in GyrA (Gly214 → Glu) that conveys tolerance to LetD confirms that gyrase is the target of this protein (Maki et al., 1992). A tight complex is formed between LetA and LetD and it is proposed that the former removes the latter from the LetD-GyrA complex in order to reactivate the
topoisomerase. Since LetD and LetA are thought to contribute to the stable maintenance of the F plasmid and LetD inhibits segregation of chromosomal DNA and cell division of the host strain, characterisation of these proteins may provide a useful handle for probing the in vivo role(s) of gyrase.

1.4.3 Similarities between gyrase and other type II topoisomerases

Comparison has been made of the sequences of bacterial, eukaryotic and viral type II topoisomerases and significant structural homology has been noted (eg. Wyckoff et al., 1989; García-Beato et al., 1992). Eukaryotic type II topoisomerases are dimers, with each subunit having an N-terminal region homologous to GyrB, a central region with homology to the N-terminal region of GyrA and a C-terminal region that is structurally divergent from the bacterial enzyme. The C-terminal region of *S. cerevisiae* topoisomerase II has been shown to serve as a regulatory domain and is the substrate for multiple phosphorylation by casein kinase II (Cardenas et al., 1992). Phosphorylation of topoisomerase II is cell cycle specific and may be important in modulating the decatenation activity of the enzyme (Heck et al., 1989; Cardenas et al., 1992).

Topoisomerase II from bacteriophage T4 consists of three subunits, the products of T4 genes 39, 52 and 60 (Huang, 1986a and 1986b). The gene 39 protein is homologous to GyrB, gene 52 protein to the N-terminal region of GyrA. The product of gene 60 is thought to have a role in stabilising the enzyme-DNA complex (Seasholtz and Greenberg, 1983). Sequence alignment reveals that a number of residues are conserved throughout the topoisomerase II family. Most significantly, a consensus sequence centred around a tyrosine residue has been shown in all type II topoisomerases (García-Beato et al., 1992). The equivalent tyrosine in GyrA is involved in breakage and reunion of the DNA backbone (Horowitz and Wang, 1987). The active tyrosine is always preceded in the sequence by an arginine. A cluster of 5 residues (Glu-Gly-Asp-Ser-Ala, residues 424 to 428) in *E. coli* GyrB is also conserved and is considered to be a signature for the topoisomerase II family. A Gly-X-X-Gly-X-X-Gly motif is common to all type II topoisomerases and forms part of the ATP-binding site (Tamura and Gellert, 1990; Wigley
et al., 1991) and the sequence (Lys or Arg)-X_{5.8}-Leu-(Tyr, Ile, Val, Phe or Leu)-h-(Val, Thr or Ala)-Glu, where h is a hydrophobic residue, is also thought to play a role in ATP-binding (eg. García-Beato et al., 1992).

Given the structural similarity between gyrase and other type II topoisomerases there is ample reason to consider closely the interaction of all enzymes of this class both with their DNA substrates and with inhibitory agents, since lessons learnt for one topoisomerase may prove to have a parallel with (or indeed significant difference from) another. The relationship between eukaryotic topoisomerases and anti-tumour drugs is given fuller coverage in Section 1.8, below.

Amongst the important differences between gyrase and eukaryotic topoisomerase II is the latter’s inability to introduce supercoils into DNA. Eukaryotic topoisomerase II can relax both positive and negative supercoils in DNA, requiring ATP in order so to do. The absence of a supercoiling capacity may be related to the manner in which topoisomerase II interacts with DNA; unlike gyrase, there is no evidence for wrapping of DNA around topoisomerase II and a duplex template as short as 16 bp can be sufficient for topoisomerase II cleavage activity (Lund et al., 1990).

1.5 Interaction of quinolones with gyrase

Investigation of the effects of nalidixic acid on bacteria predate the identification of DNA gyrase as the molecular target of the quinolone family of antibacterials. An important early observation was the dominance of the quinolone-sensitive phenotype over quinolone-resistance (Hane and Wood, 1969). NAL sensitivity could be restored in NAL-resistant strains of E. coli by the introduction of an episome carrying the nalA allele from a sensitive strain. An in vitro DNA replication system using cells lysed gently on cellophane discs confirmed this result (Bourguignon et al., 1973). Replication in a system utilising lysate from NAL-resistant cells was able to proceed efficiently in the presence of 20 μg/ml NAL. However, on addition of soluble extract from NAL-sensitive cells to this reaction, DNA synthesis was inhibited.
A year after the initial characterisation of DNA gyrase (Gellert et al., 1976a), it was reported that mutation of gyrase could confer high level resistance to NAL and that *nalA* therefore encoded a component of the enzyme (Gellert et al., 1977). Subsequent studies have therefore been able to investigate the effects of quinolones on bacteria *in vivo* and *in vitro* both at a phenotypic and a molecular level. A number of quinolone-resistant point mutations in the gyrase subunits have now been described; these are discussed at some length in Section 1.6. The occurrence of such mutations would naturally lead to the expectation that quinolones bind directly to the protein. This assumption has, however, been challenged on the basis of experiments detecting the interaction of quinolones with DNA (Shen and Pernet, 1985; Shen et al., 1989a, b and c; see Section 4.2 for details) and this has formed the basis of a current model (Shen et al., 1989a).

According to the Shen model (Figure 1.6), quinolone molecules are proposed to bind to the single-stranded regions in the 4 bp stagger between the sites of gyrase-mediated cleavage on each strand. Interaction with the bases would involve hydrogen-bonding to the 3-carboxyl and 4-oxo groups that are a feature of all antimicrobial quinolones (Chu and Fernandes, 1991). It is suggested that drug binding is cooperative, with the binding of subsequent drug molecules being favoured by drug-drug ring stacking and hydrophobic interaction. This model is not universally popular failing, in particular, to explain the significance of quinolone-resistant gyrase mutations (Section 1.6). Details of the experiments that support and refute the model can be found in the introduction to Chapter 4.

The manner in which the interaction of quinolones with gyrase and/or DNA brings about cell death has also to be clarified. Any plausible interpretation must accommodate the dominance of the quinolone sensitive phenotype, as outlined above. The observation that the concentration of drug required to inhibit the *in vitro* reactions of gyrase, eg. supercoiling, is 5-100 times higher than the Minimum Inhibitory Concentration (MIC) required to elicit cell death (Gellert et al., 1977; Zweerink and Edison, 1986; Hallett and Maxwell, 1991), a phenomenon that has been termed the "MIC paradox" (eg. Maxwell, 1992), also needs to be explained. A proposal that could satisfy both of these criteria
FIGURE 1.6: A model for the proposed interaction of quinolones with DNA (Shen et al., 1989a)

(a) Binding of quinolones to a ssDNA pocket opened by gyrase

(b) Detail of the interaction with DNA
was originally proposed over 10 years ago (Kreuzer and Cozzarelli, 1979). It was found that T7 bacteriophage DNA replication in a quinolone-sensitive host cell was inhibited by the presence of NAL. Given that the T7 genome is linear and is not, therefore, topologically constrained, there should have been no requirement for gyrase activity in this process. This was confirmed by the restoration of T7 replication on transfer of the host strain, containing temperature-sensitive gyrase, to the non-permissive temperature. The authors concluded that the effect on phage replication was a corruption, stemming from the quinolone-dependent stabilisation of gyrase on the template DNA and that the gyrase-quinolone-DNA complex may be a barrier to the passage of polymerases. Results that support this "poison" hypothesis are given in Chapter 5 and a similar explanation has been invoked to explain the cytotoxic effects of anti-tumour drugs (Liu, 1989; see Section 1.8).

In such a scenario, inhibition could be caused by the presence of a small amount of sensitive protein irrespective of the concentration of resistant protein, and hence the sensitive phenotype would be dominant. Cell death is not resulting from inhibition of gyrase activity per se, and would require only a small percentage of the gyrase population to be trapped in this way. This subtlety is not detected by an assay of DNA supercoiling since gyrase without bound quinolone can continue to introduce supercoils catalytically; to observe 50% inhibition of supercoiling would therefore require 50% or more of the supplied gyrase to be inhibited. Thus the "MIC paradox" would be explained.

1.6 Gyrase-mediated resistance to quinolones

In recent years, the molecular basis of quinolone resistance arising from a number of gyrase mutations has been determined, see Tables 1.2 and 1.3. Alteration of both the gyrA and gyrB genes has been noted, with the former being the more significant in terms of the degree of resistance and the frequency of clinical observation. In E. coli, the GyrA mutations are clustered between residues 67 and 106 of the 875 amino acid protein, and this has been termed the "quinolone-resistance determining region" of GyrA (Yoshida et
<table>
<thead>
<tr>
<th>Reference</th>
<th>Amino Acid Change</th>
<th>Resistance (amp)</th>
<th>NAT</th>
<th>CFX</th>
<th>ND</th>
<th>ND</th>
</tr>
</thead>
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<tr>
<td>1988</td>
<td></td>
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</table>

Table 1: Changes in Coxiella burnetii gene sequence. Amino acid changes in the Coxiella burnetii gene sequence.
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>SUBUNIT</th>
<th>AMINO ACID CHANGE(S)</th>
<th>REFERENCE</th>
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</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>A</td>
<td>Ser45 → Leu and Ser50 → Pro</td>
<td>Stechman et al., 1990</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>A</td>
<td>Glu49 → Lys</td>
<td>Goetz and Martin, 1991</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>A</td>
<td>Ser91 → Phe</td>
<td>Fuliang et al., 1990</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>A</td>
<td></td>
<td>Yashita et al., 1991b</td>
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<tr>
<td>Neisseria gonorrhoeae</td>
<td>A</td>
<td></td>
<td>Stahl et al., 1991</td>
</tr>
<tr>
<td>Actinobacillus actinomycetemcomitans</td>
<td>A</td>
<td></td>
<td>Hausel and Wieldermann, 1991</td>
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<td>Pseudomonas aeruginosa</td>
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</tr>
<tr>
<td></td>
<td>A</td>
<td>Ser30 → Leu</td>
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</tr>
<tr>
<td></td>
<td>A</td>
<td>Ser52 → Pro</td>
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</tr>
<tr>
<td></td>
<td>A</td>
<td>Ser50 → Pro</td>
<td>ND</td>
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</tbody>
</table>

a = Not Determined
b = sensitive sequence unknown
Proximity to Tyr_{122}, the residue responsible for the DNA breakage and reunion reaction (Horowitz and Wang, 1987), has been noted and in the absence of contradictory tertiary structure information it seems possible that these mutations are genuinely close to the catalytic site of GyrA (and hence also to the DNA). Alteration of Ser_{83} appears to be particularly significant in quinolone-resistance; mutation of Ser_{83} to Trp or Leu is common among clinical isolates and gives rise to high levels of resistance (Yoshida et al., 1988; Cullen et al., 1989). In _Staphylococcus_ species, the equivalent residue is Ser_{84} and is the most frequent site of mutation associated with clinical resistance to ciprofloxacin (Sreedharan et al., 1991; Goswitz et al., 1992).

The mutation of serine to leucine involves the replacement of a small polar residue by a bulkier aliphatic group, and the change to tryptophan sees the introduction of an aromatic side-chain at the expense of the hydroxyl group. These are, therefore, comparatively severe alterations. Employing _in vitro_ mutagenesis techniques, the more conservative change of Ser_{83} to Ala (i.e. loss of the hydroxyl group without an increase in residue size) has been engineered (Hallett and Maxwell, 1991). The resultant protein confers low level resistance to CFX and therefore suggests that the polarity of serine has a role to play in quinolone binding. Interestingly, the mutation Ser_{84} → Ala is amongst the alterations giving rise to quinolone-resistance in clinical isolates of _S. aureus_ (Goswitz et al., 1992).

A noteworthy mutation at residue 81 has recently been reported (Moniot-Ville et al., 1991; Cambau et al., 1993). The change Gly_{81} → Asp, which introduces a negative charge, apparently gives high level resistance to fluoroquinolones, up to 500-fold for some compounds, yet the sensitivity to NAL was "unaffected" (interpretation of results here has to be handled with some caution since the resistant strain has a secondary permeability mutation affecting drug uptake). Different degrees of resistance to various quinolones is not unknown, another example is the GyrB mutation Lys_{8447} → Glu, discussed below. The unusual feature of Asp_{81} is the high level resistance to the newer, and generally more potent compounds, without a decrease in sensitivity to the first quinolone, nalidixic acid. The availability of this mutation for _in vitro_ studies may provide a useful tool for
investigation of gyrase-quinolone interactions. A second mutation at this site, Gly$_{81} \rightarrow$ Cys, gave 8-fold resistance to all quinolones tested (Yoshida et al., 1990a).

As we have seen above, the GyrA mutation Ser$_{83(84)} \rightarrow$ Ala was sufficient to give resistance to quinolones in both E. coli and S. aureus. In E. coli, the reverse change at a different position, Ala$_{87} \rightarrow$ Ser, also confers a low level of resistance (4 to 8-fold) to a variety of drugs (Yoshida et al., 1988 and 1990a). Residue 84 of E. coli GyrA is normally alanine and the equivalent position, residue 85, in S. aureus is serine; it is not known if this difference contributes to the lower overall sensitivity of S. aureus gyrase to quinolones. In both organisms, mutation to proline at this position gives rise to resistance (Yoshida et al., 1990a; Sreedharan et al., 1990) though the resultant increase in resistance to CFX is very different, 8-fold for E. coli and 128-fold for S. aureus. Two different mutations that result in the introduction of a positive charge at residue 106 of E. coli GyrA, Gin$_{106} \rightarrow$ His or Arg, both give 2 to 4-fold resistance to quinolones (Yoshida et al., 1988; Hallett and Maxwell, 1991).

Only two mutations in GyrB, Asp$_{426} \rightarrow$ Asn and Lys$_{447} \rightarrow$ Glu, have been shown to result in resistance to quinolones in E. coli, and enzyme with the Glu$_{447}$ mutation is actually hypersensitive to amphoteric quinolones such as pipemidic acid and NFX (Yamagishi et al., 1986; Yoshida et al., 1991). A gyrB mutation responsible for low level NAL-resistance in Neisseria gonorrhoeae has also been noted and provisionally attributed to residue Asn$_{419}$ (analogous to Asn$_{426}$ in E. coli) although the sequence of the sensitive protein is not known (Stein et al., 1991). An unidentified gyrB mutation has also been reported in P. aeruginosa (Yoshida et al., 1990c). The Asn$_{426}$ and Glu$_{447}$ mutations are both in the C-terminal domain of GyrB known to interact with GyrA (Gellert et al., 1979), and it has been proposed that a quinolone-binding pocket may exist at the interface between the GyrA and GyrB subunits (Cullen et al., 1989; Yoshida et al., 1991). Alternatively, and based on the greater significance of GyrA mutations over GyrB, it is possible that the GyrB mutations give rise to quinolone resistance indirectly via altered protein-protein interactions with GyrA (Reece and Maxwell, 1991a).
1.7 Non-quinolone Inhibitors of DNA gyrase

1.7.1 Coumarins  The second major class of antibacterials that act upon DNA gyrase, the coumarin family was first identified as natural products of *Streptomyces* species although some semi-synthetic derivatives have now been prepared. As a rule, coumarins are more active against Gram-positive bacteria than Gram-negatives, and this is generally attributed to differences in the uptake of the drug. Interest in coumarins in a clinical context has recently been revived on the basis of their activity against methicillin-resistant *S. aureus* (Lambert and O'Grady, 1992).

Having been initially identified as inhibitors of DNA replication (Ryan, 1976), the establishment of DNA gyrase as the primary target of coumarin action was made shortly after the discovery of the enzyme (Gellert et al., 1976a and 1976b). Novobiocin (see Figure 1.7), the best known of the coumarin drugs, was shown *in vitro* to inhibit the ATP-dependent introduction of negative supercoils into closed circular DNA (Gellert et al., 1976b) without affecting the ATP-independent relaxation of negatively supercoiled DNA by gyrase (Gellert et al., 1977; Sugino et al., 1977). These observations led to the conclusion that a component of DNA gyrase, targeted by novobiocin, was responsible for the hydrolysis of ATP that provides energy for supercoiling, and that this reaction was inhibited in the presence of the drug. As understanding of gyrase structure became clearer, these features, as we have seen above (Section 1.4.2.2), were attributed to GyrB.

Steady-state kinetic experiments were interpreted to show that novobiocin and coumermycin, another compound of this class, are competitive inhibitors of both ATP hydrolysis and DNA supercoiling by gyrase (Sugino et al., 1978; Sugino and Cozzarelli, 1980). A number of factors now suggest that inhibition is not strictly competitive. Gyrase is highly specific for ATP and other nucleoside triphosphates are poor substrates/inhibitors of the enzyme (Gellert et al., 1976a). It is therefore unlikely that coumarin drugs, which have little structural resemblance to ATP, would bind to the same site on GyrB. This view is substantiated by the recent crystallisation and structural analysis of the N-terminal 43 kD fragment of *E. coli* GyrB (Wigley et al., 1991) which identified the residues involved in nucleotide binding, the characterisation of coumarin-resistant point mutations in GyrB.
FIGURE 1.7: Structures of coumarin antibiotics
and studies on the in vitro behaviour of the 43 kD fragment in the presence of nucleotide and/or coumarin drugs (Ali et al., 1993).

The 43 kD fragment has ATPase activity and binds coumarins. It has been found (Contreras and Maxwell, 1992) that twice as much novobiocin as coumermycin is required to inhibit supercoiling activity, and it is suggested that novobiocin may bind to a GyrB monomer while coumermycin, whose structure is approximately two novobiocins tail-to-tail, may bind to a GyrB dimer or simultaneously to two GyrB monomers. The latter suggestion is supported by the distance in the central hole between the two halves of the GyrB crystal being too great for coumermycin to span. Additionally, gel filtration and ultracentrifugation data looking at the sedimentation of the 43 kD fragment in the presence of novobiocin, coumermycin or the non-hydrolysable ATP analogue ADPNP (as in the crystallisation) reveal that in the presence of novobiocin the 43 kD fragment is monomeric (Ali et al., 1993). In the presence of either coumermycin or ADPNP the 43 kD is apparently dimeric, but there is a distinct and repeatable difference in the sedimentation profile, suggesting that the complex in the presence of coumermycin may indeed be different from the crystal form.

Crystallisation in the presence of ADPNP identified the residues responsible for interaction with the nucleotide. Sequencing of gyrB mutations giving rise to coumermycin resistance in E. coli (del Castillo et al., 1991; Contreras and Maxwell, 1992) and resistance to novobiocin in the halophilic archaebacteria Haloferax (Holmes and Dyall-Smith, 1991) revealed the significance of residue Arg-136 (Arg-137 in the Haloferax numbering system) in coumarin resistance. To date, all but one resistant bacteria studied have carried alteration at that residue, most frequently to Cys but His, Ser and Leu mutations have also been detected. The exception carries the temperature-sensitive mutation of Gly-164 to Val and was selected in the presence of chlorobiocin, giving only marginal resistance to coumermycin (Contreras and Maxwell, 1992). When superimposed onto the 43 kD crystal data, it is apparent that Arg-136 and Gly-164 are close to, but outside, the region of interaction with the nucleotide, although an H-bond is formed between the guanidinium
group of Arg-136 and the main chain carbonyl group of Tyr-5 (from the other subunit) which is involved in the proposed ATP-binding site. Two other mutations have been identified in *Haloferax* in conjunction with the Arg-137 alteration; these are Asp-82 to Gly and Ser-122 to Thr (again the *Haloferax* numbering system is one removed from that in *E. coli*). It is unclear whether these additional mutations play a role in increasing the resistance to novobiocin or possibly are compensatory, helping to overcome the impairment of ATPase activity shown for the mutations at position 136 of the *E. coli* protein (Contreras and Maxwell, 1992).

Two further routes to coumarin resistance warrant discussion. Firstly, the experiments of del Castillo and coworkers have shown that overproduction of a protein comprising the first 500 residues of *E. coli* GyrB can confer resistance to coumermycin, presumably by sequestering the drug, which is known to bind stoichiometrically and extremely tightly to GyrB (del Castillo *et al.*, 1991; Staudenbauer and Orr, 1981). Secondly, it is worth considering how a species that produces a coumarin can avoid committing suicide in the process. *Streptomyces sphaeroides*, a producer of novobiocin, was found to have two *gyrB* genes encoding proteins with different sensitivity to the drug (Thiara and Cundliffe, 1988 and 1989). It appears that the gene for the resistant form of the enzyme is under the control of a topology-sensitive promoter. As the bacterium produces novobiocin and poisons its own sensitive GyrB, the action of topoisomerase I without compensatory gyrase activity leads to a reduction in the negative superhelical density of the DNA, and this activates the transcription of the gene for the novobiocin-resistant GyrB. An indicator of the validity of this proposed mechanism is the activation of the novobiocin-resistance gene when an alternative gyrase poison, namely CFX, is employed (Thiara and Cundliffe, 1989).

1.7.2 Other compounds There are a limited, though increasing, number of other compounds that do not fall into the quinolone or coumarin families but have been reported to inhibit gyrase activity (see Figure 1.8).
FIGURE 1.8: Structures of gyrase inhibitors that do not fall into either the quinolone or coumarin families of antibacterials

Clerocidin

Quercetin

Cinodine, where R can be:

\( \beta \)  
\( \gamma_1 \)  
\( \gamma_2 \)
Cinodine, a glycocinnamoylspermidine antibiotic produced by species of *Nocardia*, was shown to inhibit the *in vitro* supercoiling reaction of *Micrococcus luteus* DNA gyrase, without affecting the activities of the restriction enzyme *BamH I* or calf thymus topoisomerase I (Osburne *et al.*, 1990). Cinodine apparently promotes the cleavage of plasmid DNA by *E. coli* gyrase (J. Sutcliffe, 92nd General Meeting of the ASM, New Orleans, May 1992). Although reminiscent of the influence of quinolones on gyrase, the manner in which these compounds lead to DNA cleavage is believed to be dissimilar; an assay of gyrase’s ability to religate DNA cleaved in the presence of calcium showed that CFX prevented religation occurring, but cinodine did not and is therefore considered to somehow promote the initial cleavage reaction.

Microcin B17, a glycine-rich peptide of approximately 3.2 kD, is produced by enterobacteria that harbour the pMccB17 plasmid (Baquero *et al.*, 1978). Microcin B17 has been shown to block DNA replication (Herrero and Moreno, 1986) and it was recently reported that DNA gyrase was the target (Vizán *et al.*, 1991). Once again, the mode of action appeared to be the interruption of the DNA cleavage and reunion reaction, with the accumulation of double-strand cleaved DNA in an *in vitro* system. Two independently isolated microcin B17-resistant mutants of *E. coli* were both shown to carry an AT to GC transition at position 2251 of the *gyrB* gene, resulting in a protein with a Trp to Arg substitution at residue 751 (Vizán *et al.*, 1991). Cleaved DNA was not observed in assays with the resistant protein.

Clerocidin. The microbial terpenoids, terpentecin and clerocidin were initially identified as potential anti-tumour drugs (Anderson *et al.*, 1983) and have since been shown to induce topoisomerase II-directed DNA cleavage *in vitro* at a comparable frequency to that observed with *m*-AMSA or VP-16 (Kawada *et al.*, 1991; see Section 1.8). Clerocidin has recently been reported to inhibit DNA gyrase with a potency some 10-fold greater than it exhibits against eukaryotic topoisomerase II (McCullough *et al.*, in press). Clerocidin was more efficient than CFX at killing Gram-positives. In Gram-negatives there appeared to be partial cross-resistance with fluoroquinolones, and this was mediated via GyrA. Curiously, and in contrast to the cleavable complexes formed with
gyrase and quinolones or topoisomerase II and etoposides, formation of the cleavable complexes with clerocidin was not reversible by exposure to heat (65 °C) or high salt (Kawada et al., 1991; McCullough et al., 1993). Because of the relatively small margin of clerocidin activity against gyrase over its activity against eukaryotic topoisomerase II, this compound itself would never be licensed as an antibacterial although its existence points to the terpenoids as a family of compounds that can be investigated with this purpose in mind.

Flavones: a number of naturally occurring flavones are reported to have potent activity against topoisomerases (K.A. Ohemeng, C.F. Schwender, K.P. Fu and J.F. Barrett, 92nd General Meeting of the ASM, New Orleans, May 1992; Yamashita et al., 1990; Austin et al., 1992). Quercetin and ellagic acid were the most active compounds against E. coli gyrase, inhibiting DNA supercoiling somewhat less efficiently than NFX.

1.8 The Interaction of drugs with eukaryotic topoisomerases

1.8.1 Type II topoisomerases: Several structurally diverse classes of anti-tumour agents have been shown to act upon topoisomerase II (Drlica and Franco, 1988). Acridines (eg. m-AMSA), anthracyclines (eg. doxorubicin), ellipticines (eg. 2-methyl-9-OH-ellipticine) and epipodophyllotoxins (eg. VP-16 and VM-26) promote the double-stranded cleavage of DNA in a manner analogous to that observed for quinolones with gyrase. Doxorubicin (adriamycin) is one of the most successful and widely used anti-tumour drugs, used in the treatment of acute leukemias, lymphomas and a variety of solid tumours. m-AMSA (ansacrine) is used in second-line treatment of acute myeloid leukemias that have become refractory to other agents. VP-16 (etoposide) can be effective in the treatment of small-cell lung carcinoma and advanced testicular cancer while the related VM-26 (tenoposide) has significant activity in the treatment of Hodgkin’s and non-Hodgkin’s lymphoma (therapeutic uses detailed in the British National Formulary and by Rose, 1988).

Although clinically relevant antibacterial quinolones have no significant effect on the eukaryotic counterpart, recent quinolone derivatives promote DNA cleavage by...
Drosophila topoisomerase II (Robinson et al., 1991) and the enzyme isolated from calf thymus glands (Yamashita et al., 1992). The possession of two halogens in the C-6 and C-8 positions and an N-1 cyclopropyl group appear to be the significant structural features in quinolones potent against eukaryotic topoisomerase II (Yamashita et al., 1992; Robinson et al., 1992).

Structures of anti-tumour agents are given in Figure 1.9, and their diversity should immediately be evident. The surprise that structurally distinct compounds should apparently elicit their effect via the same mechanism is compounded by their differing interaction with DNA; acridines, anthracyclines and ellipticines are intercalators but epipodophyllotoxins and quinolones are not considered to interact with DNA in that way (though both do feature planar ring structures).

Unlike DNA gyrase, cleavage of DNA by topoisomerase II can be revealed in the absence of any drug, and it is therefore possible to study the effects of the anti-tumour agents both in terms of their ability to enhance the degree of cleavage and their influence in the selection of cleavage sites. Pommier and coworkers have reported local DNA sequence requirements for cleavage by topoisomerase II from a murine leukemia cell line in the absence of any drug and in the presence of doxorubicin (Capranico et al., 1990) and of m-AMSA and VM-26 (Pommier et al., 1991a). By using a statistical approach they noted significantly high, and significantly low, occurrence of particular bases in positions around cleavage sites in SV40 DNA. In a manner identical to gyrase, cleavage of DNA by topoisomerase leaves the enzyme covalently attached by a phosphotyrosine bond to each strand, with a 4 bp stagger between the cleavage sites (Liu et al., 1983). Considering the bases on one strand only, the nucleotide attached to the enzyme is numbered +1 and the nucleotide left with a free 3'-OH group is numbered -1, see Figure 1.10. Similarly, the base paired to number +4 is covalently linked to the enzyme, and the base paired to number +5 has a free 3'-OH. In the presence of doxorubicin, a preference was observed for an A at the 3' terminus of at least one of the staggered DNA breaks, i.e. there was statistically frequent occurrence of adenine at position -1 and/or thymine at position +5. Exactly the reverse was found for sites where topoisomerase cleaved in the absence of
FIGURE 1.9: Structures of anti-tumour compounds that inhibit topoisomerases

Ellipticine

Doxorubicin

m-AMSA

Camptothecin

VP-16 (etoposide)
Panel (a) depicts the 2 subunits of topoisomerase II (light grey) to which each strand of DNA is covalently attached via a phosphotyrosine bond (black circle). Inhibitor molecules (dark grey rectangles) are believed to stabilise the gyrase-DNA complex in this "open" state by intercalation at the sites of strand breakage. According to the model, the sequence-specificity of cleavage sites, see Panel (b), reflects the interaction between drug molecules and the bases either side of the cleaved bond.
drug; adenine was significantly infrequent at -1 and thymine at +5 and the two together (A at -1, T at +5) was never observed. With VM-26 the strongest base preferences were for C at -1 and G at +5. For m-AMSA, sites were favoured that had A at +1 and/or T at +4. The authors consider that in all these cases, and despite the fact that VM-26 is not generally considered to be an intercalator, the drug molecules are stacking between bases at the cleavage site and that the observed nucleotide preferences at positions -1 and +1 reveal especially favourable drug-DNA interactions. Intercalation alone cannot be the sole determinant of inhibitory potential; not only does the intercalator ethidium bromide have no influence on topoisomerase II, but o-AMSA which intercalates into DNA as efficiently as its isomer m-AMSA (Wilson et al., 1981) does not inhibit topoisomerase II and is not an anti-tumour agent (Nelson et al., 1984).

The interaction of m-AMSA with topoisomerase II is probably the most extensively studied. m-AMSA has a bipartite structure, featuring an acridine ring that intercalates into DNA and an anilino ring that is believed to be the protein-binding domain. Baguley and coworkers have compared the interaction of m-AMSA and derivatives with a wild-type and a resistant murine leukemia cell line in which an unspecified alteration of topoisomerase II had been identified as the resistance mechanism (Drake et al., 1987; Baguley et al., 1990). The m-AMSA-resistant cell line was some 70-times more sensitive in vivo to an m-AMSA derivative in which the methoxy group had been removed from the anilino ring (see Figure 1.9).

Resistance to m-AMSA has been reported to arise in bacteriophage T4 from mutation of either the gene 39 or the gene 52 products (Huff et al., 1990), though the exact nature of the alterations and how they lead to resistance has not been described. Resistance to m-AMSA in a human leukemia cell line was recently shown to result from a point mutation in the topoisomerase II gene (Hinds et al., 1991). The change involved was replacement of Arg486 with Lys. The authors pointed out that this residue is conserved in all the topoisomerase II sequences, prokaryotic and eukaryotic, that had been sequenced thus far (Wyckoff et al., 1989), with the exception of E. coli, which already carries a lysine at this position, and is relatively resistant to m-AMSA. Interestingly, the lysine in question
is Lys$^{447}$ of the GyrB sequence and its alteration to Glu confers resistance to acidic quinolones and hypersusceptibility to amphoteric quinolones (Yamagishi et al., 1986).

Cells selected for their ability to grow in the presence of one of the anti-tumour agents that act upon topoisomerase II have exhibited a certain degree of cross-resistance to the other compounds (D'Arpa and Liu, 1989). Multiple-drug resistance (MDR) is a serious problem in chemotherapy and has principally been associated with overproduction of a 170 kD membrane glycoprotein that mediates active efflux (Endicott and Ling, 1989). Mutation of eukaryotic topoisomerase II is now recognised as an alternative route by which an MDR phenotype can arise (Morrow and Cowan, 1990).

1.8.2 Type I topoisomerases Also worthy of mention are studies of eukaryotic topoisomerase I inhibition by the plant alkaloid camptothecin. Clinical trials in the early 1970s noted potential therapeutic use for camptothecin but worries over toxicity and uncertainty over the site of action led to it being shelved (D'Arpa and Liu, 1989). With the identification of eukaryotic topoisomerase I as the target molecule (Hsiang et al., 1985; Nitiss and Wang, 1988), interest in camptothecin in chemotherapy has been revived. Camptothecin slows religation of topoisomerase I-directed DNA breaks (Porter and Champoux, 1989) and it has been proposed that camptothecin-induced stabilisation of the topoisomerase I-DNA complex leads to cell death by the arrest of replication fork movement that was uninhibited in the absence of drug (Hsiang et al., 1989). Similarly, premature termination of SP6 RNA polymerase by topoisomerase I in the presence of camptothecin has been noted in an in vitro transcription system (Bendixen et al., 1990). Camptothecin stabilises the cleavage of DNA in a single-strand, as would be anticipated with a type I topoisomerase. Transcription through topoisomerase I was only inhibited on the cleaved strand; movement of RNA polymerase past topoisomerase I along the uncleaved strand was not affected by the camptothecin-stabilised cleavage of DNA in the other strand.
1.9 Aims of project

Despite extensive research, major questions relating to the mode of quinolone action have yet to be answered satisfactorily. In particular, uncertainty has surrounded the identity of the primary site for drug binding (is it gyrase, or DNA or a complex of the two together?) and the manner in which this interaction leads to cell death.

The current study has sought, with the aid of wild-type and quinolone-resistant gyrases, to further our understanding of these matters. The binding of quinolone to gyrase and/or DNA has been analysed by rapid gel-filtration and fluorescence techniques (Chapter 4). Experiments described in Chapter 5 have investigated the influence of gyrase and quinolone on in vitro transcription and replication systems. These studies examine the proposal (Kreuzer and Cozzarelli, 1979) that cell death results from the inability of DNA processing enzymes, notably polymerases, to pass a stable gyrase-quinolone-DNA complex.

Quinolone-dependent cleavage of DNA was an early observation in the study of gyrase (Gellert et al., 1977; Sugino et al., 1977). A recent report (Shen et al., 1989c) has contradicted some of the original conclusion about the influence of reaction components on the cleavage process and this thesis has therefore looked afresh at this issue. Additionally, comparison of DNA cleavage in the presence of CaCl₂ and the drug-dependent scission of DNA has been made. The inter-relationship of DNA cleavage and inhibition of DNA supercoiling has also been examined. These experiments are reported in Chapter 3.
CHAPTER 2

Materials and Methods
2.1 Bacteriology

2.1.1 Bacterial strains

**TABLE 2.1: Genotype of strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>Δ(lac-proAB), recA1, end A1, thi-1, supE44, hsdR17, relA1, gyrA96, F′ (traD36, proAB+, lacI, lacZΔM15)</td>
</tr>
<tr>
<td>IC 38</td>
<td>tus strain obtained from Dr. K.J. Marians</td>
</tr>
<tr>
<td>EJ45</td>
<td>zeg 298: Tn10, gyrA43 (ts 42°C)</td>
</tr>
<tr>
<td>JM1acA</td>
<td>JM109 harbouring plasmid pPH3 (encoding gyrA)</td>
</tr>
<tr>
<td>JM1acB</td>
<td>JM109 harbouring plasmid pAG111 (encoding gyrB)</td>
</tr>
</tbody>
</table>

2.1.2 Bacterial media

**Luria-Bertani broth ("LB")**

Yeast extract (Difco) 5.0 g
Tryptone (Difco) 10.0 g
Sodium Chloride 10.0 g
Water to 1 litre.

**Luria-Bertani broth agar**

Yeast extract (Difco) 5.0 g
Tryptone (Difco) 10.0 g
Sodium Chloride 10.0 g
Agar (Oxoid) 15.0 g
Water to 1 litre.
Super broth
Prepared as two solutions, autoclaved separately, and combined when sterile.

Solution 1:
- Yeast extract (Difco) 24.0 g
- Tryptone (Difco) 12.0 g
- Glycerol 8.0 ml
- Water to 900 ml

Solution 2:
- K$_2$HPO$_4$ 12.5 g
- KH$_2$PO$_4$ 3.8 g
- Water to 100 ml

2.1.3 Plasmids used in this study

pAG111 (7.2 kb): Derived from cloning vector pTTQ18 (Stark, 1987) and containing the gyrB gene from *E. coli* under the control of an IPTG inducible tac promoter (Hallett *et al.*, 1990). Carries the ampicillin resistance gene.

pBR322 (4.4 kb): A multipurpose cloning vector with a number of unique restriction sites. Ampicillin and tetracycline resistance genes as selective markers.

pMECS (10.6 kb): Carries a clinically isolated *E. coli gyrA* gene in which a single point mutation (Ser$_{83}$ to Trp in the resultant protein) confers resistance to quinolones (Cullen *et al.*, 1989). Kindly donated by Dr. L.M. Fisher.

pPH3 (8.0 kb): Derived from cloning vector pTTQ18 and containing the *gyrA* gene from *E. coli* under the control of an IPTG inducible *tac* promoter (Hallett *et al.*, 1990). Confers ampicillin resistance.
pPH311.1 (8.0 kb): Derived from pPH3 by directed mutagenesis (Hallett and Maxwell, 1991). Contains a single point mutation (Ser to Ala in GyrA) which confers quinolone-resistance.

pPH383 (8.0 kb): Derived from pPH3 by directed mutagenesis (this study). Contains a single point mutation (Ser to Tyr in GyrA) which confers quinolone-resistance.

pPH483 (8.0 kb): Derived from pPH3 in this study. A fragment coding for residues 20 to 369 of GyrA replaced by the corresponding region from pMECS and therefore containing the Trp mutation.

pSTD147 (6.4 kb): Has multiple copies of a 147 bp DNA fragment that includes the preferred site for gyrase-directed cleavage of pBR322 (Dobbs et al., 1992).

pTH101 (4.2 kb): Contains a 23 bp ter termination site from the E. coli chromosome such that replication in the presence of Tus protein results in an arrested product of 1.7 kb (Hill and Marians, 1990). Based on vector pGEM3 and carrying genes for resistance to ampicillin and kanamycin, the plasmid must be maintained in a Tus-free strain. Kindly donated by Dr. K.J. Marians.

pTHN147 (4.3 kb): Derived from pTH101 during this study and containing a preferred site for gyrase-directed cleavage incorporated on a 147 bp fragment with concomitant loss of the kanamycin resistance gene.

2.1.4 Quinolones
ciprofloxacin (CFX) was obtained from Bayer
difloxacin (DFX) was obtained from Abbott
enoxacin (ENX) was obtained from Dainippon
naldixic acid (NAL) was purchased from Sigma

norfloxacin (NFX) was obtained from Merck, Sharp and Dohme

$^3$H-norfloxacin ($^3$H-NFX) was a gift from Dr. L.L. Shen (Abbott laboratories)

ofloxacin (OFX) was obtained from Hoechst

oxolinic acid (OXO) was purchased from Sigma

pefloxacin (PFX) was obtained from Rhone-Poulenc Rorer

2.2 DNA methods

2.2.1 Phenol extraction

To remove proteins from solutions of nucleic acid, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, mixed thoroughly by vortexing and the aqueous and organic phases separated by centrifugation. The upper aqueous phase was removed to a fresh tube with due caution to avoid carry-over of phenol or denatured protein from the interface.

2.2.2 Ethanol precipitation

In order to transfer DNA to a smaller volume of the same or different buffer, the nucleic acid was precipitated by the addition of sodium acetate (to a final concentration of 0.3 M) and 3 volumes of absolute ethanol.

2.2.3 Gel purification of DNA fragments

DNA fragments were separated on the basis of their size-dependent mobility in an agarose gel. Two different forms of low melting point agarose were used; for small fragments (<1 kb as a rough guide) NuSieve GTG agarose (supplied by FMC Bioproducts) was the medium of choice, while larger pieces (>1 kb) were purified from gels of ultraPURE LMP agarose (BRL).

The inclusion of EtBr in gels facilitated visualisation of the DNA under UV light and each required band was cut from the gel in as small a volume of gel as possible and
added to 100 µl of TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM Na$_2$-EDTA) in a 1.5 ml micro-centrifuge tube (Eppendorf). The tube was incubated at 67°C until the gel was melted. An equal volume of prewarmed phenol:TE (phenol equilibrated with TE until the pH of the accompanying aqueous phase was greater than 7.6) was added and the mixture vortexed (15 min). Phases were separated by centrifugation (5 min in microfuge) and an equal volume of water-saturated ether added to remove traces of phenol. The DNA was isolated by ethanol precipitation.

2.2.4 Dephosphorylation of vector DNA

Linearised vector DNA with identical or compatible sticky ends was treated with calf intestinal phosphatase (CIP) to reduce the occurrence of self-ligation. 1.5 µg DNA was incubated for 30 min at 37°C with 0.1 units of CIP in 1-Phor-All buffer (total reaction volume, 50 µl) as advised by the supplier (Pharmacia). Heating to 85°C for 15 min was sufficient to inactivate the enzyme.

2.2.5 Determination of DNA concentration

Calculation of DNA concentration was determined spectrophotometrically by light absorption of a DNA solution at 260 nM ($A_{260}$). As a rule, 5 µl of a DNA sample was added to 995 µl of TE in a clean quartz cuvette. The measured $A_{260}$ was converted into a concentration on the basis that dsDNA at 1 mg/ml has an $A_{260}$ of 20 units (40 units for ssDNA).

2.2.6 Preparation of oligodeoxynucleotides

Oligonucleotides were synthesised ‘in house’ by Debra Langton at a concentration of approximately 1 mg/ml. The oligos were purified according to the method of Sawadogo and Van Dyke (1991). 100 µl of oligo was transferred to a 1.5 ml Eppendorf tube and 1 ml of n-butanol was added. The tube was vortexed for 15 sec and then spun at 13 K for 1 min. The single butanol/water phase was removed and the pelleted DNA dried under
vacuum then resuspended in 100 μl of sterile water. DNA concentration was determined by A_{260}. The sequences of oligos used in this study are given below.

### 2.2.6.1 Oligos directed to pBR322 DNA

#### CW192(T7)

\[
\begin{align*}
\text{AAA TTA ATA CGA CTC ACT ATA GGG GCT TGC GGT} \\
\text{ATT CGG ATC TT}
\end{align*}
\]

Equivalent to CW492 with an extended region coding a T7 promoter sequence. Used in conjunction with CW292 to produce an 310 bp DNA template for transcription through the "990 preferred cleavage site".

#### CW292

\[
\begin{align*}
\text{TCA GCG GTC CAG TGA TGC AAG TTA}
\end{align*}
\]

#### CW392(T7)

\[
\begin{align*}
\text{AAA TTA ATA CGA CTC ACT ATA GGG TCA CGG GTC} \\
\text{CAG TGA TCG AAG TTA}
\end{align*}
\]

Equivalent to CW292 with an extended region coding a T7 promoter sequence. Used in conjunction with CW492 to produce an 310 bp DNA template for transcription through the "990 preferred cleavage site" in the reverse orientation to CW192(T7) & CW292.

#### CW492

\[
\begin{align*}
\text{GCT TGC GGT ATT CGG AAT CTT}
\end{align*}
\]

#### CW592

\[
\begin{align*}
\text{ATC CAG CCT GCG GTC CGG AA}
\end{align*}
\]

#### CW692

\[
\begin{align*}
\text{TTC CCC ATT ATG ATT CTT CT}
\end{align*}
\]

#### CW792

\[
\begin{align*}
\text{GCG CAT CCA GCC TCG CGF CG}
\end{align*}
\]

#### CWUV5

\[
\begin{align*}
\text{TTA GGC ACC CCA GGC TTT ACA CTT TAT GCT TCC} \\
\text{GGC TCG TAT AAT GTG TGG CTT GCG GTA TCC GGA} \\
\text{ATC TT}
\end{align*}
\]

Equivalent to CW492 with an extended region coding a lac UV5 promoter sequence for E. coli RNA polymerase. Used in conjunction with CW292 to produce an 307 bp DNA template for transcription through the "990 preferred cleavage site".

#### TXT130

\[
\begin{align*}
\text{GCA AGA CGF AGC CCA GCG CG}
\end{align*}
\]

#### TXT135

\[
\begin{align*}
\text{GCC CAG CAA GAC GTA GCC CA}
\end{align*}
\]

#### TXT140

\[
\begin{align*}
\text{GCG AAC GCC AGC AAG ACG TA}
\end{align*}
\]
A series of oligos used in conjunction with CW192(T7) to prepare templates from which T7 RNA polymerase could transcribe size marker RNAs of 130 to 150 nucleotides respectively.

2.2.6.2 Oligos directed to pPH3

**PH-15**

GCG TCG CGA ACG CCA GCA AG  
GCC TCG CCG GCC GAA GCC CA

Used in sequencing of pPH3, pPH383, pPH483 and pPH311.1 (see Figure 3.5).

**PH-17**

GCC TCG CGA ACG CCA GCA AG

**PH-18**

CAT CCC CAT GGT GAC TAG GCG GTC TAT GAC ACG

Oligos PH-17 and PH-18 used in polymerase chain reaction to generate a portion of GyrA with point mutation (Ser83 → Tyr) for subsequent cloning as pPH383.

2.2.7 Preparation of plasmid DNAs

DNA was generally prepared by growth of strains harbouring the desired plasmid in 400 ml ("maxiprep"), 5 ml ("midiprep") or 1 ml ("miniprep") of bacterial media.

**Maxiprep**: The large-scale production of plasmid DNA was performed as outlined by Maniatis et al. (1982). 400 ml of superbroth was inoculated with 5 ml overnight culture of a strain carrying the appropriate plasmid and grown for 48 hours at 37°C in the presence of 50 µg/ml ampicillin. The resulting bacteria were pelleted by centrifugation at 6 K for 10 min and resuspended in 60 ml of Solution I (500 mM glucose, 250 mM Tris-HCl (pH 7.5, all solutions in this work were adjusted to the correct pH at 25°C unless otherwise stated), 100 mM Na2-EDTA, 5 mg/ml lysozyme). The cell suspension was incubated at room temperature for 10 min before the addition of 120 ml of freshly prepared Solution II (0.2 M NaOH, 1% SDS). The resultant mixture was stirred well with a glass
pipette but was not vortexed. After 10 min on ice, 60 ml of precooled Solution III (3 M sodium acetate, brought to pH 4.8 with glacial acetic acid) was similarly stirred into the mixture which was then incubated on ice for a further 15 min. Cell debris was pelleted by centrifugation (8 K, 5 min, 4°C) and the supernatant transferred into 150 ml of propan-2-ol, mixed and spun again (8 K, 5 min, 20°C) thereby precipitating the DNA. The pellet was dried under vacuum and resuspended in 4 ml of sterile TE buffer.

Purification of supercoiled DNA from other DNAs (chromosomal, nicked and linear plasmid) was achieved by CsCl-EtBr density-gradient centrifugation. The resuspended DNA sample was weighed and 1.019 g of CsCl and 0.11 ml of EtBr per gram of sample were added. The mixed components were transferred to a polyallomer tube and spun at 48 K overnight (VTi 50 rotor, Beckman ultracentrifuge). The following morning the lower band was removed from the tube via a hypodermic needle.

Generally, supercoiled pBR322 was further purified by a second round of CsCl-EtBr density-gradient centrifugation and, as appropriate, was subsequently relaxed by incubation with chicken reticulocyte topoisomerase I for 60 min (Bates and Maxwell, 1989), or linearised by treatment with EcoR I.

Midiprep: Medium scale DNA preparation was performed according to a protocol derived from the method of Birnboim and Doly (1979). 5 ml of LB containing a suitable antibiotic (normally 50 μg/ml amp) was inoculated with a single colony. After overnight incubation at 37°C, the cells were pelleted by 10 min centrifugation (3 K, 4°C) and the supernatant discarded. The cells were resuspended in 1 ml of ice-cold TNE (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM Na₂-EDTA) and transferred to an Eppendorf tube before further pelleting (13 K, 4°C, 5 min) and the supernatant again discarded. The pellet was resuspended in 100 μl of Solution I (as in “maxiprep” above) and incubated at room temperature for 5 min before the addition of 200 μl of freshly prepared Solution II and 150 μl of Solution III.

After 15 min on ice, cell debris was pelleted by centrifugation (13 K, 4°C, 5 min) and the supernatant subjected to phenol extraction and ethanol precipitation. The pelleted nucleic acid was dried under vacuum and resuspended in 100 μl TE buffer. RNase A, to a

- 50 -
final concentration of 100 μg/ml, was added and allowed to digest RNA at 37°C for 30 min. Subsequently, 260 μl of sterile water and 40 μl of Solution III were added and the phenol extraction and ethanol precipitation steps were repeated. The final pellet was resuspended in 20 μl of TE and DNA concentration determined by A₂₆₀.

**MiniPrep:** The procedure for small-scale (1 ml) preparation of DNA was closely analogous to the midiprep protocol, except for the omission of a distinct step to remove RNA. Instead of adding RNase after phenol extraction and ethanol precipitation, the DNA pellet was rinsed with 1 ml of ice-cold ethanol (70%) and then returned to the bottom of the tube by centrifugation (13 K, 4°C, 10 min). After careful removal of the supernatant, the pellet was dried under vacuum and resuspended in 40 μl of TE containing RNase at 20 μg/ml.

**2.2.7.1 Preparation of replication template DNA:** Following a “midiprep” preparation as outlined above, 150 ng of each template DNA, pTH101 and the derivative plasmid pTHN147 (see Section 2.2.11 for construction), was further purified by sucrose gradient centrifugation. Gradients contained 20mM Tris-HCl (pH 7.5 at 4°C), 1mM Na₂-EDTA, 1mM NaCl and 5 to 20% sucrose and were spun at 19 K for 40 hours in a Sorvall ultracentrifuge. Appropriate fractions were pooled, dialysed into TE and concentrated by extraction with 2-butanol with excess butanol removed by subsequent ether extraction. Yields were 48.6 ng pTH101 and 72.8 ng pTHN147. pBR322 for replication assays was provided by Dr. K.J. Marians.

**2.2.8 Preparation of 147 bp DNA fragments**

Multiple copies of the 147 bp fragment were derived from pSTD147 by treatment with Ava I and purified by gel filtration using an FPLC Superose 6 column equilibrated with TNE1 buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM Na₂-EDTA), flow rate 0.1 ml/min. Under these conditions, the following retention times were consistently reported: vector, 66-82 min; 147 bp, 115-121 min; RNA (when not removed by density-gradient centrifugation), 167-210 min.
2.2.9 Polymerase chain reaction

PCR was performed using a Pharmacia Gene ATAQ thermal cycling apparatus. 50 µl reaction mix contained 25 pmol of each oligo, 10 ng of template DNA and 10 nmols of each dNTP (pH 7.5, purchased from Pharmacia) in appropriate buffer supplied with the Taq polymerase (Boehringer Mannheim) of which 5 units were added. 50 µl of sterile paraffin oil was overlayed on the reaction mix in a 500 µl Eppendorf. Silicon grease was smeared on the outside of the tube to improve thermal contact and the template DNA was denatured by heating to 92°C for 1 min. The primers were allowed to anneal at 55°C for 1 min and elongation occurred at 72°C for a further 1 min. The denaturation-annealing-elongation conditions were repeated for 30 cycles after which the aqueous layer was brought above the organic by the addition of 50 µl of chloroform-isoamyl alcohol (24:1). The desired product was purified away from any by-products and unincorporated primer by electrophoresis in low-melting agarose.

2.2.10 DNA sequencing

DNA sequence was determined directly from double-stranded plasmid template according to the method of Kraft et al. (1988) and drawing heavily on the recommended procedure provided with the Sequenase enzyme (a modified T7 DNA polymerase, supplied by USB). DNA was prepared by the mini-prep protocol outlined above. After ethanol precipitation, the DNA pellet was resuspended in 16.8 µl of sterile water and further purified by addition of 3.2 µl of 5 M NaCl and 20 µl of 13% PEG 8000. Incubation on ice for 30 min followed by centrifugation at 13 K for 10 min (4°C) was sufficient to pellet the DNA which was subsequently washed with 1 ml of 70% ethanol and dried under vacuum.

The DNA was redissolved in 20 µl of sterile water and denatured by incubation at room temperature for 5 min in the presence of 0.2 M NaOH and 0.2 mM Na₂-EDTA. Sequential addition of 240 mM Tris-HCl (pH 4.5) and 0.3 M sodium acetate neutralised the solution and 3 volumes of absolute ethanol was sufficient to pellet the DNA after 20 min on dry ice and 5 min centrifugation (13 K, 4°C).
After washing and vacuum drying, the DNA was resuspended in 7 µl of sterile water prior to the addition of 1 µl of appropriate sequencing primer and 2 µl of 5x sequencing buffer (supplied with enzyme kit). This annealing mix was incubated at 37°C for 30 min and at the same time 4 appropriately labelled tubes were charged with 2.5 µl of one of the termination mixes (ddATP, ddCTP, ddGTP and ddTTP) and prewarmed to 37°C. 1 µl of 0.1 M DTT, 2 µl labelling mix (diluted from 5x stock), 0.5 µl of [α-35S] dATP and 2 µl Sequenase (diluted from 8x stock) were added to the annealing mix and incubated at room temperature for 5 min. 3.5 µl of labelling reaction was added to each of the termination mixes and incubated for a further 5 min at 37°C. 4 µl stop solution was added and the samples heated to 70°C for 10 min prior to loading on a pre-warmed sequencing gel (8% polyacrylamide (19:1), 7 M Urea (USB) and TBE buffer (100 mM Tris-HCl (pH 7.5), 83 mM boric acid, 1 mM Na2-EDTA). Depending upon the distance from the primer to the sequence of interest, the gel was run at 42 watts for 1-3 hours.

2.2.11 Construction of plasmid pTHN147

Plasmid pTH101 containing the ter termination region was supplied by Dr. K.J.Marians. A 147 bp fragment with Nru I sticky ends was donated by Dr. S.T.Dobbs. pTH101 was linearised by cleavage at a unique site with Nru I (supplied by NEB) and treated with Calf Intestinal Phosphatase (CIP, Pharmacia) to reduce the level of self-religation. Linearised pTH101 was mixed in the presence on DNA ligase (NEB) with a limiting supply of Nru I-ended 147 fragment in order to avoid multiple insertion events. Successful construction of pTHN147, the derivative plasmid, was confirmed by linearisation with Eag I (NEB), an enzyme that cleaves at a unique site in the 147 bp region but not in the parent pTH101, see Figure 2.1. Orientation of the 147 bp fragment within pTHN147 was determined by sizing of the restriction fragments generated by a digest with enzymes Cla I (BRL) and Eag I.
Plasmid pTH101 contains the origin (Ori) and termination region (Ter) for DNA replication. Shaded regions represent the genes conferring resistance to ampicillin (Amp\textsuperscript{r}) and Kanamycin (Kan\textsuperscript{r}). A 147 bp fragment containing the preferred site of quinolone-directed cleavage of pBR322 by DNA gyrase was cloned into pTH101 through the utilisation of a unique \textit{Nru} I restriction site. pTHN147 was selected by the loss of resistance to Kanamycin.
2.2.12 Transformation of competent cells

Preparation of competent cells: 1 ml of LB was innoculated with a colony of an appropriate strain (normally *E. coli* JM109) and grown at 37°C for 3 hours (until a slight cloudy appearance was evident, approximate OD$_{595}$ of 0.25). Cells were pelleted by centrifugation (30 sec) and resuspended in 500 µl of solution A (20 mM RbCl, 20 mM MOPS (pH 7.0)). Following a further 15 sec spin, the supernatant was discarded and the cells resuspended in 500 µl of solution B (20 mM RbCl, 100 mM CaCl$_2$ and 200 mM MOPS (pH 6.5)).

After about 60 min on ice, the cells were pelleted by centrifugation (10 sec) and 350 µl of buffer was removed. Cells were resuspended in the remaining 150 µl and 3 µl of DMSO was added to aid permeabilisation.

Transformation: DNA (typically 25-50 ng in TE buffer or ligation mix) was added to the competent cell suspension and the tube incubated on ice for 60 min. The cells were heat-shocked (55°C for 30 sec, 0°C for 1 min), 1 ml of LB added and the mix incubated at 37°C for 60 min to allow the cells to recover prior to plating out on LB agar containing an appropriate selective antibiotic.

2.2.13 Recloning of quinolone-resistant *gyrA* genes

Plasmids pPH3 and pAG111 carry the *gyrA* and *gyrB* genes respectively, under the control of an IPTG-inducible promoter to facilitate the overproduction of the wild-type gyrase proteins (Hallett *et al*., 1990). Mutations at residue 83 of the gyrase A protein, serine in the wild-type enzyme, have been shown previously to confer quinolone-resistance (eg Yoshida *et al*., 1988; Cullen *et al*., 1989). A GyrA*Ala83* mutation had previously been cloned into pPH3 (Hallett and Maxwell, 1991) and the resultant protein exhibited a low level of quinolone resistance.

**GyrA*Trp83***: Plasmid pMEC5 containing a gene for GyrATrp83, a mutation leading to the highest level of quinolone resistance reported thus far (Yoshida *et al*., 1988; Cullen *et al*., 1989) was supplied by Dr L.M.Fisher. A fragment swap was undertaken to replace a region incorporating the codon for Trp83 from pMEC5 with the corresponding region of
pPH3. *Sac* I (BRL) and *Bst* B I (NEB) were used to liberate an 1048 bp fragment, coding for residues 20 to 369, from pMEC5 which was isolated by gel purification and mixed in excess with pPH3 cleaved using the same enzymes. RFLP analysis based on the loss of an *Hin* f I (NEB) restriction site in the codon for Trp83 was used to distinguish between wild-type and mutant clones that resulted from the subsequent ligation and transformation into *E. coli* strain JM109 of the fragment mix. The recombinant plasmid has been named pPH483.

**GyrA*<sub>Tyr</sub>*83**: A GyrA*<sub>Tyr</sub>*83 mutant was derived from the wild-type gene of pPH3 by a PCR based mutagenesis protocol devised by Dr. P. Hallett (Figure 2.2). The first oligo, a 33mer, included the recognition sequence for *Nco* I (Pharmacia) and featured a double mismatch at the codon for residue 83 (TAT instead of TCG). The second oligo, a 20mer was exactly complementary to the *gyrA* sequence some 400 bp 3' to the first and a short distance downstream of a unique *Sma* I (Pharmacia) restriction site. The region between the primers was then amplified by PCR (30 cycles). Cleavage of the PCR product with *Nco* I and *Sma* I generated a fragment with suitable ends for exchange with the corresponding region of pPH3.

The recipient plasmid was prepared by digestion with the same enzymes. *Aat* II (NEB) was also included to cleave the 400 bp *Nco* I-*Sma* I fragment into smaller pieces and thereby facilitate the subsequent gel purification of the other 378 bp *Sma* I-*Nco* I fragment generated by this digest which was required for the recloning. The remaining 7.2 kb of predominantly vector DNA was also gel purified and treated with CIP to reduce the potential for resealing of its *Nco* I ends. Protocols for gel purification and treatment with CIP are elaborated elsewhere. RFLP analysis based on the loss of an *Hin* f I restriction site was again used to distinguish between wild-type and mutant clones that resulted from the subsequent ligation and transformation into *E. coli* strain JM109. The plasmid carrying the GyrA*<sub>Tyr</sub>*83 mutation is termed pPH383.
FIGURE 2.2: Cloning strategy for producing $\text{GyrA}_{\text{Tyr83}}$

$\text{Nco I (2235)}$

$\text{Aat II (2467)}$

$\text{Sma I (2634)}$

$\text{pPH3}$

$\text{Nco I (3012)}$

$\text{PCR}$

$\text{30 Cycles}$

$\text{Nco I/Sma I/Aat II digest}$

$\text{Gel purification}$

$\text{Nco I/Sma I digest}$

$\text{CIPed}$

$\text{pPH383}$
2.3 Protein methods

2.3.1 Determination of protein concentration

The concentration of protein in a given preparation was calculated according to Bradford method (Bradford, 1976). Dilutions of the test solution and a reference series of BSA dilutions (focused around concentrations of 1-20 μg/ml) were prepared, each in a total volume of 100 μL. 1 ml of Bradford reagent (100 μg/ml Coomassie dye G, 5% ethanol and 8.45% phosphoric acid) was added to each sample, mixed by gentle inversion and allowed to stand at room temperature for 5 min in order for the colour to develop. Absorbance at 595 nm was recorded for each sample against a blank consisting of 100 μl of water and 1 ml of Bradford reagent.

A standard curve (concentration v. \(A_{595}\)) was constructed from the BSA dilutions to enable the concentration of protein in the gyrase preparations to be calculated. For absolute protein concentrations to be determined, multiplication by a conversion factor of 1.43 (for GyrA) and 0.71 (for GyrB) according to the recommendations of Gellert (unpublished data).

2.3.2 SDS-polyacrylamide gel electrophoresis of proteins

To identify fractions containing the gyrase subunits and to assess the degree of purity achieved at each step of protein preparation, samples were subjected to electrophoresis under denaturing conditions on a discontinuous polyacrylamide gel containing 0.1% SDS. Protein samples were mixed with an equal volume of sample application buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue) and boiled for 4 min. Proteins were stacked in a layer of 4% polyacrylamide (37.5:1, pH 6.8) before separation on a 12% polyacrylamide (37.5:1, pH 8.8) gel. Coomassie stain (30% methanol, 0.01% Coomassie Brilliant Blue, 12% trichloroacetic acid, 10% sulphosalicylic acid) was used to visualise protein bands after electrophoresis.
2.3.3 Purification of gyrase proteins

2.3.3.1 Gyrase A protein - Preparation of GyrA (wild-type and mutant) was performed in the following stages:

(i) Large-scale culture: 4 x 500 ml flasks of sterile LB broth were each inoculated with 5 ml of an appropriate overnight culture and grown to an OD$_{595}$ of 0.4 prior to induction with 0.05 mM IPTG. Cells were grown for a further 3 hours at 37°C and harvested by centrifugation and resuspended in Tris-sucrose (50 mM Tris-HCl (pH 7.5), 10% sucrose).

(ii) Preparation of cell extract: DTT, Na$_2$-EDTA and KCl were added to the cell suspension to final concentrations of 2 mM, 20 mM and 100 mM respectively. The resultant mixture was then sonicated for 4 x 30 sec using an ice-cooled probe. Cell debris was spun out by centrifugation for 1 hour at 34 K in a pre-cooled Sorvall VTi 50.13 rotor.

(iii) Polymin P = ammonium sulphate precipitation: extract was diluted to 9 mg/ml so that the final solution contained 0.2 M NaCl, 50 mM Tris-HCl (pH 7.5), 10% sucrose and 0.35% Polymin P (pH 7.9, supplied by BASF). The solution was stirred at 0°C for 15 min then spun at 10 K for a further 15 min. The pellet was taken and resuspended in 0.45 M NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM Na$_2$-EDTA and 1 mM DTT to a total 40% of the 9 mg/ml solution volume. After stirring at 0°C for 15 min then centrifugation at 10 K for a further 15 min, the new pellet was resuspended in 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM Na$_2$-EDTA and 1mM DTT to the same volume. After stirring at 0°C for 15 min then centrifugation at 10 K for a further 15 min, the pellet was resuspended in 100 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM Na$_2$-EDTA and 5 mM DTT to 20% of the original cell extract volume.

(iv) FPLC Mono Q 5/5: 20 ml of sample was added to the column at 1 ml/min, in TED buffer (20 mM Tris-HCl (pH 7.5), 0.2 mM Na$_2$-EDTA, 5 mM DTT) and eluted with a linear salt gradient (0-1 M NaCl in 40 min). Under these conditions, GyrA
was eluted at about 300 mM NaCl. Fractions containing GyrA were identified by SDS-
PAGE and pooled before dialysis into Enzyme Buffer ("EB"; 50 mM Tris-HCl (pH 7.5),
100 mM KCl, 5 mM DTT, 10% glycerol, 1 mM Na₂-EDTA).

2.3.3.2 Gyrase B protein -

(i) Large-scale culture: 12 litres of LB broth were sterilised at 121°C in a
fermenter and incubated at 37°C overnight to ensure sterility. The fermenter was treated
with ampicillin (to give a final concentration of 50 μg/ml) and inoculated with a 500 ml
overnight culture of JMtyacB. Stirring at 600 rpm and sparging air into fermenter at 15
litres/min, cells were grown to an optical density of 0.5 measured at 595 nm and induced
with IPTG at a working concentration of 0.05 mM. Growth was monitored by OD₅₉₅
every 30 min until a plateau was reached. Cells were harvested by centrifugation and
resuspended in Tris-sucrose (50 mM Tris-HCl (pH 7.5), 10% sucrose).

(ii) Preparation of cell extract: DTT, Na₂-EDTA and KCl were added to
the cell suspension, to final concentrations of 2 mM, 20 mM and 100 mM respectively.
This mixture was then passed twice through a pre-cooled French Press at 8,000-12,000 psi.
Cell debris was spun out by centrifugation at 1.4 x 10⁵ g in a Sorvall TFT 50.38 rotor for
60 min at 3°C, yielding approximately 100 ml of soluble cell extract (protein concentration
102 mg/ml).

(iii) Heparin-Sepharose: 2 ml of cell extract were added to a Heparin-
sepharose column (column volume, 40 ml; column dimensions: radius, 2 cm; height, 5 cm)
in TGED buffer (20 mM Tris-HCl (pH 7.5), 10% glycerol, 0.2 mM Na₂-EDTA, 5 mM
DTT) and eluted with a linear salt gradient (0-1 M NaCl in 500 ml TGED; flow rate, 2
ml/min). Under these conditions, gyrase B protein (GyrB) was eluted between 300 and
470 mM NaCl.

(iv) FPLC Mono Q 10/10: GyrB fractions from the Heparin-Sepharose
column were added to the column in TED and eluted with a linear salt gradient (0-400 mM
NaCl in 120 ml TED; flow rate, 4 ml/min). GyrB was eluted between 250 and 350 mM
NaCl. Fractions containing GyrB were pooled and dialysed into EB.
Amicon microconcentrator: GyrB solution was concentrated from 0.15 mg/ml to 1.7 mg/ml in an Amicon microconcentrator using a YM30 filter (30 kD cut-off) under a pressure of 50 psi and with continuous stirring to avoid non-specific interaction between protein and filter.

2.4 Enzyme assays

2.4.1 DNA supercoiling

DNA supercoiling by gyrase was assayed using the conditions described by Reece and Maxwell (1989). 10 µg/ml relaxed pBR322 was incubated with enzyme at 25°C for 1 hour in a 30 µl reaction mixture containing 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl₂, 1.8 mM spermidine, 0.36 mg/ml BSA, 9 µg/ml tRNA, 5 mM DTT, 1.4 mM ATP and 6.5% glycerol. Reactions were stopped by the addition of 30 µl of chloroform:isoamyl alcohol (24:1) and Stop Dye (100 mM Tris-HCl (pH 7.5), 1 mM Na₂-EDTA, 40% sucrose and 0.5 mg/ml bromophenol blue). Samples were electrophoresed through a 0.8% agarose gel, stained with ethidium bromide (2 µg/ml), then photographed under UV light.

2.4.2 DNA cleavage

Assays for the quinolone-induced cleavage of DNA were performed using the conditions described by Reece and Maxwell (1989) as standard (see Table 3.1). Conditions described by Gellert et al. (1977) and Sugino et al. (1977), and variations thereof were also employed, as outlined in the text. Investigation was also made of calcium-induced cleavage by replacement of CaCl₂ for MgCl₂ in the reaction buffers. All assays used 30 µl reaction volumes. The separation of DNA fragments generated by cleavage of the 147 bp fragment, beyond the resolution of agarose electrophoresis, was performed using an 8% polyacrylamide (19:1) gel in TBE buffer using a BioRad mini-protean II dual slab cell.
2.4.3 Gel-retardation assays

The amount of gyrase required to bind a given concentration of 147 bp DNA was investigated by gel-retardation assay. DNA and increasing concentrations of gyrase were incubated at 25°C for 1 hour in Binding Buffer (50 mM Tris-HCl (pH 7.5), 55 mM KCl, 4 mM MgCl$_2$, 5 mM DTT, 5% glycerol and 0.36 mg/ml BSA). The samples were loaded onto a 5% polyacrylamide (29:1) gel in TBM buffer (90 mM Tris-HCl (pH 7.5), 90 mM boric acid, 5 mM MgCl$_2$), without adding stop dye since bromophenol blue may disrupt the gyrase-DNA complex. Under these conditions, and provided that the gel was kept cool during electrophoresis, any gyrase-DNA complex remained intact. The gel could then be stained with EtBr (2 μg/ml) to reveal DNA and subsequently with Coomassie stain to visualise protein.

2.4.4 In vitro transcription assays

Linear DNA templates for transcription by T7 and *E. coli* RNA polymerases were generated by PCR. A region of 307 bp was amplified from around the preferred gyrase binding site at position 990 of plasmid pBR322. Promoter sequence for T7 or *E. coli* RNA polymerase was included as an extension on one of the oligos giving a total DNA fragment of 332 bp.

**T7 transcription assays:** Run-off transcription assays (5 μl total volume) were performed in buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 15 mM DTT, 0.2 mM Na$_2$-EDTA, 6 mM MgCl$_2$, 2 mM spermidine, 2% glycerol (w/v) and rATP, rCTP, rGTP (each at 0.5 mM) and unlabelled rUTP at 50 μM. 300 units of T7 RNA polymerase (kindly donated by Dr. I.C. Eperon) was added and the mixture incubated at 37°C for 30 min in the presence of [α-32P] rUTP, 9 units of RNA guard (Pharmacia) and gyrase and/or quinolone as appropriate.

**E. coli transcription assays:** Run-off transcription assays (5 μl total volume) were performed in buffer containing 32 mM Tris-HCl (pH 7.5), 120 mM KCl, 80 μM DTT, 80 μM Na$_2$-EDTA, 8 mM MgCl$_2$ and rATP, rCTP, rGTP (each at 0.5 mM) and unlabelled rUTP at 50 μM. 1 unit of *E. coli* RNA polymerase (Pharmacia) was added and the mixture
incubated at 37°C for 30 min in the presence of [α-32P] rUTP, 9 units of RNA guard (Pharmacia) and gyrase and/or quinolone as appropriate.

Analysis of transcripts After incubation, 3 μl of formamide-EDTA dye was added to each tube and the labelled transcripts analysed by electrophoresis (42 watts, 1 hour) in a 50 ml gel containing 6% polyacrylamide (19:1), 7 M urea (USB), 4.5-M formamide (Fisons, electrophoresis grade) and TBE buffer (100 mM Tris-HCl (pH 7.5), 83 mM boric acid, 1 mM Na2-EDTA). Following electrophoresis, the gel was sandwiched between two pieces of 3M paper and allowed to expose photographic film at -70°C for 1-12 hours.

2.4.5 In vitro ColEl-type replication assay

*E. coli* replication proteins were supplied by Dr. K.J. Marians; for preparation protocols see Minden and Marians (1985). Replication reaction mix (25 μl) contained 40 mM HEPES-KOH (pH 8.0), 100 μg/ml BSA, 10 mM magnesium acetate, 20 mM KCl, 400 μM UTP, CTP and GTP, 4 mM ATP, 40 μM dATP, dCTP, dGTP and 3H-dTTP, 100 μM NAD, 10 mM DTT, 4 μg/ml tRNA, 4 mM phosphocreatine and 200 μg template DNA. Typical reactions involved the following proteins; DnaB, DnaC, DnaG, DnaT, PriA, PriB, PriC, SSB, RNAP, Pol I, Pol III*, β, ligase and gyrase. Where appropriate, Tus termination protein was also included. Mixtures were assembled on ice then incubated at 37°C for 20 min. Reactions were stopped by the addition of 100 μl of 200 mM sodium pyrophosphate, 100 μl of carrier DNA (1 mg/ml heat-denatured calf thymus DNA) and 4 ml of 5% trichloroacetic acid (TCA). After 10 min on ice, acid-insoluble material was collected on glass fibre filters (ENZO Labs, NY) which were washed with 1% TCA and 95% ethanol and dried under a heat lamp. 4 ml scintillant was added to dried filters and the radioactivity retained was counted.

In experiments where the replication products were to be studied by gel electrophoresis, the label was [α-32P] dATP and reactions were stopped by addition of 1 μl of 0.5 M Na2-EDTA. 2 μl of each reaction was taken for analysis of TCA-precipitable counts as described above. 10 μl samples were examined by electrophoresis under both denaturing and non-denaturing conditions. 1% agarose slab gels were run under the
following conditions: denaturing gels were run horizontally in NE buffer (30 mM NaOH, 2 mM Na$_2$EDTA) at 20 volts, non-denaturing gels were run vertically in TAE buffer (40 mM Tris-HCl (pH 7.5) acetate, 2 mM Na$_2$EDTA) at 30 volts. Both gels were run for 14 hours, washed with 5% TCA for 10 min (denaturing gels only) and dried under vacuum prior to photographic film being exposed to the gels for an hour at -70°C.

**UpU Staged Initiation of replication:** An initial mixture containing 40 mM Hepes-KOH (pH 8.0), 100 mM KCl, 8 mM MgCl$_2$, 1mM DTT, 10% glycerol, 10 µg tRNA, 0.5 mM UpU, 2 µM GTP, CTP and ATP, 100 µg/ml BSA, 4 units RNasin, 100 µM NAD, RNAP, ligase and 200 µg template DNA was incubated for 10 min at 30°C to establish an initiation complex. 20 µg/ml rifampicin was added and the mixture incubated for a further 7 min at 30°C to prevent subsequent initiation. The remaining replication components were then included and the reaction protocol followed as outlined above.

2.5 Other methods

2.5.1 High pressure liquid chromatography

$^3$H-norfloxacin was purified by reversed-phase HPLC. Samples stored in ethanol:water (1:1) were freeze dried and redissolved in H$_2$O containing 0.1% trifluoroacetic acid. The sample was loaded onto a C18 column equilibrated with the same mobile phase and eluted with a 0-80% gradient of acetonitrile over a 40 min period; flow rate 0.5 ml/min. Elution of the drug was followed by absorption at 330 nm and fluorescence spectroscopy (excitation 340 nm, peak emission at 420 nm). Under these conditions the $^3$H-NFX had a retention time of approximately 15 min. Fractions were collected every 30 sec after a wait time of 12 min. Yield was calculated by scintillation counting. Fractions containing $^3$H-NFX were dried under vacuum and resuspended in 1:1 ethanol:water. An elution profile is shown in Figure 2.3.
FIGURE 2.3: Trace showing purification of $^3$H-NFX by HPLC
2.5.2 Spin-column binding experiments

2.5.2.1 Standard procedure The set-up for spin-column experiments is outlined in Figure 2.4. A column of G50 Sephadex, equilibrated in Binding buffer, was preformed in a 1 ml syringe. Reaction mixtures were incubated with $^3$H-NFX (2.5 µg/ml) for 1 hour in Binding Buffer (total volume 120 µl) before the application of 50 µl samples, in duplicate, to the column. During centrifugation at 1500 g for 2 min, macromolecule(s) and bound ligand pass through the column and are collected in a decapped Eppendorf tube, whilst free ligand is retained by the Sephadex beads. Samples before and after centrifugation were analysed by scintillation. After subtraction of the control values, the amount of $^3$H-NFX counts coming through the column, and therefore bound to macromolecule, was calculated as a percentage of the total counts added. This figure was subsequently converted into a drug concentration in nM.

2.5.2.2 Spin-column cleavage assays Using the same centrifugation procedure but with unlabelled quinolone, reaction mixtures were incubated for varying times before and after passage through a G50 sephadex column. The interaction of drug with gyrase, DNA and a complex of both together was then assessed by the cleavage assay outlined above.

2.5.3 Fluorescence techniques

Using a SLM 8000c fluorimeter (SLM instruments), investigation was made of the influence of gyrase and/or DNA upon the fluorescence spectrum of CFX and of the polarity of emitted light. Excitation and emission resolution (slit width) were each set at 4 nm throughout. Excitation (260-400 nm) and emission (360-500 nm) spectra were studied, in increments of 1 nm. Details of channel parameters are given in the text (Section 4.4).
FIGURE 2.4: Set-up for spin-column binding experiments

Protein and/or DNA after 1 hr incubation with $^3$H-NFX

Free ligand retained by beads

1 ml syringe
G50 Sephadex
glass wool
1.5 ml eppendorf tube
10 ml sterilin tube

Macromolecule(s) and bound ligand
CHAPTER 3

Investigating the cleavage reaction of DNA gyrase
3.1 Summary

Addition of CFX causes rapid inhibition of DNA supercoiling but DNA cleavage occurs more slowly, implying that there is a two-step interaction of quinolones with DNA-bound gyrase. The principal site for gyrase-mediated cleavage of linearised pBR322 in the presence of CaCl$_2$ is not the preferred position for quinolone-directed cleavage. Mutations at residue 83 of GyrA giving rise to quinolone-resistance do not always affect calcium-dependent cleavage.

3.2 Introduction

The supercoiling activity of DNA gyrase involves breakage of the DNA backbone in both strands, passing of a double-stranded segment through the gap and subsequent resealing of the breakage points. Under certain conditions, quinolone antibacterials can be shown to stabilise the gyrase-DNA complex with the DNA in a broken or cleaved state. To discern the quinolone-dependent cleavage of DNA it is necessary to disrupt the gyrase-DNA complex by treatment with a protein denaturant, such as SDS (Gellert et al., 1977; Sugino et al., 1977). Experiments of this kind have revealed a 4 bp stagger between the cleavage site in each strand (Morrison and Cozzarelli, 1979) and shown that the protruding 5'-DNA is covalently attached to the gyrase A subunits (Sugino et al., 1980) via a phosphotyrosine bond to GyrA residue Tyr$_{122}$ (Horowitz and Wang, 1987). Treatment with SDS leaves the protein attached to the DNA and in order to analyse the cleaved DNA by gel electrophoresis it is necessary to add a protease (eg. proteinase K) to remove the protein.

Interestingly, DNA cleavage is not detected when addition of protein denaturant is preceded by disruption of the quinolone-gyrase-DNA complex by heating to 80°C for 2 min or treatment with 2 M NaCl. There are two possible explanations for this observation; either the DNA was cleaved in the quinolone-gyrase-DNA complex and became resealed during the heat and salt treatments, or alternatively DNA might only become cleaved upon addition of SDS. As a result of this uncertainty, some researchers have preferred to refer
to the quinolone-gyrase-DNA complex as being in a "cleavable" rather than a "cleaved" state.

3.3 Cleavage of plasmid DNA

3.3.1 Influence of reaction components on DNA cleavage  A short while before the commencement of this work, a report suggested that only certain topological forms of DNA could be cleaved by gyrase (Shen et al., 1989c). In particular, it was stated that relaxed plasmid DNA could not be cleaved in the absence of either ATP or the non-hydrolysable analogue ADPNP. This result ran contrary to expectations from earlier studies (eg. Gellert et al., 1977) and investigation was initiated into the ability of gyrase to perform quinolone-dependent cleavage of relaxed, linear (cut with EcoRI), nicked circular and supercoiled pBR322 templates in the presence and absence of nucleotide.

3.3.1.1 Significance of DNA topology and ATP  All four forms of pBR322 proved readily cleavable in the presence and in the absence of nucleotide, as indicated by the appearance of linear plasmid from circular substrates and by the cutting of the linear template into smaller fragments (Figure 3.1). It was noted that the reaction mixture used in these experiments, the standard supercoiling conditions employed in this laboratory (see Reece and Maxwell, 1989 and Table 3.1, below), differed in several respects from the conditions quoted in the reference work (Shen et al., 1989c report that they followed the recommendations of Gellert et al., 1977 and Sugino et al., 1977). In fact, there are several differences between the reaction components in the two studies cited, see Table 3.1, and both were subsequently employed for this re-examination. Quinolone-dependent cleavage of relaxed and supercoiled DNA (the only forms tested) was possible under both Gellert-type and Sugino-type conditions, in the presence of ATP or ADPNP and in their absence (data not shown). The choice of conditions did, however, appear to influence the extent of cleavage and subsequent investigation therefore focussed on the principle differences, namely the concentrations of spermidine and MgCl₂ in the reactions.
FIGURE 3.1: Influence of DNA structure and addition of adenine nucleotide on quinolone-induced cleavage by DNA gyrase

(a) relaxed and linear pBR322

<table>
<thead>
<tr>
<th></th>
<th>ADPNP</th>
<th>ATP</th>
<th>CFX</th>
<th>Gyrase</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>l</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>linear</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>relaxed</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>s</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>r</td>
</tr>
</tbody>
</table>

Gyrase-mediated cleavage of nicked circular (n), linear (l), supercoiled (s) and relaxed (r) pBR322 DNA was investigated. With the exception of the factors under investigation, the reactions were performed according to the standard supercoiling conditions described by Reece and Maxwell (1989). The concentration of ATP and ADPNP was 1.67 mM, CFX was 10 μg/ml (27.2 μM) and gyrase, 67 nM.
FIGURE 3.1 (continued): Influence of DNA topology and addition of adenine nucleotide on quinolone-induced cleavage by DNA gyrase

(b) supercoiled and nicked circular pBR322

<table>
<thead>
<tr>
<th></th>
<th>ADPNP</th>
<th>ATP</th>
<th>CFX</th>
<th>Gyrase</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n</td>
</tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>l</td>
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<td>+</td>
<td>+</td>
<td>supercoiled</td>
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<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>s</td>
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<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>r</td>
</tr>
</tbody>
</table>
TABLE 3.1: Conditions for supercoiling and cleavage reactions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Standard</th>
<th>Sugino et al.</th>
<th>Gellert et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris.HCl (pH)</td>
<td>35 mM (7.5)</td>
<td>35 mM (7.6)</td>
<td>35 mM (7.6)</td>
</tr>
<tr>
<td>KCl</td>
<td>24 mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KH₂PO₄/K₂HPO₄</td>
<td>-</td>
<td>18 mM</td>
<td>18 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>4 mM</td>
<td>6 mM</td>
<td>1.6 mM</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1.8 mM</td>
<td>5 mM</td>
<td>1.8 mM</td>
</tr>
<tr>
<td>BSA</td>
<td>0.36 mg/ml</td>
<td>50 mg/ml</td>
<td>0.36 mg/ml</td>
</tr>
<tr>
<td>tRNA</td>
<td>9 μg/ml</td>
<td>90 μg/ml</td>
<td>9 μg/ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>6.5 %</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DTT</td>
<td>5 mM</td>
<td>5 mM</td>
<td>5 mM</td>
</tr>
<tr>
<td>ATP (or ADPNP)</td>
<td>1.67 mM</td>
<td>1.4 mM</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>DNA</td>
<td>0.3 μg</td>
<td>0.2 μg</td>
<td>0.3 μg</td>
</tr>
<tr>
<td>Quinolone</td>
<td>10 μg/ml</td>
<td>28 μg/ml</td>
<td>200 μg/ml</td>
</tr>
<tr>
<td>DNA gyrase</td>
<td>4 units</td>
<td>1 unit</td>
<td>4 units</td>
</tr>
</tbody>
</table>

3.3.1.2 The influence of nucleotide, spermidine and MgCl₂ Setting the other components at concentrations described by Gellert and coworkers, the influence of a variety of MgCl₂ and spermidine concentrations upon the cleavage of relaxed and supercoiled pBR322 was investigated. The importance of ATP was also considered. The results are summarised in Table 3.2. Consistently, increasing the MgCl₂ concentration led to enhanced DNA cleavage; this was true for both relaxed and supercoiled DNA and in the presence and absence of ATP. A plateau was reached at about 3 mM MgCl₂ above which cleavage did not increase (up to 10 mM, the highest concentration tested). As a rule, an increase in the spermidine concentration led to a reduction in DNA cleavage. This effect was clearer with relaxed than with supercoiled DNA, and was most evident in the absence of nucleotide. Cleavage of relaxed DNA was always possible in the absence of ATP (the original contention under study in this series of experiments) but was consistently greater when nucleotide was added. It appeared that ATP could either enhance or inhibit cleavage of supercoiled pBR322 dependent upon the other reaction components. For example, in the presence of 6 mM MgCl₂, 5 mM spermidine and 0.25 μg/ml NFX cleavage was
stimulated by ATP, but inclusion of ATP inhibited cleavage with 1.6 mM MgCl₂, 1.8 mM spermidine and 8 μg/ml NFX. This may in part have been due to ATP binding magnesium and thus destabilising the gyrase-DNA complex.

**TABLE 3.2: Effects of DNA topology and assay conditions upon quinolone-induced DNA cleavage**

<table>
<thead>
<tr>
<th>Variable</th>
<th>DNA topology</th>
<th>Effect(s) observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Relaxed</td>
<td>Increased [spermidine] led to reduced cleavage</td>
</tr>
<tr>
<td></td>
<td>Supercoiled</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Relaxed</td>
<td>Increased [MgCl₂] gave increased cleavage</td>
</tr>
<tr>
<td></td>
<td>Supercoiled</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>Relaxed</td>
<td>Cleavage greater with ATP than without</td>
</tr>
<tr>
<td></td>
<td>Supercoiled</td>
<td>ATP may stimulate or inhibit cleavage dependent upon other components</td>
</tr>
</tbody>
</table>

3.3.2 Comparison of quinolone and calcium-induced cleavage of pBR322

Cleavage of linear DNA was performed under a variety of conditions to consider if the selection of sites within the plasmid was influenced by the choice of divalent cation (MgCl₂ and CaCl₂) or by inclusion of ATP in the reactions. In the presence of a great excess of linear DNA, each gyrase should interact with a different molecule, facilitating interpretation of results that would be complicated if more than one cleavage event had occurred within any one molecule. From this experiment (Figure 3.2) it appears that the selection of a cleavage site is influenced by the divalent cation, but not by the inclusion of ATP. Both of these results are contrary to previous observations, which have shown that CaCl₂ directs cleavage to the same site as CFX (L.M. Fisher, M.H. O’Dea and M. Gellert, unpublished observation) and that ATP does alter site usage (Morrison et al., 1980).
Cleavage of linear pBR322 was compared in the presence of combinations of CFX (2.5 μg/ml, 6.8 μM), MgCl$_2$ or CaCl$_2$ (each at 4 mM), and ATP (1.7 mM). The sizes of resultant fragments were estimated by comparison with the mobility of marker DNAs, a 1 kB standard ladder (left) and a *Hinf* I digest of plasmid pAJ1 (right).
In the presence of CFX, the most prominent band was estimated to be 3.6 kb in length. Since the gel-based analysis appears to be over-estimating the size of DNA (see below), this fragment is likely to be the 3371 bp piece that results from cleavage at the 990 bp site, shown previously to be the preferred site for quinolone-directed cleavage of pBR322 (Fisher et al., 1981 and 1986). The smaller fragment resulting from scission at this site is not, unfortunately, visible in the gel. Since band intensity is proportional to the concentration of ethidium bromide bound to the DNA which is, in turn, determined by the length of the DNA, it is not uncommon for shorter fragments to be undetectable. This situation has not been aided by the migration of the loading dye with DNA of about 1 kb. Under the influence of calcium, cleavage appears to be occurring most frequently at a different position; the major fragments are estimated to be 3 kb and 1.7 kb (a total calculated size of about 4.7 kb set against the true size of 4361 bp).

In a separate experiment (Figure 3.3), CFX and OXO were both found to direct cleavage to a site that is plausibly the same position seen here with CaCl$_2$; the sizes of the most prominent bands was estimated from the gel to be 3 kb and 1.9 kb (compared with 3 kb and 1.7 kb). This does not readily correspond with any of the “strong” OXO-directed cleavage reported by Lockshon and Morris (1985), from which fragments would be 990/3371, 1460/2901, 2384/1977, 2472/1889 or 3689/672 (these finding were, incidentally, from studies of cleavage in vivo). Any thorough comparison would clearly require use of a more accurate mapping procedure than afforded by agarose gel electrophoresis.

It is interesting to consider why the major cleavage site in this experiment (Figure 3.3) has not corresponded either with the literature or with the previous experiment (Figure 3.2). With hindsight, an explanation may arise from differences in the reaction conditions. The experiment described in Figure 3.2 was performed under standard conditions, as outlined in Table 3.1, which are similar to those quoted by Fisher and coworkers (Fisher et al., 1986). The comparison of cleavage with CFX and OXO (Figure 3.3) was carried out with “Binding Buffer” conditions (50 mM Tris.HCl (pH 7.5), 55 mM KCl, 4 mM MgCl$_2$, 5 mM DTT, 0.36 mg/ml BSA and 5% glycerol (w/v)) more commonly used in this study.
Comparison was made of cleavage site selection by DNA gyrase under the influence of CFX (2.5 μg/ml) and OXO (500 μg/ml). Linear pBR322 was incubated with gyrase at 25°C for 1, 5, 20 and 60 min prior to treatment with SDS and proteinase K in the normal way. The approximate size of fragments resulting from cleavage events was determined by reference to the mobility of DNA markers, a 1 kb standard ladder (shown on left) and a Hinf I digest of plasmid pAJ1 (shown on right).
for experiments with small DNA substrates (see Sections 3.5 and 4.3). Unlike the other conditions mentioned here, Binding Buffer does not contain any spermidine (present at a concentration of 1.8 mM in the other reactions). It is shown later in this study that spermidine can influence cleavage site selection (see Figure 3.8, Section 3.5.1) and it is therefore not unreasonable to consider that the effects observed in Figure 3.3 may result from this difference. Clarification of this matter would require further investigation.

3.4 Studies using quinolone-resistant mutants

3.4.1 Preparation of mutants  Plasmid pPH3 contains the wild-type gyrA gene from E. coli under the control of the IPTG-inducible tac promoter and a protocol had been developed for purification of GyrA from a strain harbouring this plasmid (Hallett et al., 1990). A GyrASer83 → Ala point mutation had previously been introduced into pPH3 by site-directed mutagenesis (Hallett and Maxwell, 1991) and in the course of this study, two further GyrA mutants were prepared. The gyrA gene from a clinical isolate of E.coli had previously been cloned as plasmid pMEC5 and a high degree of quinolone-resistance was shown to result from a GyrATSP83 mutation (Cullen et al., 1989). A SstI-BstBI I fragment, coding for residues 20 to 369 of GyrA, was taken from pMEC5 and used to replace the corresponding region of pPH3, see Section 2.2.13. The resultant plasmid was termed pPH483. An additional mutant, GyrATyr83, was prepared by a PCR-based mutagenesis strategy to form pPH383 (Section 2.2.13). Since the alteration of a codon encoding serine to a tyrosine codon requires a dinucleotide substitution, this mutant is unlikely to arise spontaneously.

Mutation of the codon for residue 83 leads to the loss of a HindI restriction site and thus facilitates restriction fragment length polymorphism (RFLP) analysis (Fisher et al., 1989). This technique was used in the preliminary screening of transformed colonies for both pPH383 and pPH483. Loss of the HindI site resulted in the combining of fragments of 535 bp and 99 bp into one fragment of 634 bp, see Figure 3.4. The exact nature of the alteration(s) was subsequently confirmed by DNA sequencing (Figure 3.5).
FIGURE 3.4: RFLP confirmation of directed mutagenesis

Panel (a) shows the pattern of fragments resulting from *Hinf* I digestion of plasmids pPH3 (encoding wild-type GyrA<sub>Ser83</sub>), pPH311.1 (GyrA<sub>Ala83</sub>) and pPH383 (GyrA<sub>Tyr83</sub>). Mutation of the codon for residue 83 leads to loss of a *Hinf* I restriction site, see Panel (b), and the appearance of a novel fragment of 634 bp.
Sequencing through the quinolone-resistance determining region of gyrA was performed in order to confirm the genetic basis for resistance in the resultant proteins. In each case, changes were mapped to the codon for residue 83. The wild-type (Ser83) sequence is shown alongside the altered forms. Additional mutations noted in the gene for Trp83 GyrA, which is derived from a clinical isolate, proved to be silent.
3.4.2 All mutants were capable of supercoiling DNA  As a preliminary investigation of the properties of the GyrA mutants, their ability to support supercoiling of pBR322 under standard conditions, and with the replacement of MgCl$_2$ by CaCl$_2$ was studied (Figure 3.6). Although this experiment shows only a snapshot of the extent of supercoiling after 1 hour at 25°C, it is apparent that all four proteins (GyrA$\text{Ser}_{83}$, GyrA$\text{Ala}_{83}$, GyrA$\text{Thr}_{83}$ and GyrA$\text{Yry}_{83}$, each in combination with wild-type GyrB) are capable of supercoiling pBR322. It was separately determined that each GyrA protein had a similar specific activity, $-10^6$ units/mg (data not shown).

In study of the concentration of drug required to inhibit $in$ $vitro$ DNA supercoiling by 50% (IC$_{50}$), gyrase composed of wild-type GyrB and GyrA$\text{Yry}_{83}$ was found to be 60 times more resistant to CFX than the wild-type protein (Sue Critchlow, personal communication). For comparison, GyrA$\text{Ala}_{83}$ was 10 times as resistant, and GyrA$\text{Thr}_{83}$ 100 times more resistant, than the wild-type enzyme in the same assay.

3.4.3 Comparison of CFX and Ca$^{++}$-directed cleavage with gyrase mutants

Gyrase-mediated cleavage of relaxed DNA was investigated in the presence of 2.5 $\mu$g/ml (6.8 $\mu$M) CFX or 4 mM CaCl$_2$. Samples were incubated at 25°C for 2 hours prior to treatment with SDS and proteinase K and incubation at 37°C for 30 min in the normal way. The assay has only looked at one timepoint and at one enzyme concentration (Figure 3.7) but the following tentative conclusions can be made. The response to CFX is in agreement with the known resistance characteristics from study of supercoiling inhibition with these proteins; the DNA had been cleaved into small pieces by wild-type and GyrA$\text{Ala}_{83}$ gyrase, less cleavage has occurred with the more quinolone-resistant GyrA$\text{Yry}_{83}$ and less still with GyrA$\text{Thr}_{83}$.

It had previously been reported that wild-type and GyrA$\text{Ala}_{83}$ gyrase caused comparable cleavage in the presence of calcium (Hallett and Maxwell, 1991). This result was in apparent contradiction to an experiment where GyrA$\text{Thr}_{83}$ gyrase was found to be resistant to calcium-induced as well as quinolone-dependent cleavage (L.M. Fisher, personal communication). Both of these results are, however, endorsed in the present study. In the presence of calcium, similar extents of cleavage are seen with wild-type and
The DNA substrate (relaxed pBR322) is also shown in the left hand track of the gel. In all cases equilibration wild-type GyrB was provided and reaction mixtures were incubated at 25°C for 1 hour. The DNA substrate (relaxed pBR322) is also shown in the left hand track of the gel.

The capacity of GyrA mutants carrying substitution of the wild-type residue 83 (serine) by alanine, tyrosine and tryptophan was investigated. Comparison was made of supercoiling in the presence of MgCl₂ and CaCl₂ (each 4 mM) in all cases equilibrated. Wild-type GyrB was provided and reaction mixtures were incubated at 25°C for 1 hour. The DNA substrate (relaxed pBR322) is also shown in the left hand track of the gel.

**FIGURE 3.6:** Confirmation that quinolone-resistant gyrase are capable of supercoiling plasmid DNA
Supercoiled pBR322 was incubated with 260 nM gyrase for 2 hours at 25°C in the presence or absence of either 4 mM MgCl₂ and 6.8 mM CFX or 4 mM CaCl₂ without quinolone. The mobility of the substrate DNA is shown in the control track.

<table>
<thead>
<tr>
<th></th>
<th>Tp</th>
<th>Trp</th>
<th>AIA</th>
<th>Ser</th>
<th>Tp</th>
<th>Trp</th>
<th>AIA</th>
<th>Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>calcium</td>
<td></td>
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<tr>
<td>Ciprofloxacine</td>
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</tr>
</tbody>
</table>

**Figure 3.7:** Comparison of CFX and calcium-directed cleavage of supercoiled pBR322 by wild-type and quinolone-resistant gyrase
GyrA_{Ala83} enzyme, the latter is possibly more reactive, yet the GyrA_{Tyr83} enzyme is apparently as resistant to calcium-directed cleavage as it was to cleavage with CFX. An intriguing result comes with GyrA_{Ytr83} gyrase which, despite being highly quinolone-resistant (like GyrA_{Tyr83}), has cleaved DNA like the wild-type enzyme in the presence of calcium. It therefore appears that both quinolone- and calcium-directed cleavage may be affected by the amino acid at residue 83 of GyrA, but in different ways. There is clearly scope for further study on this subject.

3.5 Cleavage of a small DNA substrate

3.5.1 Selection of appropriate DNA. 3 potential substrate DNAs were available for this study. Fragments of 207 and 172 bp, originally derived from the sea urchin 5S ribosomal gene and cloned as multiple tandem repeats (Simpson et al., 1985) were two of the possibilities, the third was a 147 bp fragment that incorporates a 123 bp region of pBR322 DNA centered around the preferred site of quinolone-directed cleavage at position 990 (Fisher et al., 1981, Dobbs et al., 1992).

Several factors led to the ultimate selection of the 147 bp fragment as the template for study of cleavage (this section) and binding (chapter 4). A test cleavage assay revealed that the 147 bp fragment was cleaved at a unique position (Figure 3.8). Although the larger fragments each had a preferred cleavage site, they were unsuitable for the intended studies because of the existence of alternative cleavage positions (Figure 3.8). The 172 bp fragment had a second prominent cleavage site and when spermidine was omitted from the reaction a third site of cleavage was also apparent.

Secondly, since the 147 bp was principally derived from pBR322, it was felt that use of this fragment might facilitate comparison between cleavage of the same sequence in two distinct situations; as part of a small DNA and of the larger parent molecule. The principal factor in favour of using either the 207 or 172 bp substrates was their availability in mg quantities. This advantage was negated with the successful cloning of the 147 bp fragment in a multiple copy plasmid (Dobbs et al., 1992).
Comparison was made of the quinolone-dependent cleavage (6.8 μM CFX in each reaction) of three linear DNA fragments under a range of conditions. By comparison with the mobility of the Msp I digested pBR322 size markers (622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76 and 67 bp), it was estimated that the 207 bp template was cleaved by gyrase into pieces of approximately 104 and 115 bp. In the absence of spermidine, additional products of about 58 and 149 bp were observed. Cleavage of the 172 bp fragment gave pieces of about 74 and 109 bp, 87 and 98 bp, and in the absence of spermidine further products of 83 and 101 bp were noted. The 147 bp fragment was cleaved at a unique site into pieces estimated to be 75 and 82 bp. The true sizes of these products are known to be 69 and 78 bp (Dobbs et al., 1992). Spermidine apparently had no effect on cleavage site selection with the 147 bp fragment.
3.5.2 Cleavage of the 147 bp fragment is a slow process. In order to determine conditions under which all of the 147 bp fragment was gyrase-bound, a gel-shift assay was carried out, observing retarded mobility of a constant concentration of 147 bp DNA in the presence of increasing gyrase (Figure 3.9). Selecting conditions in which complete retardation had been observed, the influence of incubation time on quinolone-induced DNA cleavage was then examined (Figure 3.10). Cleavage was shown to be a slow process, with incubation for 4 hours prior to the addition of SDS and proteinase K being required to ensure all the DNA had been cleaved. Analysis with $^{32}$P end-labelled DNA has confirmed that cleavage occurs in the 147 bp molecule at exactly the same position as in the parent pBR322 (Fisher et al., 1981; Dobbs et al., 1992) and generates pieces of 69 and 78 bp. A CFX concentration of 2.5 μg/ml was required to ensure that complete cleavage could be observed, and raising the quinolone concentration above this level did not speed up the process (data not shown). Heating the reaction mixture to 80°C for 2 min prior to addition of SDS disrupted the gyrase-DNA-quinolone complex in a manner that left the DNA uncleaved in subsequent treatment with the detergent and proteinase K (Figure 3.10). To establish that the resealed DNA had not been modified by the process, restriction digestion of the heat resealed 147 bp DNA was performed. The base sequence at the cleavage site is T↓GGCCT of which the central 4 bp are a $Hae$ III restriction site. Scission of the DNA was as efficient at this position as at the two further $Hae$ III sites within the 147 bp sequence and at all three sites of 147 bp DNA that had not been treated with gyrase (data not shown), implying that no significant modification had occurred.

3.5.3 The effect of temperature on the cleavage reaction. Although gyrase assays in this laboratory are generally carried out at 25°C, other research groups study supercoiling and cleavage at different temperatures, notably 30°C (eg. Morrison and Cozzarelli, 1981) and 37°C (eg. Walton and Elwell, 1988). CFX-dependent cleavage by gyrase of the 147 bp fragment was therefore studied at 25°C, 30°C and 37°C to see what influence an increase in temperature had upon the cleavage process (Figure 3.11). Samples from each reaction mixture were taken after incubation for 1, 2 and 3 hours and
FIGURE 3.9: Gel-retardation assay to determine the [gyrase] required to bind all available 147 bp DNA (72 nM per track)
FIGURE 3.10: Gyrase-mediated cleavage of the 147 bp DNA fragment is a slow process

(a) The 147 bp fragment is composed of a 123 bp region of pBR322 (shaded grey), flanked by sequences containing restriction sites for EcoR I (E) and Ava I (A), and is cleaved by gyrase into fragments of 69 and 78 bp as shown in (b) where [CFX] was 2.5 µg/ml in all cases. The time of incubation (min) at 25°C prior to SDS treatment is given above each track. "Heat" indicates that sample was heated to 80°C for 2 min between 240 min incubation and SDS treatment.
FIGURE 3.11: The influence of temperature on gyrase-mediated cleavage of the 147 bp DNA substrate

<table>
<thead>
<tr>
<th>25°C</th>
<th>30°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

147 bp DNA was incubated with gyrase and CFX (2.5 μg/ml) for 3 hours at 25°C, 30°C and 37°C. Samples were taken each hour during the incubation and treated with SDS and proteinase K in the normal way.
treated with SDS and proteinase K in the normal manner (30 min incubation at 37°C in the presence of 0.2% SDS and 1 mg/ml proteinase K). As one might expect, DNA cleavage occurred more rapidly at the higher temperatures; at 37°C conversion of the 147 bp fragment into the smaller pieces was almost complete in 1 hour. There was not, however, any indication that cleavage occurs at any different or additional sites when the temperature is increased.

3.5.4 CaCl₂-directed cleavage of the 147 bp fragment was not detected

Cleavage of plasmid DNA has been shown to occur in the absence of quinolone when MgCl₂ in the reaction mixture is replaced by CaCl₂ (e.g. Reece and Maxwell, 1989). It did not, however, prove possible to detect cleavage of the 147 bp fragment under any of the conditions tested, which included all of the combinations discussed in Section 3.3.2. Linear, relaxed, supercoiled and nicked circular pBR322 were all cleaved under "binding buffer" and standard cleavage conditions (data not shown). This result agrees with a previous study which reported failure to detect calcium-directed cleavage of the 147 bp fragment under "binding buffer" conditions (Dobbs et al., 1992). Cleavage of ³²P end-labelled DNA was, however, observed by Dobbs and coworkers when standard cleavage conditions were employed and it is possible that calcium-directed cleavage is occurring at a low level, detectable by autoradiography but below the sensitivity of ethidium staining. Since the 990 bp preferred site for quinolone-induced cleavage of pBR322 is not the major position for calcium-directed cleavage (see Figure 3.2, Section 3.3.2), difficulty in detecting cleavage of the 147 bp fragment, which contains a 123 bp region around the 990 bp preferred site, may reflect the importance of DNA sequence in cleavage-site selection.

3.6 Comparison of quinolone-dependent inhibition of supercoiling and DNA cleavage

In the light of the slow cleavage of the 147 bp DNA fragment, an investigation was made of the time required for quinolone to inhibit supercoiling of relaxed pBR322 and to direct cleavage of the DNA (see Figure 3.12). A concentration of gyrase was incubated with relaxed pBR322 in the absence of quinolone for sufficient time (15 min at 25°C) for
FIGURE 3.12: Procedure for comparison of quinolone-dependent inhibition of supercoiling and onset of DNA cleavage

Reaction Mix
(without gyrase or quinolone)

- Take sample and STOP (-ve control)

Add gyrase

15 min incubation at 25°C

- Take 3 samples:
  a. STOP
  b. Incubate with SDS/proteinase K for 30 min at 37°C, then STOP
  c. Incubate at 25°C for further 45 min, then STOP (+ve control)

Add CFX
(final concentration 2.5 µg/ml)

- Take samples (2 for each timepoint) at various times:
  a. STOP
  b. Incubate with SDS/proteinase for 30 min at 37°C, then STOP

Final timepoint 240 min after initial addition of gyrase

STOP indicates treatment with an equal volume of chloroform: isoamyl alcohol (24:1) and addition of Stop Dye. At each timepoint 2 samples were taken; one to monitor the CFX-dependent inhibition of supercoiling (treatment a), the other to study the onset of DNA cleavage (treatment b). The results are given in Figure 3.13.
supercoiling to be underway but not complete. Prior to the addition of 2.5 μg/ml CFX, three samples were taken from the reaction mix. The first was treated directly with chloroform:isoamyl alcohol and retained to show the extent of supercoiling before the addition of the inhibitor. The second was treated with SDS and proteinase K to show how much DNA cleavage occurred in the absence of quinolone and the third was incubated at 25°C for a further 45 min to show that the supercoiling reaction would go to completion if drug had not been added. After the addition of CFX, the reaction mixture was incubated for a further 225 min during which samples were taken at various times and treated in the same manner as the first and second samples taken at the 15 min stage. These samples were taken to investigate the inhibition of supercoiling and the onset of DNA cleavage respectively. The results are shown in Figure 3.13.

Unlike the 147 bp fragment, cleavage of pBR322 did not continue to increase for the complete 4 hour incubation, appearing instead to reach a maximum level of cleavage in around 2 hours. This was still, however, markedly longer than the time required to inhibit supercoiling; the assay revealed that supercoiling was inhibited within a minute of the addition of drug. It therefore appears that the effect of quinolone on the interaction of gyrase with DNA acts at two levels; the initial binding of drug to the gyrase-DNA complex is sufficient to cause inhibition of supercoiling but does not support DNA cleavage. This ternary complex is hereafter termed “Complex I” (Figure 3.14). Subsequent cleavage of the DNA is a slow process and may involve a further conversion to “Complex II” in which the DNA strands have been broken.

3.7 Discussion

Experiments described in this chapter have focussed upon the gyrase-mediated cleavage of DNA. Investigation of the significance of reaction conditions showed that ATP, MgCl₂ and spermidine can all affect the extent of DNA cleavage. Although quinolone-directed cleavage of relaxed DNA is enhanced by ATP, the presence of nucleotide was not a requirement for cleavage to occur, contradicting a proposal made in a previous study (Shen et al., 1989c). The authors of the original work have now reassessed
FIGURE 3.13: Comparison of quinolone-dependent inhibition of supercoiling and onset of DNA cleavage

Panel (a) shows that supercoiling is inhibited within a minute of the addition of CFX whereas panel (b) shows that the onset of CFX-induced DNA cleavage occurs over a longer timescale. The track labelled "-ve" in panel (a) shows the relaxed DNA at the start of the experiment. Supercoiling was allowed to proceed for 15 min and a sample, taken prior to the addition of CFX, was incubated for a further 45 min, see "+ve" in panel (b). Details of the experimental procedure are given in Figure 3.12.
conversion to Complex II in which the DNA is cleaved, but the DNA has not been cleaved. Complex I subsequently undergoes slower conversion to Complex II. The three are thought to rapidly form Complex I in which supercoiling is inhibited, a "G". The enzyme is represented as a light grey ovoid. DNA is parallel lines and quinolone drug as a ring.
their data and observe that some linearisation of relaxed DNA was occurring in the absence of cofactor, concluding that it would be better to say "the cleavage was more efficient in the presence of the cofactor" (L.L. Shen, personal communication). This revised result is endorsed by the present study.

As well as affecting the extent of cleavage (Section 3.3.1.2), spermidine can influence cleavage site selection (Section 3.5.1). Spermidine is a polyanion included in supercoiling reactions because it is believed to reduce phosphate-phosphate interference and so allow tighter packing of the DNA backbone. It is therefore possible that the effects described here occur indirectly and involve changes in DNA structure.

Whilst CaCl$_2$ and quinolone both promote gyrase-mediated cleavage of pBR322, DNA scission need not be occurring at the same location(s) with both effectors (Section 3.3.2). Some analogy can be drawn to experiments investigating cleavage by eukaryotic topoisomerase II in the presence and absence of anti-tumour compounds (Capranico et al., 1990; Pommier et al., 1991; See Section 1.8.1). It has been shown that cleavage site selection is influenced by local DNA sequence and that the base requirements are different for each class of drug and in the absence of any inhibitor. The factors determining sites for cleavage in the presence of doxorubicin were exactly the reverse of those influencing cleavage by topo II alone, making the two sets mutually exclusive. (These experiments are considered again in Section 6.2 with reference to the binding studies of Chapter 4).

Unlike eukaryotic topoisomerase II, gyrase cannot readily be shown to cleave DNA without addition of quinolone. The exception, as shown in this chapter, occurs when CaCl$_2$ is used in place of MgCl$_2$ in the reaction mixture. It is possible that, in the presence of CaCl$_2$, gyrase is revealing "drug-free" cleavage site preferences that are different from determinants for quinolone-dependent scission. At present such suggestions are pure speculation; clarification of this matter would require careful comparison of CaCl$_2$ and quinolone-dependent cleavage.

Analysis of CFX-directed cleavage of a 147 bp DNA fragment revealed that incubation for up to 4 hours at 25°C was required in order to see complete scission of the DNA (Section 3.5.2). Subsequent investigation of cleavage of relaxed pBR322 confirmed
that cleavage is a slow process taking, in this case, up to 2 hours for maximal linearisation to have occurred (Section 5.6). In the same experiment, DNA supercoiling was inhibited within a minute of addition of quinolone (Figure 3.13). From this result it is concluded that quinolones interact with DNA-bound gyrase in two distinct ways. The initial formation of a quinolone-gyrase-DNA ternary complex, termed Complex I, is sufficient to inhibit the supercoiling reaction but does not promote DNA cleavage. Later conversion of Complex I to Complex II is apparently required for breakage of the DNA.

Does addition of detergent and protease reveal or cause DNA cleavage? These experiments have varied the time of incubation of drug with gyrase and DNA, but kept the SDS/protease K treatment identical throughout. The observed increase in cleavage with longer incubations implies that the DNA has been cleaved before the addition of denaturant. It would, however, be useful to perform similar experiments in which a fixed pre-incubation time was followed by treatment with detergent and protease for various times. Since heating the reaction mix to 80°C for two min prior to the addition of SDS abolishes cleavage (Figure 3.10), this step must somehow allow rejoining of the broken ends.
CHAPTER 4

Investigation of the binding of quinolones to gyrase and DNA
4.1 Summary

Detection of quinolone binding to the complex of gyrase and DNA has been observed in studies using a rapid gel filtration ("spin-column") technique. This interaction was greatly reduced when the gyrase A subunits carried mutation of residue 83 from serine to tryptophan (GyrATp83) or alanine (GyrAAl83). These mutations are known to confer quinolone resistance (Yoshida et al., 1988; Cullen et al., 1989; Hallett and Maxwell, 1991). No binding of NFX or CFX to DNA alone was observed. Similarly, neither the wild-type nor resistant gyrases were able to form detectable interactions with quinolone in the absence of DNA. Use of the natural fluorescence of quinolones as an indicator of binding lends some support to these results, but interpretation is limited by poor reproducibility.

4.2 Introduction

It is now widely accepted that DNA gyrase is the intracellular target of quinolone antibacterials. There has, however, been some contention about the nature of the interactions that result in inhibition of gyrase, with separate proposals that quinolones bind to the enzyme itself, to DNA, or to a gyrase-DNA complex (Gellert et al., 1977; Shen and Pernet, 1985; Shen et al., 1989a).

To date, the strongest evidence for the direct involvement of gyrase in the binding of quinolones has been the existence of quinolone-resistant protein arising from point mutations in the GyrA or GyrB subunits. These were considered at length in the introductory chapter, see Section 1.6. Evidence for binding of quinolones to DNA has come principally from Shen and coworkers at Abbott laboratories (Shen and Pernet, 1985; Shen et al., 1989a, b and c). In the initial experiments, equilibrium dialysis and membrane filtration were used to monitor the binding of 3H-NFX and revealed apparent binding of two drug molecules per CoIE1 plasmid (6646 bp). This binding was not altered by the presence of gyrase, and no binding to the enzyme alone was observed (Shen and Pernet, 1985). Later work suggested that gyrase did, in fact, facilitate the stable binding of NFX (Shen et al., 1989c). In a spin-column assay, where a reaction mixture is passed under
centrifugation through a column of G50 Sephadex, there was detectable binding of \(^3\)H-NFX to the gyrase-DNA complex, but not to gyrase, or indeed DNA, alone. Examination of NFX interaction with a variety of DNAs suggested that binding was greater to single-stranded than to double-stranded DNA, interpreted to show that binding is non-intercalative (Shen et al., 1989b). Furthermore, it appeared that significantly greater binding was occurring to single-stranded homopolymers of poly(dG) or poly(G) than to any other homopolymers, including poly(dI). From these results it was concluded that the significant difference is the possession of an additional hydrogen-bond donor per guanine base and that binding was occurring through hydrogen-bonding of NFX to the DNA; this became a central tenet of the binding model (Shen et al., 1989a; see Figure 1.6).

According to the Shen model, gyrase binds to DNA and in the course of its catalytic activity cleaves the DNA in both strands. The strand cleavage is staggered by 4 bp (Morrison and Cozzarelli, 1979) and generates a ssDNA "bubble" to which a quinolone molecule can hydrogen bond. Binding of further drug molecules is co-operative and is favoured by the additional interactions, ring stacking and tail-to-tail hydrophobic interactions, that can be made with the first drug molecule. The C-3 carboxyl and C-4 keto groups, common to all antimicrobially active quinolones (see generalised structure, Figure 1.3), are responsible for hydrogen-bonding to appropriate acceptors and donors on the DNA. Interaction with the enzyme, if any, would be afforded through the C-7 group.

This model has not received universal approval and a number of reservations have been documented (Reece and Maxwell, 1991a; Yoshida et al., 1991). Several other reports into the interaction of quinolones with DNA and/or gyrase have come to contradictory conclusions. An attempt to detect binding of NAL to calf thymus DNA by absorption spectroscopy concluded that no interaction was occurring (Bourguignon et al., 1973). Similarly, Palu' and coworkers found no binding of NFX or OFX to DNA (single- or double-stranded) by either fluorescence spectroscopy or equilibrium dialysis (Palu' et al., 1988). Le Goffic noted alterations in the \(^{19}\)F nuclear magnetic resonance signal of PFX in the presence of relatively small amounts of gyrase but no change in the presence of DNA alone (Le Goffic, 1985). Admittedly, the validity of these data has been questioned (Shen,
1989), but they do reveal that the current model must be considered only as a working hypothesis.

The assignment of the C-7 position as the only interaction with the protein has caused surprise (Reece and Maxwell, 1991a). Enzyme-ligand interactions are generally noted for their specificity, yet throughout the quinolone family the substituent group at C-7 is highly variable being, for example, a piperazine ring in NFX, a methyl group in NAL and an alkoxy group in OXO (exhaustive coverage of the C-7 variations is given by Chu and Fernandes, 1991) and it is unlikely that so much variability would be tolerated. It would be more in keeping with other examples of ligand binding for the H-bonding capacity of the C-3 carboxyl and C-4 keto groups to be directed towards the enzyme.

Consider two examples of antibacterial-enzyme interaction that have been studied by X-ray crystallography; the binding of chloramphenicol to chloramphenicol acetyltransferase (CAT) and of trimethoprim to dihydrofolate reductase (DHFR).

Analysis of CAT crystals in the presence of chloramphenicol (Leslie, 1990) reveals that interaction involves two direct hydrogen bonds and a third via a bridging water molecule with further contact made by hydrophobic interactions. It is interesting to note that chloramphenicol binds at the interface of two subunits (CAT is a trimer of identical monomers) and while most of the contacts maintaining binding are provided by one subunit, the catalytic residue (His195) involved in the transfer of an acetyl group to the substrate is provided by the other polypeptide.

Trimethoprim binds to the active site of *E. coli* DHFR and contact is maintained by a combination of van der Waals, hydrogen bonding and ionic interactions (Matthews et al., 1985). 4 hydrogen bonds are made between the enzyme and trimethoprim, 3 bonds are direct between DHFR and ligand while the fourth is mediated by a water molecule. A plasmid-encoded form of DHFR which confers trimethoprim-resistance has been partially sequenced (Thomson et al., 1990). Although crystal details of the resistant DHFR are not yet available (and the protein not fully sequenced), it appears that mutation at two positions is sufficient to confer resistance. The substitutions, Ala6 → Leu and Leu29 → Met result in the loss of Van der Waals contacts between trimethoprim and the enzyme.
Detail of the forces that attract quinolones to gyrase and DNA have not been established. Hydrogen bonding is likely to be a feature of quinolone-gyrase interactions; several mutations giving rise to quinolone resistance occur at residues that were hydrophilic amino acids in wild-type GyrA, eg. Ser33, Asp87 and Glu106. Although the GyrAAla83 mutation does not confer as great resistance as, for example, GyrATyr83 or GyrALeu83 it is the subtlety of the alteration that makes it particularly significant. Serine and alanine are very similar in size, occupying 89 Å³ and 88.6 Å³ respectively (compared with 227.8 Å³ for tryptophan). The 10-fold resistance to quinolones that results from this change is therefore entirely attributable to the loss of a hydroxyl group, and therefore of hydrogen-bonding potential (Hallett and Maxwell, 1991).

While the H-bonding of quinolones to DNA may be a genuine phenomenon with idealised DNA substrates, it does not necessarily follow that this is of any physiological significance. In any mixing of H-bond donors with H-bond acceptors there is likely to be some degree of interaction. Also, if H-bonding to ssDNA is indeed the mode of interaction, how is the ssDNA in the gyrase cleavage-site different from that revealed, for example, during DNA replication (Minden and Marians, 1985)? Why are "strong" gyrase-binding sites, where the enzyme has been shown to exhibit preference for quinolone-induced cleavage of DNA, not especially characterised by a high GC content (Lockshon and Morris, 1985)?

4.3 Binding analysis using a rapid gel-filtration ("spin column") approach

4.3.1 Establishing the technique

A method for rapid gel-filtration, commonly referred to as a spin-column, has proven useful in the separation of populations of small ligands that are bound to macromolecules from those that remain unbound (details can be found in Section 2.5.2). For example, this procedure is often used in the purification of radiolabelled DNA to remove unincorporated nucleotide.
In the experiments reported in this chapter (Section 4.3), a spin-column approach has been used to probe the interaction of quinolones with gyrase and/or DNA. Drug bound to macromolecule should pass through the column of G50 Sephadex while free quinolone molecules will be trapped in the beads and retained. This method has previously revealed binding of NFX to a complex of gyrase and DNA together but not to either component alone (Shen et al., 1989c). Here, interaction has been monitored in two ways; by scintillation counting of $^3$H-NFX (Section 4.3.2) and indirectly by quinolone-induced DNA cleavage (Section 4.3.3). The $^3$H-NFX, a gift from Linus Shen (Abbott labs) was repurified by reverse phase HPLC according to the recommended procedure (Shen et al., 1989; see Section 2.5.1).

### 4.3.1.1 Selection of gyrase:DNA ratio

Previous studies on quinolone binding have generally involved plasmid DNAs of 4-7 kb. Each drug molecule is, very approximately, the size of just a purine base and given this size differential, there exists the possibility of non-specific interactions. In an attempt to reduce the probability of non-specific binding, this study has exploited the availability in mg quantities of a 147 bp DNA fragment containing the major site from pBR322 for quinolone-induced cleavage by gyrase (Fisher et al., 1981; Dobbs et al., 1992). Gyrase is known to protect approximately 100-155 bp of DNA from nuclease digestion (Morrison and Cozzarelli, 1981; Fisher et al., 1981; Kirkegaard and Wang, 1981; Rau et al., 1987) and each 147 bp DNA fragment binds one, and only one, gyrase holoenzyme (Dobbs et al., 1992).

Prior to work on the binding of quinolones, a suitable gyrase:DNA ratio was determined by gel-shift assay. A range of gyrase concentrations were incubated with a fixed concentration of 147 bp fragment for one hour at 25°C. Samples were subjected to electrophoresis in a 5% polyacrylamide gel in TBM buffer, under non-denaturing conditions. The gel was then stained by two processes; firstly with ethidium bromide to reveal the position of DNA and secondly with Coomassie stain to determine the location of gyrase. In this way, it was shown that DNA runs in two positions, with gyrase-bound DNA moving more slowly through the gel matrix than the free form. An example "move-
up" gel is shown in Figure 3.9. A concentration of gyrase sufficient to cause all of the DNA to be in the bound position was used for the quinolone binding experiments. It was necessary to repeat this procedure each time a different preparation of either gyrase subunit was introduced.

4.3.1.2 Selection of centrifugation speed Under optimal conditions, the rapid gel-filtration procedure should allow for complete transfer of gyrase-DNA complex through the spin column without dissociation. A variety of conditions close to those recommended (2 min at 1000 x g, Maniatis et al., 1982) were considered. On the basis of comparative band intensity in samples that had or had not been centrifuged prior to electrophoresis in a non-denaturing gel, a 2 min spin at 1500 x g was selected (data not shown).

4.3.2 Studies with radiolabelled norfloxacin

Because the $^3$H-NFX was redissolved in a 1:1 water:ethanol mix after repurification, a total of 1.5% of the final reaction mixture was alcohol. Control experiments were performed to ensure that this was not affecting the interaction of gyrase, DNA and quinolone and it was found that up to 5% ethanol had no effect on the extent of quinolone-induced DNA cleavage by gyrase (data not shown).

4.3.2.1 Detection of $^3$H-NFX binding to gyrase-DNA complex Investigation was made of the interaction of $^3$H-NFX with gyrase, with DNA and with complex of both together. The gyrase:DNA ratio was determined by gel shift assay as described above and the overall NFX concentration was 2.5 μg/ml (7800 nM) which had been shown previously to induce maximal DNA cleavage. Reaction mixtures were incubated at 25°C for 1 hour prior to centrifugation. The volume of eluants was recorded and then a standard volume was taken for scintillation counting (30 min per sample, overnight). When necessary, values were adjusted to correct for changes in volume (with 50 μl put onto a column, 40 to 82 μl were eluted, though on any one occasion the variation was only a fraction of this range).
A summary of the results (averaged from 2-6 repeats) is given in Table 4.1. Significant binding to gyrase-DNA complex has been noted. Under the same conditions, no binding of \(^3\text{H}-\text{NFX}\) to DNA alone could be detected, above the background levels for drug alone. When the concentration of DNA was increased 10-fold there was little increase in \(^3\text{H}-\text{NFX}\) coming through the column. With gyrase alone, a borderline degree of interaction was observed. Initially, it was unclear whether this level of radioactivity represented genuine binding, but subsequent experiments (Section 4.3.2.2) have not detected binding to gyrase alone.

This set of results indicate that stable quinolone binding requires the presence of both the enzyme and the DNA. Since, however, the technique involves centrifugation the reactions have not been performed under true equilibrium conditions. It is therefore possible that some weak interaction may be occurring between NFX and either gyrase or DNA alone but any bound drug is dissociated during centrifugation.

Table 4.1: Binding of \(^3\text{H}-\text{NFX}\) to gyrase-DNA complex

<table>
<thead>
<tr>
<th>[DNA]</th>
<th>[A(_2)B(_2)]</th>
<th>[NFX](_{\text{Total}})</th>
<th>[NFX](_{\text{Bound}}) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72(^a)</td>
<td>380</td>
<td>7800</td>
<td>17.8</td>
</tr>
<tr>
<td>-</td>
<td>380</td>
<td>7800</td>
<td>2.2</td>
</tr>
<tr>
<td>72</td>
<td>-</td>
<td>7800</td>
<td>0</td>
</tr>
<tr>
<td>720</td>
<td>-</td>
<td>7800</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\(^a\) all concentrations quoted in nM

\(^b\) after deduction of NFX alone values

4.3.2.2 Influence of GyrA point mutations on NFX binding A number of point mutations in GyrA have been shown to confer resistance to quinolones (see Section 1.6). Included amongst these are alterations of serine at residue 83 in the sensitive enzyme to tryptophan (Yoshida \textit{et al.}, 1988; Cullen \textit{et al.}, 1989) and the more conservative change to alanine (Hallett and Maxwell, 1991). The effect of these mutations has been assessed both in terms of the concomitant increase in the minimum inhibitory concentration (MIC)
required to kill cells with altered gyrase (Yoshida et al., 1988; Cullen et al., 1989; Hallett and Maxwell, 1991) and in the relative abilities of the mutant enzyme to perform the supercoiling of relaxed DNA in vitro. By the latter test, enzyme prepared from GyrB with GyrA Ala83 is 10-fold, and GyrATrp83 100-fold more resistant to inhibition by CFX than the sensitive form (Sue Critchlow, personal communication). *A priori*, it is feasible that these mutations could act either to reduce the level of interaction with quinolone drugs (the most likely scenario) or alternatively they could somehow facilitate the gyrase to continue its catalytic activity despite the presence of bound drug. The influence of both GyrA Ala83 and GyrATrp83 on quinolone binding has been studied using the spin-column approach. Results from these experiments are given in Tables 4.2 and 4.3.

<table>
<thead>
<tr>
<th>Table 4.2 : Comparison of binding of NFX to complex of DNA with wild-type and GyrAAla83 gyrases</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>[DNA]</th>
<th>[AwtB2]</th>
<th>[AAla83B2]</th>
<th>[NFX]Total</th>
<th>[NFX]Bound b</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 a</td>
<td>380</td>
<td>-</td>
<td>7800</td>
<td>4.3</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>380</td>
<td>7800</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* a all concentrations quoted in nM  
  
  b after deduction of NFX alone values

According to this experiment, the binding of NFX to gyrase-DNA complex is reduced at least 4-fold by the Ser83 → Ala mutation. The data are not ideal since the binding to sensitive enzyme-DNA complex is only a quarter of the average observed on other occasions. Unfortunately the experiment has not been repeated, and the limited availability of the labelled quinolone does not allow for imminent reassessment. Nevertheless, it is apparent that the loss of a hydroxyl group associated with mutation of serine to alanine has been sufficient to cause a measurable reduction in quinolone binding to the gyrase-DNA complex. It was felt that the more resistant GyrATrp83 mutant would give a clearer impression of the influence of point mutations on NFX binding, and work proceeded with this enzyme.
Experiments comparing binding to complex of DNA with sensitive and GyrATrp83 gyrase were preceded by the discovery that complete cleavage of the 147 bp DNA fragment at the expected site required incubation at 25°C for at least 3 hours (see Section 3.5.2). By increasing the time of incubation in binding assays from 1 to 3.5 hours, it was hoped to increase the observed binding of NFX to the wild-type gyrase-DNA complex, on the assumption that for complete double-stranded cleavage, at least one, and more likely two, quinolone molecules per gyrase-DNA complex would be required.

Since both the wild-type GyrA and GyrATrp83 were new preparations, gel shift assays to establish the concentration of gyrase required to give complete retardation of the DNA were performed (data not shown). It transpired that a lower concentration of the mutant enzyme than of this preparation of sensitive GyrA (each in combination with GyrB) was required to "move-up" all of the 147 bp DNA. To have used a lower concentration of the resistant protein in binding assays would have left open the possibility that reduced binding was attributable to a reduction in the enzyme concentration rather than, or in addition to, the significance of the point mutation. The concentration of GyrATrp83 was therefore increased to match the concentration of GyrAwild-type. The results of binding assays are given in Table 4.3.

Table 4.3: Investigating the significance of GyrASer63→Trp mutation on the binding of NFX

<table>
<thead>
<tr>
<th>[DNA]</th>
<th>[A\textsuperscript{wr2}B\textsubscript{2}]</th>
<th>[A\textsuperscript{Trp83}B\textsubscript{2}]</th>
<th>[NFX]\textsubscript{Total}</th>
<th>[NFX]\textsubscript{bound}\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>86\textsuperscript{a}</td>
<td>700</td>
<td>-</td>
<td>7800</td>
<td>11.9 (1.6)</td>
</tr>
<tr>
<td>-</td>
<td>700</td>
<td>-</td>
<td>7800</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>86</td>
<td>-</td>
<td>700</td>
<td>7800</td>
<td>0.2 (0.2)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>700</td>
<td>7800</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>86</td>
<td>-</td>
<td>-</td>
<td>7800</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} all concentrations quoted in nM

\textsuperscript{b} after deduction of NFX alone values; standard deviations in parentheses
Binding was detected to the complex of wild-type gyrase with DNA but not to either the protein or the DNA alone. Unfortunately, and despite the optimisation of conditions to those giving full DNA cleavage at the expected site, the binding to the gyrase-DNA complex was not improved and was, if anything, proportionally lower than in the earlier studies. Possible explanations for this observation are given in the discussion below. The GyxA<sup>T983</sup> mutation was sufficient to reduce binding to the gyrase-DNA complex to an undetectable level. The clear implication is that quinolone resistance can be conferred by a reduction in binding of the drug to gyrase-DNA complex solely on the basis of a point mutation in the Gyrase A subunits.

Not surprisingly, in the light of the result with the gyrase-DNA complex, no binding could be detected to A<sup>T983</sup>B<sub>2</sub> alone. In agreement with the previous studies, no binding to DNA alone was observed.

4.3.2 Effect of [NFX]<sub>Total</sub> on elution of NFX from the column According to the current model of quinolone interaction with gyrase and DNA, the binding of drug is cooperative (Shen et al., 1989a). Cooperativity is believed to stem from ring stacking and hydrophobic interaction between the first and subsequent quinolone molecules. In such a scenario, raising the overall quinolone concentration should result in a sigmoidal binding curve; as the concentration becomes high enough for the binding of one molecule this should lead to more rapid binding of further molecules with a levelling off if and when saturation occurs. The [NFX]<sub>Total</sub> in all the spin-column experiments reported thus far has been a constant 2.5 μg/ml (7800 nM), a concentration known to be sufficient to give maximal cleavage in an hour (data not shown).

To investigate the effect of the overall [NFX] on the quantity of NFX passing through the column, a series of experiments was performed in which increasing [NFX]s were incubated in the presence and absence of wild-type gyrase (380 nM) and 147 bp DNA (72 nM) for 1 hour at 25°C before centrifugation through a spin-column in the normal manner. The results are given in Table 4.4 and Figures 4.1 and 4.2.
FIGURE 4.1: Comparison of NFX passing through a spin-column in the
presence and absence of gyrase-DNA complex.
FIGURE 4.2: Determination of NFX1 WHDl complex (NFX1 WHDl complex) - NFX1 WHDl complex.

Gyrase-DNA complex (NFX1 WHDl complex - NFX1 WHDl complex)
Table 4.4: The influence of [NFX]_{Total on} on [NFX] passing through column

<table>
<thead>
<tr>
<th>[NFX]_{Total on}, μM</th>
<th>[NFX] through column under conditions stated, nM</th>
<th>(i) drug alone</th>
<th>(ii) with complex</th>
<th>(ii) - (i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.31</td>
<td>0.03</td>
<td>0.32</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>0.78</td>
<td>(0.08)(^a)</td>
<td>0.71</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>1.60</td>
<td>0.24</td>
<td>1.58</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>3.90</td>
<td>(0.52)</td>
<td>4.16</td>
<td>3.64</td>
<td></td>
</tr>
<tr>
<td>7.80</td>
<td>(1.07)</td>
<td>6.59</td>
<td>5.52</td>
<td></td>
</tr>
<tr>
<td>15.6</td>
<td>2.93</td>
<td>10.93</td>
<td>8.90</td>
<td></td>
</tr>
<tr>
<td>31.2</td>
<td>4.37</td>
<td>18.25</td>
<td>13.88</td>
<td></td>
</tr>
<tr>
<td>62.4</td>
<td>8.74</td>
<td>30.58</td>
<td>21.84</td>
<td></td>
</tr>
<tr>
<td>124.8</td>
<td>16.22</td>
<td>78.62</td>
<td>62.4</td>
<td></td>
</tr>
<tr>
<td>156</td>
<td>34.32</td>
<td>76.44</td>
<td>42.12</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Values in parentheses have not been determined experimentally but are calculated from the other "drug alone" data, assuming a linear relationship.

Figure 4.1 shows that the increase in the concentration of NFX passing through the column in the absence of gyrase-DNA is directly proportional to the initial concentration added. With the possible exception of the highest concentration tested (156 μM NFX), the graph reveals a linear relationship between [NFX]_{on} and [NFX]_{off}. The breakthrough of NFX would be expected to increase dramatically when the Sephadex beads became saturated with drug and it is possible that this is beginning to occur at about 150 μM, which is some 20-fold higher than the standard NFX concentration used in the binding assays above (7.8 μM).
It was initially unclear whether the [NFX] binding to the gyrase-DNA complex is increasing linearly throughout the experiment or is beginning to plateau (Figure 4.1). Subtraction of the drug alone values from the [NFX] carried through the column in the presence of gyrase-DNA complex (Figure 4.2) supports the latter conclusion, serving to accentuate the plateauing of NFX binding. It is probably coincidental that saturation of binding is apparently occurring at approximately one NFX molecule per A$_2$B$_2$-DNA complex (72 nM). As outlined above, it would be anticipated that at least two quinolone molecules per complex would be required to cause double-stranded cleavage and this is supported, in part, by the observation of single- rather than double-strand breaks at low quinolone concentrations (Snyder and Drlica, 1979). Additionally, complete double-stranded cleavage of the 147 bp DNA fragment can occur at 7.8 μM NFX (see Section 3.5.2) well below the concentration where saturation is apparently occurring here. There is no evidence for co-operativity of NFX binding in this work.

4.3.3 Studies monitoring DNA cleavage

As an alternative to recording binding of $^3$H-NFX, and prompted in part by the limited availability of radiolabelled quinolone, DNA cleavage was used as an indirect monitor of binding. Cleavage can only occur when gyrase, DNA and drug are all present. The spin-column procedure was followed by addition of SDS (to a final concentration of 0.2%) and 1 mg/ml proteinase K. Samples were incubated at 37°C for 30 min, the reactions were stopped by addition of an equal volume of chloroform:isoamyl alcohol (24:1) and a half volume of stop dye. Products were analysed by electrophoresis in an 8% polyacrylamide (19:1) gel and visualised by staining with ethidium bromide (see Section 2.4.2).

4.3.3.1 Stability of gyrase-quinolone-DNA complex during centrifugation In order to investigate whether gyrase-quinolone-DNA complex was significantly disrupted by the spin-column technique, comparison was made between the cleavage that occurred in samples that had been subject to rapid gel-filtration and those that had not. A 300 µl
reaction mixture containing 380 nM gyrase, 72 nM DNA and 7800 nM NFX was incubated for 1 hour at 25°C and then split into 6 equal aliquots (50 μl each). 2 samples were treated directly with SDS and proteinase K according to the standard procedure. The remaining aliquots were passed through a spin-column. Eluant from two of the columns was collected in Eppendorf tubes that had been primed with 5 μl of 2% SDS prior to centrifugation; the other two had 5 μl of water. In this way it was intended to show whether any significance could be attached to the time that material spent in the bottom of the tube whilst centrifugation continued. When the rotor had come to rest, 5 μl of 2% SDS was added to the "water" tubes, and vice versa. This was followed by addition of proteinase K, incubation and subsequent electrophoresis in the normal way.

The results are given in Figure 4.3. The extent of cleavage may be slightly lower in the samples that were passed through a spin-column, but there is certainly no evidence of disruption of the gyrase-quinolone-DNA ternary complex in a manner that would explain the apparent sub-stoichiometric binding of ^3H-NFX reported in Section 4.3.2.2. Comparable cleavage was observed for the samples that were centrifuged directly into SDS and those that had the detergent added once the rotor had come to rest, implying that no significant breakdown of ternary complex was occurring in this phase.

4.3.3.2 Investigation of the stability of the gyrase-quinolone-DNA complex
Since the vast majority of quinolone added to a spin-column is retained within the Sephadex beads, any DNA cleavage mediated by gyrase after centrifugation must have been induced by drug brought through the column in association with the gyrase-DNA complex. By continuing the incubation of such a reaction after centrifugation it should be possible to use the extent of cleavage as an indicator of the stability of the gyrase-quinolone-DNA complex. A 160 μl mixture of gyrase (700 nM), 147 bp DNA (60 nM) and CFX (20 μg/ml, 60 μM) was incubated for 2 hours at 25°C. The reaction mixture was then split into 4 equal aliquots, each was passed through a spin-column and the aliquots were then repooled (this slightly convoluted procedure was followed in order to avoid overloading any one column). At time intervals from 5 min to 3 hours after spinning, a
Controls were performed in order to ensure that significant dissociation of quinolone from the gyrase-DNA-complex was not occurring during centrifugation. Comparison was made of the extent of cleavage resulting from treatment with SDS and proteinase K in samples that had been passed through a spin-column and in samples that had not been subject to centrifugation. Two spin-column conditions were investigated; samples were either passed directly into SDS or into an equal volume of water followed by addition of SDS when the rotor had come to a halt. This approach was taken to ensure that dissociation was not occurring in the spin-column eluant during the 2 min of centrifugation. Duplicate assays are shown.
sample was taken and treated with SDS and proteinase K in the normal way. Each sample
was incubated for 30 min, then the reactions stopped with chloroform:isoamyl alcohol
(24:1) and stop dye. The results of electrophoresis are shown in Figure 4.4.

The constant level of DNA cleavage in all tracks indicates that the ternary complex
is stably maintained for the full 3 hours after spinning, although the 147 bp band at the
final timepoint was beginning to show signs of degradation. Since rebinding of CFX to
the gyrase-DNA complex is unlikely to occur in the greatly reduced [CFX]_{overall} after
centrifugation, it may be fair to assume that the quinolone binds tightly. This result is
different from those reported in Section 3.5.2 where, in the absence of a spin-column step,
the extent of cleavage increased with prolonged incubation.

4.3.3.3 Pairwise mixing of gyrase, DNA and CFX A series of assays was
performed in which one component (gyrase, DNA or CFX) was omitted from the initial
reaction mixture and added after the other two components had been incubated for 2 hours
and passed through a spin-column. The new reaction mixture was then incubated for a
further 2 hours prior to addition of 0.2% SDS and 1 mg/ml proteinase K and 30 min
incubation at 37°C. The results are given in Table 4.5 and Figure 4.5.

When gyrase and DNA were incubated together and CFX (20 µg/ml, 60 µM) was
added following centrifugation, cleavage was observed, but when gyrase alone was
incubated with quinolone and DNA added following centrifugation, no cleavage was
detected. Similarly, no cleavage was observed when DNA and CFX were incubated
together and enzyme added subsequently. These results support the findings with \(^3\)H-NFX
(Section 4.3.2.1), ie. detection of binding to gyrase-DNA complex but not to either gyrase
or DNA alone.

4.4 Binding analysis using fluorescence techniques

A number of studies have attempted to exploit the intrinsic fluorescence of
quinolones as a handle for monitoring drug-target interactions. Shen and coworkers have
used fluorescence in conjunction with membrane filtration to study binding of OXO, NAL
FIGURE 4.4: Use of DNA cleavage as an indicator of quinolone-gyrase-DNA complex stability after centrifugation.

147 bp DNA was incubated with gyrase and CFX for 120 min at 25°C prior to passage through a spin-column. After centrifugation, incubation was continued at 25°C for the time indicated (min) before treatment with SDS and proteinase K in the normal manner. For purposes of comparison, one sample was not passed through a spin-column but was treated with SDS and proteinase K immediately after the initial 120 min incubation (track "before").
FIGURE 4.5: Investigation of CFX binding to gyrase and/or DNA by monitoring of DNA cleavage after rapid gel-filtration

DNA cleavage was used as an indicator of quinolone binding. Combinations of 147 bp DNA (D), gyrase (G) and CFX (C) were incubated together for two hours and then passed through a spin-column. After centrifugation, any component that had been omitted from the initial reaction mixture was added. Curvy brackets are used in the shorthand notation to indicate those components that were included in the initial incubation and subsequent centrifugation, any reagent added afterwards is listed outside the bracket. Samples of untreated 147 bp DNA (D) and cleavage in ternary complex that had not been centrifuged (D+G+C) are also shown.
and NFX to DNA (Shen et al., 1989b). Another study failed to see any change in fluorescence spectra when a variety of different DNAs were added to a solution containing NFX (Palu' et al., 1988). In a reply to that report, Shen argues that the intrinsic fluorescence of NFX is quenched by addition of thermally denatured calf thymus DNA in a manner proportional to the concentration of DNA added and that this is another indicator of quinolone binding to DNA (Shen, 1989).

Table 4.5: Ciprofloxacin-induced cleavage of DNA by gyrase

<table>
<thead>
<tr>
<th>Incubation prior to centrifugation (2 hours)</th>
<th>Subsequent Addition (2 hours incubation)</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gyrase (700 nM)</td>
<td>DNA (60 nM)</td>
<td>CFX (60 μM)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The work presented here in Section 4.4 has attempted to employ two fluorescence techniques to investigate quinolone binding. Of the two, fluorescence spectroscopy (Section 4.4.1) is the more commonly used technique in studying biological systems; fluorescence depolarisation (Section 4.4.2) requires a more sophisticated machine.
4.4.1 Fluorescence spectroscopy

4.4.1.1 Principle and Theory  The first step in the generation of a fluorescent signal is the absorption of light by a fluorophore which results in the raising of an electron from the "ground state" to a higher energy "excited" state, a process that is common to all absorption spectroscopy (Campbell and Dwek, 1984). The excited electron must eventually return to the ground state, and for non-fluorescent molecules this generally involves the dissipation of the excess energy to the surroundings in the form of heat or vibrational energy. In the case of a fluorophore, the energy can be re-radiated (emitted) in the form of light, and this is termed fluorescence. There is a finite time, typically $10^{-10}$ seconds, during which the electron is in an excited state prior to its return to the ground state. During this time some energy will be lost as heat or vibration and the re-emitted light therefore has less energy, i.e. it has a longer wavelength than the light that caused the excitation.

Two kinds of fluorescence spectra are studied, these are excitation and emission spectra. An excitation spectrum is measured by monitoring the intensity of the emission signal at a single wavelength as a function of the excitation wavelength. To see an emission spectrum, excitation occurs at a single wavelength and the intensity of emission is scanned as a function of emission wavelength. As a result of the loss of energy during the lifetime of the excited species, as outlined above, the peak(s) of an emission spectrum will appear at a longer wavelength than the peak(s) of the excitation spectra.

4.4.1.2 Application to study of quinolone binding  With quinolones being intrinsically fluorescent, the hope would be to see an alteration in the intensity, or preferably in the wavelength, of the emitted light. Conditions for the initial experiments were identical to those for the later spin-column assays with wild-type gyrase and 147 bp DNA, i.e. 86 nM DNA, 700 nM gyrase and 7800 nM NFX with the reaction volume being increased from 120 μl to 200 μl. An excitation wavelength of 340 nm and an emission wavelength of 420 nm were selected in accordance with previous work (Paltu' et al., 1988; Shen, 1989). Because depolarisation experiments were being conducted in parallel with
the fluorescence spectroscopy studies, there were polarising filters in the light beam both before and after its passage through the sample cuvette. Each polarising filter can be considered to reduce the intensity of the light by approximately an order of magnitude. This concentration of NFX was however found to swamp any interaction resulting from the DNA and/or enzyme (data not shown) and for further studies the conditions were therefore altered in the following way. The concentration of quinolone was reduced from 2.5 μg/ml (7800 nM for NFX) to 0.25 μg/ml (760 nM for CFX) and this was accompanied by a shift to using CFX instead of NFX, since the former is thought to have a higher affinity for gyrase and DNA. The concentrations of gyrase and DNA were increased to 1150 nM and 140 nM respectively, thereby maintaining the molar ratio determined previously.

Excitation and emission spectra for CFX alone and in the presence of gyrase, of DNA and of both together were compared (Figures 4.6 and 4.7). The samples were mixed directly in the cuvette and where necessary the standard volume and concentration of buffer components was maintained by addition of enzyme buffer or water. The transfer of light through the sample was monitored in three directions. Channels A and B are required for the detection of fluorescence depolarisation (see below) and monitor light fluorescing from the sample perpendicular to the direction of the original beam. Channels A and B have polarising filters orientated at 90° to one another. Channels A and C are responsible for detection of the fluorescence spectra. Under perfect conditions only Channel A would be required, but comparison with Channel C, which detects light heading back in the reverse direction to the original beam, facilitates screening of the data for any “results” that have erroneously arisen from “flicker” in the light source. For this experiment, the detectors were set up in the following way:

- Channel A: Gain 100  hu 1000
- Channel B: Gain 10   hu 800
- Channel C: Gain 1    hu 270

In agreement with Shen, comparison of emission spectra (Figure 4.6) shows that DNA can quench the intensity of the fluorescence signal. The addition of gyrase alone,
Figure 4.6: CFX Fluorescence Emission Spectra
conversely, led to marked enhancement of the signal, and in the presence of both gyrase and DNA the signal intensity was intermediate, and closer to that observed for drug alone. In no instance did interaction result in a noticeable shift of the wavelength of emitted light. When the excitation spectra were compared, different trends were apparent, Figure 4.7. CFX alone had peak values at about 270 nm and particularly at about 330 nm. On addition of gyrase, both peaks were enhanced. When DNA was added in the absence of gyrase, the peak at 330 nm was quenched but the intervening range between the 270 and 330 nm peaks was enhanced. Subsequent addition of gyrase served to increase this enhancement (and to counter the quenching at 330 nm). A smaller peak at about 365 nm was present in the CFX alone and CFX plus gyrase samples, but not in the presence of DNA.

4.4.1.3 Comparison of sensitive and quinolone-resistant gyrases Since no binding had been detected by fluorescence depolarisation (see Section 4.4.2.2), the polarising filters were removed in order to allow a more intense signal from a lower CFX concentration, and this was dropped to 0.05 µg/ml (152 nM). The gyrase and DNA concentrations were 700 nM and 140 nM respectively. The resulting emission spectra are shown in Figure 4.8. In this instance, the spectrum of CFX was only marginally quenched by DNA. The effects of wild-type gyrase alone, and of GyrA<sub>Tp83</sub> gyrase both in the presence and absence of DNA, were apparently identical, giving the same enhancement of the peak but no shift in its wavelength. Addition of wild-type gyrase and DNA, however, gave both enhancement and a shift in the wavelength of the peak from about 400 nm to about 420 nm. Unfortunately, a repeat experiment failed to reproduce these results.

4.4.2 Fluorescence depolarization

4.4.2.1 Principle and Theory Light can normally be considered to have a vertical and a horizontal component. By the use of polarizing filters orientated in one of these directions, it is possible to effectively remove the perpendicular component and the light passing through the filter is then said to be "plane-polarised". When plane-polarised light is directed at a fluorescent molecule, the outcome will be dependent on the values of \( t \), the lifetime of the excited electronic state and \( \chi \), the speed at which the molecule is rotating. If
FIGURE 4.8: Comparison of CFX emission spectra in the presence of wild-type and quinolone-resistant gyrase.
t is very short or x very fast, the passage of light through the solution will apparently be
unaffected, and comparison of the light beam before and after the transmission will reveal
that the light remains orientated in the original plane. If t is very long or x very slow, the
movement of the molecule will disrupt the passage of the light beam so that light is as
likely to be detected in the original plane or at 90° to it. In this case, the light is said to be
totally depolarised. The interesting, and potentially analytical, situation occurs when t and
x are of the same order of magnitude. If a fluorophore rotates in isolation at a speed that
does not affect the plane of polarisation, but is slowed by binding to a larger molecule in a
manner that leads to depolarisation of the light, this can be used as evidence that an
interaction has occurred.

In practice, polarisation is studied by measuring the fluorescence signal in two set
directions (A and B) whilst alternating the excitation polariser, i.e. the filter preceeding the
passage of light through the sample, between the vertical (0°) and the horizontal (90°)
positions. Polarisation (P) is then derived electronically according to the equation:

\[
\text{Polarisation (P)} = \frac{[(R_{\text{vert}}/R_{\text{horiz}}) - 1]}{[(R_{\text{vert}}/R_{\text{horiz}}) + 1]} = \frac{R_{\text{corr}} - 1}{R_{\text{corr}} + 1}
\]

Where: 
- \( R_{\text{vert}} \) is the ratio A/B when the excitation polariser is set vertical (0°)
- \( R_{\text{horiz}} \) is the ratio A/B when the excitation polariser is set horizontal (90°)

An alternative measure of depolarisation, termed anisotropy (r) is derived from the
polarisation value P by the equation:

\[
r = \frac{2P}{3-P}
\]

4.4.2.2 Application to study of quinolone binding
Given the clear difficulties
posed by the concomitant enhancement of fluorescent signal with gyrase and quenching by
DNA, it was hoped that depolarisation would allow determination of the target. With
quinolones being small fluorescent ligands and with both gyrase and 147 bp DNA being
relatively large molecules, this would appear to be an ideal case for fluorescence
depolarisation work. The anticipated difficulties, if any, would stem from the bound drug
being only a small percentage of the overall quinolone concentration and it was feared that this could cause a high background signal and mask any increase in depolarisation.

Unfortunately, these reservations proved to be well-founded; despite attempts with a variety of conditions no detectable change in polarisation could be recorded. As with the fluorescence spectroscopy studies, the initial depolarisation work was conducted under the same conditions as the spin-column experiments of Section 4.3.2.2; i.e. 86 nM DNA, 700 nM gyrase and 7800 nM NFX. It was quickly evident that unbound drug was swamping any change in signal (data not shown) and a switch to using 140 nM DNA, 1150 nM gyrase and 760 nM CFX ensued. A typical result is shown in Table 4.6. These data are derived from integrations of 120 secs with the filter in both positions for 6-10 repetitions.

The channel settings were: A: Gain 100 hu 1000
B: Gain 10 hu 800
C: Gain 1 hu 270.

<table>
<thead>
<tr>
<th>Components</th>
<th>Correction (C)</th>
<th>Polarisation (P)</th>
<th>Anisotropy (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFX alone</td>
<td>0.785 +/- 0.003</td>
<td>0.042 +/- 0.001</td>
<td>0.028 +/- 0.001</td>
</tr>
<tr>
<td>CFX + DNA</td>
<td>0.785 +/- 0.004</td>
<td>0.043 +/- 0.003</td>
<td>0.029 +/- 0.002</td>
</tr>
<tr>
<td>CFX + Gyrase</td>
<td>0.722 +/- 0.010</td>
<td>0.045 +/- 0.003</td>
<td>0.030 +/- 0.002</td>
</tr>
<tr>
<td>CFX + Gyrase + DNA</td>
<td>0.769 +/- 0.005</td>
<td>0.044 +/- 0.002</td>
<td>0.030 +/- 0.001</td>
</tr>
</tbody>
</table>

In no instance were the polarisation or the anisotropy values significantly altered by the presence of gyrase and/or DNA. Since the components of these assays were being mixed directly in the cuvette and were not, therefore, being afforded the preincubation time that was a feature of the spin-column experiments, it was important to ensure that this difference was not contributing to the failure to detect interaction. Reactions containing 7.6 μM and 0.15 μM CFX were prepared in the presence and absence of gyrase-DNA
complex and incubated at 25°C for 4 hours prior to fluorescence analysis. The channel settings were:

A: Gain 100, hv 800; B: Gain 10, hv 625 for the 7.6 μM CFX samples and
A: Gain 100, hv 1100; B: Gain 10, hv 850 for the 0.15 μM CFX samples.

The results are given in Table 4.7. Once again, there was no detectable alteration in the polarisation of the signal emerging from the samples, and there therefore seemed little point in using depolarisation to compare the effect of wild-type and GyrA_{Tyr83} gyrase. This decision had the benefit that the polarising filters could be removed for comparison of fluorescence spectra, enhancing the intensity of the signal, as discussed above. This line of investigation was therefore closed.

Table 4.7: Measurement of depolarisation after 4 hour incubation

<table>
<thead>
<tr>
<th>[CFX], μM</th>
<th>Components</th>
<th>Correction (C)</th>
<th>Polarisation (P)</th>
<th>Anisotropy (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.60</td>
<td>CFX alone</td>
<td>1.052</td>
<td>0.028</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td>+/- 0.003</td>
<td>+/- 0.001</td>
<td>+/- 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFX + DNA</td>
<td>1.130</td>
<td>0.030</td>
<td>0.020</td>
</tr>
<tr>
<td>+ Gyrase</td>
<td>+/- 0.003</td>
<td>+/- 0.001</td>
<td>+/- 0.001</td>
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<tr>
<td>0.15</td>
<td>CFX alone</td>
<td>0.998</td>
<td>0.100</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>+/- 0.009</td>
<td>+/- 0.005</td>
<td>+/- 0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CFX + DNA</td>
<td>0.906</td>
<td>0.095</td>
<td>0.066</td>
</tr>
<tr>
<td>+ Gyrase</td>
<td>+/- 0.011</td>
<td>+/- 0.005</td>
<td>+/- 0.004</td>
<td></td>
</tr>
</tbody>
</table>
4.5 Discussion

Determination of the primary site of interaction of quinolone antibacterials with gyrase and/or DNA has been a contentious issue. A current hypothesis suggests that quinolones bind by hydrogen bonding to a single-stranded DNA pocket at the active site of the enzyme (Shen et al., 1989a). The rapid gel-filtration experiments reported in this chapter support the suggestion that stable binding of drug requires the presence of both DNA and gyrase, but have found no evidence for binding to either alone. In conflict with the model, a single point mutation in the gyrase A subunits, GyrASer83 → Trp, was able to abolish binding to the gyrase-DNA complex. Supercoiling assays (Section 3.4.2) reveal that enzyme with this mutation is able to perform the introduction of negative supercoils into closed-circular DNA as efficiently as the wild-type enzyme. In so doing, it is required to open up the DNA in a similar manner to its quinolone-sensitive counterpart. It would appear, therefore, that the major determinant of binding to the gyrase-DNA complex is the GyrA subunit. This agrees with the frequent occurrence of quinolone-resistant point mutations in the region between residues 67 and 106 of GyrA, and particularly at residue 83.

The spin-column technique involves centrifugation and the reactions are not under true equilibrium conditions. It is therefore possible that quinolones form weak interactions with either gyrase or DNA alone. Preliminary experiments using alteration of the fluorescence of CFX could be interpreted to show that the drug can indeed interact with both gyrase and DNA in isolation; the presence of gyrase leads to enhancement of the peak in a CFX emission spectrum while DNA leads to a quenching of the fluorescent signal. On another occasion, and under different experimental conditions, a shift in the peak wavelength in an emission spectrum was observed with wild-type gyrase-DNA complex but not with the GyrATrp83 form.

A problem associated with the use of fluorescence to monitor biological systems stems from the extreme sensitivity of the technique. Vigilance therefore needs to be exercised in the interpretation of all fluorescence experiments to avoid false-positive results. Since apparently identical reaction mixtures have failed, on occasions, to
reproduce the results cited it is therefore wise to consider them with a certain reserve. There may be scope for further study involving fluorescence.

The greatest worry from experiments performed using the spin-column approach to studying binding stems from the apparent sub-stoichiometric binding of NFX to gyrase-DNA complex. It may be that despite HPLC repurification, the \(^3\)H-NFX contained other tritiated material that gave the impression that more counts of \(^3\)H-NFX were being added than was actually the case. Such an interpretation is certainly uncomfortable, and every effort has been made to rule it out as the correct explanation. The later spin-column experiments were carried out under conditions that were known to lead to full cleavage of the 147 bp DNA fragment at the expected site. To do so would require the influence of at least one, and more likely two, quinolone molecules per gyrase-DNA complex. If the binding data prove to be correct and there really was less than one NFX molecule present per gyrase-DNA complex, another explanation would have to be sought. It is feasible, though unlikely, that the complex could be permanently modified by quinolone so that the drug could dissociate away but its effect would persist.

Further experiments using the spin-column technique could certainly be worthwhile. The availability of a different radiolabelled quinolone at high specific activity would aid the discernment of whether binding to gyrase or DNA is a real phenomenon, and could also be put to use for other binding methods, such as equilibrium dialysis. It would be particularly interesting to study the effect of a Gyra\(_{\text{Tyr122 \to Phe}}\) mutation using this approach. This alteration removes a hydroxyl group from the residue responsible for the breakage and reunion of DNA. It has been shown previously that Gyra\(_{\text{Phe122}}\) gyrase can bind to DNA but is unable to carry out the catalytic reactions (Wilkinson and Wang, 1990). By employing this mutant it should be possible to show whether or not quinolone binding to the gyrase-DNA complex requires the breakage of the DNA strands.

Binding could also be studied indirectly in cleavage assays. The authors of an experiment comparing the selection by gyrase of cleavage sites on linearised pBR322 under the influence of a range of different quinolones concluded that the same sites were chosen irrespective of which drug was used (Walton and Elwell, 1988). Whilst it is true
that all of the quinolones direct cleavage to the same absolute positions on the linearised plasmid, they fail to comment on the different intensity of the cleaved DNA fragment. While all of the drugs have directed cleavage to the same set of sites, say for simplicity A, B and C, some of the drugs appear to favour cleavage most frequently at site B and to a lesser extent at sites A and C (eg. OFX in their Figure 2), while others offer no distinction, cleaving A, B and C with equal frequency (CFX). A third drug may cleave at site C more frequently than at A or B (NFX), while a fourth does exactly the reverse (CI-934). Since it is fair to assume that the enzyme and plasmid in each of these assays is identical, then it must be a feature of drug itself that is influencing this selection. By carrying out a series of cleavage assays with quinolones that differ at only one substituent or functional group it should be possible to make some headway in assigning functionality to the different components of the drug molecules. Clearly if high enough drug concentrations were used, the preference of resistant proteins could also be investigated.

Ultimately, crystallography is going to be the most useful source of information. The recent work on the N-terminal fragment of GyrB (Wigley et al., 1991) and the preliminary characterisation of the N-terminal fragment of GyrA (Reece et al., 1990) are encouraging pointers to this being a fruitful avenue. If quinolones really do require both the enzyme and DNA in order to bind, the formation of crystals of such a large assembly is likely to be many years away. Assignment of residues in the N-terminal fragment of GyrA, including the ‘quinolone-resistance determining’ region between residues 67 and 106 and of the catalytic residue Tyr122 is a much more realistic prospect and could reveal tertiary structure information into which binding of quinolone molecules could be modelled.
CHAPTER 5

The influence of DNA gyrase and quinolones on DNA replication and RNA transcription
5.1 Summary

It has been proposed that quinolone drugs may initiate cell death by acting to stabilise the interaction of gyrase with DNA in a manner that blocks the passage of polymerases along the template. To investigate the validity of this "poison" hypothesis the effect of quinolones and gyrase on in vitro DNA replication (Section 5.3) and transcription systems (Section 5.4) has been investigated. Despite technical difficulties encountered with the in vitro pBR322 replication system, it was clear that quinolones inhibited the synthesis of DNA by DNA polymerase III in a gyrase-dependent manner. This process was less sensitive to CFX when the gyrase A subunit carried a Ser83 to Trp point mutation that is known to confer quinolone resistance. When DNA synthesis was studied in the presence of both GyrA_{Ser83} and GyrA_{Trp83}, an intermediate response was observed.

Transcription by T7 and E.coli RNA polymerases of a linear template, derived from pBR322 by PCR, is unaffected by the presence of gyrase or CFX alone, but the passage of polymerase along the template is blocked by the formation of a stable gyrase-quinolone-DNA complex. This result has implications for the manner in which quinolone antibacterials initiate cell death. In the presence of CFX, gyrase was found to protect a region of approximately 26 base pairs from transcription by T7 RNA polymerase, with the strongest protection for a core of 20 bp around the site of gyrase-mediated DNA cleavage.

5.2 Introduction

A paradoxical feature of the action of quinolones against bacteria is their capacity to elicit cell death at concentrations some 5-100 fold lower than that required to inhibit the major reactions of DNA gyrase, for example DNA supercoiling, in vitro (Gellert et al., 1977; Zweerink and Edison, 1986). Such an observation could be explained if an active import system was in operation, resulting in the true intracellular drug concentration being higher than the overall concentration supplied. Although there may be some quinolone accumulation occurring, an internal quinolone concentration up to 4 times higher than the external concentration has been noted (Bedard et al., 1987; Hooper et al., 1989), this would not be sufficient to account for this "MIC paradox".
5.2.1 The "poison" hypothesis

An alternative explanation is available in the form of the "poison" hypothesis which proposes that cell death will occur when only a small proportion of the intracellular gyrase population has quinolone bound, possibly as a result of the blocking of replication fork movement by a ternary complex in which the presence of quinolone has somehow "anchored" gyrase to the template DNA (Kreuzer and Cozzarelli, 1979). The in vitro replication of bacteriophage T7 DNA was investigated in the presence of a temperature-sensitive gyrase, in the presence and absence of NAL. At the permissive temperature, T7 replication was inhibited by addition of NAL, even though the T7 genome is linear and should therefore have no topological constraints requiring gyrase activity. At the restrictive temperature, viral DNA replication was restored, irrespective of the addition of quinolone. It was concluded that inhibition of replication resulted from the quinolone-dependent stabilisation of the gyrase-DNA complex in a manner that prevented passage of the polymerase. This idea is supported by filter-binding studies, which have shown that the presence of oxolinic acid stabilises gyrase-DNA complex (Higgins and Cozzarelli, 1982) and the detection of quinolone binding to gyrase-DNA complex reported elsewhere (Shen et al., 1989c; Section 4.3 of this study). Although the model was formulated to explain the effect of gyrase and quinolone on DNA replication, it can be applied with equal validity to transcription, or indeed to any situation in which a macromolecular assembly tracks along the DNA.

A "poison" hypothesis of this kind could explain the dominance of a quinolone-sensitive (Qs) phenotype over quinolone-resistance (Qr), even when Qr GyrA protein is provided in great excess from an expression vector (Hane and Wood, 1969; Hallett and Maxwell, 1991). Only a few sensitive complexes would need to be quinolone-bound in order for replication to be blocked and cell death initiated. In keeping with this suggestion is the observation that the minimum inhibitory concentration required to kill 50% of cells (MIC50) correlates more closely to the quinolone concentration required for the first detection of inhibition of supercoiling activity than with the IC50, the concentration for
50% inhibition (Walton and Elwell, 1988). The "poison" hypothesis has also been invoked to explain the action of anti-tumour drugs against eukaryotic topoisomerases (Liu, 1989).

5.2.2 Requirements for in vitro DNA replication

If such a hypothesis were correct, investigation using a reconstituted in vitro DNA replication system might be expected to reveal firstly, inhibition of replication at a concentration equivalent to the MIC and secondly, a mixture in which both Q1 and Q8 gyrase are provided would be expected to exhibit behaviour characteristic of Q8 gyrase.

An in vitro system for ColEl-type DNA replication reconstituted from purified proteins has been developed by Marians and co-workers (Minden and Marians, 1985). Replication is initiated by the de novo synthesis of a primer RNA (RNA II) by RNA polymerase (RNAP), see Figure 5.1. The formation of this stable hybrid between RNA II and the leading-strand DNA template leads to specific unwinding of the DNA duplex in the origin region, with the displaced DNA strand (which will become the template for lagging-strand synthesis) being stabilised and prevented from forming intrastrand secondary-structure by a coating of single-strand binding protein (SSB). RNase H processes the hybrid, cleaving RNA II at one of three consecutive AMP residues, defined as the origin of DNA replication, within a cluster of five AMP residues, creating a 3'-OH terminus for the initiation of DNA synthesis by DNA polymerase I (Pol I) which, in keeping with all known cellular DNA polymerases, is incapable of de novo (unprimed) DNA synthesis.

Pol I extends the primer for 200-400 nucleotides in a 5' to 3' direction after which DNA polymerase III (Pol III HE) takes over synthesis. As Pol I passes a region 150 nucleotides downstream of the origin, the binding of SSB to the displaced ssDNA strand activates a primosome assembly site (PAS) at which a seven-protein complex known as a primosome will assemble in order to commence lagging-strand synthesis. A second PAS is activated on the leading-strand template approximately 400 nucleotides downstream of the origin. It is speculated that this second PAS may be involved in the switch from Pol I to Pol III HE-mediated DNA synthesis.
FIGURE 5.1: Model for ColE1-type replication
(reproduced from Minden and Marians, 1985)

Primosome assembly is understood to proceed in the following manner; coating of the PAS with SSB enables the PriA protein (also known as n' and factor Y) to recognise and bind the site, with PriB (formerly n protein) subsequently associating with the PriA-DNA complex. DnaT (i protein) transfers the DnaB helicase from a DnaB-DnaC complex to the PriA-PriB-DNA complex. PriA, PriB, PriC, DnaB, DnaC and DnaT are together termed the pre-primosome, the multiprotein unit being completed by the inclusion of DnaG (primase). Both PriA and DnaB possess helicase activity, operating in a 3' to 5' and 5' to 3' direction respectively. DnaB unwinds the DNA duplex in advance of the replicative machinery utilising energy derived from the hydrolysis of ATP. The function of PriC (n") remains unclear.

DnaG is a rifampicin-resistant RNA polymerase that synthesises primers for lagging-strand synthesis, with Pol III HE subsequently adding dNMPs directly onto the RNA primer without the involvement of Pol I. The combination of two conflicting features of DNA replication, namely that DNA is synthesised only in a 5' to 3' direction and that the DNA strands in any duplex run antiparallel is a conundrum resolved by the retrograde synthesis of Okazaki fragments. Lagging-strand DNA is prepared in fragments of 500-2000 nucleotides in the opposite direction to the overall movement of the replication fork, though there is some evidence for the machinery involved in this process being physically coupled to the components synthesising the leading strand. Because of the difficulties presented by lagging-strand synthesis, the process of DNA replication is therefore described as semi-discontinuous.

DNA polymerase III is itself a multiprotein complex, consisting of seven different subunits. α, ε and δ subunits constitute the core enzyme which has only feeble synthetic activity and low processivity, with both features being enhanced considerably by the addition of γ,δ,ε and β subunits. The β-subunit is thought to dissociate from the rest of the complex (known as Pol IIIε), with reassociation favoured by the presence of nucleotide. In practice, this tends to mean that Pol IIIε and β-subunit are purified separately. With the exception of the Pol III subunits, the other proteins required for the replication system have all now been cloned in expression vectors.
A system devised by Tomizawa and Itoh (1982) exploits the unusual 5'-pppUpU dinucleotide at the start of the RNA II primer to establish a limited initiation complex for ColE1-type (e.g. pBR322) DNA replication. RNA synthesis performed by RNA polymerase (RNAP) in the presence of UpU and the three rNTPs (CTP, GTP, ATP) but not UTP leads to the production of a stable 23 nucleotide RNA-template-RNAP ternary complex. Addition of rifampicin to the reaction mix inhibits further initiation of RNA synthesis, but has no effect on elongation of established primers once the fourth nucleotide is supplied.

Termination of replication can be studied using the in vitro replication system provided that the plasmid template (for example, pTH101) has, in addition to an appropriate origin, a 23 bp terminator site (ter) in the correct orientation and with the inclusion of Tus in the protein cocktail. Tus protein binds to ter and is sufficient to arrest the movement of Pol III HE-primosome replication forks (Hill and Marians, 1990). In the presence of Tus, replication of pTH101 is terminated at ter, leading to a product some 1650 nucleotides in length. A 147 bp DNA fragment incorporating the preferred site of quinolone-directed cleavage by gyrase of pBR322 (Fisher et al., 1986; Dobbs et al., 1992) was inserted into pTH101 between the ori and ter regions. The derivative plasmid, termed pTHN147, may provide a means to observe a defined-length product from blockage of replication by a gyrase-quinolone-DNA ternary complex.

DNA gyrase is believed to play a role in the initiation, elongation and termination stages of DNA replication (reviewed by McMacken et al., 1987). It is possible that the role of gyrase in initiation is indirect, reflecting the need for a negatively supercoiled DNA template rather than a direct interaction with any replication proteins. It is likely that gyrase is required to overcome the torsional difficulties resulting from movement of the replication machinery along the template. A conceptually similar problem in transcription has been described by Liu and Wang in terms of a twin supercoiled domain model, with positive superhelicity building up in front, and negative supercoils behind, the transcription assembly (Liu and Wang, 1987). At the termination of replication, daughter chromosomes need to be segregated. Observation of aberrant segregation in temperature-sensitive gyrase
mutants has been seen to require the decatenation activity of gyrase (Steck and Drlica, 1984) although a role in decatenation has been attributed to topoisomerase III (Digate and Marians, 1988).

5.2.3 Influence of DNA-bound protein on replication and transcription

The Tus-dependent termination of replication at ter is an example of the effect that a DNA-bound protein can have on replication fork progression (Hill and Marians, 1990). This does not indicate that replication will always be interrupted by confrontation with other proteins on the template; for example, in an in vitro T4 replication system, replication forks were found to pass through nucleosomes (Bonne-Andrea et al., 1990). Some evidence was found for fork pausing prior to reading through nucleosomes, but it was not necessary for histones to be displaced for replication to continue. Undoubtedly, the stability of the "roadblock" protein-DNA complex will have significant bearing on the outcome. It was recently reported that the Tus-ter complex is very stable; dissociation of Tus from a 37 bp oligonucleotide containing terB was found to be 50-100 times slower than the dissociation of lac repressor from a 36 bp operator fragment (Gottlieb et al., 1992).

A study on the effect of the eukaryotic topoisomerase I inhibitor camptothecin on the in vitro replication of SV40 DNA is particularly interesting (Ishimi et al., 1991). Camptothecin severely inhibited DNA synthesis in the reaction containing wild-type human topoisomerase I, but not with topoisomerase I purified from a camptothecin-resistant human lymphoblastoid cell line. The mutant enzyme, containing the alterations Asp533 to Gly and Asp533 to Gly, was over 125-fold resistant to camptothecin. It appears that replication fork movement is arrested when a drug-stabilised "cleavable" complex is formed, in which the topoisomerase is attached covalently to the DNA by a phosphotyrosine bond. Since the effect of camptothecin on topoisomerase I is analogous to the action of quinolones on gyrase, it does not seem unreasonable to expect that the latter might yield a similar outcome in an in vitro prokaryotic replication system.
Present evidence would suggest that a range of responses may occur when RNA polymerase encounters a protein bound in its path. There may be transient or partial release of the protein complex accompanying passage of the polymerase, or there may be stalling or termination of transcription. Two groups found that RNA polymerase from bacteriophage SP6 was able to read through a nucleosome core without detectable pausing, though they disagreed on whether the process displaces histones from the template DNA (Lorch et al., 1987) or not (Losa and Brown, 1987). A recent review has proposed a compromise scenario in which there is progressive displacement of nucleosome subunits (Van Holde et al., 1992). According to the model, H2A:H2B dimer is displaced by a transcription bubble as it approaches one side of the nucleosome, allowing the DNA around the first half of the nucleosome to be read. At this point the dissociated H2A:H2B dimer reestablishes contacts with the remaining units of the nucleosome core, before the H2A:H2B dimer from the other side is transiently separated from the core in a similar fashion.

Elongation by E. coli RNA polymerase has been reported to be blocked by the lac repressor-operator complex (Deuschle et al., 1986) and by an EcolI mutant that binds to the recognition sequence without cleaving the DNA (Pavco and Steege, 1990). Deuschle and coworkers showed that lac repressor blocks transcribing RNA polymerases in vitro and in vivo. Comparison of mRNA levels resulting from transcription of a CAT reporter gene in the presence and absence of the repressor protein showed that termination was 90% efficient and is proposed by the authors as a means of gene regulation by operator-bound proteins. In a hybrid system, the same group later showed that eukaryotic RNA polymerase II, but not T3 or T7 RNAP, was similarly blocked by lac repressor (Deuschle et al., 1990).

A single amino acid substitution at position 111 of EcolI (Glu to Gin) results in a mutant that retains high affinity for the wild-type EcolI recognition sequence but is defective in cleavage activity (Wright et al., 1989). Pavco and Steege investigated the ability of the mutant EcolI to block elongation by E. coli RNAP (Pavco and Steege, 1990) and T7 and SP6 phage RNAPs (Pavco and Steege, 1991). It appears that E. coli
RNAP is efficiently halted by EcoR I<sub>Gin111</sub>, with the transcription complex stably maintained in an active form that can resume elongation when the blocking protein is displaced by an increase in ionic strength. T7 and SP6 RNAP, however, while being partially blocked by the mutant, exhibit large levels of read-through transcription.

Westergaard and coworkers have investigated the influence of eukaryotic topoisomerase I and II on in vitro transcription by SP6 RNA polymerase (Bendixen et al., 1990; Thomsen et al., 1990). It was found that transcription was blocked by human topoisomerase I, but only in the presence of the inhibitor camptothecin and then only when cleavage occurred in the coding strand; the RNA polymerase transcribed unimpeded through complexes in which the topoisomerase was covalently attached to the non-coding strand. With topoisomerase I linked to the coding strand, the premature termination of transcription was observed 10 base pairs upstream of the nucleotide attached to the enzyme. Similarly, calf thymus topoisomerase II blocked transcription by SP6 RNA polymerase in the presence of an inhibitory agent, Ro 15-0216 (Thomsen et al., 1990). A protected region of 28 bp with a highly protected central core of 22 bp located symmetrically around the topoisomerase II-mediated cleavage site was detected. In the absence of any inhibitor, transcription was not impeded by the topoisomerase.

5.3 The effects of quinolones and gyrase on DNA replication in vitro

5.3.1 Gyrase mediates the inhibition of DNA replication by quinolones

The effect of CFX on DNA replication in the presence of wild-type and mutant gyrases was investigated. In the absence of gyrase, a significant background level, about 8 pmol, of incorporation was observed (see Figure 5.2) which was unaffected by the addition of CFX. On addition of gyrase, a significantly higher concentration of <sup>3</sup>H-dTMP, about 25 pmol, was incorporated in a quinolone-sensitive manner. With wild-type gyrase, an increase in the concentration of CFX led to a reduced level of incorporation; this was not the case for GyrAT<sub>Trp83</sub> gyrase, which did not appear to be significantly inhibited by the concentrations tested here. The insensitivity of replication to quinolone in the absence of
The effect of ciprofloxacin (CFX) on DNA replication was monitored in the presence and absence (open circles) of DNA gyrase. Wild-type (Ser83, closed squares) and mutant (Trp83, open triangles) GyrA proteins were combined with wild-type GyrB. DNA replication was measured by the incorporation of 3H-dTMP.
gyrase implies that this enzyme is the sole component of the replication system that is inhibited by the drug. The background level of gyrase-independent incorporation has been deducted from raw data in assessing the effect of quinolone on replication with wild-type and mutant gyrases, see Figures 5.3 and 5.4 and Table 5.1. Comparison was made of inhibition of replication by CFX and the less potent NFX with wild-type gyrase (Figure 5.4, Table 5.1).

The initial hypothesis that replication would be inhibited at concentrations equivalent to the minimum inhibitory concentration (MIC) required to elicit cell death was not supported by the results seen here. The [quinolone] required for inhibition of 50% of replication (IR$_{50}$) were actually some 2-5 fold higher than the requirements for 50% inhibition of supercoiling (IC$_{50}$), the most frequently assayed property of gyrase (eg. Hallett and Maxwell, 1991).

Reflection on the data of Staudenbauer (1976) reveals a similar outcome with NAL using a crude in vitro replication system. Employing cells lysed gently on cellophane discs or permeabilised with high sucrose, replication was inhibited by 50% with ~20 µg/ml NAL. These experiments preceded the characterisation of DNA gyrase (Gellert et al., 1976). It has subsequently been shown that, the MIC$_{50}$ for NAL is 3.13 µg/ml and the IC$_{50}$ for inhibition of supercoiling by purified gyrase is 20 µg/ml.

5.3.2 The Involvement of DNA Polymerase III

The role of Pol III HE in the observed replication reaction was confirmed by comparison of incorporation of $^3$H-dTMP in the presence and absence of the enzyme (Table 5.2).

In the absence of Pol III HE, approximately 3 pmol of $^3$H-dTMP was incorporated in a gyrase-independent manner that was unaffected by CFX. The involvement of Pol III HE in the remaining 5 pmol of background incorporation reported previously was confirmed by the addition of the holoenzyme to the assay. In the presence of Pol III HE, replication occurred at the higher level with both wild-type and resistant gyrases, with only the former being inhibited by 1.0 µg/ml CFX.
FIGURE 5.3: Comparison of Replication inhibition by CFX in the presence of wild-type and resistant gyrases

DNA replication was measured by the incorporation of $^3$H-TMP in the presence of wild-type or mutant gyrases. Mutations at residue 83 of GyrA lead to quinolone resistance. In combination with wild-type GyrB, Ala83 GyrA exhibited a low level of resistance to CFX and Trp83 a higher level. In all cases a level of gyrase-independent incorporation that was not sensitive to CFX has been deducted from raw incorporation values prior to calculation of percentages.
Inhibition of DNA replication in the presence of wild-type gyrase holoenzyme by CFX (open triangles) and NFX (closed triangles). Incorporation in the absence of gyrase has been deducted prior to calculation of percentages.
### TABLE 5.1: Comparison of quinolone concentration required to inhibit gyrasedependent reactions by 50%

<table>
<thead>
<tr>
<th>Quinolone</th>
<th>GyrA in ( A_2B_2 ) (cell death)</th>
<th>MIC(_{50})(^a) (supercoiling)</th>
<th>IC(_{50})(^b) (replication)</th>
<th>IR(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser83 (wt)</td>
<td>0.0125(^c)</td>
<td>0.05</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Ala83</td>
<td>0.06</td>
<td>0.5</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Trp83</td>
<td>0.39</td>
<td>5</td>
<td>&gt; 10</td>
<td></td>
</tr>
<tr>
<td>NFX</td>
<td></td>
<td>0.05</td>
<td>0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\(^a\) Reece and Maxwell, 1991a  
\(^b\) Hallett and Maxwell, 1991; Sue Critchlow, personal communication  
\(^c\) All drug concentrations in \( \mu \)g/ml

### TABLE 5.2: Involvement of Pol III HE in replication reactions

<table>
<thead>
<tr>
<th>Pol III</th>
<th>[CFX], ( \mu )g/ml</th>
<th>Buffer alone</th>
<th>Excess wt GyrB with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GyrA(_{Ser83})</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>2.9(^a)</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>8.1</td>
<td>20.6</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>7.4</td>
<td>9.7</td>
</tr>
</tbody>
</table>

\(^a\) \(^3\)H-dTMP incorporated, pmol
5.3.3 UpU initiation system and Tus-termination

Utilising the UpU system for targeting initiation to the RNA II, an attempt was made to study quinolone-dependent polymerase blocking using pTHN147. Tus protein was included to give a defined length product of ~1600 nucleotides representing the region from ori to ter. Figure 5.5 shows the results obtained. The major product in each case was Form I replicated plasmid; this should not have been the case, since in the presence of a 50-fold excess of Tus protein, all forks should have been halted at ter. In the presence of gyrase, the formation of a Tus-terminated product was inhibited by increasing [CFX], initially with the wild-type enzyme and at higher concentrations this inhibition was also observed with the resistant form. This was the *a priori* expectation for all of replication. Coupled with the absence of Tus-terminated product when gyrase was not included in the reaction, these results suggest that the gyrase-independent incorporation of ^H-dTMP observed does not reflect replication directed from the proper origin, a conclusion supported by the absence of RNase H dependence and the aberrant production of rolling-circle products in other assays (data not shown). No band corresponding to the termination of replication at the strong gyrase-binding site introduced on the 147 bp fragment was detected. Coupled with the failure to halt forks at ter, it is proposed that the RNA II-DNA hybrid was extending beyond its normal limitations in a manner that disrupted ter, and the gyrase-binding site. This is not unreasonable, since RNA II-DNA hybrids up to 3 kb have been reported (Parada and Marians, 1989).

5.3.4 Behaviour of a mixed gyrase population

If replication was inhibited by binding of quinolone to sensitive gyrase complexes, a reaction in which equimolar Q^s and Q^r enzyme were provided might be expected to be inhibited in a manner akin to Q^s alone. If the sole requirement for gyrase in DNA replication is the maintenance of appropriate superhelical density both at initiation and during elongation, the level of Q^r gyrase activity provided by the mutant enzyme in the mix should be sufficient to allow replication to proceed to a level equal to that seen for Q^r alone.
The product of termination of replication by the Tus protein is indicated. Synthesis of this DNA requires gyrase and is inhibited by increasing [CFX]. The major product in each case is pTHN147, but replication should not have been able to pass Tus bound at the ter site. This result therefore reflects inappropriate synthesis. The $^{32}$P-dATP labelled products are visualised by gel electrophoresis under denaturing conditions.
Figure 5.6 reveals that a mixture of Q<sup>e</sup> and Q<sup>f</sup> gyrase was actually found to give an intermediate level of <sup>3</sup>H-dTMP or <sup>32</sup>P-dATP incorporation. This may reflect the exchange of GyrA subunits between A<sub>2</sub>B<sub>2</sub> complexes. If rapid exchange of subunits occurs in the absence of DNA, it is conceivable that during preparation, a shuffling has occurred that results in the true ratio of gyrase holoenzymes being closer to 2 A<sup>e</sup>A<sup>f</sup>B<sub>2</sub>, 1 A<sup>f</sup>2B<sub>2</sub>, and 1 A<sup>e</sup>2B<sub>2</sub>. If the requirement for activity in the presence of quinolone is the possession of two A<sup>f</sup> subunits, then the population would be in an overall ratio of 3 Q<sup>e</sup> to 1 Q<sup>f</sup> gyrase. Alternatively, it may be that replication blockage does occur and the intermediate result reflects the processivity of A<sup>f</sup>2B<sub>2</sub> allowing templates receiving a Q<sup>f</sup> gyrase to proceed until a Q<sup>e</sup> gyrase-DNA-drug complex is encountered.

This latter proposal is largely undermined by an examination of the products resulting from such a mixture (Figure 5.7). If blockage was occurring, an accumulation of truncated products with increasing [quinolone] might be anticipated. This was not the case, with increasing [CFX] leading to a global reduction in incorporation.

Curiously, the observation of an intermediate phenotype from a mixture of Q<sup>e</sup> and Q<sup>f</sup> gyrase fits with results of Bourguignon et al., 1973. Addition of soluble extract from NAL-sensitive cells (NAL<sup>e</sup>) conferred a degree of NAL sensitivity on DNA synthesis from NAL-resistant (NAL<sup>f</sup>) lysates, but it proved impossible to confer complete sensitivity (Bourguignon et al., 1973).

5.4 The effect of gyrase and quinolones on RNA polymerases

5.4.1 Construction of transcription templates

With the development of the polymerase chain reaction (PCR) it has become possible to generate desired DNA fragments without the need for appropriate restriction enzyme sites. PCR technology has been exploited here to prepare templates for transcription through the preferred site for quinolone-directed cleavage at position 990 of pBR322 (Fisher et al., 1981). A promoter for T7 RNA polymerase (Osterman and
DNA replication was studied by the incorporation of $^3$H-dTMP (closed points) or $^{32}$P-dAMP (open points) in the presence of wild-type GyrA (triangles), Trp83 (squares) or a mixture of both together (circles), each in combination with wild-type GyrB. To facilitate comparison, the mixed gyrase population had as much Ser83 and Trp83 enzyme as the assays with each alone, i.e. the overall concentration was doubled. Each form of GyrA was preincubated with GyrB and then combined.
The $\alpha^{32}$P-dATP labelled products of replication were analysed by gel electrophoresis under denaturing conditions. The effect of CFX on replication in the presence of wild-type (Ser83) and resistant (Trp83) GyrA were compared with a reaction in which both were present. Wild-type GyrB was provided in all reactions.
Coleman, 1981) and the lac UV5 promoter for *E. coli* RNA polymerase (Straney and Crothers, 1985) were positioned adjacent to the DNA region of interest by inclusion of promoter sequence as a non-homologous extension from one primer (Chabot, 1993). This DNA will not hybridise in the first round of PCR but is incorporated in subsequent rounds. Oligonucleotide sequences and thermocycle conditions are given in Sections 2.2.6.1 and 2.2.9 respectively. Since DNase I footprinting has revealed protection by gyrase of some 100-155 bp of DNA (Morrison and Cozzarelli, 1981; Fisher *et al.*, 1981; Kirkegaard and Wang, 1981; and Rau *et al.*, 1987), sufficient distance was included between the promoter and the anticipated position of bound gyrase to ensure that the latter did not interfere with transcription initiation.

Full length templates equivalent to pBR322 sequence spanning from nucleotides 838 to 1145, were prepared by PCR (Figure 5.8). Under the direction of the T7 promoter, in both orientations, the full length transcript was 310 nucleotides (3 bases of the primer oligo were also transcribed). The lac UV5 promoted template was only prepared in the forward (that is from nucleotide 838 towards 1145) direction, giving a transcript of 307 nucleotides. Shorter DNAs, truncating at the preferred cleavage site on the template strand (transcript length 159 nucleotides) and to the staggered cleavage position on the other strand (154 nucleotides) were also prepared, as well as a series of templates to produce a marker RNA ladder of 130-150 nucleotides in increments of 5 nucleotides. All of the shorter DNAs were under the direction of the T7 promoter, and only in the forward direction.

5.4.2 Gyrase acts as a quinolone-dependent blockage to the passage of T7 and *E. coli* RNA polymerases

Transcription was performed in the presence and absence of gyrase, with or without CFX. The progression of both T7 (Figure 5.9) and *E. coli* RNA polymerase (Figure 5.10) along the template DNA was hindered by the presence of gyrase and CFX together, resulting in a shorter transcript. This result was not seen with either gyrase or CFX alone; in both instances the full length transcript was observed. Further analysis
FIGURE 5.8: Preparation of transcription templates by the polymerase chain reaction

Schematic representation of the transcription templates generated by PCR (not to scale). DNA was amplified between two primers, one of which carried an RNA polymerase promoter sequence as an extension. The darker central region represents DNA between the Eag I (939 bp) and BspM I (1063 bp) restriction sites of pBR322 that is common to these templates and to the 147 bp fragment used in the binding experiments of Chapter 4. Arrowheads indicate the startpoint and direction of transcription.
The influence of gyrase and quinolone (CFX) upon transcription by T7 RNA polymerase was investigated. Assays contained (+) or did not contain (-) 1.4 μM gyrase and/or 25 μg/ml CFX. Premature termination of transcription was observed only when gyrase and CFX were both present; in their absence, or with either alone, the full length transcript of 310 nucleotides was produced. The approximate size of transcripts was determined by comparison with Hpa II-digested pBR322 (kindly donated by Dr. I.C. Eperon).
The influence of gyrase and quinolone (CFX) upon transcription by *E. coli* RNA polymerase was investigated. Assays contained (+) or did not contain (-) gyrase (0.7 μM) and/or CFX (10 μg/ml). Premature termination of transcription was observed only when gyrase and CFX were both present; in their absence, or with either alone, the full length transcript of 307 nucleotides was produced. The positions of full length and blocked transcripts are indicated.
confirmed that the relative proportion of truncated and full-length transcripts was dependent on both the concentration of gyrase (Figure 5.11) and CFX (Figure 5.12).

The implication is that RNA polymerase can normally pass DNA-bound gyrase, but it remains unclear whether the polymerase is displacing or somehow 'reading-through' the gyrase. The bactericidal activity of quinolones may stem, therefore, from the formation of a stable gyrase-quinolone-DNA ternary complex that acts as a "roadblock" to the passage of polymerases.

Figure 5.12 shows that with increasing CFX, a point is reached where the intensity of the full-length transcript decreases in a manner that cannot be accounted for by enhanced blockage, since the shorter RNA also becomes less intense. One possible explanation would be the direct inhibition of polymerase activity by quinolone except that, in the presence of quinolone-resistant gyrase, transcription is still possible at 500 μg/ml (Figure 5.13, Section 5.4.3). It therefore appears that this inhibition is gyrase dependent, and may stem from quinolone-mediated stabilisation of gyrase at a second, weaker, site upstream of the preferred cleavage site in a manner that disrupts initiation of transcription. The total DNA length of 330 bp could be sufficient to bind two gyrases simultaneously and move-up controls appeared to indicate two species of gel-retarded DNA with the second, slower moving, form becoming evident at higher concentrations (data not shown).

5.4.3 A higher CFX concentration is required to achieve blockage by GyraTrp83 gyrase

Gyrase holoenzyme composed of GyraTrp83 subunits (with wild-type GyrB) has been shown above (Section 5.2.2) to support DNA replication at quinolone concentrations that inhibited the wild-type enzyme. Additionally, a spin-column assay failed to detect binding of 3H-NFX to a complex of ATrp83B2 with DNA (Section 4.3.2). Here, the effect of the Trp83 mutation on transcription blockage was investigated. At 5 μg/ml CFX, more than 50% of transcripts were blocked by wild-type gyrase, but ATrp83B2 did not appear to be appreciably affected (Figure 5.13). The initial interpretation that 500 μg/ml CFX was also too low to inhibit transcription was not borne out when a range of CFX concentrations
Transcription by T7 RNA polymerase was performed in the presence of increasing [gyrase]. The enzyme concentration, in μM, is given above each track. The CFX concentration in all reactions was 25 μg/ml (67 μM). Hpa II-digested pBR322 DNA markers were used to give an approximate indication of the transcript length.
FIGURE 5.12: The influence of increasing [CFX] on transcription by T7 RNA polymerase in the presence of 0.7 μM gyrase

<table>
<thead>
<tr>
<th>[CFX], μM</th>
<th>0</th>
<th>0.008</th>
<th>0.027</th>
<th>0.08</th>
<th>0.27</th>
<th>0.8</th>
<th>2.7</th>
<th>8.1</th>
<th>27</th>
<th>81</th>
</tr>
</thead>
</table>

Transcription by T7 RNA polymerase was compared in the presence of wild-type (GyrA<sub>Ser83</sub>) and quinolone-resistant (GyrA<sub>Trp83</sub>) gyrase. In each case the gyrase concentration was 1.38 µM. The concentration of CFX in each reaction is given in µM.
was studied (Figure 5.14). Curiously, a peak of inhibition was achieved at about 100 
μg/ml; at higher concentrations the quantity of stopped product fell away. This 
observation may only be a reflection of the overall reduction in transcription that is evident 
from the lower levels of full length product. According to this assay, even the optimal 
concentration of CFX inhibited only a fraction of the overall transcripts. This was not 
indicative of lower gyrase activity since a control supercoiling experiment showed the 
ATp832B2 enzyme to be as active as the wild-type (data not shown).

5.4.4 Novobiocin did not lead to transcription blockage by gyrase

Novobiocin is a coumarin that inhibits the ATPase reaction of GyrB; 5 μM drug is 
sufficient to abolish supercoiling (Gellert et al., 1976b). At the concentrations tested (up 
to 10 μM) transcription by T7 RNA polymerase was unaffected by novobiocin (Figure 
5.15). The gyrase concentration in each reaction was 0.4 μM. In no instance was any 
stopped product observed, implying that it is a particular feature of the action of 
quinolones, rather than inhibition of gyrase per se, that is responsible for the blockage of 
RNA polymerase progression along the template.

5.4.5 Analysis of site(s) of transcription termination

One hypothesis would explain the quinolone-dependent termination of 
transcription in terms of the RNA polymerase reaching a point at which the template DNA 
has been cleaved. To investigate this scenario, template DNAs, for the subsequent 
synthesis of RNA markers, were prepared by PCR. Under the action of T7 RNA 
polymerase, markers of 159 nucleotides (to the 994 bp cleavage site on the template 
strand), 155, 150, 145, 140, 135 and 130 nucleotides were transcribed.

Comparison of the length of the gyrase-quinolone blocked products of transcription 
from the T7 promoter, in both directions (Figures 5.16 and 5.17), and from the lac UV5 
promoter (forward direction only, Figure 5.18) with the size markers reveals that 
interruption of transcription occurs some short distance upstream of the cleavage site. This 
result indicates that the termination of transcripts involves steric contact between the
FIGURE 5.14: The influence of [CFX] on transcription by T7 RNA polymerase in the presence of quinolone-resistant gyrase.

Transcription by T7 RNA polymerase was compared in the presence of quinolone-resistant (GyrA<sub>Trp83</sub>) gyrase. In each case the gyrase concentration was 1.38 μM. The concentration of CFX in each reaction is given in μM.
FIGURE 5.15: Novobiocin does not cause blockage of transcription by T7 RNA polymerase

The concentration of novobiocin (µM) in each reaction is given. As a positive control, inhibition by CFX (8.1 µM) is shown in the neighbouring track.
FIGURE 5.16: Determining the length of RNA transcripts formed by the gyrase-dependent blockage of T7 RNA polymerase

(a) and the right-hand side of panel (b) show the position of RNA size markers (130-159 nucleotides) after exposure of photographic film to the gel for 10 and 180 min respectively. The concentration of gyrase in each assay is indicated in panel (b); the concentration of CFX was 16 µg/ml throughout. Dashes indicate the position of the major gyrase-stopped products, and their locations relative to the sites of CFX-dependent cleavage by gyrase can be seen in Figure 5.19.
The effect of increasing [gyrase] on transcription by T7 RNA polymerase was investigated. The template DNA was in the reverse orientation to the other experiments reported thus far. The concentration of CFX in all assays was 27 μM. The size of RNA markers is given in nucleotides. The bold arrow indicates the position of the full transcript (307 nucleotides), the thinner arrow the position of the blocked transcript and dotted arrows the position of apparent additional stopped products that appeared at higher gyrase concentrations.
In order to determine accurately the length of the truncated transcripts, and hence their positions relative to the site of quinolone-dependent cleavage by gyrase (Figure 5.20), it was necessary to expose photographic film to the gel for two different times. Panel (a) was exposed for 20 min and shows the position of size markers (130-310 nucleotide). Panel (b) depicts a longer exposure, 210 min, in which the markers are overexposed to allow the gyrase-blocked transcripts to be seen. Where present, the concentration of gyrase was 0.7 μM and CFX was 5 μg/ml.
polymerase and gyrase enzymes rather than polymerase running out of template, and into covalently linked GyrA, at a point where the DNA backbone has been breached by gyrase. The action of eight different quinolones, CFX, NFX, NAL, OXO, PFX, DFX, OFX and ENX, all led to transcript blocking at the same position (data not shown).

Figure 5.19 gives a summary of the experiments mapping the 3' ends of RNA transcripts. Three distinct products are evident for transcription by both T7 and E.coli RNAP in a forward direction. Such diversity of ends is not uncommon in studies of RNA polymerase interaction with proteins bound in its path. Sellitti and coworkers noted that in encounters between E. coli RNAP and lac repressor in vivo, there “is variation in the number and spacing of the 3' ends produced” (Sellitti et al., 1987). In a study of the blocking of T7 and SP6 RNAP progression by the EcoRI Gin111 mutant described in Section 5.2.3, it was found that the phage polymerases could transcribe DNA to within 3, 2 and even 1 nucleotide of the EcoRI recognition sequence (Pavco and Steege, 1991). This suggested a notably closer interaction between T7 RNAP and bound EcoRI Gin111 than they had previously observed with E. coli RNAP and the mutant endonuclease (Pavco and Steege, 1990) where transcription was terminated, at a unique site, 14 nucleotides upstream of the recognition sequence, or indeed with Exonuclease III (Exo III), which was able to digest dsDNA to within 7 nucleotides of EcoRI Gin111.

In the experiments reported in this chapter, it appears that T7 RNAP is stalled in a manner that gives transcripts with 3' ends 9, 12 and 15 nucleotides away from the cleavage sequence (note that numbering is based from the centre of the cleavage sequence). RNA transcripts generated by the E. coli enzyme stopped further from the cleavage centre (15, 17 and 21 nucleotides). In the reverse orientation, only one site for gyrase-mediated stoppage of transcription by T7 RNAP was detected (Figure 5.17, Figure 5.19). This site is 10 nucleotides from the cleavage centre and indicates that a region of approximately 20 nucleotides around the breakage and reunion site is strongly protected by gyrase interaction with the DNA. To date, the experiment with E. coli RNAP in this orientation has not been attempted. A number of previous studies have investigated the boundaries of gyrase-DNA complex by protection from DNase I, Exo III and staphylococcal nuclease.
Figure 5.19: Transcriptional footprint of DNA region bound by gyrase
DNase I and staphylococcal nuclease "footprints" revealed protection of approximately 100-155 bp from nuclease attack, with a central region of 40-50 bp particularly well protected.

The footprint of protection against Exo III differs from the pattern given by DNase I. In the absence of ATP/ADPNP or quinolone, barriers to Exo III were observed 83, and to a lesser extent 94, nucleotides upstream of a preferred DNA-breakage site of gyrase in ColE1 (Morrison and Cozzarelli, 1981) and a separate study reports a similar site (Fisher et al., 1981). Both groups comment on the apparent asymmetry of Exo III protection; Fisher and coworkers noted Exo III protection 15 and 18 nucleotides to the right of a gyrase cleavage site, Morrison and Cozzarelli reported a blockage 43 nucleotides upstream of the cleavage site which was overcome in the presence of ADPNP to give a new specific barrier 16 nucleotides beyond the cleavage site. For a different DNA sequence they showed a stable oxolinic-acid dependent protection point 15 nucleotides upstream of the gyrase cleavage centre, a distance reminiscent of the experiments reported here for quinolone-dependent RNAP stalling 9-21 nucleotides from the centre. In the absence of quinolone, no Exo III footprint was detected at this sequence, a result that also agrees with the RNAP experiments.

Figure 5.17 gives, incidentally, the clearest example of an apparent accumulation of shorter transcripts behind a gyrase-quinolone blockage of RNA polymerase. This phenomenon has been observed previously for transcription interrupted by lac repressor (Sellitti et al., 1987) and EcoR I
g111 (Pavco and Steege, 1990) and the shorter RNA species has been interpreted to be the product of a second RNA polymerase stalled behind the first stalled RNA polymerase.

The transcriptional footprint reported here is in good agreement with results found for eukaryotic topoisomerases (Bendixen et al., 1990; Thomsen et al., 1990). Premature termination of transcription by SP6 RNA polymerase was found to occur 10 nucleotides upstream of the cleavage site in a camptothecin-stabilised topoisomerase I-DNA complex (Bendixen et al., 1990). In the presence of an inhibitor, topoisomerase II protected a
region of 28 bp from polymerase, with a central core of 22 bp being particularly well protected (Thomsen et al., 1990). In the presence of CFX, I have found that gyrase protected a region of approximately 26 bp from T7 RNA polymerase with the strongest protection for a core of 20 bp centred around the cleavage site.

5.4.7 Is gyrase displaced by a passing polymerase?

Attempts thus far have failed to establish if gyrase is displaced from the template by a passing polymerase. The intention has been to establish gyrase-DNA complexes in the presence and absence of CFX, prior to addition of RNAP and excess competitor DNA. The competitor DNA is radiolabelled and is generated by PCR with primers that do not have the promoter sequence. Contrary to the a priori prediction, this DNA stably interacts with E. coli RNAP (data not shown). It is interesting to note that the papers that have studied the interaction of RNAP and bound protein frequently comment upon the fact that polymerase must either be reading past or displacing the protein in its path (eg. Pavco and Steege, 1990; Giordano et al., 1989), yet reports determining which of the situations is occurring are notably rare. This may be a reflection of the difficulty of successfully analysing for displacement and those studies that have attempted to do so have proved contradictory (eg. Losa and Brown, 1987; Lorch et al., 1987).

Results with eukaryotic topoisomerase I suggest that transcription may not require displacement of bound proteins in the path of RNA polymerase (Bendixen et al., 1990). Since topoisomerase I cleaves DNA in one strand only, it is possible to assess the ability of RNA polymerase to read along the coding strand while topoisomerase is anchored to the DNA by covalent interaction with the other strand and cannot therefore be displaced. It was found that the passage of RNA polymerase was not impeded by the covalent attachment to topoisomerase I to the non-coding strand. The authors do point out that the phage RNA polymerase used in these experiments is relatively small compared with the eukaryotic equivalent and caution that read-through transcription of this type may not be possible with the larger enzyme. Additionally, the dissociation of one subunit only has not strictly been ruled out. Nevertheless, this result strengthens the case for RNA
polymerase's ability to read-through a protein in its path without requiring the latter to be displaced.

5.4.5 Effect of preincubating gyrase, CFX and DNA

Blocked transcripts were observed at lower CFX concentrations when gyrase and DNA were preincubated with the drug for 1-3 hours at 25°C prior to addition of T7 RNAP (Figure 5.20). This fits with the time-dependence of the DNA cleavage reaction (Section 3.5.2), although in no experiment has 100% blockage of transcription been observed. The inability of topoisomerase I in the presence of camptothecin to completely inhibit transcriptional elongation has been noted in both in vivo (Stewart et al., 1990) and in vitro studies (Bendixen et al., 1990). This phenomenon was attributed to the reversible nature of the camptothecin-stabilised topoisomerase I-DNA complex.

On reflection, it is possible that the manner in which these experiments have been performed masks the efficiency of gyrase-quinolone-DNA complex to interrupt transcription. Despite precautionary move-up controls to ensure that all of the template DNA was protein-bound, it is nevertheless feasible that a small subpopulation of unbound DNA could remain. In the 30 min of each transcription assay, many rounds of transcription could be performed on unbound templates, leading to anomalous significance in comparison with gyrase-bound templates where only one round of transcription may be possible.

5.5 Discussion

Results of in vitro experiments studying DNA replication and, more conclusively, RNA transcription suggest that the passage of a macromolecular protein assembly along the template DNA is inhibited by the formation of a stable gyrase-quinolone-DNA complex. In the absence of drug, gyrase does not impede the progress of RNA polymerase, implying that the topoisomerase is either displaced from the DNA or that the polymerase is capable of transcribing through the bound protein. There are precedents for each of these explanations from in vitro studies with other proteins (eg. Losa and Brown,
FIGURE 5.20: The effect of preincubation of gyrase and DNA on transcription inhibition

Template DNA (original orientation) was incubated at 25 °C with 0.84 μM gyrase for 0, 1 or 3 hours prior to addition of T7 RNA polymerase. The concentration of CFX, in μM, is given above each track. It is apparent that with increasing pre-incubation, blocked product appears at a lower [CFX].
1987; Lorch et al., 1987) and it remains to be determined which outcome is occurring when polymerase and gyrase meet. Experiments with eukaryotic topoisomerase I and camptothecin (Bendixen et al., 1990) may lend support for the proposal that gyrase remains bound during transcription. When topoisomerase I was covalently attached to the non-coding strand and could not therefore be displaced, SP6 RNA polymerase was still capable of reading uninterrupted along the coding strand.

The point mutation GyrAser83 → Trp is known to confer quinolone resistance (Yoshida et al., 1988; Cullen et al., 1989). It has been shown elsewhere in this study (Section 4.3.2.2) that negligible binding could be detected to gyrase-DNA complex in which the GyrA subunits carried this alteration. The requirement of increased [CFX] in order to inhibit transcription through this protein correlates well with this result. Since novobiocin did not cause gyrase to block transcription by T7 RNA polymerase, it is therefore a feature of quinolone action rather than inhibition of gyrase activity per se that leads to the formation of a gyrase-DNA complex that is capable of interrupting the passage of polymerases. It is probable that gyrase is becoming covalently attached to the DNA as a result of quinolone-mediated interruption of the breakage and reunion reaction.

The transcriptional footprint observed for the CFX-dependent protection of DNA by gyrase is in good agreement with the studies on eukaryotic topoisomerase II in the presence of an inhibitor (Thomsen et al., 1990). Gyrase protected a region of approximately 26 bp, making especially strong contact with a core of 20 bp centred on the preferred cleavage site. This study has not sought to show that gyrase is cleaving the 307 (310) bp template DNAs at the anticipated site. Cleavage has previously, however, been shown to be directed to this site in longer (pBR322; Fisher et al., 1986) and shorter (147 bp fragment; Dobbs et al., 1992) DNAs that share with the transcription templates a central region of 124 bp. Coupled with the centrality of the staggered cleavage site within the gyrase-protected footprint it seems reasonable to assume that cleavage is occurring at the expected bonds. This should, perhaps, be confirmed in any further experiments on this system.
CHAPTER 6

Conclusions
6.1 Introduction

Several factors have contributed to the enthusiasm with which quinolones have been received by the clinical fraternity. As entirely synthetic compounds, acting via a novel inhibitory mechanism, quinolones offer much in the battle against increasingly widespread resistance to the "traditional" antibiotics, such as penicillins, aminoglycosides and tetracyclines. It is a bonus that the newer members of the class, the fluoroquinolones, have potent activity against a broad spectrum of bacteria, have excellent absorption after oral administration and rarely give rise to significant side effects at efficacious concentrations (Walker and Wright, 1991). Regrettably, such alluring characteristics have contributed to indiscriminate and inappropriate use of these compounds and, in turn, to significant resistance development. The continued success of the quinolone family will now depend upon improved understanding of their mode of action in order to facilitate rational drug design and means to overcome resistance. Identification of the quinolone binding site and the manner in which interaction of quinolones with gyrase results in cell death are two of the outstanding problems that require resolution. This concluding chapter will consider the contribution made by the present study to our knowledge on these important issues and will move on to outline possible future work.

6.2 What is the binding site for quinolone antibacterials?

The nature of the primary binding site of quinolone antibacterials has been the subject of some contention, with separate proposals that drug molecules bind to DNA gyrase (Gellert et al., 1977), to DNA (Shen and Pernet, 1985) or to a gyrase-dependent site within the DNA (Shen et al., 1989a). In rapid gel-filtration experiments (reported in Chapter 4), it was found that stable quinolone binding required the presence of both gyrase and DNA; no binding was found to DNA or to gyrase alone. Furthermore, in experiments where the wild-type GyrA subunits were replaced with a quinolone-resistant mutant form (GyrASer83 \rightarrow Trp), drug binding could no longer be measured. What do these results reveal about the interaction of quinolones with gyrase and DNA and how do they correlate with current models of drug binding to topoisomerases?
In the model of quinolone binding drawn up by Shen and coworkers, drug molecules bind by hydrogen-bonding to the bases in a single-stranded DNA pocket at the catalytic site of gyrase (Shen et al., 1989a). Although both gyrase and DNA are therefore required for stable interaction, the Shen model suggests that it is the DNA that plays the significant role in quinolone binding with the function of gyrase largely reduced to generation and maintenance of the single-stranded DNA pocket in a suitable configuration to facilitate hydrogen-bonding of drug molecules to the exposed bases. Despite the identification of several quinolone-resistant point mutations in GyrA, the existence of quinolone-resistance mutations in GyrB is deemed by Shen to negate the possibility of direct quinolone-GyrA contacts and gyrase mutations are thought to give rise to quinolone-resistance indirectly by altering the configuration of the DNA pocket. The ability of mutant gyrases to perform DNA supercoiling (Section 3.4.2) implies, however, that the DNA configuration at the active site is not significantly different with wild-type and resistant enzymes; indeed mutation of an essential protein to overcome the influence of an inhibitor would be without purpose if the new form was incapable of performing the required tasks. Since the quinolone-resistant gyrases can catalyse the breakage and rescaling reactions that are central to DNA supercoiling, significant alteration in the orientation of the DNA seems unlikely.

The observation that a GyrA_{Ser83 → Trp} point mutation can substantially reduce binding of quinolone to the gyrase-DNA complex (Section 4.3.2.2) lends credence to the existence and importance of direct interaction between drug and enzyme (to the A subunits at least). It is important to remember, however, that no quinolone binding to gyrase could be detected in the absence of DNA and any valid model for drug binding must therefore incorporate a role for DNA, and also indeed for GyrB, as evidenced by resistance arising from mutations in this subunit.

DNA could be involved indirectly in quinolone binding if interaction of gyrase with DNA elicited a conformational change in the enzyme and hence created the correct orientation for a drug target site. Evidence from biophysical studies argues against this proposal; small angle neutron scattering and dynamic light scattering data suggest that
there is no significant difference in the shape of gyrase in the presence and absence of DNA (Krueger et al., 1990), though it is pertinent to point out that techniques of this kind would not detect subtle alterations. A more direct role is also implied by the influence of quinolones on cleavage site selection; different drugs appear to have determined the efficiency of scission at a variety of sites within pBR322 (Walton and Elwell, 1988). Pommier and coworkers have investigated the correlation between local base sequence and cleavage site selection with a variety of eukaryotic topoisomerase II-inhibitors (Capranico et al., 1990; Pommier et al., 1991). They have revealed the importance of bases directly adjacent to the cleaved bond(s) and propose a general model in which anti-tumour drugs interrupt topoisomerase II by intercalating into the DNA at the cleavage site (see Figure 1.10). Similarities between the influence of these agents on Topo II and the effect of quinolones on gyrase potentiate consideration of antibacterial action in the light of this model. Despite the planar nature of the bicyclic ring of quinolones, studies (performed in the absence of gyrase) have failed to detect intercalation by these compounds (Tornaletti and Pedrini, 1988; Shen et al., 1989c). The situation envisaged at the cleavage site would be somewhat different; with a backbone bond broken and a phosphotyrosine bond formed between the DNA and the enzyme, the available interactions for an intercalating drug molecule will be altered. Pommier's studies with topoisomerase II suggest that "non-intercalators" such as the epipodophyllotoxins, eg. VM-26, are capable of stacking with a base pair within a topoisomerase II complex in a manner that is similar, if not identical, to that found with intercalators such as doxorubicin and m-AMSA (Pommier et al., 1991).

Topoisomerase-drug interactions could still make vital contributions to the stable binding of drug molecules, explaining the relevance of resistance mutations within the protein. Quinolone-resistance mutations in gyrase are clustered within a 40-residue region of GyrA and the two sites in GyrB that have been shown to affect sensitivity to quinolones are both within the region of the subunit that is known to interact with GyrA (Gellert et al., 1979). It has previously been noted that these quinolone-resistance determining residues of GyrA and GyrB may conceivably be located on the surface of the protein, at the GyrA-GyrB interface (Cullen et al., 1989) and in close proximity to the DNA. There are
precedents for the involvement of more than one protein subunit in the formation of a ligand binding site, the interaction of chloramphenicol with CAT is one example (Leslie, 1990) and the contact to both GyrB subunits in the binding and hydrolysis of ATP (Wigley et al., 1991) is another.

Experiments to compare the onset of supercoiling inhibition and of DNA cleavage in the presence of CFX (Section 3.6) indicate that there may be two stages to quinolone interaction with a gyrase-DNA complex. The ability of gyrase to supercoil DNA was completely inhibited within a minute of the addition of CFX but DNA cleavage apparently occurred over a longer timescale; linearisation of DNA continued to increase for up to two hours after addition of the drug (Figure 3.13). Study of the quinolone-directed cleavage by gyrase of a 147 bp DNA fragment confirms that cleavage is a slow process; cleavage of the 147 bp DNA into pieces of 69 and 78 bp took up to 4 hours at 25°C to reach completion (Figure 3.10).

An explanation of the physical alteration that could be accompanying transition from a supercoiling-inhibiting to a DNA-cleaving interaction of quinolone with gyrase and DNA is not immediately apparent.

6.3 How do quinolones initiate cell death?

In addition to understanding the nature of quinolone interaction with gyrase and DNA it is important to determine how that interaction can come to initiate cell death. There are good reasons to believe that abolition of gyrase activity is not in itself sufficient to explain the bactericidal properties of quinolones. If cell killing simply involved inactivation of sensitive gyrase, provision of a drug-resistant form should allow restoration of the necessary activity and hence cell viability. The dominance of the quinolone-sensitive phenotype (Hane and Wood, 1969), even in the presence of an excess of resistant protein, implies that the relationship is more subtle. Similarly, the requirement of a lower concentration of quinolone to kill bacteria (MIC) than to inhibit supercoiling in vitro (Gellert et al., 1977; Walton and Elwell, 1988) suggests that a further element may be involved.
Investigation of transcription by T7 and E. coli RNA polymerases (Section 5.4) supports a hypothesis in which quinolone-mediated stabilisation of a gyrase-DNA complex prevents passage of polymerase along the template (Kreuzer and Cozzarelli, 1979). Inhibition of transcription required the presence of gyrase and quinolone together; RNA polymerase was unaffected by either quinolone or gyrase alone; implying that polymerase can normally pass or displace gyrase. In such a scenario, cell death might require stabilisation of only one gyrase-DNA complex. This would not be evident in an in vitro assay of supercoiling inhibition since quinolone-free gyrase can continue to act catalytically upon the DNA.

The quinolone-dependent inhibition of transcription by gyrase correlates well with recent studies on other topoisomerases. Blockage of transcription by eukaryotic topoisomerase I was found to require the presence of camptothecin (Bendixen et al., 1990) and similar inhibitor-dependent interruption of transcription was seen with eukaryotic topoisomerase II (Thomsen et al., 1990).

Given the intimate coupling of DNA replication and cell division, inhibition of DNA polymerases would be particularly significant in mediating the bactericidal effect of quinolones. Technical difficulties have meant that experiments to monitor the effect of quinolone and gyrase on DNA replication (Section 5.3) have been less conclusive than the transcription studies. It would be valuable to prove conclusively that DNA polymerase is inhibited by gyrase-quinolone-DNA complex in an analogous manner.

6.4 Further experiments

The nature of scientific research inevitably means that questions will lie partially answered or unanswered at the end of an investigation. This predicament is especially apparent in the limited duration of postgraduate study and the following paragraphs outline experiments that would add to our understanding of the interaction of quinolone drugs with DNA gyrase.

The basis of quinolone binding to gyrase and DNA would be greatly clarified by crystallisation in the presence of drug. The recent achievement of obtaining detailed
structural information from a crystal of the 43 kDa N-terminal fragment of GyrB (Wigley et al., 1991) and the preliminary characterisation of the N-terminal fragment of GyrA (Reece et al., 1990) indicate real progress in this field. It is a general principle of crystallography that small molecules form regular (and therefore informative) crystals more readily than larger ones. For this reason, it would be naïve to think that crystallisation of gyrase holoenzyme in the presence of DNA and quinolone is imminent. A feasible development, however, would be the assignment of residues within the N-terminal fragment of GyrA. This portion of the enzyme contains both the “quinolone-resistance determining region” (Yoshida et al., 1990), between residues 67 and 106, and the catalytic residue, Tyr122. Crystallisation of this fragment could provide tertiary structure information and, in conjunction with molecular modelling techniques, reveal details of quinolone binding and illuminate the significance of resistance mutations.

In the absence of suitable crystals, a number of important questions can be addressed by further binding studies using rapid gel-filtration and additional approaches (eg. equilibrium dialysis). Investigation with a GyrATyr122 → Phe mutant, known to bind but not cleave DNA, could reveal whether quinolone binding occurs prior to formation of a phosphotyrosine bond between enzyme and DNA. Detection of quinolone binding to a complex composed of 147 bp DNA and GyrAPhe122 gyrase would rule out an intercalation model of the kind proposed for anti-tumour drugs and eukaryotic topoisomerase II. Availability of radiolabelled CFX at a high specific activity would facilitate binding studies.

Polymerase blocking experiments have shown that a quinolone-gyrase-DNA complex can interrupt the passage of a macromolecular protein assembly along the DNA. It is not yet clear, however, if blockage requires cleavage of the DNA. Formation of the truncated RNA has occurred without pre-incubation of gyrase with the template DNA (eg. Section 5.4.2) but pre-incubation did result in blockage occurring at lower [CFX] (Section 5.4.8). The slow rate of quinolone-dependent cleavage (Sections 3.5.2 and 5.6) may imply that cleavage is not required. This question could be answered directly by analysis of
transcription in the presence of CFX and GyrA{Phe}122 gyrase with observation of blocked product confirming that cleavage is not required.

To date the most exhaustive study of transcription inhibition has been performed with RNA polymerase from the T7 bacteriophage. Elaboration of the preliminary experiments carried out with E. coli RNA polymerase would more closely mimic the situation in vivo. Inevitably it will be useful to scrutinise the influence of gyrase and quinolones on DNA polymerases from E. coli, especially polymerase III. Investigation need not employ the full in vitro replication system, and strategies requiring fewer of the replication proteins can be devised. With careful planning, an assay for the conversion of ssDNA to the replicative form could exploit the inability of gyrase to bind to ssDNA and use an appropriate oligonucleotide to localise gyrase to a pre-determined position on the template. This would facilitate analysis of anticipated results in a similar way to the study of transcription through a known gyrase-binding site allowed testing of a priori predictions.
CHAPTER 7

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APPENDIX
A Single Point Mutation in the DNA Gyrase A Protein Greatly Reduces Binding of Fluoroquinolones to the Gyrase-DNA Complex

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Binding of the quinolone drug norfloxacin to gyrase and DNA has been investigated. We have detected binding to gyrase-DNA complex but find no significant binding to either gyrase or DNA alone. Enzyme containing gyrA protein with the mutation Ser-83 to Trp (conferring quinolone resistance) showed greatly reduced drug binding.

The target of the quinolone antibacterial agents is thought to be DNA gyrase. The enzyme from Escherichia coli consists of two A and two B subunits (the products of the gyrA and gyrB genes, respectively) and is responsible for catalyzing topological changes in DNA (for a review, see reference 11). A recent model for the mechanism by which gyrase catalyzes topological changes in DNA (for a review, see reference 11) was prepared from plasmid pSTD147 as described previously (12).  

The DNA used was a 147-bp fragment containing the major gyrase cleavage site from pBR322 (3), which has been shown to be cleaved by gyrase at a single site into fragments of 69 and 78 bp (2). Incubation of the 147-bp fragment (86 nM) with gyrase (700 nM) and NFX (2.5 μg/ml; 7,800 nM) at 25°C for 30 min at 25°C to form the AgB^ supercoiled complex. Typical specific activities were — 10¹⁰ U/mg for GyrA (10). In addition, we used a rapid gel-filtration (spin column) technique (7) as a means of studying binding; free ligand is retained on the column, and ligand bound to macromolecule is eluted. This approach has previously been used to address the question of quinolone interaction with gyrase and DNA (12).  

Quinolone-resistant protein, GyrA^i^, is bound approximately threefold less drug than wild-type complex did (data not shown). In addition, very little binding to either the wild-type DNA gyrase or the enzyme carrying the GyrA^-^ mutation was found. Significantly, this binding was virtually abolished for complexes of the GyrA^-^-B^-^- complex and DNA. Other experiments showed that enzyme-DNA complex prepared with another quinolone-resistant protein, GyrA^-^- (6), bound approximately threefold less drug than wild-type complex did (data not shown).

In a second series of experiments, DNA cleavage (4) was used as an indirect monitor of binding. When the related quinolone ciprofloxacin (CFX) was used, no binding to either wild-type gyrase or the DNA alone was found (Table 2). A series of assays was performed in which one component (gyrase, DNA, or CFX) was omitted from the initial reaction mixture and added after the other two components had been incubated for 2 h and passed through a spin column. The new reaction mixture was then incubated for a further 2 h prior to the addition of 0.2% SDS and 1 mg of proteinase K per ml and 30 min of incubation at 37°C. When gyrase and DNA were incubated together and CFX (20 μg/ml; 60 μM) was added following centrifugation, cleavage was observed (Table 2), but when gyrase alone was incubated with quinolone and DNA was added following centrifugation, no cleavage was detected. Similarly no cleavage was observed when DNA and CFX were incubated together and enzyme was added subsequently.

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It should be pointed out that the centrifugation involved in a spin column assay means that the experiments are not performed under true equilibrium conditions. These results do not, therefore, rule out the possibilities that some weak interaction may be occurring between quinolones and either gyrase or DNA alone and that dissociation may occur during centrifugation. Control experiments have revealed comparable extents of DNA cleavage with and without centrifugation. The stability of the gyrase-DNA-quinolone complex following centrifugation was also confirmed. After incubation of gyrase and DNA with unlabelled CFX for 2 h, the reaction mixture was passed through a spin column and incubation continued for a further 0 to 3 h prior to the addition of SDS and proteinase K. The extent of cleavage in samples 3 h after incubation was also confirmed. After incubation of gyrase and DNA cleavage with and without centrifugation. The stability was also confirmed. After incubation of gyrase and DNA cleavage with and without centrifugation. The stability of the gyrase-DNA-quinolone complex following centrifugation was also confirmed. After incubation of gyrase and DNA with unlabelled CFX for 2 h, the reaction mixture was passed through a spin column and incubation continued for a further 0 to 3 h prior to the addition of SDS and proteinase K. The extent of cleavage in samples 3 h after incubation after the second 2-h incubation. SDS and proteinase K were added; the final incubation was at 37°C for 30 min.

### Notes

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We support the proposal that both the enzyme and the DNA are required for stable interaction with quinolones but contend that efficiency of binding is primarily determined by the gyrase A subunits.

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