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The Effects of Norfloxacin on Pseudomonas Aeruginosa and Its DNA Gyrase

Doris M. Benbrook
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THE EFFECTS OF NORFLOXACIN ON PSEUDOMONAS AERUGINOSA
AND ITS DNA GYRASE

by

Doris M. Benbrook

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A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

December

1985

Science is a partial means to the understanding of the ultimate question of life, the universe, and everything.

I dedicate this thesis to my family:

Jeffrey P. Benbrook who married me in my second year of graduate school and offered me love, companionship, and understanding during my trial years.

Nick and Dawn Mangiaracina, my parents, who offered me their encouragement, pride, and generous support throughout my entire academic career.

Mary Ann Lufkin, Nick Mangiaracina, and Mark Mangiaracina, my sister and brothers, whose encouragement and pride in my accomplishments made it seem all worthwhile.

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Lastly, I thank every teacher that I have ever been a student of; for no person is an island.

VITA

Doris Benbrook was born to Nick and Dawn Mangiaracina in Chicago, Illinois, on May 4, 1959. She attended Maternity B.V.M. Elementary School and Madonna High School, both located in Chicago. In 1975, her family moved to Addison, Illinois.

After graduating from Addison Trail High School in 1977, she attended North Central College in Naperville. In 1981, she received her Bachelor of Arts degree with majors in Biology and Chemistry and a minor in German. That year, she began working for her Ph.D. in Biochemistry at Loyola University Medical Center. In 1982, she began her research in the lab of Robert V. Miller, Ph.D.

She married Jeffrey P. Benbrook in June, 1982. She is presently working as a post-doctoral researcher at La Jolla Cancer Research Foundation.

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INTRODUCTION

This dissertation is a study of the effects of pyridonecarboxylic acid derivatives (quinolones) on Pseudomonas aeruginosa. These drugs are important to study because the newer, more potent derivatives are effective against gram negative infections (30,34) such as P. aeruginosa which is an opportunistic pathogen of burn wounds and the lungs of children with cystic fibrosis (2,13,14,32,66). Norfloxacin, a newer derivative, which is more potent than oxolinic acid and nalidixic acid, has not yet been studied in P. aeruginosa. Since the effects of oxolinic acid and nalidixic acid on Escherichia coli have been well studied (e.g., 19), it is beneficial to compare the effects of norfloxacin, oxolinic acid, and nalidixic acid on P. aeruginosa.

Pyridonecarboxylic acids act by binding to DNA (70) and inhibiting the activities of DNA gyrase and of one of its two subunits (termed A) which also interacts with another protein to form Topo II' (6). Therefore, in order to understand the mechanism of drug action, the in vitro effects of these derivatives on P. aeruginosa DNA gyrase and its A subunit will be studied.

REVIEW OF THE RELATED LITERATURE

SUPERHELICAL DNA

The bacterial chromosome consists of a closed circular double helix of DNA that is maintained in an underwound topological state. The twisting of the DNA helix either into an underwound or an overwound state is termed negative supercoiling or positive supercoiling, respectively. The degree of supercoiling is described by the following relationship:

$$T=L-L_0 \qquad 1.$$

where T is the number of titratable superhelical turns, L is the number of times that one strand of the double helix crosses over the other strand, termed the linking number, and L_0 is the linking number of relaxed (not twisted) DNA.

In nature, DNA is found in a negatively supercoiled state ($L < L_0$) which promotes single strandedness. This negative superhelicity has been shown to be required for replication, transcription, recombination, repair of DNA, and plasmid maintenance (reviewed in 10, 24, and 85). Positively supercoiled DNA ($L > L_0$) can be generated in vitro, however no biological significance has been found for overwound DNA.

DNA TOPOISOMERASES

How does the chromosome become negatively supercoiled? Environmental parameters such as ionic strength, temperature, and the concentrations of certain DNA binding ligands affect the number of primary twists per DNA molecule. Therefore, the value of L_0 in equation 1 will vary depending on the environmental conditions in which the DNA is formed (reviewed in 18).

The other factor that determines superhelicity of DNA is the concerted activity of a class of enzymes termed DNA topoisomerases. Topoisomerase I relaxes negative supercoils by breaking one strand of the duplex and releasing one supercoil (L) at a time. Topoisomerase II (DNA gyrase) supercoils the chromosome by binding DNA at two places, breaking both strands of one of the bound portions and passing the other bound portion through the break (Figure 1), thus decreasing L by two for each reaction (24).

The activities of topoisomerases I and II in the cell are balanced to maintain an optimal level of superhelicity of the chromosome. Mutations in Escherichia coli topoisomerase I result in an increase in negative superhelical density in both the bacterial chromosome and plasmid pBR322. Some strains containing topoisomerase I mutations, however, have decreased negative superhelical density. These mutants have developed secondary mutations in DNA gyrase that compensate for the presumably intolerable level of superhelical density caused by the deficiency in topoisomerase I (66).

The homeostatic control of DNA supercoiling by topoisomerase I and DNA gyrase regulates the expression of the E. coli DNA gyrase gene.

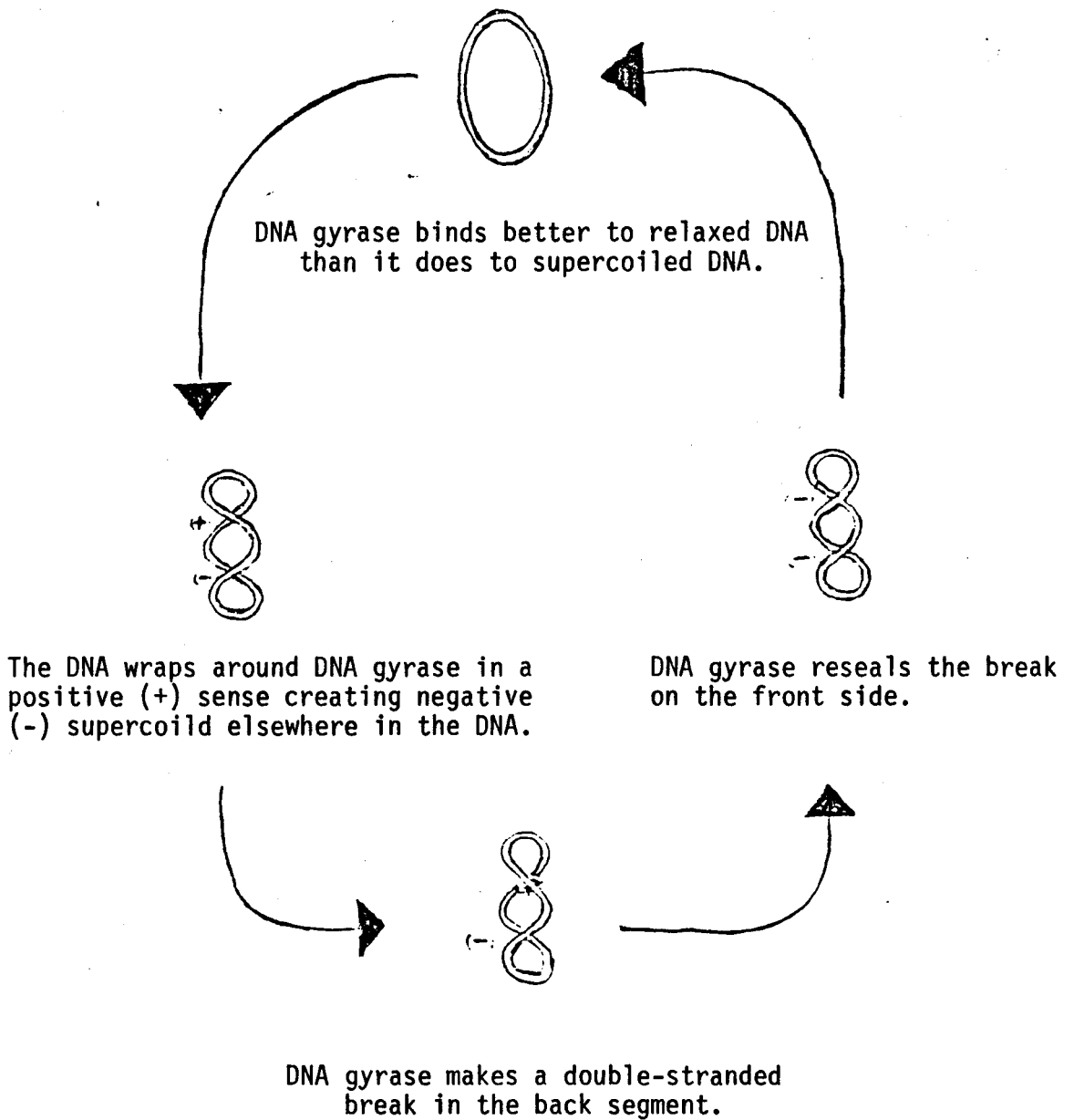


Figure 1. The Supercoiling Reaction of DNA Gyrase

Addition of exogenous topoisomerase I to an S-30 in vitro protein synthesizing system stimulates synthesis of DNA gyrase (52). This stimulation is probably due to a decrease in the negative superhelical density and not to any direct role of E. coli topoisomerase I as a regulatory protein, since addition of exogenous HeLa cell topoisomerase I (a eukaryotic enzyme) to this system also stimulates DNA gyrase synthesis (52). Treatments that block DNA gyrase activity and decrease the superhelical density of intracellular DNA result in as much as a 10-fold increase in the in vivo synthesis of both the A and B subunits of DNA gyrase (52). Thus, relaxation of the chromosome stimulates the synthesis of DNA gyrase and results in an increased capability of the cell to supercoil DNA. Conversely, increasing the superhelical density of the chromosome decreases the synthesis of DNA gyrase and results in a decreased capability of the cell to supercoil DNA. It is not known if the expression of the topoisomerase I gene is affected by the degree of supercoiling as well.

DNA gyrase is made up of two subunits (A and B) which map separately on the E. coli chromosome (29,62). The holoenzyme, which has a molecular weight of 400,000, consists of two A subunits (MW 105,000) and two B subunits (MW 95,000) (29,62). In vitro there are seven different reactions catalyzed by DNA gyrase as a holoenzyme or by one of the subunits alone (Figure 2):

1. Both subunits are required for negative supercoiling of a closed DNA duplex in a processive reaction that required ATP and Mg^{++} , and that is stimulated by spermidine (10,21,22,29).

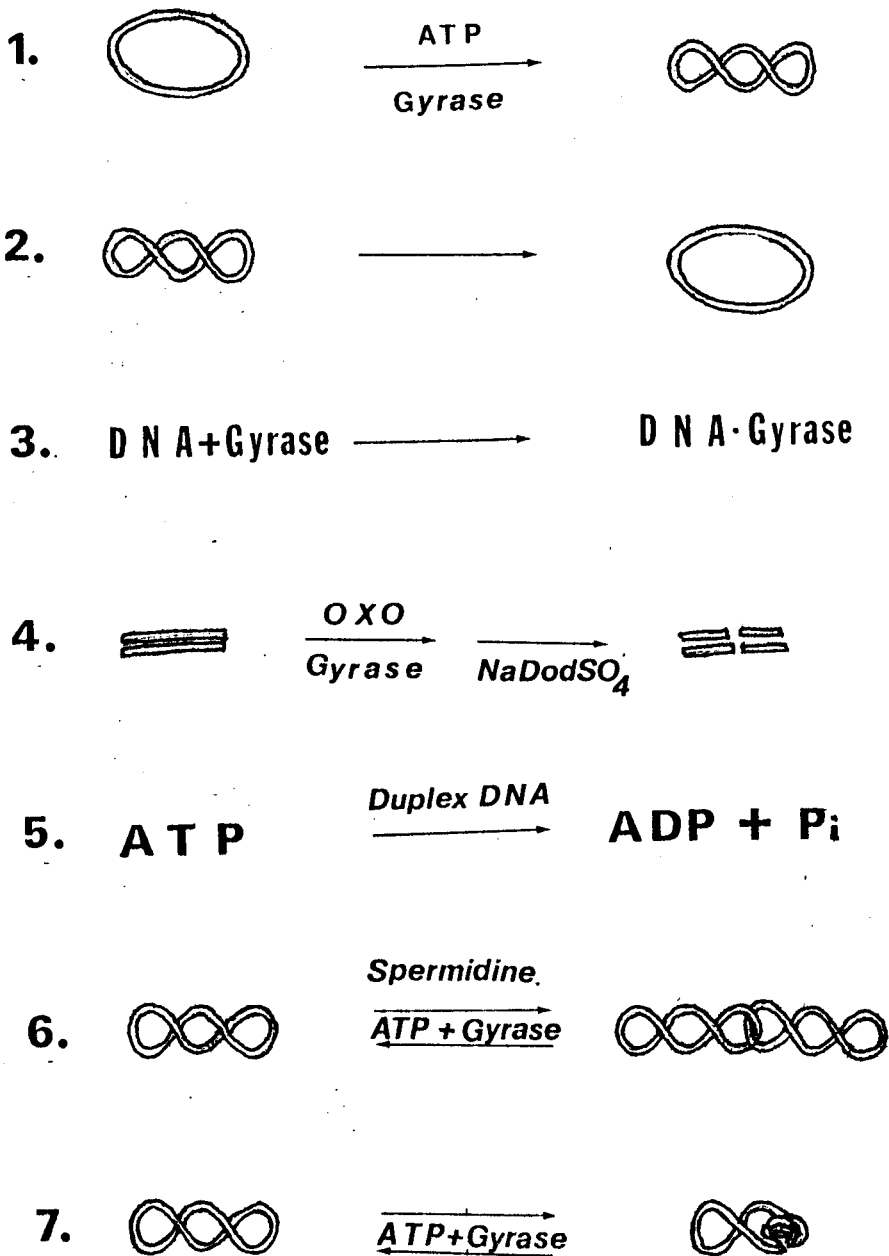


Figure 2. The Activities of DNA Gyrase.

The reactions illustrated are supercoiling (reaction 1), relaxation (reaction 2), binding (reaction 3), cleavage (reaction 4), ATPase (reaction 5), catenation and decatenation (reaction 6), and knotting and unknotting (reaction 7). The DNA substrate is shown as relaxed, closed circular DNA for reaction 1, negatively supercoiled DNA for reaction 2, 6, and 7, and as linear DNA for reaction 4. Oxolinic acid is abbreviated OXO and sodium dodecyl sulfate as NaDodSO₄.

2. The A subunit will relax negatively supercoiled DNA in the absence of ATP (22,23,46,77). In the presence of oxolinic acid (an inhibitor of the A subunit), less than one percent of the supercoiling reaction products consists of cleaved linear DNA (77).

3. The holoenzyme binds to DNA about every 100 kilobase pairs. About 140 base pairs of DNA wrap around the outside of the enzyme in a positive coil creating counterposing negative supercoils elsewhere in the DNA (29,39,62). DNA gyrase binds better to relaxed DNA than it does to supercoiled DNA (10). Using electron microscopy, the A subunit has been visualized to bind to DNA in the absence of the B subunit (62). Electron micrographs of DNA gyrase from M. luteus shadowed with platinum show a heart shaped molecule. When these molecules are complexed with DNA, two binding modes are observed, one in which the length of the bound DNA is shortened, termed the full binding mode, and a second in which the length of the bound DNA is unaltered, termed the incomplete binding mode (38).

4. The holoenzyme cleaves the DNA by forming a phosphotyrosine bond between the 5'-hydroxyl of each strand and the A subunit (79). This can be seen in vitro by incubating DNA gyrase and DNA with oxolinic acid, followed by treatment with sodium dodecyl sulfate (SDS). Cleavage is observed in the absence of SDS, but SDS treatment increases the fraction of cleaved molecules from less than one percent to 41 percent. The same pattern of cleavage is observed when DNA gyrase isolated from E. coli, Micrococcus luteus, Bacillus subtilis, or a combination of subunits from E. coli and M.

luteus is used (6,61,76,77). ATP alters the cleavage pattern by stimulating cleavage at some sites and reducing it at others (77).

5. The B subunit hydrolyzes ATP into ADP and P_i in the presence of duplex DNA (58,77).

6. The holoenzyme can catenate and decatenate DNA rings in the presence of ATP. Catenation requires spermidine and decatenation requires the absence of spermidine (2).

7. The holoenzyme can also unknot and knot DNA in the presence of ATP (10,43).

The subunits of DNA gyrase are most likely in equilibrium with the complete protein, since the addition of the A subunit to DNA gyrase stimulates its supercoiling activity and addition of drug resistant A subunit to DNA gyrase renders the supercoiling activity resistant to the drug.

TOPO II'

The A subunit of DNA gyrase can also bind with another protein, termed v, to form topo II'. The v subunit which consists of a single 50,000 dalton polypeptide appears to be a proteolytic product of the B subunit, since their polypeptide maps show substantial similarity (6). Topo II' has three activities in common with DNA gyrase; relaxation of negative supercoils, wrapping of the DNA substrate around it in a positive coil, and cleavage of DNA at specific sites in the presence of oxolinic acid (reference 4, and activities 2, 3, and 4 from the previous section). Although the cleavage patterns of topo II' and DNA gyrase are the same, topo II's pattern is not altered by ATP as is the pattern of

DNA gyrase (6,77). Topo II' possesses one activity that DNA gyrase lacks; it will relax positive supercoils. This activity does not require ATP. Neither subunit A or v by themselves will relax positive supercoils (6). It appears that subunit v results from the cleavage of the portion of the B subunit that contains the ATPase activity.

Brown et al. (6) noticed that DNA relaxed by topo II' has a higher electrophoretic mobility than DNA gyrase-relaxed DNA. This increased mobility is due to positive supercoils present in the topo II' relaxed DNA to an extent depending stoichiometrically on the ratio of enzyme to DNA. They suggest that the reason for this is that topo II', like DNA gyrase, wraps the DNA substrate around itself in a positive coil which creates counterposing negative supercoils elsewhere in the DNA. Topo II' unlike DNA gyrase will relax these negative supercoils, leaving positively supercoiled DNA.

It is suspected that less pure preparations of DNA gyrase are contaminated with this relaxing enzyme, because as the purity of DNA gyrase is increased, the ratio of oxolinic acid-sensitive relaxation activity to oxolinic acid-sensitive supercoiling activity declines to a nonzero plateau value (29,62,77; i.e., there is an excess of relaxation activity over supercoiling activity). The contaminating relaxing activity cannot be due to topoisomerase I, since this enzyme is not sensitive to oxolinic acid (24). Since topo II' can relax positive supercoils, the ability of less pure DNA gyrase preparations to relax positive supercoils (22,77) and the disappearance of this activity upon further purification (6) can be explained.

There are three known E. coli topoisomerases (compared in table 1): topoisomerase I, topoisomerase II (DNA gyrase), and topoisomerase II'.

Table 1. *E. coli* Topoisomerases

Property	Topo I (w)	Topo II (gyrase)		Topo II'	
Subunits	Monomer	A	B	A	v
Protomer Mw. (x 10 ³)	110	105	95	105	50
Structural gene	Unknown	<u>gyrA</u>	<u>gyrB</u>	<u>gyrA</u>	<u>gyrB?</u>
Activities:					
Relaxation of (-) Supercoils	Yes	Yes		Yes	
Relaxation of (+) Supercoils	No	No		Yes	
Introduction of (-) Supercoils	No	Yes		No	
Stoichiometric (+) Supercoil Introduction	No	No		Yes	
DNA Wrapping around Enzyme	None	(+)		(+)	
DNA Site- specific Binding	Some	Yes		Yes	
Denaturant- induced DNA breaks	Single- strand	Double- strand		Double- strand	

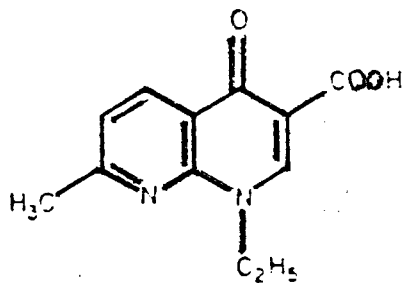
All relax negative supercoils, with topoisomerase I possessing the greatest total detectable activity, topo II' the next most, and DNA gyrase the least (6). Only DNA gyrase can negatively supercoil DNA, and only topo II' can relax positively supercoiled DNA (6).

ANTIBIOTICS THAT AFFECT DNA GYRASE AND TOPO II'

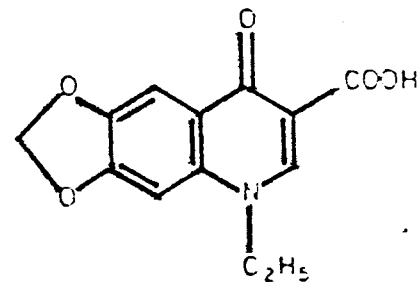
The activities of the B subunit are inhibited by coumermycin A1 and novobiocin (reviewed in 20 and 24). These antibiotics cause a reduction in the superhelical density that closely parallels an inhibition of DNA synthesis, indicating that the decrease causes the inhibition of DNA synthesis (16).

Pyridonecarboxylic acid derivatives (e.g., nalidixic acid, oxolinic acid, and norfloxacin, Figure 3) are antimicrobial drugs effective against most gram negative bacteria. These drugs inhibit DNA synthesis by inhibiting the activities of the A subunit (10). This inhibition is readily reversed by removal of the drug. Engle et al. (19) have suggested that these drugs inhibit DNA replication by forming a tight complex between the A subunit and DNA which inhibits replication fork migration. Upon treatment with the drug, there is an immediate inhibition of DNA synthesis. Recovery synthesis following inhibition is possible in wild type strains of E. coli but not in mutants deficient in recombinational repair of DNA (19).

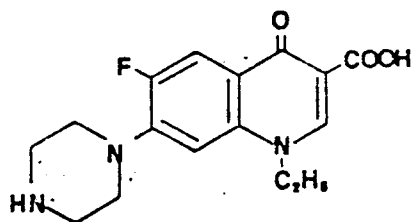
Recent evidence shows that pyridonecarboxylic acids bind to DNA and not the DNA gyrase (70). They bind preferentially to single-stranded DNA rather than the double-stranded DNA. The calculated apparent K_d values of the different derivatives correlate well with their K_i values



Nalidixic Acid
Mw. 232.2



Oxolinic Acid
Mw. 261



Norfloxacin
Mw. 319.33

Figure 3. The Chemical Structures of Pyridonecarboxylic Acid Derivatives.

(i.e., the more potent the inhibitor, the greater it binds to DNA). Shen et al. (70) proposed that DNA gyrase translocates along the DNA or DNA segments translocate through gyrase until the DNA gyrase comes to the bound drug molecule and forms a ternary complex that is more stable than the binary DNA gyrase-DNA complex.

Inhibition of DNA synthesis in E. coli by pyridonecarboxylic acids has been shown to have biphasic kinetics in the absence of DNA repair or initiation of new rounds of DNA replication (17). There is an initial rapid rate of inhibition followed by a slower rate of inhibition. If the drug-DNA gyrase complexes occurred only at 100 kbp intervals, then monophasic kinetics would be expected. The fact that oxolinic acid and SDS treatment induces additional cleavage in pulse-labeled DNA as opposed to long term-labeled DNA (17), indicates that DNA gyrase binds at or near the replication fork in addition to its binding at about 100 kbp intervals. The initial rapid inhibition observed in pyridonecarboxylic acid treated E. coli cells may be due to inactivation of gyrase at or near the replication forks. Some replication forks may have escaped the drug and the second slower rate of inhibition may be due to these replication forks proceeding to the terminus of replication. Increasing the number of inactivated DNA gyrase sites by increasing the concentration of pyridonecarboxylic acids causes an increase in the initial rapid rate of inhibition but does not change the slower rate of inhibition (17). This suggests that the replication forks are not stopped by drug-DNA gyrase complexes located at 100 kbp intervals (17).

DNA REPAIR AND RECOVERY SYNTHESIS IN E. COLI

There are four DNA repair pathways known to exist in E. coli (82). These pathways are called photoreactivation, SOS processing, excision repair, and postreplication recombination (Table 2).

Photoreactivation specifically repairs the most common effect of ultraviolet irradiation on DNA, the thymine dimer. A thymine dimer, results from the covalent cross-linking of two thymine bases located adjacent to each other on the same strand of DNA. The photoreactivation enzyme binds to a dimer and directly cleaves the crosslink in a light-dependent reaction (82). Therefore, in order to study the other three DNA repair pathways without interference of photoreactivation, experiments must be conducted in the dark.

The three dark repair pathways have the recA gene in common. The product of the recA gene is a protein that regulates the expression of SOS processing and one of the two modes of excision repair. It is also an integral part of recombinational repair. A double mutant deficient in both excision repair and the recA function has virtually no DNA repair capacity in the absence of visible light (82).

SOS processing is part of the SOS response that occurs when the protease activity of recA protein is induced by a DNA damaging agent such as ultraviolet light or nalidixic acid. The recA protease acts by cleaving a repressor protein coded for by the lexA gene. This lexA repressor binds to the control regions of the genes involved in the SOS response (Table 2) and prevents their expression. Cleavage of the lexA repressor by the recA protein allows the expression of the SOS genes until the DNA damaging signal is gone and the recA protease function is

Table 2. The Genes Involved in the SOS Response

DNA Repair Pathways	<u>E. coli</u> Genes	Removes Lesions	Replication Dependent	Inducible	Mutagenic
Photoreactivation	<u>phr</u>	Yes	No	No	No
Excision Repair	<u>uvrA</u> , <u>uvrB</u> , <u>uvrC</u> , <u>uvrE</u>	Yes	No	Yes*	No
Postreplication Recombination	<u>recA</u> , <u>recB</u> , <u>recC</u> , <u>sbcB</u> , <u>sbcB</u> , <u>recE</u> , <u>recF</u> , <u>recJ</u> , <u>recK</u>	No	Yes	Yes	No
SOS Processing	<u>umuC</u> , <u>umuD</u> , <u>lon</u> , <u>mut</u> , <u>dam</u> , <u>lexA</u>	No	Yes	Yes	Yes

*The short patch repair mode is constitutive.

no longer active. Not much is known about the SOS processing repair pathway except that it is error-prone (or mutagenic) and that it occurs during replication. It appears that during replications, SOS processing functions will pair any base with a damaged base. Since there is a three out of four chance that the wrong base will be paired with the damaged base, mutations will result from this type of repair (82).

The excision repair pathway repairs DNA by removing a portion of the strand of DNA that contains the damage. Ninety nine percent of the time, a section of approximately 20 bases is cleaved in the short patch repair mode, which is a constitutive function of the cell. In the other one percent of the time, a section of about 1500 bases will be cleaved in long patch repair, a mode which must be induced by the recA protease. This repair must occur before replication can continue since the damage poses as a barrier to the migration of the replication fork (79).

Postreplication recombination is useful when the cell has acquired a large amount of damage and does not have enough time to repair the DNA before replication has to be completed. DNA polymerase will bypass the damage and leave a gap in the complementary strand. The damage and resulting gap will be repaired later when the cell has the time and energy. The gap is filled with a strand from the complementary DNA molecule, leaving one molecule with the damage and the other with a gap. Now each DNA molecule has one good strand that can be used as a template for synthesizing a complementary strand (82).

The recovery synthesis seen in E. coli after inhibition of DNA synthesis by pyridonecarboxylic acid derivatives is not affected by mutations that render the cell deficient in excision repair (19). Recovery synthesis does not occur in mutants with defects in the recA or

lexA genes (19). Since the short patch repair mode of excision repair is not regulated by the recA protein (82), and since the drug-DNA gyrase complex is longer than twenty nucleotides (the length of DNA excised in the short patch repair mode), the short patch repair mode can be ruled out as the repair process used to remove the drug-DNA gyrase complex.

Nalidixic acid has been shown to induce protein X, which has been identified as the protein product of the E. coli recA gene (25,26,29,30). It also has been shown to induce error-prone repair of UV-irradiated phage ϕ X174 DNA in E. coli (67). However, little or no induction of recA occurs at low concentrations of oxolinic acid where inhibition and recovery synthesis still occur (19). Perhaps there are enough low constitutive levels of recA protein present in the wild type cell to remove the drug-DNA gyrase complex from the DNA. The exact DNA repair mechanism that E. coli uses to recover from inhibition of DNA synthesis by pyridonecarboxylic acid derivatives is still unknown.

BACTERICIDAL ACTIVITY OF PYRIDONECARBOXYLIC ACID DERIVATIVES

Inhibition of DNA synthesis may not be the cause of death in cells treated with pyridonecarboxylic acid derivatives. Optimal bactericidal concentrations of these drugs cause almost 100% inhibition of DNA synthesis but still allows some RNA and protein synthesis to occur (76). At higher concentrations, RNA and protein synthesis are inhibited to a greater extent and the drug is no longer as potent. Competent RNA and protein synthesis are required for killing, since their inhibition will reverse the bactericidal effects of pyridonecarboxylic acids (76,83). These results have led Stevens (76) to propose that the mechanism of

drug-induced death was due to an imbalance between DNA and protein biosynthesis which leads to filamentation. This drug-induced filamentation results from the growth of the cell due to continued RNA and protein synthesis when cell division is inhibited due to inhibition of DNA replication. In support of this model, he noted that drug resistant mutants did not experience inhibition of DNA synthesis until high enough concentrations of the drug that RNA and protein synthesis were also inhibited. He also noted that cell death may be due to a more specific event since hydroxyurea also induces filamentation by inhibiting DNA synthesis while allowing RNA and protein synthesis to continue but does not cause cell death (76).

Additional research has also suggested that the mechanism of drug-induced cell death may be more complex. A norfloxacin treated culture of logarithmically growing E. coli cells will almost immediately lose their viability, but some will grow and divide, while others will filament (11). This lethal unbalanced growth could result from the prevention of the completion of a round of chromosome replication by norfloxacin, since a round of chromosome replication may trigger the initiation of septation which precedes cell division (15). Lethal unbalanced growth is also seen in B. subtilis cells during thymine starvation or treatment with ionizing radiation (5). These treatments inhibit DNA replication but do not inhibit RNA or protein synthesis (5).

Several laboratories have shown that inhibition of DNA gyrase causes differential effects on specific gene expression (summarized in table 1 or reference 18). The differential effects on gene expression may be the actual cause of drug-induced death. For instance, inhibiting DNA gyrase results in a decrease in the synthesis of outer membrane

proteins as opposed to inner membrane proteins (28). The rate of synthesis of inner membrane proteins increases exponentially throughout the cell cycle (63,64). The rate of synthesis of the outer membrane proteins is constant in the early part of the cell cycle, but doubles abruptly just before cell division (63,64). It has been proposed that this abrupt increase might be sufficient to initiate surface invagination and trigger septum formation and cell division (63,64). Therefore, by inhibiting the expression of outer membrane proteins, pyridonecarboxylic acid derivatives may prevent cell division.

DNA GYRASE AND GENE EXPRESSION

Genes coding for membrane proteins are not the only ones affected when DNA gyrase is inhibited. Inhibition of gyrase with nalidixic acid causes a decrease in expression of catabolite-regulated operons (66).

A catabolite-regulated operon in E. coli is one that is turned off when glucose is present in the medium. E. coli metabolizes glucose in preference to other sugars such as lactose, galactose, and arabinose. In the presence of glucose, E. coli will repress the expression of the operons that code for the enzymes that metabolize these other sugars. The binding of a protein termed catabolite activator protein (CAP) to the control region of catabolite-regulated operons is required for their expression. The CAP protein, in turn, must interact with cyclic AMP (cAMP) to be able to bind to DNA. Glucose prevents the expression of catabolite-regulated operons by reducing the levels of cAMP in the cell. The reduction in expression of catabolite-regulated operons by nalidixic acid does not seem to be mediated through alterations of the cAMP

concentrations in the cell, since addition of exogenous cAMP does not reverse the effects of nalidixic acid (71).

The more sensitive an operon is to catabolite repression, the more its expression is reduced in the presence of nalidixic acid. The expression of the tryptophanase gene and the three maltose operons, which are all very sensitive to catabolite repression, is also strongly inhibited by nalidixic acid, whereas the lactose operon displays a lower sensitivity to both catabolite repression and nalidixic acid. The threonine and tryptophan operons, which are insensitive to catabolite repression, are also insensitive to nalidixic acid (68).

Smith et al. (73) found that expression of the tryptophan operon is inhibited by novobiocin and nalidixic acid when it is under the control of the lambda phage P_L promoter, but not when it is under the control of its own promoter. Transcription from P_L is inhibited by phage repressor (71). This is another instance where an operon whose transcription is affected by a regulatory protein is repressed by nalidixic acid, while an operon whose transcription is unaffected by regulatory proteins is not repressed by nalidixic acid.

Genes under the control of the tryptophan promoter require coupling between the translational machinery and RNA polymerase for continued transcription, while genes under the control of the P_L promoter do not require this coupling (33). This indicates that there may be a relationship between the repression of a gene by pyridonecarboxylic acids and how closely it is coupled to translation.

Novobiocin and pyridonecarboxylic acids have been shown to have a similar effect on transcription from specific operons (69,72,85), but they have different effects on the synthesis of the protein products of

these specific operons (81). In B. subtilis, novobiocin greatly inhibits the synthesis of alkaline phosphatase and histidase. It inhibits the synthesis of protease to a lesser extent and has no effect on amylase synthesis (81), whereas, pyridonecarboxylic acid severely inhibits only alkaline phosphatase synthesis (81). This difference between the two types of drugs indicates that they may effect gene expression through different mechanisms.

The selective inhibition of gene expression by novobiocin and coumermycin A1 is most likely a result of the decrease in negative superhelical density caused by these drugs (37). As was mentioned earlier, decreasing the superhelical density of the E. coli chromosome by addition of exogenous topoisomerase I will stimulate the expression of the DNA gyrase gene in vitro (52). DNA gyrase synthesis can also be stimulated up to 10-fold by treatment with coumermycin A1 or novobiocin, which block DNA gyrase activity and decrease the supercoiling of intracellular DNA (52). It appears that changing the degree of supercoiling by inhibiting DNA gyrase will have a positive effect on the expression of some genes and a negative effect on others. The change in superhelical density may alter the shape of binding sites of regulator proteins, such as control regions and promoters, and thus alter the affinity of the control proteins, such as CAP and RNA polymerase, for these sites.

Pyridonecarboxylic acid derivatives may effect gene expression through a different mechanism, because the reduction of gene expression by oxolinic acid does not parallel the decrease in superhelical density caused by the drug (51). The selective inhibition of gene expression

may be caused by the drug-DNA gyrase complexes spread throughout the genome (74).

PSEUDOMONAS AERUGINOSA DNA GYRASE AND TOPO II'

P. aeruginosa is not inhibited by most pyridonecarboxylic acids (34), and DNA gyrase from P. aeruginosa is more resistant to inhibition by nalidixic acid than is gyrase from E. coli (56). Norfloxacin, however, has been shown to be an effective antimicrobial agent against P. aeruginosa (30,34). In general, norfloxacin is more potent and acts against a broader spectrum of bacteria than other pyridonecarboxylic acids (30,34). Also, norfloxacin treatment reduces the amount of enterotoxin transported into the periplasm of E. coli cells (11). Often, the symptoms of patients with bacterial infections are caused by specific proteins such as enterotoxin, which are excreted by the invasive bacteria. Therefore, norfloxacin may also have a value in the symptomatic relief of patients.

In this dissertation, norfloxacin will be studied and compared to other pyridonecarboxylic acid derivatives. Previous studies with pyridonecarboxylic acid derivatives have dealt mostly with E. coli, a bacterium commonly found in the human gut. This dissertation will deal with P. aeruginosa, a soil bacterium that has been increasingly detected as an opportunistic pathogen in patients suffering from trauma (2), burns (14), and cystic fibrosis (13,14,31,32). The A subunit of DNA gyrase and topo II' from E. coli has been very well characterized (10,16,17,18,20,21,22,23,24,29,37,39,43,48,49,52,58,59,60,61,62,75,77) while little is known about P. aeruginosa A subunit (56).

METHODS

STRAINS AND MEDIA

All strains (Table 3) have been derived from the prototrophic strain PA01 (67), and were maintained in Luria broth (55). Rec^- strains are deficient in recombinational repair and Les^- strains are deficient in prophage lysogeny establishment (Table 3). The norfloxacin resistant strains are spontaneous mutants (i.e., not exposed to mutagens). They were isolated by incubating strain RM9 in 10 tubes containing high concentrations of norfloxacin. One clone was isolated out of each of the four tubes exhibiting growth. E. coli DNA gyrase was isolated from strain H560 (polA, endA) which was a gift from Dr. Martin Gellert (22). All experiments were carried out in Pseudomonas Minimal Medium (57) containing 0.4% glucose and 0.01% arginine. All drugs were freshly prepared from each experiment at 100-fold greater than the desired final concentration. Norfloxacin (gift of Merck, Sharp, and Dohme), nalidixic acid (Sigma), and oxolinic acid (Sigma) were dissolved in .01 N NaOH and novobiocin in water. Chloramphenicol and rifamycin were dissolved in ethanol. Phage D3 and plasmid pME299 were gifts from Dr. Bruce W. Holloway and Dr. Dieter Haas, respectively. ϕ X174 was purchased from Amersham.

Table 3. P. aeruginosa Strains Used in This Study

Strain	Genotype	Relevant Phenotypes
PA0303	argB21	Rec ⁺
RM8	argB21, lesB908	Rec ⁻
PA02001	arg-32, spr-39	Rec ⁺
PA02003	arg-32, spr-39, and rec-1	Rec ⁻

MIC'S AND MBC'S

The minimal inhibitory concentrations (MIC's) and minimal bactericidal concentrations (MBC's) were determined as follows. A series of test tubes containing varying concentrations of antibiotic in Mueller-Hinton broth were inoculated with 10^6 bacteria per ml and incubated at 37° C. The minimum concentration at which growth visible with the naked eye could be seen after 23 hours was designated the MIC. One hundred microliters of each culture which exhibited no visible growth was plated out in triplicate and incubated at 37° C for 23 hours. The concentration which allowed no growth on the plates was designated the MBC.

RATE OF PROTEIN SYNTHESIS

A growing culture of bacteria in early log phase was treated with antibiotic(s) (norfloxacin, nalidixic acid, and/or chloramphenicol). The rate of DNA or protein synthesis was determined by a modification of the method of Engle et al. (19). A flask containing 60 ml of Luria broth was inoculated with P. aeruginosa to 9 Klett units (OD_{660}) and incubated in a 37° C water bath with shaking until the culture grew to 20 Klett units (early log phase). The culture was then split into three flasks (20 ml each), one of which was treated with norfloxacin, another with nalidixic acid, and the other with buffer containing no drug (100 microliters of .01 N NaOH). At various times, 0.2 ml samples were removed and protein labeled with L-[4,5- 3 H]-leucine (1 microcurie/ml). After 4 minutes, NaOH (final concentration 0.3 N) and EDTA (final

concentration 0.1%) was added. The protein was precipitated with 40 micrograms/ml of bovine serum albumin (BSA) and five percent trichloroacetic acid (final concentrations). The protein precipitates were collected on nitrocellulose filters, and counted in a Beckman LS 7500 scintillation counter.

RATE OF DNA SYNTHESIS

The rate of DNA synthesis was measured in the same way that the rate of protein synthesis was measured except that [³H]-adenine (1 microcurie/ml) was used and RNA was hydrolyzed by incubating the samples at 37⁰ C overnight.

KILLING EFFECTS OF ANTIBIOTICS

A growing culture of bacteria in early log phase was divided into several aliquots and added to flasks containing various combinations of antibiotics (2700 micrograms/ml nalidixic acid, 16 micrograms/ml norfloxacin, 100 micrograms/ml rifamycin, and 500 micrograms/ml chloramphenicol). These were incubated with shaking at 37⁰ C and the number of colony forming units (CFU's) /ml counted.

WEIGLE REACTIVATION AND MUTAGENESIS

A concentration of 10⁶ phage D3/ml was exposed to ultraviolet radiation at 30 Joules/meter². A growing culture of bacteria in early log phase was treated with antibiotic. At various times 0.2 ml samples

of bacteria were removed and exposed to UV-treated phage. After 7 minutes at room temperature, λ top agar was added and the mixture plated out on Luria agar plates. After incubating at 37⁰ C overnight, the number of plaque forming units were counted to determine Weigle reactivation. Also, to determine mutagenesis, the number of D3 wild type plaques (cloudy phenotype) which reverted to the D3 mutant plaques (clear phenotype) was counted.

PLASMID MAINTENANCE

PA0303 and RM8 were made competent by MgCl₂ treatment (47) and transformed with plasmid pME292 which carries resistance to carbenicillin. A concentration of 10⁶ plasmid-containing cells/ml were exposed to varying concentrations of norfloxacin, nalidixic acid, or novobiocin up to the MBC and incubated with shaking at 37⁰ C. After 23 hours, bacteria were diluted and plated on Luria agar plates. After incubating at 37⁰ C overnight, the plates were replica plated onto Luria agar plates containing 500 micrograms/ml of carbenicillin. After incubation at 37⁰ C, the number of carbenicillin-resistant colonies was counted to determine how many still contained the plasmid.

PURIFICATION OF DNA GYRASE

Three Luria agar plates (40 cm by 24 cm each) were inoculated with P. aeruginosa strain PA0303 or E. coli strain H560 and incubated overnight at 37⁰ C. Cells were harvested (wet weight 25 grams) and suspended in 100 ml of 0.85% NaCl, incubated with shaking for 10 minutes

and centrifuged at 10,000 xg for ten minutes. The pellet was resuspended at a concentration of 0.5 g/ml of a 10% sucrose solution prepared in 50 mM Tris-HCl (pH 8.0). The suspension was frozen in a dry ice-ethanol bath and thawed in a 25^o C water bath. The volume was measured and 0.1 ml of a solution of lysozyme (2 mg/ml) was added for each ml. The mixture was allowed to incubate at 0^o C for 60 minutes. All subsequent reactions were carried out at 4^o C. The solution was centrifuged for 30 minutes at 35,000 xg. The supernatant was decanted (Fraction I) (56).

The magnesium acetate concentration of this crude extract was adjusted to 5 mM and the extract was centrifuged for 4 hours at 30,000 rpm in a Beckman Ti50 rotor. The supernatant (Fraction II) was loaded onto a novobiocin-Sepharous (75) column and washed with buffer A (0.2 M KCl, 5 mM dithiothreitol, and 10% glycerol) until the OD₂₈₀ of the eluant reached a minimum. DNA gyrase was eluted from the column with 5 M urea in buffer A. One ml fractions were collected and dialyzed against buffer B (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol) for 4 hours. The fractions were then dialyzed for an additional 20 hours against buffer B in the presence of 5 mM dithiothreitol. Each fraction was tested for supercoiling activity and the active fractions pooled.

PREPARATION OF RELAXED DNA

Col E1 DNA was isolated from E. coli strain JC 411 by growing a one liter culture in Luria broth to 40 Klett units and amplifying the plasmid by adding 200 micrograms/ml of chloramphenicol and incubating in

a 37⁰ C water bath with shaking overnight. The cells were then harvested at 10,000 xg for 10 minutes, resuspended in saline and incubated in a 37⁰ C water bath with shaking for 15 minutes in order to deplete the pools of ATP in the cells. The cells were then harvested again at 10,000 xg for 10 minutes and resuspended in 33 ml of cold 25% sucrose in 50 mM Tris-HCl (pH 8.0) and 6.6 mls of solution containing 2 mg/ml of lysozyme in 0.25 M Tris-HCl (pH 8.0) was added. After the mixture was swirled gently for 5 minutes in a 0⁰ C ice bath, 14 ml of cold .25 M ethylenediaminetetraacetic acid (EDTA, pH 8.0) was added. After mixing at 0⁰ C for another 5 minutes, 40 ml of cold two percent Sarkosyl in 0.05 M Tris-HCl (pH 8.0) and 0.0625 M EDTA was added. Five molar NaCl was added to a final concentration of one molar after another five minutes of mixing at 0⁰ C. Mixing was then continued at 4⁰ C for four hours to overnight. The supernatant was collected after centrifugation at 17,000 xg for 30 minutes and DNA was precipitated at 20⁰ C overnight after adding potassium acetate to a final concentration of 0.3 M and two volumes of 95% ethanol.

DNA was collected by centrifuging at 12,000 xg at 0⁰ C for 30 minutes, discarding the supernatant and drying the pellet. The pellet was dissolved in 16 mls of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE) after which 17.6 grams of cesium chloride and 1.6 mls of 5 ml/ml ethidium bromide was added. This mixture, which was kept in the dark from this point on in order to avoid nicking of the DNA, was centrifuged in a VTI65 rotor at 40,000 xg for 16 hours in a Beckman L5-50 ultracentrifuge. Under ultraviolet (UV) light, the plasmid band could be visualized below the chromosomal band. The plasmid band was isolated by inserting a 38.1 mm hypodermic needle just under the band and one at

the top of the tube. The liquid which flowed out of the tube was collected until the band almost disappeared and the chromosomal band was nearing the needle. Ethidium bromide was extracted three times with isopropanol saturated in a solution of cesium chloride. Potassium acetate was added to a concentration of 0.3 M and then four volumes of ethanol was added. After keeping at -20° C overnight, DNA was collected by centrifuging at 12,000 xg for 15 minutes at 0° C, discarding the supernatant, drying the pellet and resuspending in TE. The DNA was examined and the concentration determined by electrophoresis of differing amounts of the DNA on a 0.8% agarous gel at 50 volts for 17 hours with appropriate size and concentration controls. The DNA was visualized by staining the gel in 1 mg/ml ethidium bromide and placing on a UV light box.

Supercoiled phage ϕ X174 (Amersham) or Col E1 DNA was relaxed with topoisomerase I (Amersham). Twenty-five micrograms of DNA and 1.7 units of topoisomerase I were incubated at 37° C overnight in a 25 microliter volume containing 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, 1 mM EDTA, and 0.1 mg/ml of bovine serum albumin (BSA). Topoisomerase was inactivated by incubating at 70° C for 10 minutes and extracted with chloroform and isopropanol at a ratio of 24:1. The product was examined by electrophoresis as described above.

Amersham observed no nuclease activity in the topoisomerase I after incubation of 1 microgram of pBR type 1 DNA with 40 units of enzyme for 30 minutes at 37° C in the relaxation buffer described above containing 1-10 mM $MgCl_2$.

Relaxed DNA was also prepared with a nicking-closing extract which was prepared from chicken red blood cells as described by Comerini-Otero

and Felsenfeld (8). Red blood cells were harvested from whole chicken blood by centrifuging at 10,000 xg for 10 minutes. The pellet was washed four times with phosphate buffered saline (PBS buffer) by centrifugation and resuspension. The cells were then resuspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), and 1 mM magnesium acetate and washed twice. The cells were washed five times in the same sucrose solution and 0.5% (v/v) Triton X-100. The resulting nuclei were then washed an additional three times in the absence of Triton. The nuclei were swollen by washing them in 10 mM Tris-HCl (pH 8.0), 0.5 mM EDTA. In all cases, a Dounce homogenizer (loose pestle) was used for resuspension. The nuclei, swollen to about five to ten times their original volume, were then mixed with an equal volume of 300 mM phosphate buffer (pH 7.5), homogenized with a Dounce homogenizer, and centrifuged at 25,000 xg for 15 minutes. The supernatant, which is the nicking-closing extract, was monitored for relaxing activity by measuring the conversion of supercoiled Col E1 DNA to the relaxed closed-circular form as demonstrated by agarose gel electrophoresis (described above).

The nicking-closing extract was used to prepare relaxed DNA substrate in a 50 microliter reaction volume which contained 42 microliters of nicking-closing extract and 30 micrograms of supercoiled Col E1 DNA or ϕ X174 DNA in 100 mM NaCl, 10 mM Tris-HCl (pH 7.8), 0.25 mM EDTA, and 2.5% (v/v) glycerol. The reaction was carried out at 37⁰ C for 30 minutes. Enzyme was inactivated by heating at 70⁰ C for 10 minutes followed by extraction in chloroform:isopropanol at a ratio of 24:1. The product was examined by agarose gel electrophoresis as described above.

SUPERCOILING REACTION

I determined the units of activity of my preparations of DNA gyrase as the amount of enzyme required to completely supercoil 0.5 micrograms of relaxed DNA at 37⁰ C in 30 minutes in a 25 microliter reaction volume containing 50 mM Tris-HCl (pH 7.6), 20 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, 1.5 mM ATP, 5 mM spermidine-Cl₃, and 50 micrograms/ml BSA. If the units were determined using relaxed Col E1 DNA, then this same DNA was used for all subsequent experiments. The same holds true for when relaxed ϕ X174 DNA was used.

Three other buffers were also used in order to perfect the supercoiling conditions, however, they were not used in any of the final experiments. The other buffers were: buffer 1 (35 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 1.5 mM DTT, 1.5 mM spermidine-HCl, 1.5 mM ATP, and 50 micrograms/ml of BSA), buffer 2 (35 mM Tris-HCl (pH 7.5), 1.6 mM MgCl₂, 19 mM KPO₄ (pH 7.5), 5 mM spermidine-HCl, 1.4 mM ATP, 90 micrograms/ml E. coli tRNA, and 3.6 mg/ml BSA), and buffer 3 (35 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 20 mM KCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM spermidine-HCl, 1 mM ATP, 40 micrograms/ml BSA, and 10% (w/v) glycerol).

To stop the reaction, a mixture of 20% Ficoll and 400 micrograms/ml of bromophenol blue was added. The total volume of the samples was loaded onto a 0.8% agarous gel and electrophoresed at 50 volts for at least 14 hours in TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA). The gels were stained with ethidium bromide (0.5 micrograms/ml) for 15 minutes and destained in water for 15 minutes before being photographed under ultraviolet light. DNA's supercoiled to different extents migrated as individual bands with the most greatly supercoiled DNA

migrating farthest (lowest band), the completely relaxed DNA migrating the least (highest band), and with the linear DNA migrating as a band in between the completely relaxed and completely supercoiled DNA.

RELAXATION REACTION

One unit of the DNA gyrase preparation was incubated with 0.5 micrograms of supercoiled ϕ X174 DNA at 37⁰ C for 2 hours in a 25 microliter volume containing 35 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 18 mM potassium phosphate, 1.8 mM spermidine-Cl₃, 9 micrograms/ml *E. coli* tRNA, 0.36 mg/ml BSA, and 5 mM dithiothreitol. The reaction was stopped, electrophoresed, and photographed the same as the supercoiled reaction.

The velocity of the relaxation reaction in the presence and absence of drug was measured by taking densitometric tracings of negatives of gels. As the DNA is relaxed, a ladder of bands between the completely supercoiled DNA and completely relaxed DNA bands is observed on the gel. As the A subunit decreases the superhelical density by two, the DNA molecule will migrate one band up. Each band of DNA was assigned an even number depending on where it migrated on the gel. The fastest migrating band was assigned zero and the bands above this were given even numbers from two up. The total decrease in superhelical density was determined by multiplying the percentage of DNA in each band as determined by densitometric tracings of the negatives of pictures of the gels by its assigned number and adding up the products.

The velocity was derived by plotting the total decrease in superhelical density against the time that the reaction was allowed to

continue. The slope of the line is equal to the velocity. Linear regression was performed on all the data using a Texas Instruments T155 calculator in order to determine the slope of the line. Table 4 shows one example of the data used to calculate the velocity for various substrate concentrations. Since each experiment did not always result in the same number of topoisomer bands, the velocities in the presence of different antibiotics cannot be directly compared and the maximal velocity determined from these velocity units would be arbitrary.

The Michaelis constant (K_m) of the relaxation reaction was derived by a Hanes plot (27), the equation of which is:

$$s/v = K_m/V + s/V \quad 1.$$

where s is the substrate (supercoiled DNA) concentration, v is the velocity, and V is the maximal velocity. This shows that a plot of s/v against s should be a straight line with slope $1/v$ and X-intercept $-K_m$. The Hanes plot is preferred over the other straight line plots because multiplying $1/v$ by s lessens the large errors that result from taking the reciprocal of small v values that have even slight errors in them.

The inhibition constants (K_i 's) of the different pyridonecarboxylic acid derivatives were derived by a Dixon plot (12), the equation for which is:

$$\frac{1}{v} = \frac{K_m + s}{V_s} + \frac{[K_m/K_i + s/K_i']i}{V_s}$$

where i is the inhibitor (pyridonecarboxylic acid) concentration. This shows that a plot of $1/v$ against i at a constant value of s is a straight line. When two or more such lines are drawn for two or more different s values, the point of intersection along the y axis is equal to $-K_i$.

Table 4. Calculation of the Velocity of the Relaxation Reaction.

Substrate Concentration ($\times 10^{-9}$ mMoles/ml)	Time (min)	Decrease in Superhelical Density	Slope	Correlation Coefficient
0.85	30	62.34	0.122095	0.944
	60	69.63		
	120	73.99		
1.7	30	75.54	0.1347619	0.984
	60	77.49		
	120	87.25		
3.4	60	76.12	0.1531917	0.980
	120	81.94		
	180	94.40		
6.9	30	68.63	0.231	1.0
	60	75.56		

An exactly similar derivation to the Dixon plot by Cornish-Bowden (9) shows that by plotting s/v against i at several s values, a set of straight lines that intersect at $-K_i$ is again obtained. However, in this case, if the lines are parallel the inhibition is competitive.

CLEAVAGE REACTION

One unit of the DNA gyrase preparation was incubated with ϕ X174 DNA under the same reaction conditions which were used to determine the units of supercoiling activity except that one mg/ml of pyridonecarboxylic acid and, in some cases, 1.5 mM ATP or 1.5 mM adenylyl-imidodiphosphate (AppNp) was added. After 30 minutes, 11 mM EDTA, 2 mg/ml SDS, and in one case 90 micrograms/ml of proteinase K was added. The incubation was continued for 60 minutes. The reaction was electrophoresed and photographed the same as the supercoiling reaction.

CATENATION REACTION

This reaction was not specifically tested for, but was observed when the supercoiling reaction products were examined. On the gels that contained the supercoiling reactions products, bands that migrated much higher than the relaxed DNA band appeared. In some cases, the reaction was extracted with a 24:1 mixture of chloroform and isopropanol before loading the gel.

ATPase REACTION

ATPase activity was measured in the supercoiling reaction mixture (described in the Supercoiling Reaction section) after the 30 minute incubation by measuring the release of inorganic phosphate as described by Lanzetta et al. (46). Two control reactions carried out in the absence of DNA gyrase or ATP were also measured for release of inorganic phosphate.

The following stock solutions were prepared with distilled water in glassware that was well rinsed in order to remove any detergents that may have been left over from the dishwashing process: 0.045% malachite green hydrochloride (MG), 4.2% ammonium molybdate in 4 N HCl (AM), 34% sodium citrate-2H₂O (w/v), and 10 mM KH₂PO₄ (salt was dried several hours at 100⁰ C); appropriate dilutions were made from this stock and used for standards. The color reagent was prepared from the above solutions as follows: a 3:1 mixture of MG and AM solutions was mixed at least 20 minutes and then passed through Whatman No. 5 filter paper (MG/AM), and 100 microliters of Sterox (purchased from Coleman Instruments) was added to 5 ml of MG/AM solution (MG/AM/St). To 25 microliters of sample or standard, 400 microliters of the MG/AM/St solution was added mixed. After one minute, 100 microliters of the citrate solution was added and mixed. The OD₆₆₀ of this solution was read after allowing it to sit at room temperature for 2 hours. A standard calibration curve was prepared from the standards previously described every time the experiment was performed. The OD₆₆₀ of the supercoiling reactions was compared to this standard curve in order to

determine the amount of inorganic phosphate released (the amount of ATP hydrolyzed) as compared to the controls.

EFFECTS OF ANTIBIOTICS

The reactions of DNA gyrase were carried out as described above in the presence of norfloxacin, nalidixic acid, oxolinic acid, or novobiocin at concentrations ranging from the MIC to one mg/ml.

RESULTS

POTENCY OF PYRIDONECARBOXYLIC ACID DERIVATIVES ON P. AERUGINOSA

The potency of norfloxacin and nalidixic acid as bacteriostatic and bactericidal agents for the strains to be used in this study was determined by measuring the MIC and MBC of both drugs (Table 5). These concentrations were consistent with those determined for other strains of P. aeruginosa (30). Norfloxacin was several hundred to a thousand fold more potent than nalidixic acid for all strains tested. Strains deficient in recombination (Rec^-) were studied because Rec^- E. coli strains were found to be unable to recover from inhibition of DNA synthesis (19). Our Rec^- strain RM8 was slightly more resistant than its parent, strain PA0303, to both norfloxacin and nalidixic acid. Another Rec^- strain, PA02002 was slightly less resistant to nalidixic acid and exhibited the same level of tolerance to norfloxacin. Since the exact reasons for the Rec^- phenotypes of these strains is unknown, speculation as to why the mutations cause differences in pyridonecarboxylic acid resistance is left for a later date. However, it does appear that recombination is somehow involved in drug resistance.

Spontaneous mutants of PA0303 resistant to norfloxacin (RM166, RM167, and RM169) were isolated as described in Materials and Methods and found to also be resistant to nalidixic acid. RM4000, a spontaneous mutant of RM8 resistant to nalidixic acid which happened to be available

Table 5. The MIC's and MBC's for Norfloxacin and Nalidixic Acid in P. aeruginosa Strains

Strain	Nalidixic Acid		Norfloxacin	
	MIC (micrograms/ml)	MBC (micrograms/ml)	MIC (micrograms/ml)	MBC (micrograms/ml)
PA0303	350	2000 ^a	0.4	16.0
RM166 (Nor ^r)	2000	3000 ^b	6	20.0
RM167 (Nor ^r)	2700	3000 ^b	9	20.0
RM168 (Nor ^r)	3000	3000 ^b	8	20.0
RM169 (Nor ^r)	2500	3000 ^b	6	15.0
RM8 (Rec ⁻)	700	2000 ^a	1.6	16.0
RM4000 (Nal ^r)	3000	3000 ^b	6 ^a	20 ^a
PA02001	175	700 ^a	1.6	2.5 ^a
PA02002 (Rec ⁻)	44	700 ^a	1.6	2.5 ^a

^aThis is an approximate value.

^bThis is a minimum value due to the insolubility of nalidixic acid at concentrations above 3000 micrograms/ml.

in our lab, was found to also be resistant to norfloxacin. This indicates that the two drugs act similarly in that they have the same mechanism of transport into the cells, they have the same targets of action, or both.

The optical density of a culture of cells is a measure of the number of cells in that culture. The optical density of a growing culture of *P. aeruginosa* cells increases exponentially over time (Figure 4). When a culture in exponential (log) growth phase was treated with norfloxacin at a concentration equal to the MIC (0.4 micrograms/ml) or four times the MIC (1.6 micrograms/ml), the rate of growth decreased for about 100 minutes after which there was no more growth. When a concentration equal to the MBC (16 micrograms/ml) was used, there was an immediate inhibition of growth. A culture in late log phase, however, exhibited no significant decrease in the growth rate when treated with the MBC of norfloxacin (Figure 5).

When PA0303 was exposed to nalidixic acid or norfloxacin at a concentration equal to the MBC for these drugs (16 micrograms/ml and 2800 micrograms/ml), the number of colony-forming units (CFU's) recovered was drastically reduced (Figures 6A and 7A). When chloramphenicol or rifamycin was also present in the culture medium, it eliminated the bactericidal effects of norfloxacin and nalidixic acid. Any reduction in CFU's/ml could be attributed to the bacteriostatic effect of chloramphenicol or rifamycin alone. When these experiments were repeated with the Rec^- strain RM8 (Figures 6B and 7B), chloramphenicol did not alleviate the bactericidal effects of norfloxacin or nalidixic acid.

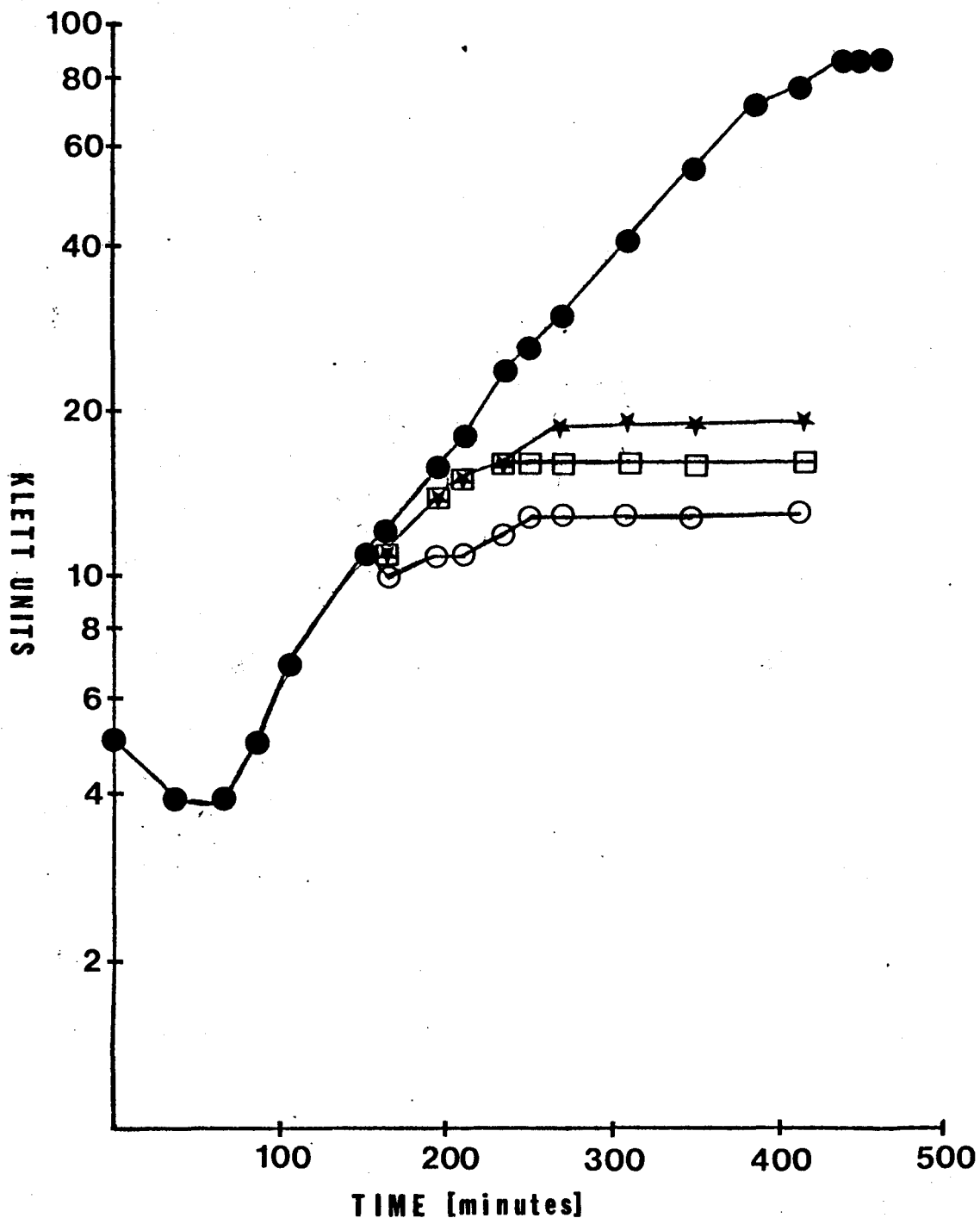


Figure 4. Inhibition of Mid-Log Phase Growth by Norfloxacin.

The growth of a culture of PA0303 was followed by measuring the Klett (OD66) at various times. At 150 minutes, the culture was divided into four portions. Each portion was treated with buffer containing norfloxacin at the MBC (○), four times the MIC (□), the MIC (★), or buffer containing no drug (●).

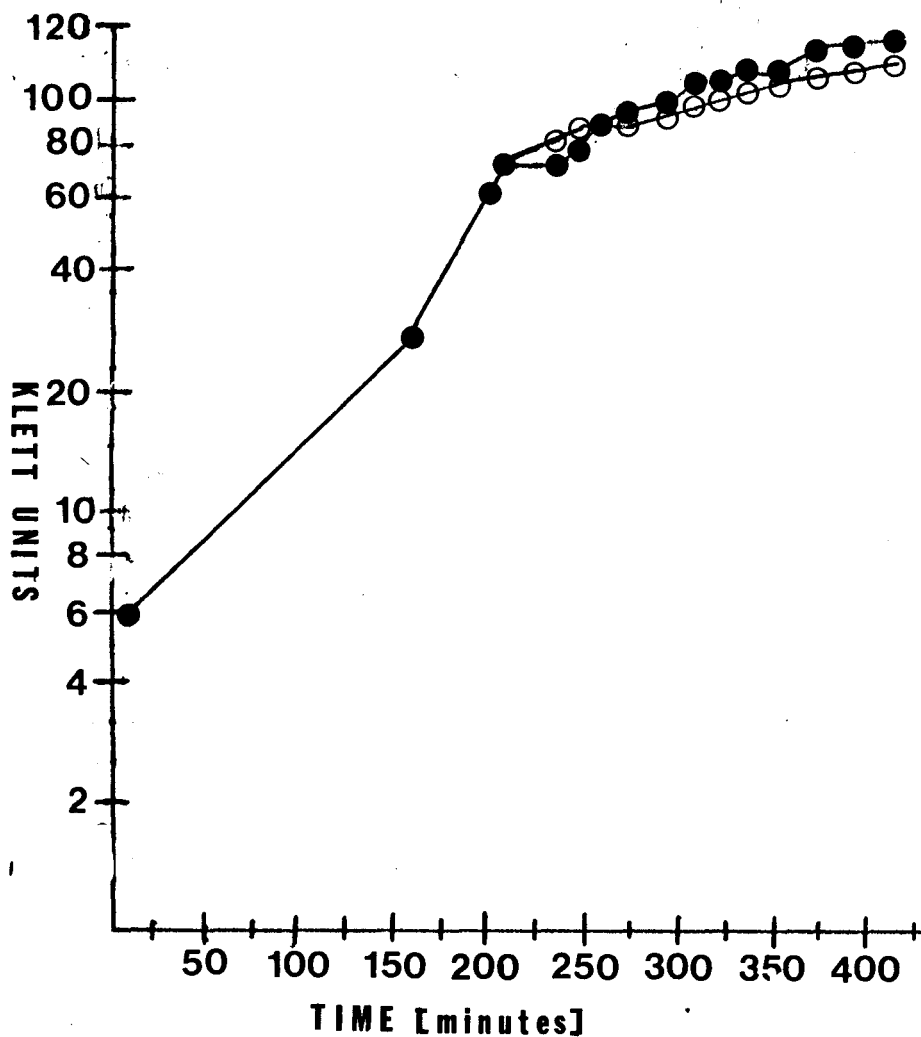


Figure 5. The Effect of Norfloxacin on Late-Log Phase Growth.

The growth of a culture of PA0303 was followed by measuring the Klett (OD660) at various times. At 200 minutes, the culture was divided. Half was treated with the MBC of norfloxacin (○) and half was treated with buffer containing no drug (●).

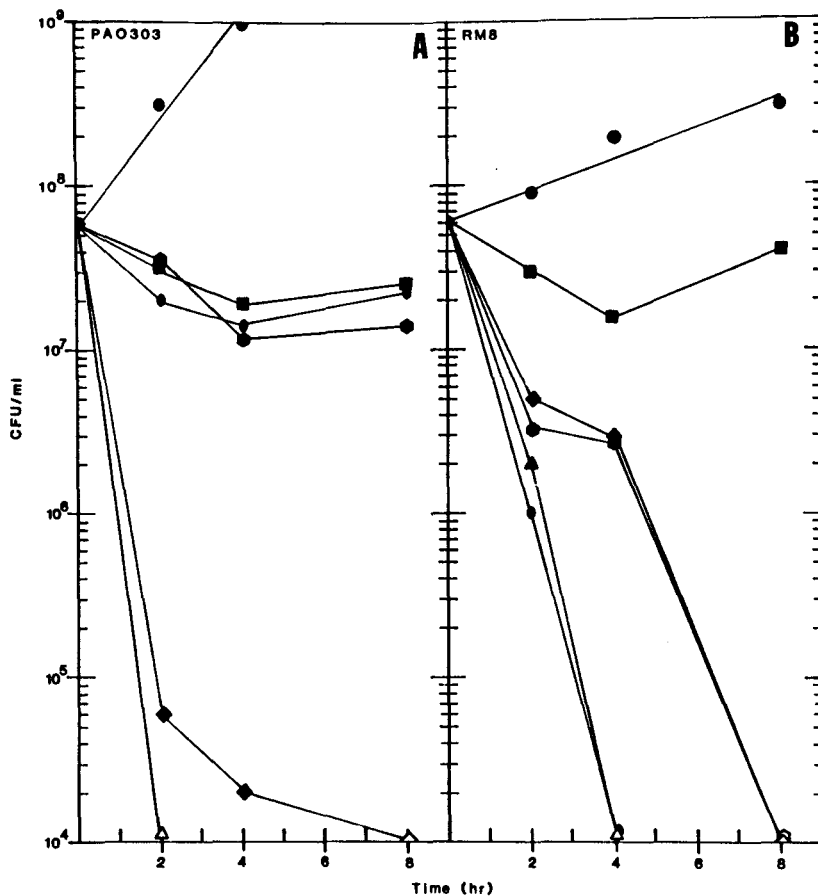


Figure 6. The Bactericidal Effect of Pyridonecarboxylic Acid and Chloramphenicol.

Logarithmically growing cultures of PAO303 (A) and RM8 (B) were divided. Portions were treated with norfloxacin (▲), nalidixic acid (◆), chloramphenicol (■), norfloxacin and chloramphenicol (◻), nalidixic acid and chloramphenicol (◐), or buffer without added drug (●) as described in the text. At various times, samples were taken and the CFU/ml of the culture determined.

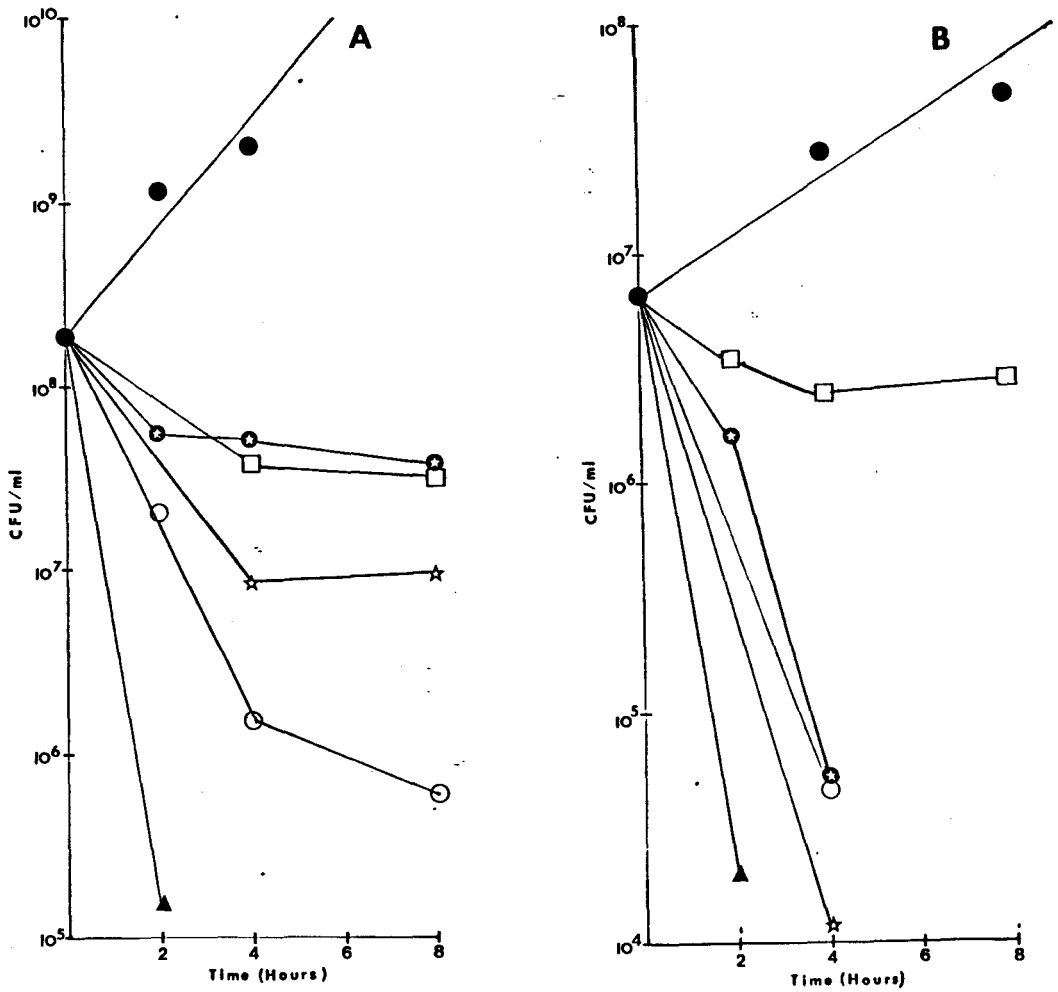


Figure 7. The Bactericidal Effect of Pyridonecarboxylic Acids and Rifamycin.

Logarithmically growing cultures of PA0303 (A) and RM8 (B) were divided. Portions were treated with norflaxacin (▲), nalidixic acid (○), rifamycin (□), rifamycin and norflaxacin (☆), rifamycin and nalidixic acid (★), or buffer without added drug (●) as described in the Methods section. At various times, samples were taken and the CFU/ml of the culture determined.

FILAMENTATION

Filamentation was not seen when PA0303 was treated with nalidixic acid or norfloxacin (Figure 8A and C, respectively). Cell division events were observed even after 23 hours of treatment when the number of CFU's/ml indicate that the cells are no longer viable (Figures 6 and 7, 8A and 8C). Simultaneous treatment with both chloramphenicol and pyridonecarboxylic acid did not stop the cell division events from taking place (Figure 8E).

Many of the Rec⁻ strain (RM8) cells treated with nalidixic acid filamented (Figure 8B), while only very few of the cells treated with norfloxacin showed even a slight increase in size (Figure 8D). Some of the cells treated simultaneously with nalidixic acid and chloramphenicol filamented also (Figure 8F). In all cases, cell division events were observed.

RATE OF PROTEIN SYNTHESIS

The rate of protein synthesis in an exponentially growing culture of P. aeruginosa increases logarithmically with time (Figure 9). Nalidixic acid inhibited protein synthesis about 99 percent, while norfloxacin inhibited protein synthesis only about 50 percent.

RATE OF DNA SYNTHESIS

The rate of DNA synthesis in a growing culture of P. aeruginosa cells (PA0303) was inhibited by norfloxacin (Figure 10). Increasing the

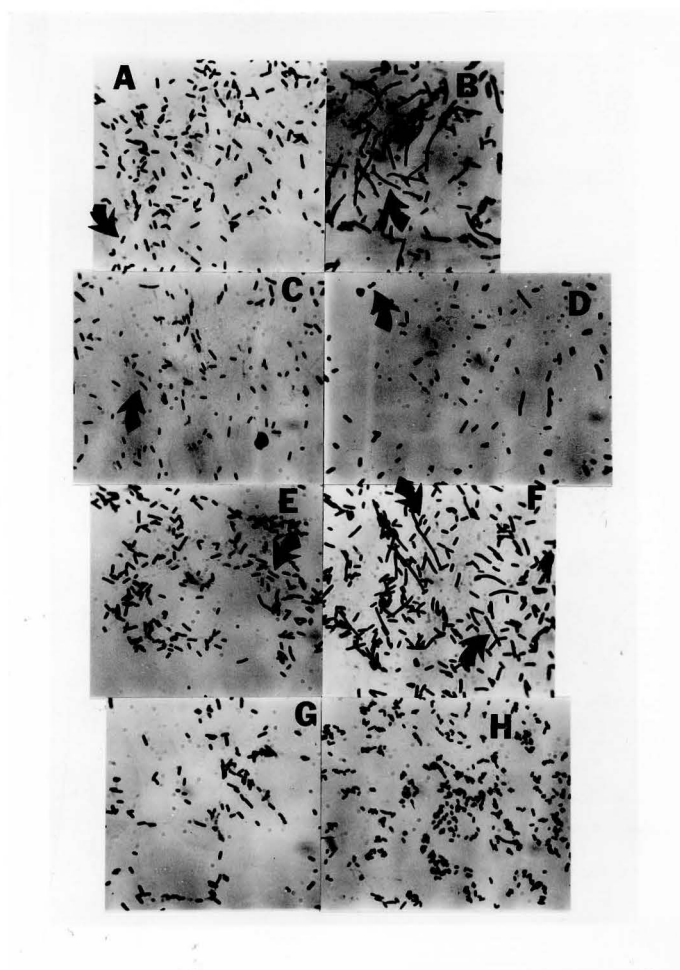


Figure 8. Lethal Unbalanced Growth in Pyridonecarboxylic Acid Treated Cells.

The cultures described in Figure 5 were photographed at a magnification of x1800 after 24 hours of incubation. Strain PA0303 (A, C, E, and G) or RM8 (B, D, F, and H) were treated with norfloxacin (C and D), nalidixic acid (A and B), nalidixic acid and chloramphenicol (E and F) or buffer containing no drugs (G and H). Arrows point out cells that appear to have just completed cell division.

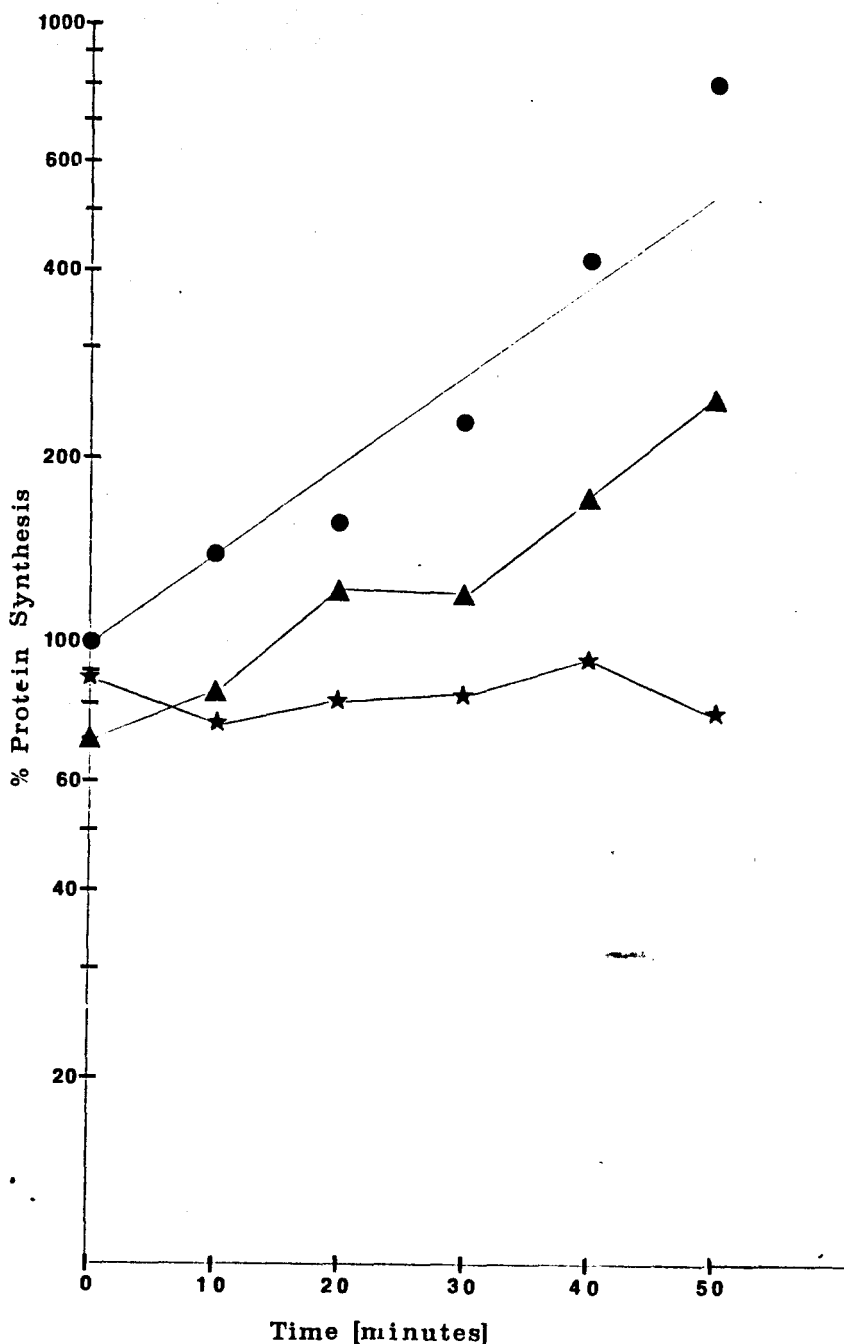


Figure 9. Inhibition of Protein Synthesis by Pyridonecarboxylic Acids.

Logarithmically growing cultures of RM8 were divided, and portions were treated with four times the MIC of norfloxacin (▲), four times the MIC of nalidixic acid (★), or buffer containing no drug (●). At various times, samples were taken and the rate of protein synthesis measured as described in the text.

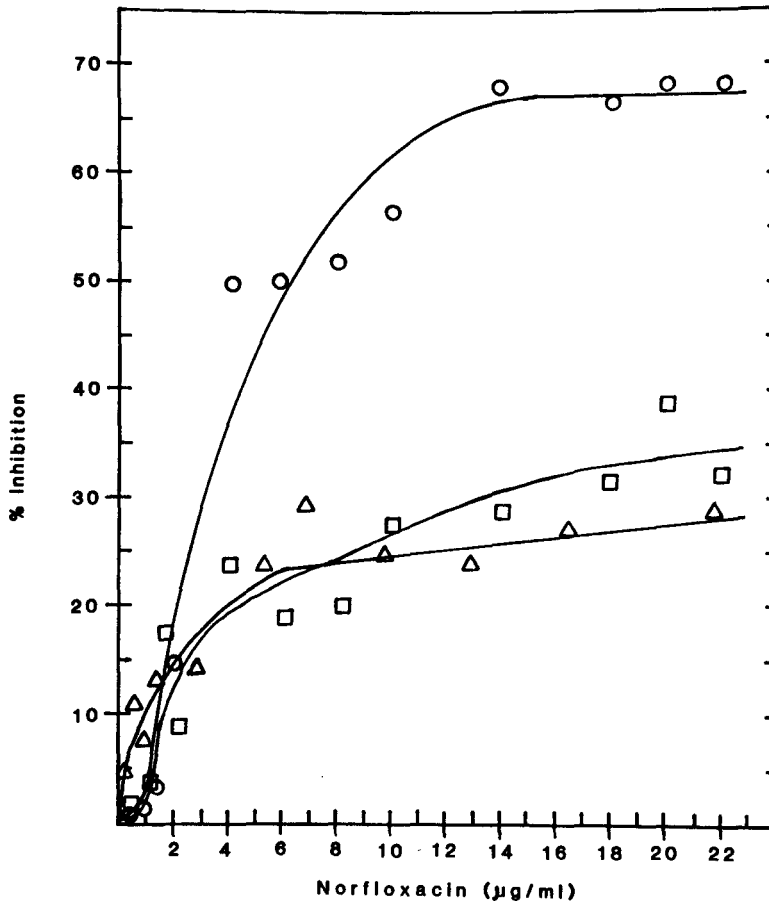


Figure 10. Inhibition of the Rate of DNA Synthesis as a Function of Norfloxacin Concentration.

Logarithmically growing cultures of strain PA0303 (○), RMB (□), or RM168 (△) were divided and exposed to various concentrations of norfloxacin. The rate of DNA synthesis was measured after four minutes of exposure as described in the text.

concentration of norfloxacin resulted in an increase in the amount of inhibition up to a plateau that occurred around the MBC (16 micrograms/ml). RM8 (the Rec⁻ mutant) and RM168 (the spontaneous mutant resistant to norfloxacin) were inhibited to a lesser extent than their parent PA0303 (Rec⁺, Nor^S). In order to keep these experiments consistent with similar experiments performed on *E. coli* (20), a drug concentration of four times the MIC was used.

The rate of DNA synthesis of a culture of *P. aeruginosa* in log phase increased exponentially over time (Figure 11). Treatment of PA0303 (Rec⁺) with either norfloxacin or nalidixic acid (Figure 11A and 12, respectively) at a concentration of four times the MIC caused an immediate inhibition of DNA synthesis followed by recovery synthesis that was also exponential and that occurred within 5 minutes of drug treatment. No recovery synthesis was observed at concentrations equal to the MBC of norfloxacin (Figure 11A). Inhibition of protein synthesis simultaneously with norfloxacin treatment did not allow recovery synthesis (Figure 13A).

When RM8 was treated with nalidixic acid at a concentration of four times the MIC (Figure 12B), recovery synthesis occurred but could not be sustained, whereas it could be sustained if norfloxacin was used (Figure 11B). Concentrations as high as the MBC for norfloxacin allowed recovery synthesis that could be sustained (Figure 11B). This difference observed between nalidixic acid and norfloxacin in the Rec⁻ mutant may be because nalidixic acid inhibits protein synthesis 99%, while norfloxacin inhibits protein synthesis only 50%. If protein synthesis was inhibited simultaneously with norfloxacin treatment (Figure 13A), the recovery synthesis could no longer be sustained in the

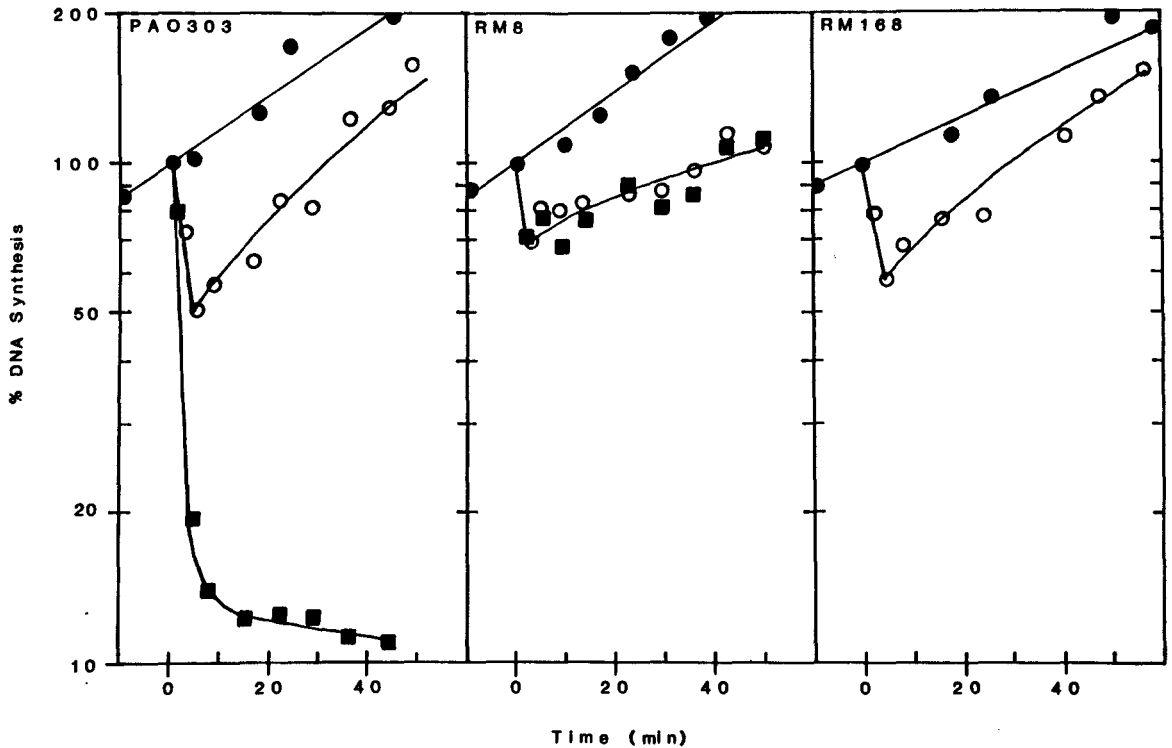


Figure 11. The Effects of Norfloxacin on the Rate of DNA Synthesis in PA0303 and RM8 Cultures.

Logarithmically growing cultures of PA0303 (A), RM8 (B), and RM168 (C) were divided and portions were exposed to four times the MIC of norfloxacin (○), the MBC or norfloxacin (□), or buffer containing no drug (●). At various times, samples were taken and the rate of DNA synthesis measured as described in the text.

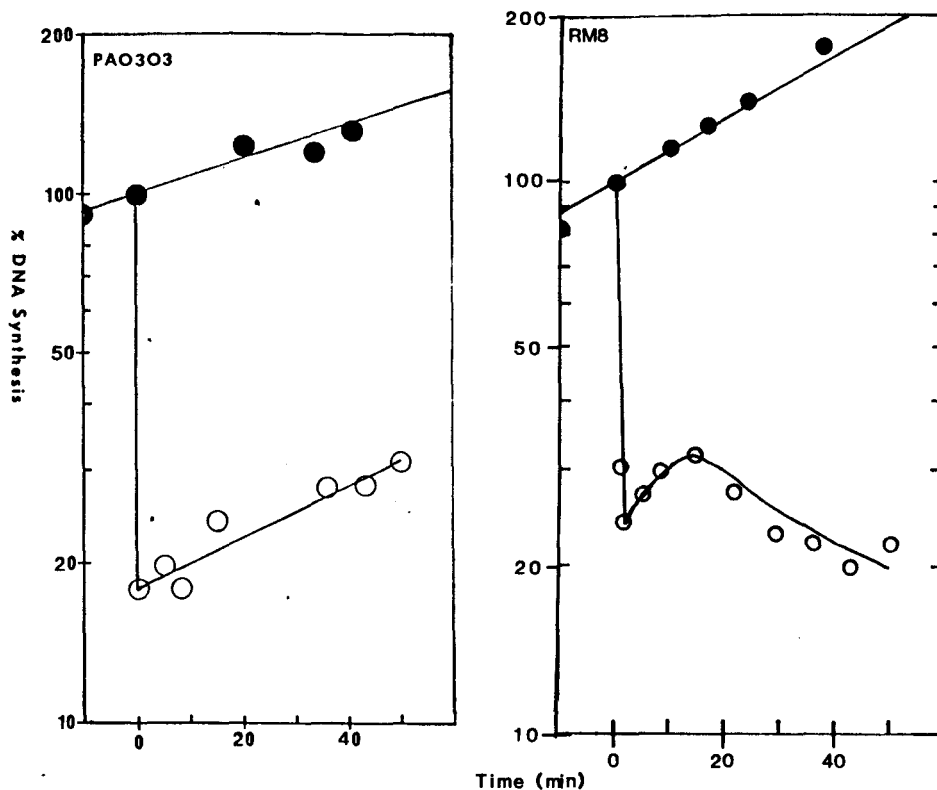


Figure 12. The Effects of Nalidixic Acid on the Rate of DNA Synthesis in PA0303 and RM8 Cultures.

Logarithmically growing cultures of PA0303 (A) and RM8 (B) were divided, half was treated with four times the MIC of nalidixic acid (○), and half was treated with buffer containing no drug (●). At various times, samples were taken and the rate of DNA synthesis measured as described in the text.

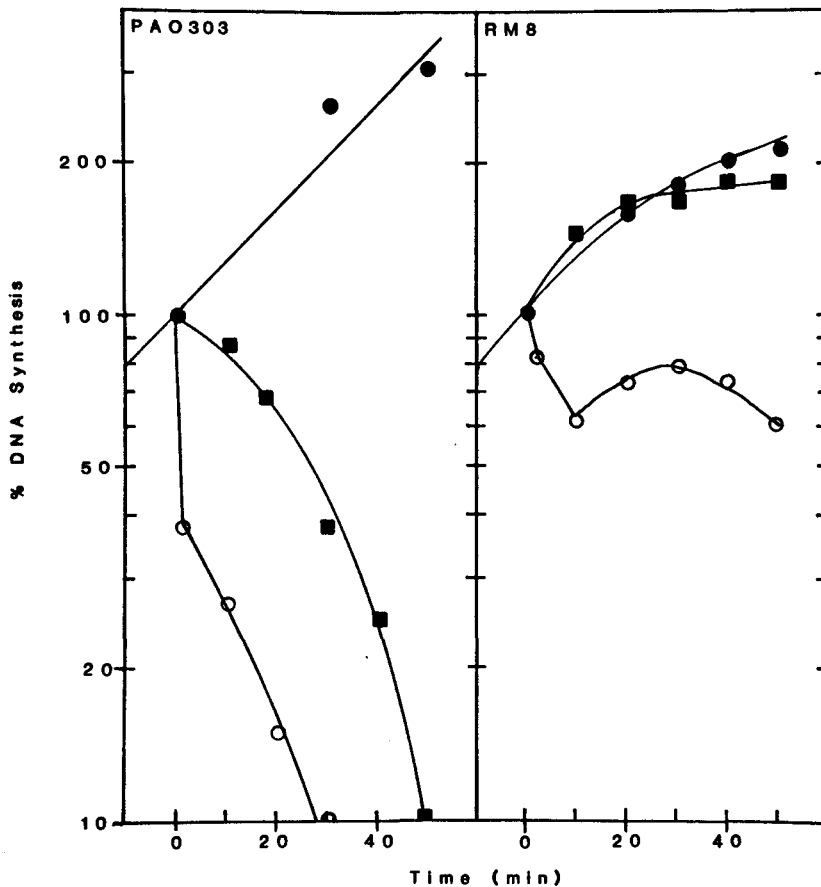


Figure 13. The Effects of Simultaneous Treatment with Norfloxacin and Chloramphenicol on the Rate of DNA Synthesis in PA0303 and RM8 Cultures.

Logarithmically growing cultures of PA0303 (A) and RM8 (B) were divided and portions were exposed to chloramphenicol (□), chloramphenicol and norfloxacin (○), or buffer without added drugs (●). At various times after introduction of the drug(s), samples were taken, and the rate of DNA synthesis measured as described in the text.

Rec⁻ mutant. Inhibition of protein synthesis simultaneously with nalidixic acid treatment had no effect on the recovery synthesis in either the Rec⁺ or Rec⁻ strains.

The rate of DNA synthesis of another Rec⁻ mutant (PA02002) and its parent (PA02001) were measured (Figure 14). These strains have a very long generation time of 75 minutes as compared to the generation time of 30 minutes for strain PA0303, accordingly, their rate of DNA synthesis is much slower than strain PA0303. Norfloxacin or nalidixic acid treatment caused an immediate inhibition of DNA synthesis followed by recovery synthesis in the parent (Figure 14A and C) or by a slower rate of inhibition in the Rec⁻ mutant (Figure 14B and D). Since the slopes of these lines were so small, linear regression was performed on these lines to determine if their slopes were indeed positive in the case of strain PA02000 and negative in the case of strain PA02001. These slopes are given in the figure legend.

RM168, a spontaneous mutant of PA0303, resistant to norfloxacin, was inhibited to a lesser extent than its parent (Figure 10). However, its inhibition and recovery of DNA synthesis occurred within the same time frame as the wild type's (Figure 11C).

RM4000, a spontaneous mutant of RM8, resistant to nalidixic acid, exhibited no recovery synthesis when treated with norfloxacin or nalidixic acid (Figures 15A and B, respectively).

INDUCTION OF DNA REPAIR PATHWAYS

Inducible DNA repair systems in a bacterium can be studied by treating cells with an inducing agent such as ultraviolet radiation or

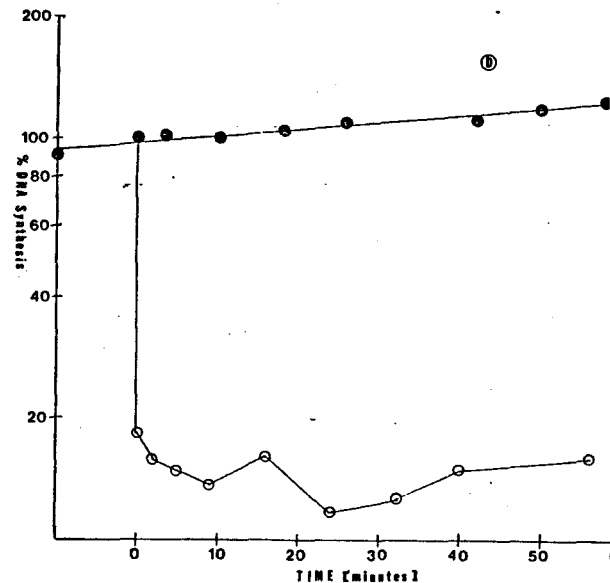
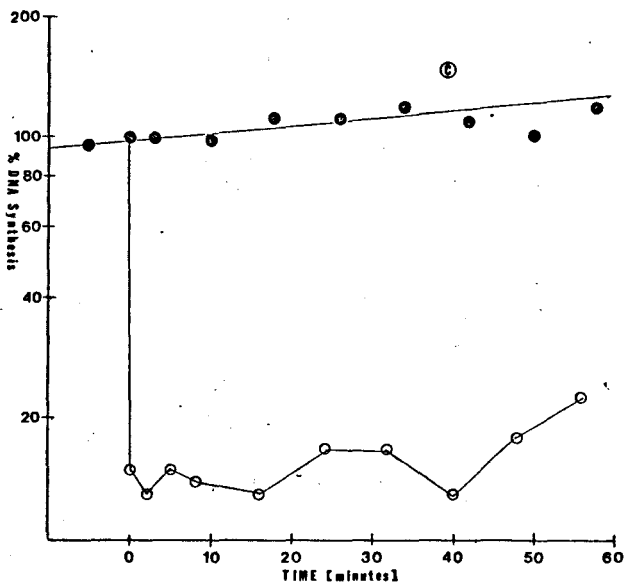
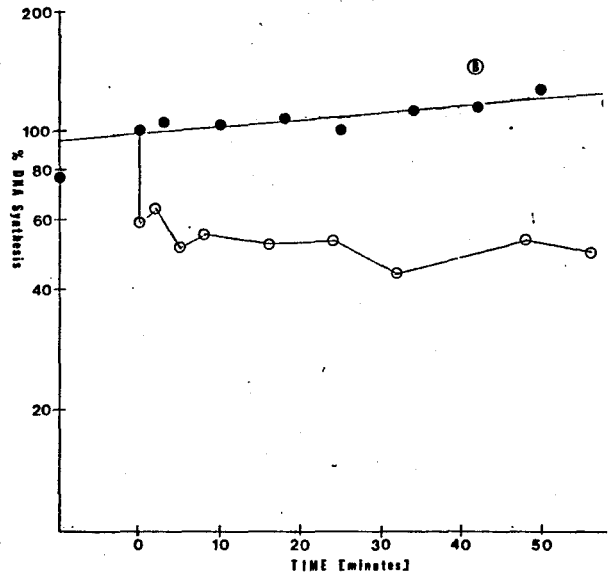
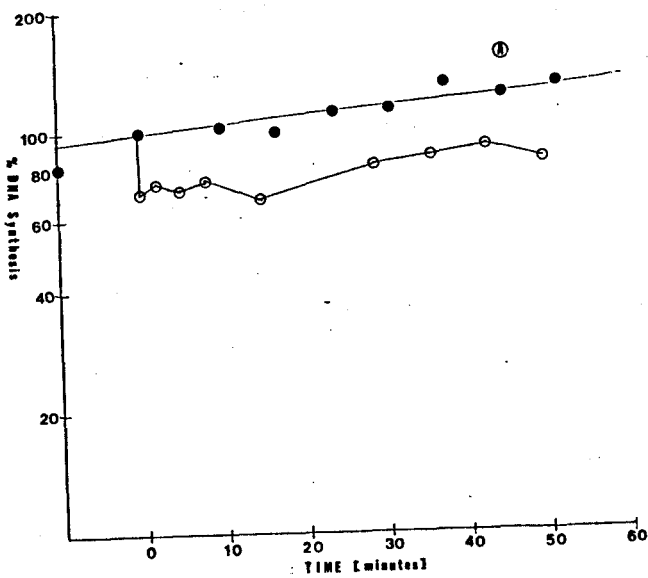


Figure 14. The Effects of Norfloxacin and Nalidixic Acid on the Rate of DNA Synthesis in PA02001 and PA02002 Cultures.

Logarithmically growing cultures of PA02001 (A and C) and PA02002 (B and D) were divided, half was treated with norfloxacin (A and B) or nalidixic acid (C and D) at a concentration of four times the MIC (○) and half was treated with buffer containing no added drug (●). At various times, samples were taken and the rate of DNA synthesis measured as described in the text. The slopes of the lower lines are +0.376 for A, -0.16 for B, +0.111 for C, and -0.029 for D.

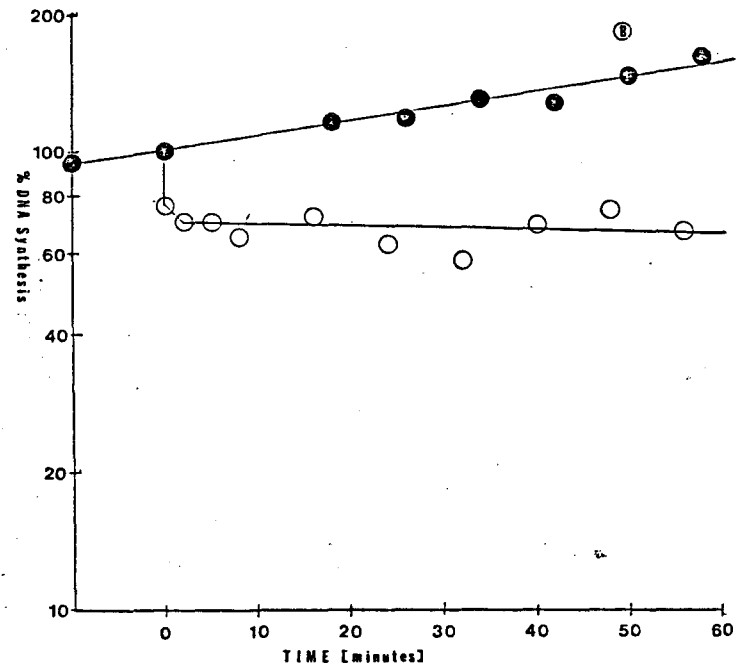
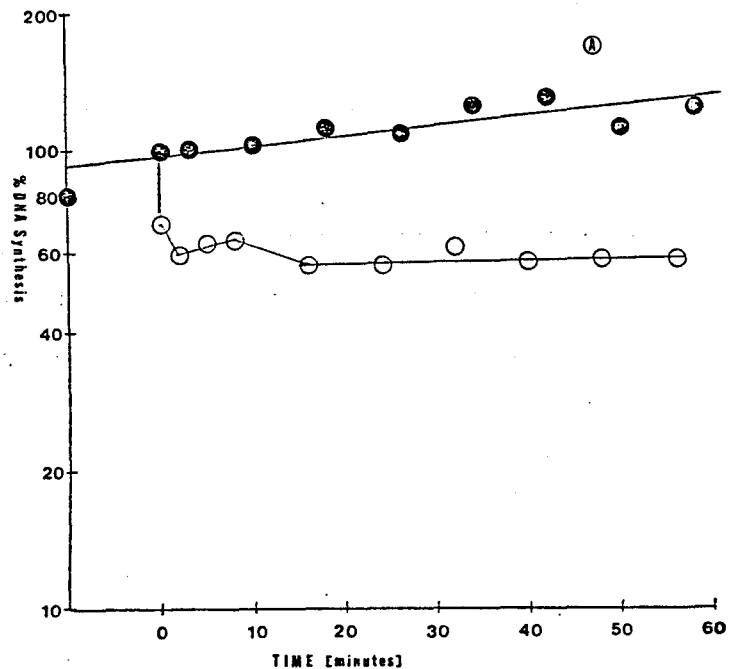


Figure 15. The Effects of Norfloxacin and Nalidixic Acid on the Rate of DNA Synthesis in Strain RM4000.

Logarithmically growing cultures of RM4000 were divided, half was treated with norfloxacin (A) or nalidixic acid (B) at a concentration of four times the MIC (○), and half was treated with buffer containing no added drug (●). At various times, samples were taken and the rate of DNA synthesis measured as described in the text.

nalidixic acid and subsequently infecting the treated cells with UV-damaged phage DNA. The cells are plated out and the number of plaque forming units (PFU's) counted. If a DNA repair system has been induced by the agent, then the number of PFU's in the treated culture will be exponentially greater than the number of PFU's in the control culture. This phenomenon, termed Weigle reactivation, is observed in E. coli cells treated with nalidixic acid (69).

Mutagenesis can be measured during the same experiment. The Pseudomonas phage D3 has a high frequency of mutation to the clear plaque phenotype as opposed to the wild type cloudy plaque. If a DNA damaging treatment is inducing an error-prone repair system, then the percentage of clear plaques in the treated culture will be greater than the percentage of clear plaques in the control culture.

Neither Weigle reactivation (Figure 16) nor mutagenesis (Table 6) was observed when PA0303 (Rec⁺) or RM8 (Rec⁻) cells were treated with norfloxacin or nalidixic acid. This is not surprising, since treatment with ultraviolet light does not induce these pathways in the Rec⁺ or Rec⁻ strains of P. aeruginosa (66).

PLASMID MAINTENANCE

Treatment of E. coli strains containing plasmid pBR322 or plasmid pMG110 with coumermycin A1, an inhibitor of the DNA gyrase B subunit, results in loss of these plasmids from the cells (85). The plasmid pME292 was transformed into PA0303 and RM8 made competent by MgCl₂ treatment (53). Treatment with varying concentrations of norfloxacin,

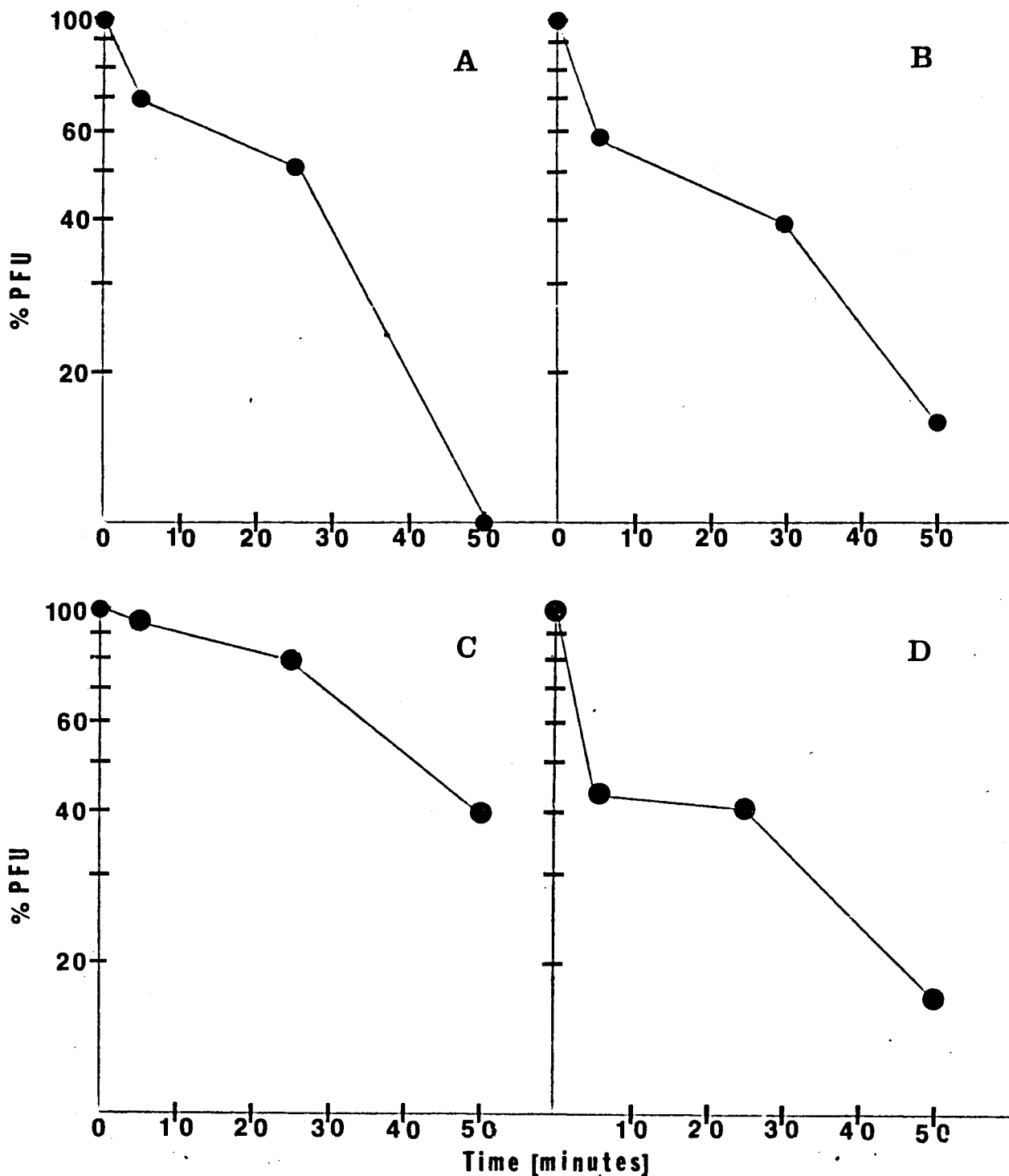


Figure 16. The Absence of Weigle Reactivation in Strains PA0303 and RM8.

At time zero, a culture of PA0303 (A and C) and RM8 (B and D) was treated with either norfloxacin (A and B) or nalidixic acid (C and D) at a concentration of four times the MIC. At various times, samples were taken, and infected with UV-irradiated (30 J/m²) phage D3. The titer of phage (PFU/ml) relative to the titer at time zero was determined.

Table 6. Frequency of Mutagenesis in the Presence of Norfloxacin or Nalidixic Acid.

Drug	Time of Exposure (min)	Mutation Frequency (mutants/10 ⁵ PFU)
Norfloxacin	0	10
	5	11
	30	11
	50	8
Nalidixic	0	17
	5	9
	30	11
	50	6

nalidixic acid, or novobiocin up to the MBC's did not cause any loss of plasmid from either strain (Table 7).

PSEUDOMONAS AERUGINOSA DNA GYRASE

DNA gyrase was isolated from strain PA0303 by affinity chromatography (Table 8) on a novobiocin-Sepharous column (75) and found to consist of two subunits whose molecular weights were determined to be $92,000 \pm 3,000$ and $108,000 \pm 3,000$ by SDS polyacrylamide gel electrophoresis. Depending on the preparation, either two or four bands were observed on the SDS gel of the final purified fraction. The 92,000 and 108,000 bands, which are presumably the two subunits of DNA gyrase, appeared consistently. Two other less intense bands of molecular weights 40,000 and 50,000 were sometimes observed. Unfortunately, with the equipment available and with my lack of expertise in the area of photography, I was unable to obtain a photograph that could distinguish these bands very well (Figure 17).

The two smaller, less intense bands may consist of proteins which bind to novobiocin since the preparation eluted off of a well washed novobiocin-Sepharous affinity column. Staudenbauer and Orr (75) also observed two smaller additional proteins other than the DNA gyrase subunits eluting off of the novobiocin-Sepharous affinity column loaded with an E. coli extract. One of these additional proteins, which has a molecular weight of 85,000, has an unknown function. They speculate that the other protein of molecular weight 40,000 may be generated from the same proteolytic cleavage event that generates the v subunit of molecular weight 50,000. The two smaller, less intense bands which I

Table 7. Plasmid Maintenance

Drug	Concentration (micrograms/ml)	Plasmid Maintenance (percent)
Norfloxacin	0	99.9
	0.2	99.9
	0.4	99.9
	1.6	99.9
Nalidixic Acid	0	99.9
	350	99.9
	700	99.9
Novobiocin	0	99.9
	1	99.9

The data is exactly the same for strains PA0303 and RM8.

Table 8. Purification of P. aeruginosa DNA Gyrase.

Fraction	Total Volume (ml)	Total Protein (mg)	Total Enzyme Activity	Specific Activity units/mg
I	22.5	4.896	*	*
II	20	4.096	*	*
III	5	0.29	2000	34,483

*No enzyme activity could be determined in these fractions.

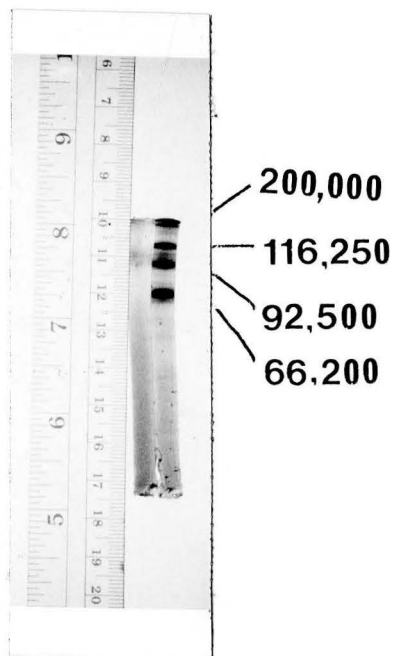


Figure 17. SDS Polyacrylamide Gel of Purified P. aeruginosa DNA Gyrase.

Purified P. aeruginosa DNA gyrase (lane 1) and SDS-PAGE high molecular weight standards (lane 2) were electrophoresed in 10% SDS polyacrylamide tube gels.

observed, also might consist of proteolytic products of the B subunit and maybe even the A subunit. The proteolysis may have occurred before, during or after the purification procedure. The B subunit may also autoproteolyze, a phenomenon which is also observed for the E. coli lexA protein (46). This autoproteolysis is suspected of my purified P. aeruginosa DNA gyrase because of the disappearance of the supercoiling activity with time or freezing, as will be described in the next section.

SUPERCOILING OF RELAXED CLOSED CIRCULAR DNA

The supercoiling of relaxed DNA by my purified P. aeruginosa DNA gyrase exhibited many irregularities. First of all, there were problems with the substrate (relaxed Col E1 or ϕ X174 DNA). After many unsuccessful attempts at increasing the superhelical density of the substrate with my purified P. aeruginosa DNA gyrase, I sent a sample of my substrate to Dr. Martin Gellert. His purified E. coli DNA gyrase was also unable to supercoil my substrate. In order to relax supercoiled DNA for substrate, I had been using a chicken nicking-closing extract which was available in our lab and was about three or four years old. I therefore prepared fresh nicking-closing extract, which I used to relax supercoiled DNA. This also led to relaxed DNA that was not supercoilable (Figure 18). I then attempted to improve the reaction conditions by carrying out the reaction in different buffers (described in the Methods section), none of which improved the results. E. coli DNA purified using the exact same procedure as used for P. aeruginosa DNA gyrase was also unable to supercoil the substrate prepared with

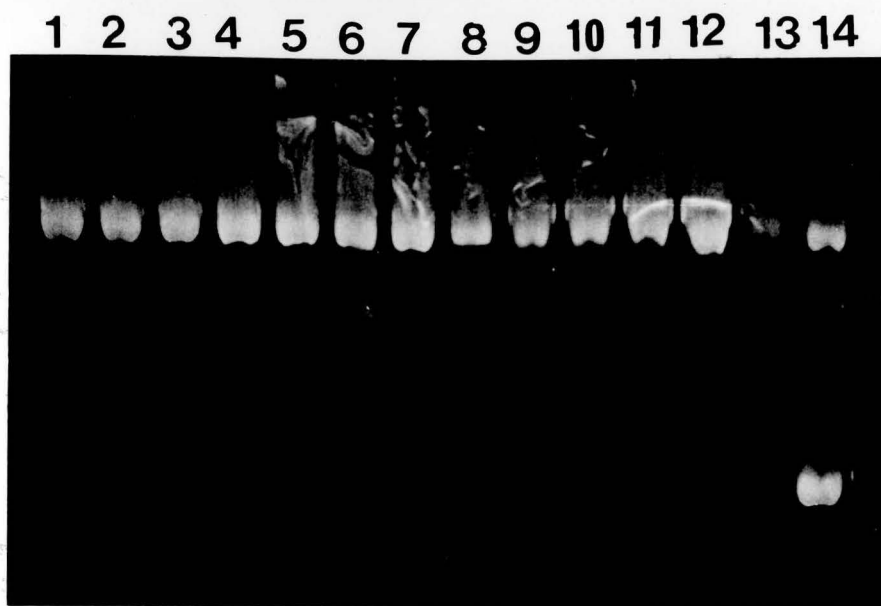


Figure 18. Lack of Supercoiling Activity of *P. aeruginosa* and *E. coli* DNA Gyrase on Nicking-Closing Extract-Relaxed DNA.

Col E1 DNA relaxed with nicking-closing extract was incubated with *P. aeruginosa* DNA gyrase (lanes 9-13) or *E. coli* DNA gyrase (lanes 2-8) as described in the Methods section. Each lane contains a different fraction collected from the novobiocin-Sepharose column. Lane 1 contains relaxed DNA incubated in the absence of DNA gyrase, and lane 14 contains supercoiled DNA.

fresh nicking-closing extract (Figure 18). Finally, I utilized topoisomerase I as a relaxing agent. This resulted in relaxed DNA that was supercoilable (Figures 19 and 21).

I only attained supercoiling on a fresh preparation of DNA gyrase that was less than 24 hours off of the column and that had never been frozen (Figure 19). After the preparation was one day old or had been frozen, I attained cut linear DNA. Figure 19 shows the agarose gel of the reaction products obtained when relaxed Col E1 DNA was used with a fresh preparation of DNA gyrase, and Figure 20 shows the reaction products from the same preparation that had been frozen. It appears that the circular Col E1 DNA treated with the older preparations obtained a double-stranded cut resulting in linear DNA. Figures 21 and 22 exhibit the reaction products obtained when relaxed ϕ X174 DNA was used with a fresh or frozen preparation, respectively. It appears that the circular ϕ X174 DNA treated with the older preparation obtained two double-stranded cuts resulting in two linear bands which migrate to the same location as the linear DNA of the cleavage reaction (see Cleavage Reaction section). The double-stranded cleavage may result from proteolytic products of the B subunit that are similar to the E. coli v subunit. Since the v subunit is a natural protein found in E. coli and P. aeruginosa possesses a DNA gyrase very similar to the E. coli enzyme, it is not unlikely that P. aeruginosa also possesses a v subunit similar to the E. coli v subunit. The loss of the supercoiling activity and the gain of cleavage activity may be due to the v subunit being more stable than the B subunit.

When I used relaxed Col E1 DNA, bands of topoisomers that were increased in superhelical density occurred, but a completely supercoiled

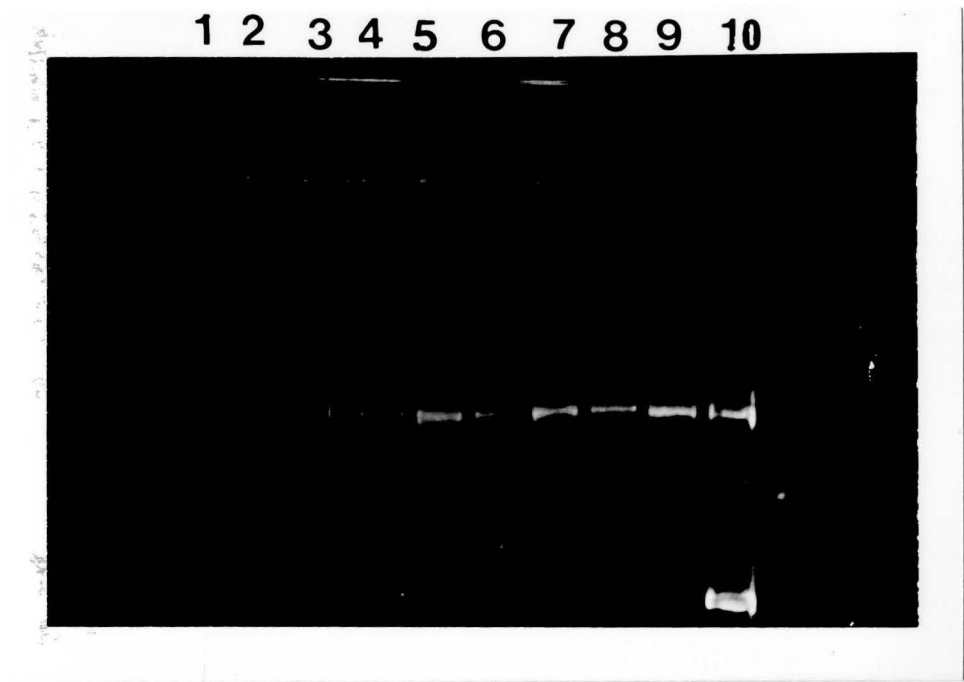


Figure 19. Supercoiling of Topoisomerase I-Relaxed Col E1 DNA by Fresh P. aeruginosa DNA Gyrase Preparation.

Col E1 DNA, which was relaxed with topoisomerase I, was incubated with P. aeruginosa DNA gyrase (lanes 2-9) as described in the Methods section. Each lane contains a different fraction collected from the novobiocin-Sepharose column. Lane 1 contains relaxed DNA incubated in the absence of DNA gyrase, and lane 10 contains supercoiled DNA.

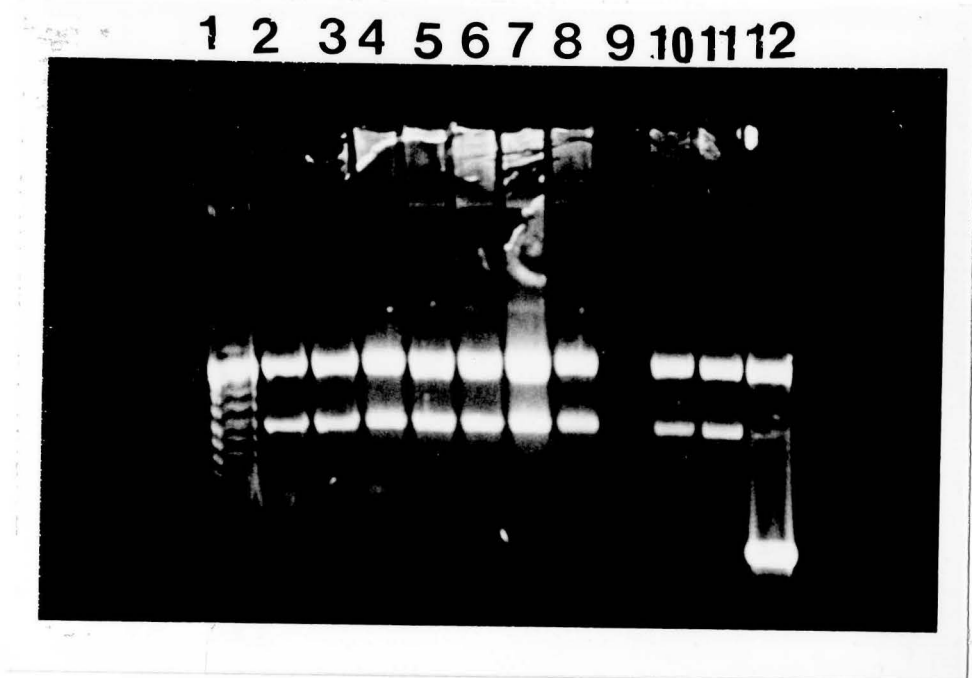


Figure 20. Effects of Frozen *P. aeruginosa* DNA Gyrase Preparation on Topoisomerase I-Relaxed Col E1 DNA.

Col E1 DNA, which was incompletely relaxed with topoisomerase I, was incubated with *P. aeruginosa* DNA gyrase preparations which had been stored frozen as described in the Methods section (lanes 2-8 and 10-11). Each lane contains a different fraction collected from the novobiocin-Sepharose affinity column. Lane 1 contains incompletely relaxed DNA incubated in the absence of DNA gyrase, lane 9 contains no DNA, and lane 12 contains supercoiled DNA.

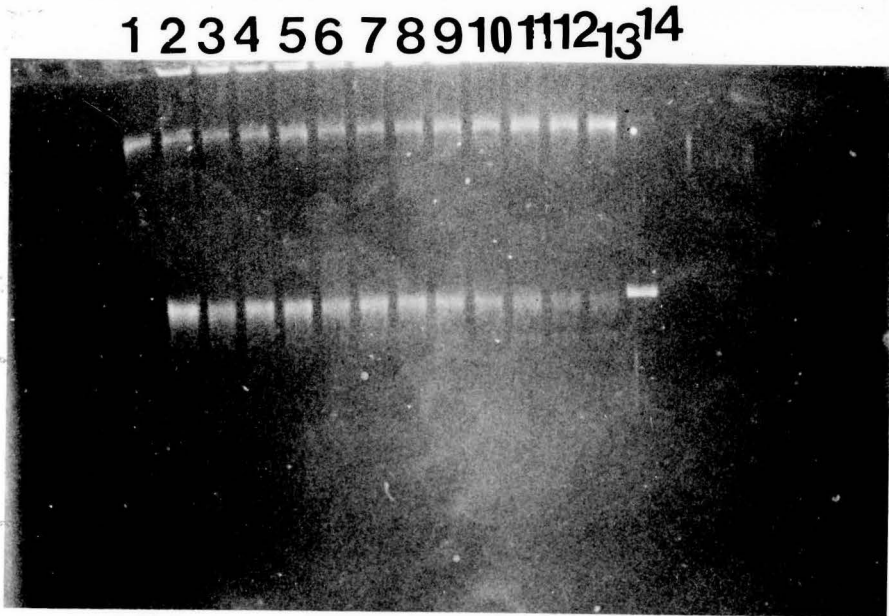


Figure 21. Supercoiling of Topoisomerase I-Relaxed ϕ X174 DNA by Fresh Preparations of *P. aeruginosa* DNA Gyrase.

Topoisomerase I-relaxed ϕ X174 DNA was incubated with a fresh preparation of DNA gyrase as described in the Methods section. Each lane contains a different amount of preparation (12 μ l in lane 2, 11 μ l in lane 3, 10 μ l in lane 4, 9 μ l in lane 5, 8 μ l in lane 6, 7 μ l in lane 7, 6 μ l in lane 8, 5 μ l in lane 9, 4 μ l in lane 10, 3 μ l in lane 11, 2 μ l in lane 12, and 1 μ l in lane 13 - for a 25 μ l total reaction volume). Lane 1 contains relaxed DNA incubated in the absence of DNA gyrase and lane 14 contains supercoiled DNA.

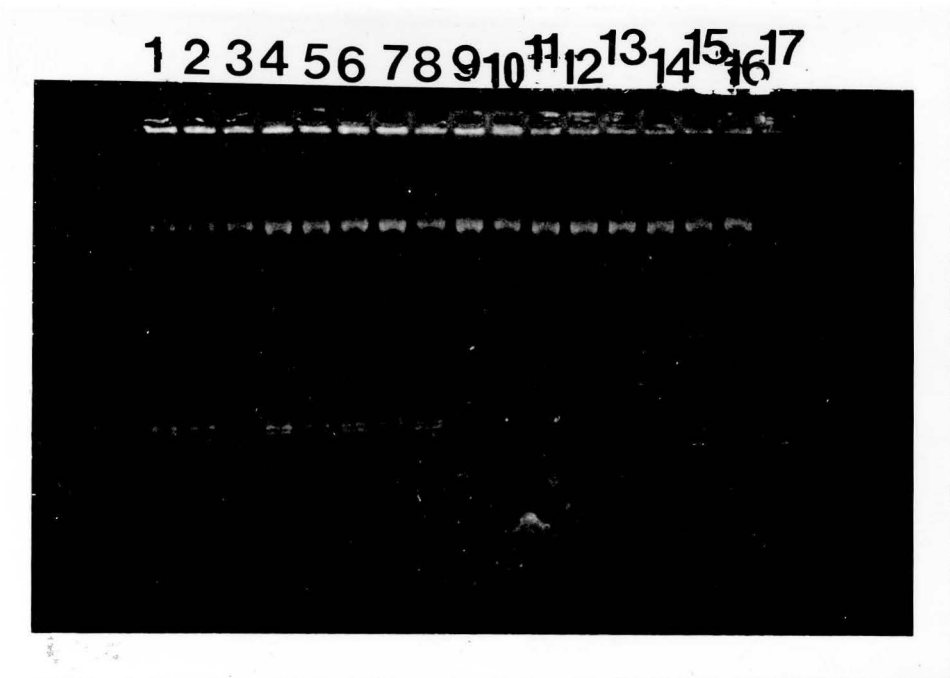


Figure 22. Effect of Frozen *P. aeruginosa* DNA Gyrase Preparations on Topoisomerase I-Relaxed ØX174 DNA.

Topoisomerase I-relaxed ØX174 DNA was incubated with preparations of DNA gyrase which were stored frozen for 24 hrs as described in the Methods section. Of the 25 μ l reaction volume, DNA gyrase preparation represented 11 μ l in lane 1, 10 μ l in lane 2, 9 μ l in lane 3, 8 μ l in lane 4, 7 μ l in lane 5, 6 μ l in lane 6, 5 μ l in lane 7, 4 μ l in lane 8, 3 μ l in lane 9, 2 μ l in lane 10, 1 μ l in lane 11, 1/2 μ l in lane 12, 1/10 μ l in lane 13, and 0 μ l in lane 16. Lanes 14 and 15 contain 11 μ l of DNA gyrase preparation which were stored frozen for a week and longer. Lane 17 contains supercoiled DNA.

product was never observed. When I used ϕ X174 DNA, completely supercoiled DNA appeared but no intermediate topoisomers were ever observed (Figure 21). Intermediate topoisomers were observed, however, when supercoiled ϕ X174 DNA was relaxed with new or old DNA gyrase preparations (see next section).

Inhibition of supercoiling by pyridonecarboxylic acids was not observed. An unexpected phenomenon did occur with topoisomerase I-relaxed Col E1 DNA (Figure 23); supercoiling was not observed in the absence or at low concentrations of pyridonecarboxylic acids, but intermediate topoisomer bands appeared in reactions carried out in the presence on MBC concentrations of norfloxacin (16 micrograms/ml) and nalidixic acid (2800 micrograms/ml). This phenomenon can be explained if the relaxing activity of the topo II' is greater in this preparation than the supercoiling activity of DNA gyrase. In the absence of pyridonecarboxylic acids, the ν subunit relaxes DNA more efficiently than DNA gyrase supercoils this same DNA, thus preventing the appearance of a supercoiled band. At higher pyridonecarboxylic acid concentrations, the relaxing activity of topo II' may be inhibited to a greater extent than the supercoiling activity of DNA gyrase and intermediates between the completely supercoiled DNA (DNA gyrase reaction products), and completely relaxed DNA (topo II' reaction products) are observed.

RELAXATION OF SUPERCOILED DNA

P. aeruginosa DNA gyrase relaxed supercoiled DNA in the absence of ATP (Figure 24, lane 2), but not in the presence of ATP (lane 3). In

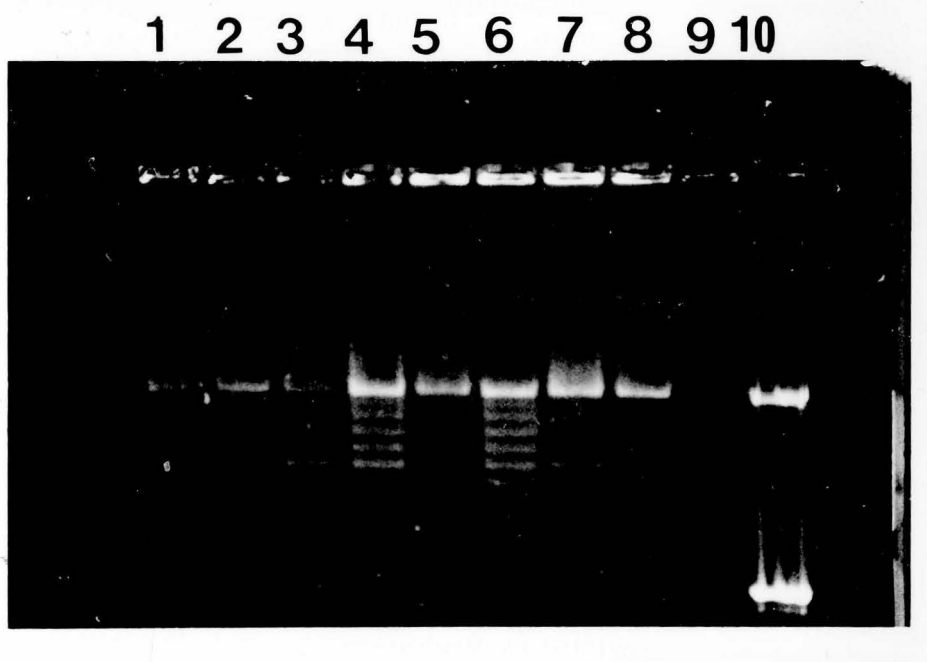


Figure 23. Inhibition of the Supercoiling Reaction by Pyridonecarboxylic Acids.

Topoisomerase I-relaxed Col E1 DNA was incubated with a fresh preparation of DNA gyrase as described in the Methods section (lane 8). Norfloxacin was added at a concentration of $0.4 \mu\text{g/ml}$ in lane 4. Nalidixic acid was added at a concentration of $350 \mu\text{l/ml}$ in lane 5 and $1400 \mu\text{l/ml}$ in lane 6. Novobiocin was added to lane 7 at a concentration of $10 \mu\text{l/ml}$. Lane 9 contains no DNA and lane 10 contains supercoiled DNA.

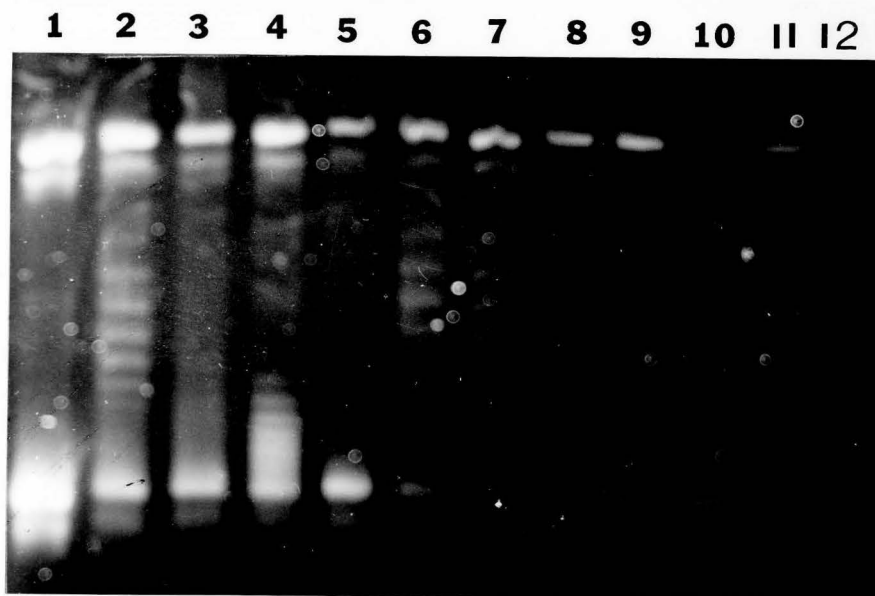


Figure 24. The Relaxation of Supercoiled DNA by *P. aeruginosa* DNA Gyrase.

Supercoiled ϕ X174 DNA (lane 1, lower band) containing some relaxed DNA (upper band) was incubated with *P. aeruginosa* DNA gyrase in the absence of ATP (lane 2), in the presence of AppNp (lane 4), in the absence of magnesium (lane 5), in the presence of norfloxacin at concentrations of 1.6 μ l/ml (lane 6), 6.4 μ g/ml (lane 7), 16 μ g/ml (lane 8), and 100 μ g/ml (lane 9), in the presence of nalidixic acid at concentrations of 350 μ g/ml (lane 10, and 700 μ g/ml (lane 11), and in the presence of 100 μ g/ml of novobiocin. The ladder of bands in between the relaxed and supercoiled bands consists of topoisomers differing by two linking numbers. Lanes 1-5 contain 0.5 μ g of DNA, and lanes 6-12 contain 0.3 μ g of DNA.

the presence of adenylyl-imidodiphosphate (AppNp) nonhydrolyzable analog of ATP (lane 4), the relaxation occurred to a lesser extent than in its absence. The velocity of the relaxation reaction was measured as described in the Methods section. By measuring the velocity over time for different substrate (supercoiled ϕ X174 DNA) concentrations and graphing a Hanes plot of the results as described in the Methods section (Figure 25), the K_m of the relaxation reaction was determined to be $1.1 \pm 0.5 \times 10^{-9}$ Molar ($3.82 \pm 1.2 \times 10^{-3}$ g/l).

The relaxation reaction was inhibited by norfloxacin (Figure 24, lanes 6-9), and nalidixic acid (lanes 10 and 11), but not by novobiocin (lane 12). Increasing the concentration of norfloxacin or nalidixic acid caused an increase in the extent of inhibition (lanes 6-9 and lanes 10 and 11, respectively). Carrying out the relaxation reaction in the presence of increasing concentrations of pyridonecarboxylic acid resulted in an increase in the electrophoretic mobility of the ladder of topoisomer bands (i.e., smaller extent of relaxation) and an increase in the fraction of products located in the completely supercoiled band (i.e., less molecules relaxed). Figure 26 demonstrates this phenomenon for relaxation on supercoiled ϕ X174 DNA in the presence of increasing concentrations of norfloxacin. The inhibition constants (K_i 's) of the pyridonecarboxylic acid derivatives on the relaxation reaction were determined by carrying out the reaction in increasing concentrations of drugs for sets of reactions containing differing concentrations of supercoiled ϕ X174 DNA and calculating the velocities as described in the Methods section. Dixon plots were drawn from the resulting data as described in the Methods section. The K_i for norfloxacin was derived to be 2.0×10^{-3} Molar (650 micrograms/ml, Figure 27), 3.7×10^{-3} Molar

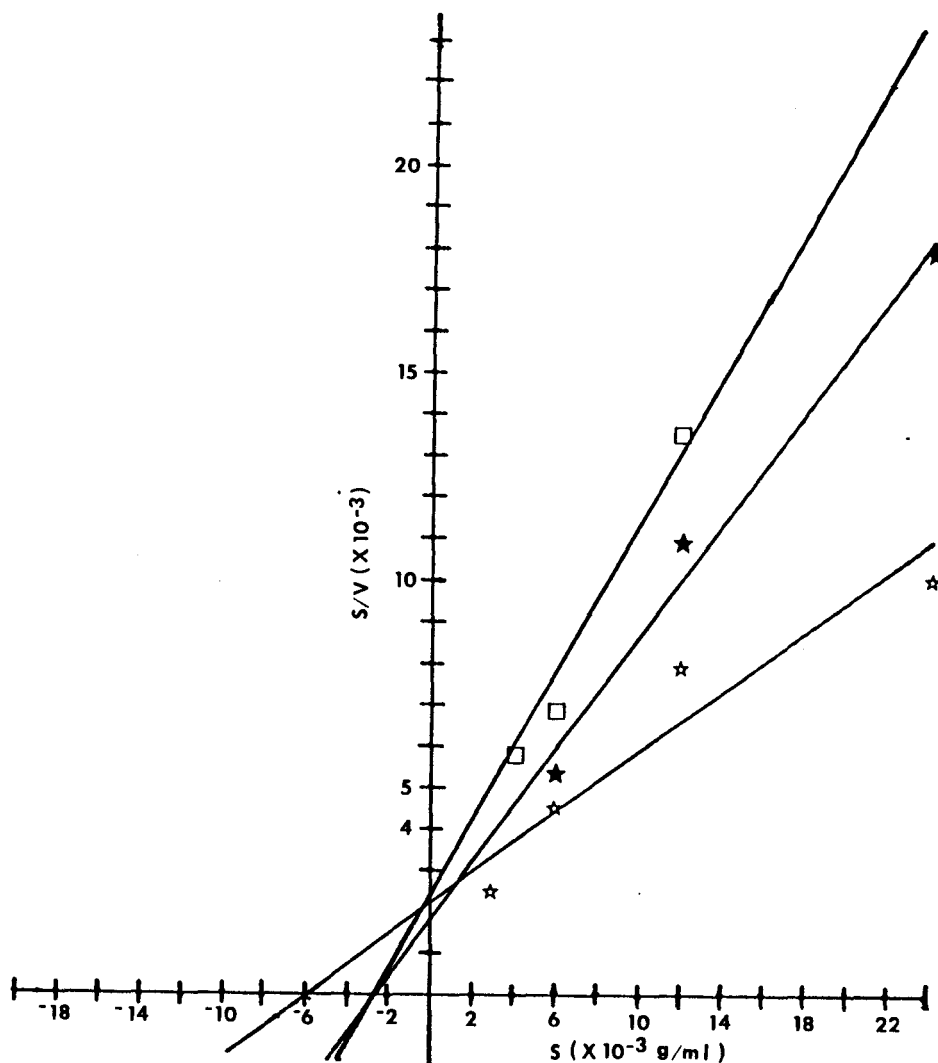


Figure 25. Hanes Plot of the Relaxation Reaction.

The velocity (v) of the relaxation reaction was determined at varying substrate concentrations (s) as described in the Methods section. s/v is plotted against s and the x-intercept is equal to $-K_m$. The three different lines represent three different experiments performed on two separately isolated preparations. The average of the three x-intercepts is $3.82 \pm 1.2 \times 10^{-3}$ g/l since the molecular weight of $\phi X174$ is 3.5×10^6 , the K_m is equal to $1.1 \pm 0.5 \times 10^{-9}$ Molar.

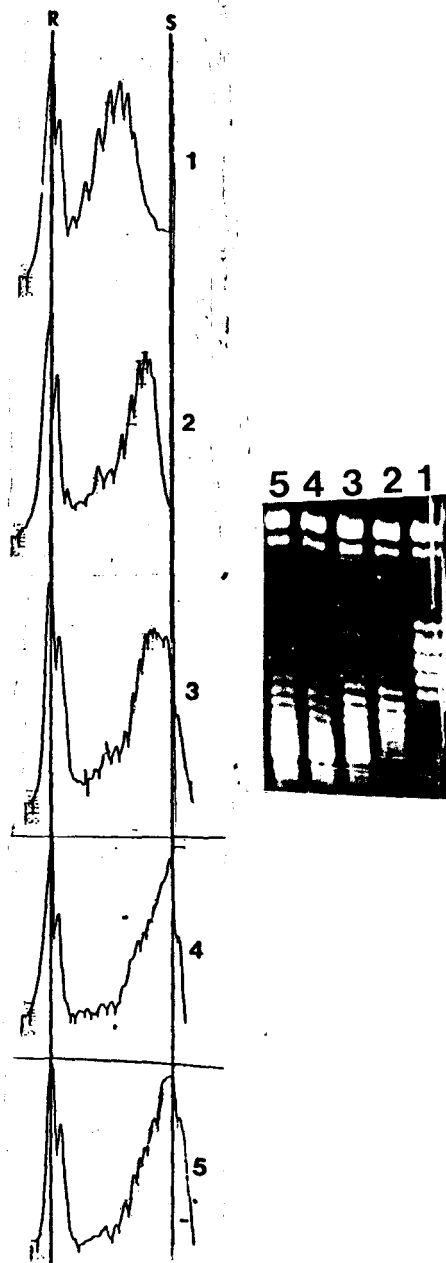


Figure 26. Increasing Concentrations of Pyridonecarboxylic Acid Result in Greater Inhibition of the Relaxation Reaction.

The relaxation reaction was carried out in the presence of norfloxacin at concentrations of 1 $\mu\text{g/ml}$ (lane 1), 250 $\mu\text{g/ml}$ (lane 2), 500 $\mu\text{g/ml}$ (lane 3), 750 $\mu\text{g/ml}$ (lane 4) and 1000 $\mu\text{g/ml}$ (lane 5). An agarose gel of the reaction products is on the right and a densitometric tracing of the negative of the picture of this gel is on the left. R is the distance migrated by completely relaxed DNA, and S is the distance migrated by completely supercoiled DNA.

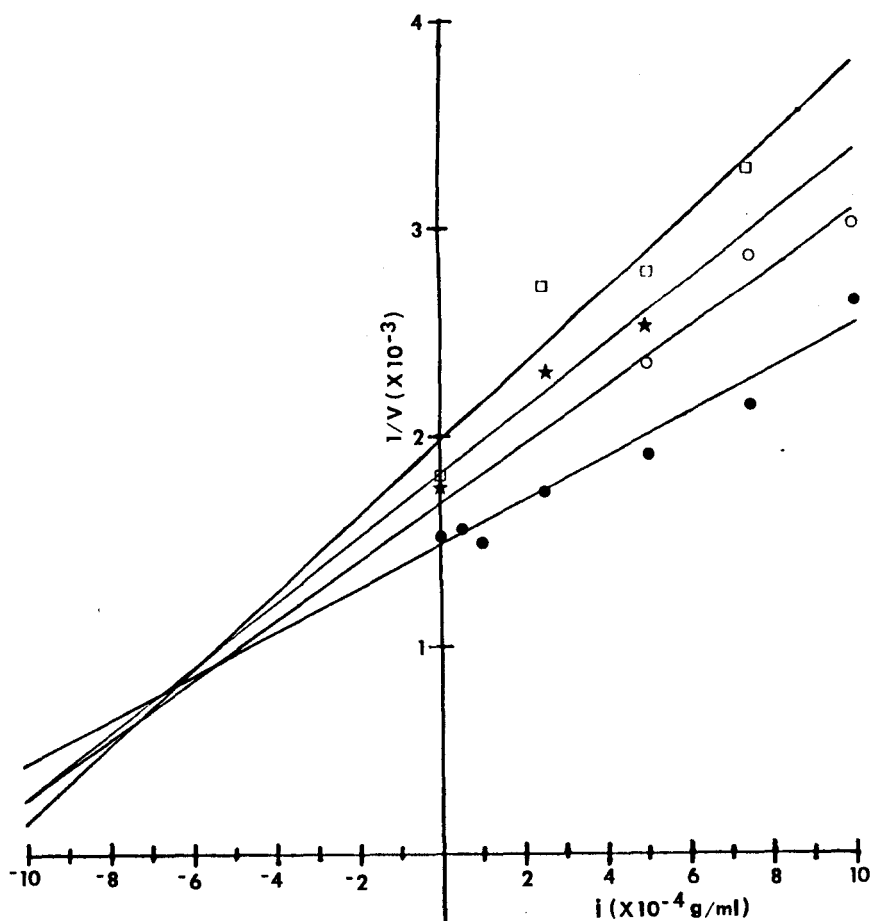


Figure 27. Dixon Plot of the Inhibition of *P. aeruginosa* DNA Gyrase-Catalyzed Relaxation by Norfloxacin.

The relaxation reaction was carried out in various concentrations of norfloxacin and substrate concentrations of 1.7×10^{-9} mMoles/ml (□), 3.4×10^{-9} mMoles/ml (★), 6.9×10^{-9} mMoles/ml (○), and 1.8×10^{-8} mMoles/ml (●) as described in the Methods section.

(975 micrograms/ml) for oxolinic acid (Figure 28), and 10.8×10^{-3} Molar (2500 micrograms/ml) for nalidixic acid (Figure 29).

A Cornish-Bowden plot (9) of the substrate concentration (s) divided by the velocity (v) versus the inhibitor concentrations (i) plotted at several (s) values will reveal what type of inhibition is occurring. If the lines are parallel, the inhibition is competitive. If the lines intersect below the x axis, the inhibition is mixed, and if the lines intersect above the x axis, the inhibition is uncompetitive. Cornish-Bowden plots of the inhibition of relaxation by norfloxacin (Figure 30), nalidixic acid (Figure 31), and oxolinic acid (Figure 32) resulted in lines that appear to be parallel except for the lines at low substrate concentrations. Analysis of covariance tests of the slopes of all of the lines for each derivative indicate that the lines are not parallel (Tables 9, 10, and 11). The type of inhibition exerted by these drugs may be different at low and high substrate concentrations, however. An analysis of covariance tests on all of the lines for norfloxacin excluding the line of lowest substrate concentration indicates that the lines are significantly parallel ($p < .01$; Table 12). This indicates that the type of inhibition is competitive at higher substrate concentrations and uncompetitive at lower substrate concentrations.

Increasing concentrations up to 1 mg/ml of novobiocin did not inhibit the relaxation reaction (Figure 22). This is expected, since novobiocin is an inhibitor of the B subunit of DNA gyrase and the relaxation reaction is a property of the A subunit.

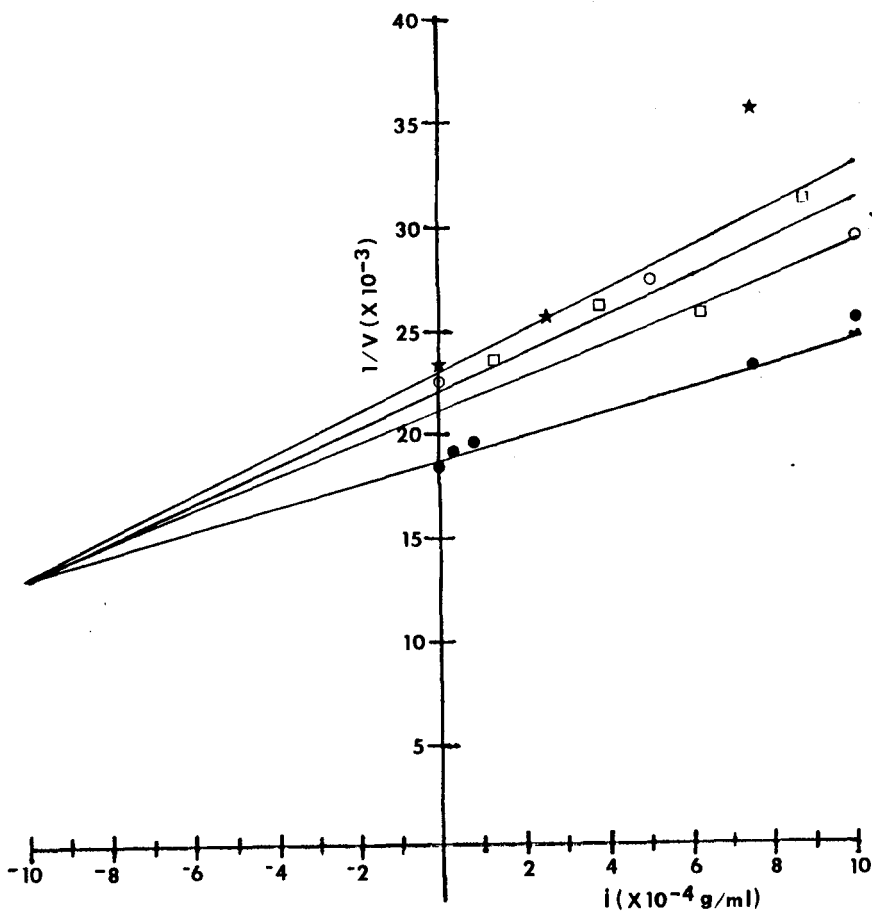


Figure 28. Dixon Plot of the Inhibition of *P. aeruginosa* DNA Gyrase-Catalyzed Relaxation by Oxolinic Acid.

The relaxation reaction was carried out in various concentrations of oxolinic acid and substrate concentrations of 1.7×10^{-9} mMoles/ml (★), 3.4×10^{-9} mMoles/ml (□), 6.9×10^{-9} mMoles/ml (○), and 1.8×10^{-8} mMoles/ml (●) as described in the Methods section.

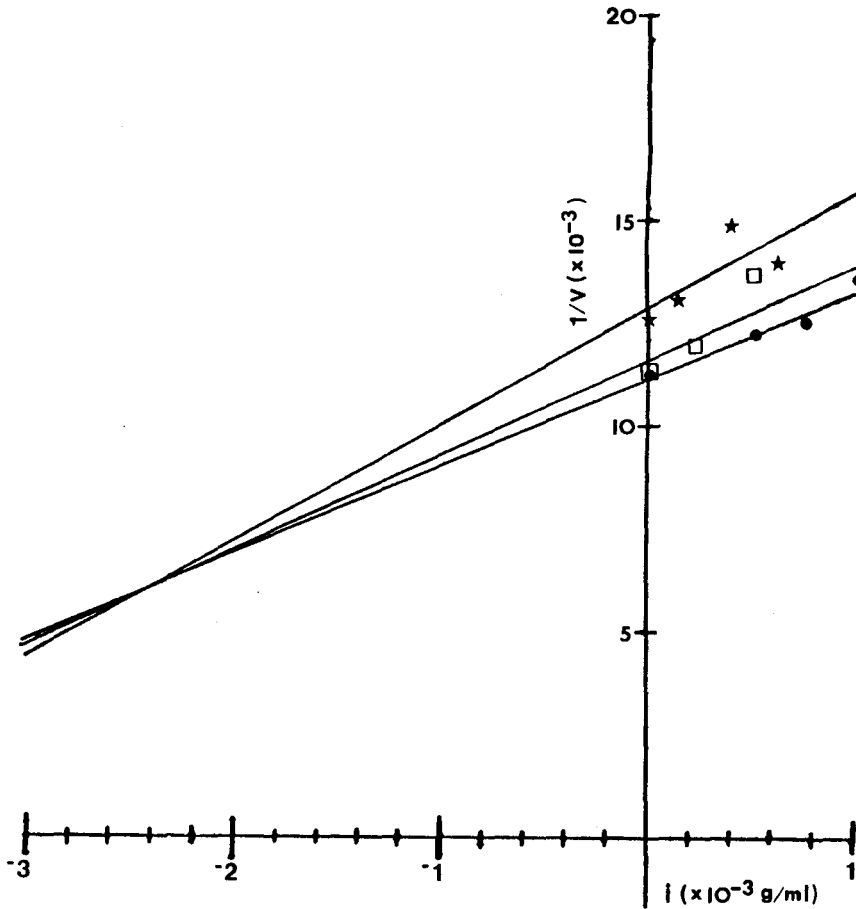


Figure 29. Dixon Plot of the Inhibition of *P. aeruginosa* DNA Gyrase-Catalyzed Relaxation by Nalidixic Acid.

The relaxation reaction was carried out in various concentrations of nalidixic acid and substrate concentrations of 1.7×10^{-9} mMoles/ml (\star), 3.4×10^{-9} mMoles/ml (\square), and 6.9×10^{-9} mMoles/ml (\bullet) as described in the Methods section.

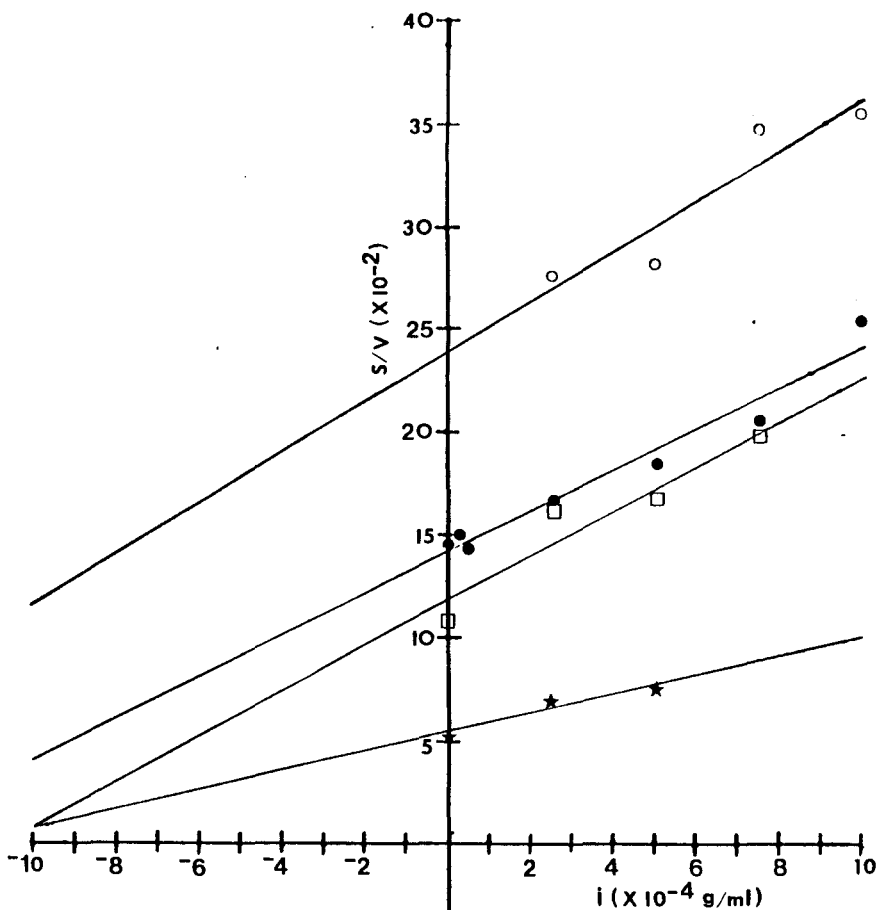


Figure 30. Cornish-Bowden Plot of the Relaxation Reaction in the Presence of Norfloxacin.

The concentration of supercoiled ϕ X174 DNA (s) divided by the velocity of the relaxation reaction (v) catalyzed by *P. aeruginosa* DNA gyrase is plotted against the concentration of norfloxacin (i) added. The different lines represent different concentrations of substrate: (★) 1.7×10^{-9} mMoles/ml, (□) 3.4×10^{-9} mMoles/ml, (○) 6.9×10^{-9} mMoles/ml, and (●) 1.8×10^{-8} mMoles/ml.

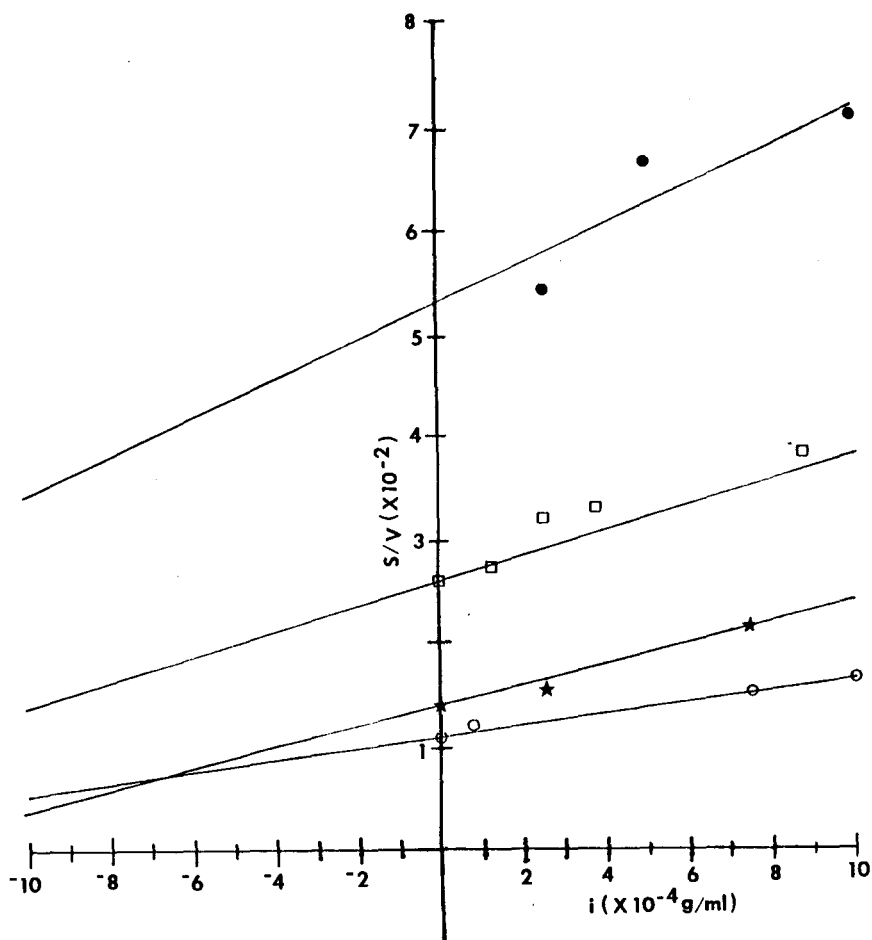


Figure 31. Cornish-Bowden Plot of the Relaxation Reaction in the Presence of Oxolinic Acid.

The concentration of supercoiled ϕ X174 DNA (s) divided by the velocity of the relaxation reaction (v) catalyzed by *P. aeruginosa* DNA gyrase is plotted against the concentration of oxolinic acid (i) added. The different lines represent different concentrations of substrate: (\star) 1.7×10^{-9} mMoles/ml, (\square) 3.4×10^{-9} mMoles/ml, (\bullet) 6.9×10^{-9} mMoles/ml, and (\circ) 1.8×10^{-8} mMoles/ml.

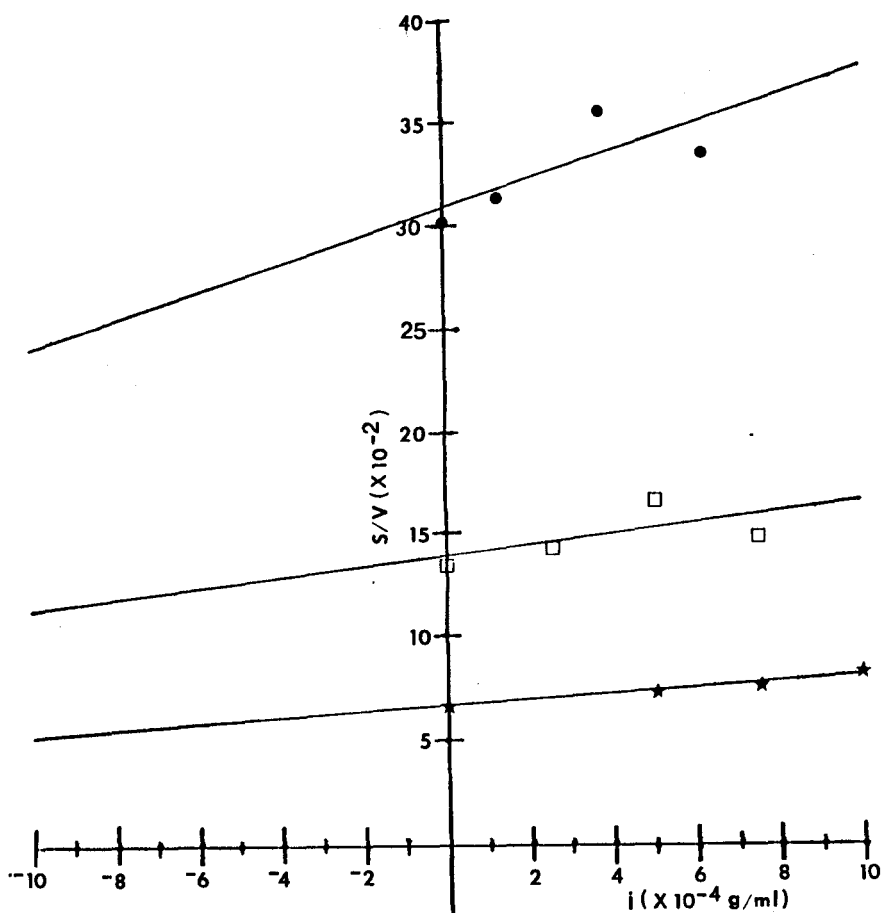


Figure 32. Cornish-Bowden Plot of the Relaxation Reaction in the Presence of Nalidixic Acid.

The concentration of supercoiled ϕ X174 DNA (s) divided by the velocity of the relaxation reaction (v) catalyzed by *P. aeruginosa* DNA gyrase is plotted against the concentration of nalidixic acid (i) added. The different lines represent different concentrations of substrate: (\star) 1.7×10^{-9} mMoles/ml, (\square) 3.4×10^{-9} mMoles/ml, and (\circ) 6.9×10^{-9} mMoles/ml.

Table 9. Analysis of the Covariance on the Slopes of the Cornish-Bowden Plots for Norfloxacin

	Group I		Group II		Group III		Group IV		SUM
	X	Y	X	Y	X	Y	X	Y	
Obs 1	0	5.3154	0	10.82	0	17.79	0	14.581	
Obs 2	250	6.9412	250	16.41	250	24.42	10	14.514	
Obs 3	500	7.6278	500	16.88	500	28.18	25	15.895	
Obs 4			750	19.83	750	34.79	50	15.101	
Obs 5					1000	35.17	75	13.368	
Obs 6							100	14.222	
Obs 7							250	16.601	
Obs 8							500	18.406	
Obs 9							750	20.484	
Obs 10							1000	25.451	
[X=	750		1500		2500		2760		7510
[X**2=	312500		875000		1875000		1893850		4956350
[Y=		19.8844		63.94		140.35		168.623	392.7974
[Y**2=		134.61706		1064.5238		4154.2059		2966.6433	8319.9901
[XY=	5549.2		27415		81457.5		57889.615		172311.31
Mean \bar{X} =	250		375		500		276		341.36363
Mean \bar{Y} =		6.6281333		15.985		28.07		16.8623	17.854427
N=	3		4		5		10		22
Byx=	0.0046248 ^{NS}		0.011 ^{NS}		0.018052*		0.0100254**		
Fb=	2.7313321		8.3566657		17.252357		11.825165		
[X*2-C _{xx})	125000		312500		625000		1132090		
[XY-C _{xy})	578.1		3437.5		11282.5		11349.667		
[Y*2-C _{yy})	2.8206129		42.4429		214.5814		123.27177		
C _{xxw} =	2194590	C _{xxb} =	198119.09	C _{xxt} =	2392709.0	FN 1=	3		
C _{xyw} =	26647.767	C _{xyb} =	11576.799	C _{xyt} =	38224.566	FN 2=	14		
C _{yyw} =	383.11668	C _{yyb} =	923.70085	C _{yyt} =	1306.8175				
bw=	0.0121424	S ₁ =	34.372857	S ₂ =	25.173827	F=	6.3719620**		

Table 9. Analysis of Covariance on the Slopes of the Cornish-Bowden Plots for Norfloxacin.

The line labelled Byx represents the slope for the individual groups. The line below, labelled Fb, provides the F-statistics to test whether these individual slopes differ significantly from zero. The critical information is the F-statistics in the lower right hand corner of the table. If that statistic is significant, the slopes vary significantly among the groups. The null-hypothesis assumes that the regressions based on the separate groups are the same as that based on the total group, except for sampling variability, and that therefore the effects of different concentrations of substrate can be disregarded. If the F-statistics is significant, then the slopes vary significantly among the groups, and from the common slope, bw, hypothesized to exist for the groups combined. The number in the left bottom corner is the common slope bw. Thus the hypothesis being tested is: $b_1 = b_2 = b_3 = b_4 = b_w$. NS indicates that a particular F-statistic is not significant, the symbol * indicates that it is significant at the .05 level, and ** indicates significance at the .01 level.

Table 10. Analysis of the Covariance on the Slopes of the Cornish-Bowden Plots for Oxolinic Acid

	Group I		Group II		Group III		Group IV		SUM
	X	Y	X	Y	X	Y	X	Y	
Obs 1	0	13.9	125	27.6	250	54.4	0	11.576	
Obs 2	250	15.4	375	31.6	500	66.7	10	12.212	
Obs 3	750	21.5	625	29.6	1000	70	25	12.628	
Obs 4	1000	50.8	875	38.5			75	12.486	
Obs 5							750	14.066	
Obs 6							1000	16.105	
Obs 7									
Obs 8									
Obs 9									
Obs 10									
[X=	2000		2000		1750		1860		7610
[X**2=	1625000		1312500		1312500		1568850		5818850
[Y=		101.6		127.3		191.1		79.073	499.073
[Y**2=		3473.26		4118.73		12308.25		1055.7266	20955.966
[XY=	70775		67487.5		116950		28028.77		283241.27
Mean X=	500		500		583.33333		310		
Mean Y=		25.4		31.825		63.7		13.178833	
N=		4 ^{NS}		4 ^{NS}		3 ^{NS}		6 ^{NS}	17
Byx=	0.03196 ^{NS}		0.01228 ^{NS}		0.0187714 ^{NS}		0.0035436 ^{NS}		
Fb=	15.778014		10.700624		5.5211064		3.6092507		
[X*2-Cxx)	625000		312500		291666.66		992250		
[XY-Cxy	19975		3837.5		5475		3516.14		
[Y*2-Cyy	892.62		67.4075		135.18		13.636792		
Cxxw=	2221416.6	Cxxb=	190839.21	Cxxt=	2412255.8	FN_1=	3		
Cxyw=	32803.64	Cxyb=	27029.069	Cxyt=	59832.709	FN_2=	9		
Cyyw=	1108.8442	Cyyb=	5195.7188	Cyyt=	6304.5631				
bw=	0.0147669	S_1=	316.34781	S_2=	308.08541	F=	3.0804556 ^{NS}		

Table 10. Analysis of Covariance on the Slopes of the Cornish-Bowden Plots for Oxolinic Acid.

The line labelled Byx represents the slope for the individual groups. The line below, labelled Fb, provides the F-statistics to test whether these individual slopes differ significantly from zero. The critical information is the F-statistics in the lower right hand corner of the table. If that statistic is significant, the slopes vary significantly among the groups. The null-hypothesis assumes that the regressions based on the separate groups are the same as that based on the total group, except for sampling variability, and that therefore the effects of different concentrations of substrate can be disregarded. If the F-statistics is significant, then the slopes vary significantly among the groups, and from the common slope, bw, hypothesized to exist for the groups combined. The number in the left bottom corner is the common slope bw. Thus the hypothesis being tested is: $b_1 = b_2 = b_3 = b_4 = b_w$. NS indicates that a particular F-statistic is not significant, the symbol * indicates that it is significant at the .05 level, and ** indicates significance at the .01 level.

Table 11. Analysis of Covariance on the Slopes of the Cornish-Bowden Plots for Nalidixic Acid

	Group I		Group II		Group III		SUM
	X	Y	X	Y	X	Y	
Obs 1	0	6.8	0	13.5	0	30.144	
Obs 2	500	7.4	250	14.3	125	31.368	
Obs 3	750	7.52	500	16.6	375	35.6856	
Obs 4	1000	29.92	750	14.8	625	33.7392	
Obs 5							
Obs 6							
Obs 7							
Obs 8							
Obs 9							
Obs 10							
[X=	2250		1500		1125		4875
[X**2=	1812500		875000		546875		3234375
[Y=		51.64		59.2		130.9368	241.7768
[Y**2=		1052.7568		881.34		4304.4078	6238.5046
[XY=	39260		22975		38390.1		100625.1
Mean-X=	562.5		375		281.25		
Mean-Y=		12.91		14.8		32.7342	
N=	4		4		4		12
Byx=	0.0186742 ^{NS}		0.00248 ^{NS}		0.0067867 ^{NS}		
Fb=	9.4048879		4.3387025		3.3488856		
[X*2=Cxx	546875		312500		230468.75		
[XY=Cxy	10212.5		775		1564.125		
[Y*2=Cyy	386.0844		5.18		18.296425		
Cxxw=	1089843.7	Cxxb=	164062.5	Cxxt=	1253906.2	FN 1=	3
Cxyw=	12551.625	Cxyb=	-10148.35	Cxyt=	2403.275	FN 2=	4
Cyyw=	409.56082	Cyyb=	957.60871	Cyyt=	1367.1695		
bw=	0.0115169	S ₁ =	58.692558	S ₂ =	206.31241	F=	0.3793118 ^{NS}

Table 11. Analysis of Covariance on the Slopes of the Cornish-Bowden Plots for Nalidixic Acid.

The line labelled Byx represents the slope for the individual groups. The line below, labelled Fb, provides the F-statistics to test whether these individual slopes differ significantly from zero. The critical information is the F-statistics in the lower right hand corner of the table. If that statistic is significant, the slopes vary significantly among the groups. The null-hypothesis assumes that the regressions based on the separate groups are the same as that based on the total group, except for sampling variability, and that therefore the effects of different concentrations of substrate can be disregarded. If the F-statistics is significant, then the slopes vary significantly among the groups, and from the common slope, bw, hypothesized to exist for the groups combined. The number in the left bottom corner is the common slope bw. Thus the hypothesis being tested is: $b_1 = b_2 = b_3 = b_4 = b_w$. NS indicates that a particular F-statistic is not significant, the symbol * indicates that it is significant at the .05 level, and ** indicates significance at the .01 level.

Table 12. Analysis of Covariance on the Slopes of the Cornish-Bowden Plots for Norfloxacin at Higher Substrate Concentrations Only

	Group I		Group II		Group III		SUM
	X	Y	X	Y	X	Y	
Obs 1	0	5.3154	0	10.82	0	17.79	
Obs 2	250	6.9412	250	16.41	250	24.42	
Obs 3	500	7.6278	500	16.88	500	28.18	
Obs 4			750	19.83	750	34.79	
Obs 5					1000	35.17	
Obs 6							
Obs 7							
Obs 8							
Obs 9							
Obs 10							
[X=	750		1500		2500		4750
[X**2=	312500		875000		1875000		3062500
[Y=		19.8844		63.94		140.35	224.1744
[Y**2=		134.61706		1064.5238		4154.2059	5353.3467
[XY=	5549.2		27415		81457.5		114421.7
Mean-X=	250		375		500		
Mean-Y=		6.6281333		15.985		28.07	
N=	3 ^{NS}		4 ^{NS}		5		12
Byx=	0.0046248		0.011		0.018052*		
Fb=	2.7313321		8.3566657		17.252357		
[X*2=Cxx	125000		312500		625000		
[XY=Cxy	578.1		3437.5		11282.5		
[Y*2=Cyy	2.8206129		42.4429		214.5814		
Cxxw=	1062500	Cxxb=	119791.66	Cxxt=	1182291.6	FN_1=	3
Cxyw=	15298.1	Cxyb=	10387.9	Cxyt=	25686	FN_2=	4
Cyyw=	259.84491	Cyyb=	905.65505	Cyyt=	1165.499		
bw=	0.0143982	S_1=	23.892503	S_2=	15.687126	F=	2.0307525 ^{NS}

Table 12. Analysis of Covariance on the Slopes of the Cornish-Bowden Plots for Norfloxacin at Higher Substrate Concentrations Only

The line labelled Byx represents the slope for the individual groups. The line below, labelled Fb, provides the F-statistics to test whether these individual slopes differ significantly from zero. The critical information is the F-statistics in the lower right hand corner of the table. If that statistic is significant, the slopes vary significantly among the groups. The null-hypothesis assumes that the regressions based on the separate groups are the same as that based on the total group, except for sampling variability, and that therefore the effects of different concentrations of substrate can be disregarded. If the F-statistics is significant, then the slopes vary significantly among the groups, and from the common slope, bw, hypothesized to exist for the groups combined. The number in the left bottom corner is the common slope bw. Thus the hypothesis being tested is: $b_1 = b_2 = b_3 = b_4 = b_w$. NS indicates that a particular F-statistic is not significant, the symbol * indicates that it is significant at the .05 level, and ** indicates significance at the .01 level.

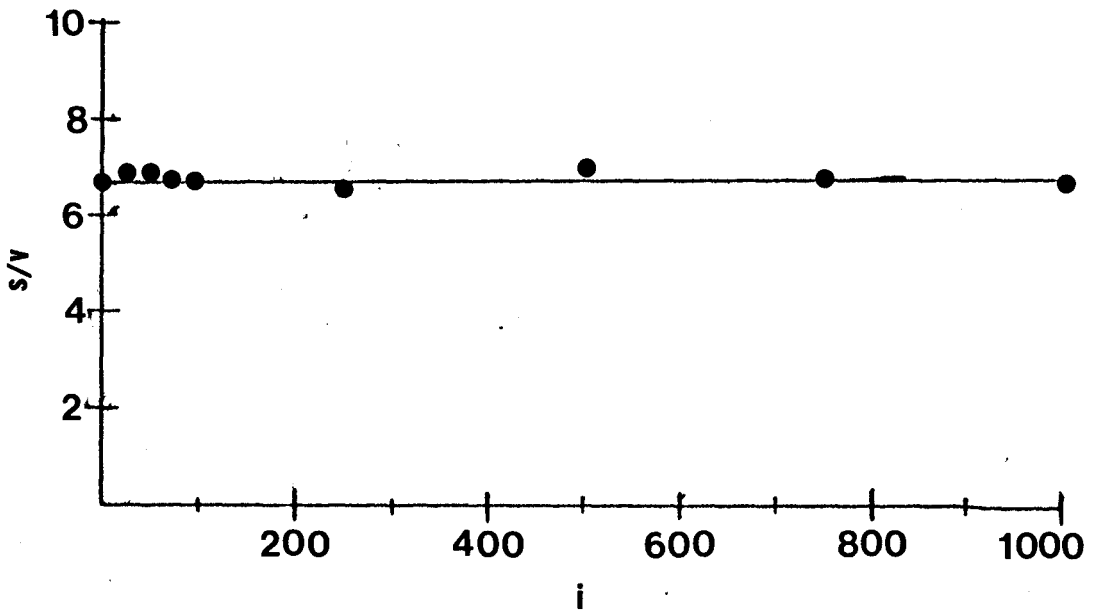


Figure 33. Plot of the Velocity of the Relaxation Reaction in the Presence of Novobiocin.

The velocity of the relaxation reaction catalyzed by *P. aeruginosa* DNA gyrase is plotted against the concentration of novobiocin (i) added.

CATENATION REACTION

An agarose gel of the products from the supercoiling reaction exhibited a band of lower electrophoretic mobility (Figure 34). This band may consist of concatamers of the circular DNA, since catenated circular DNA has been shown to have lower electrophoretic mobility than relaxed DNA on an agarose gel (42). This band may not be due to protein bound DNA since these reactions were extracted with a mixture of chloroform and isopropanol before loading the gel.

CLEAVAGE OF DNA

Incubation of DNA gyrase with pyridonecarboxylic acid for one hour at 37⁰ C with subsequent incubation in the presence of SDS resulted in cleavage of the relaxed and linear DNA with little if any cleavage of the supercoiled DNA (Figure 35, lanes 3-8). ATP (lane 7) and AppNp (lane 8) did not prevent the cleavage but inhibited it slightly. The DNA migrated to the same distance in the presence and absence of proteinase K digestion (lanes 3 and 4, respectively). This suggests that no protein is attached to the cleaved DNA, but the gel may not be sensitive enough to show the slight difference in migration. The fact that the cleavage product consists of two bands (which are hard to distinguish in Figure 35 but can be seen in the densitometric tracing) which migrate faster than linear DNA (lane 10) indicates that DNA gyrase cleaves the DNA in two places.

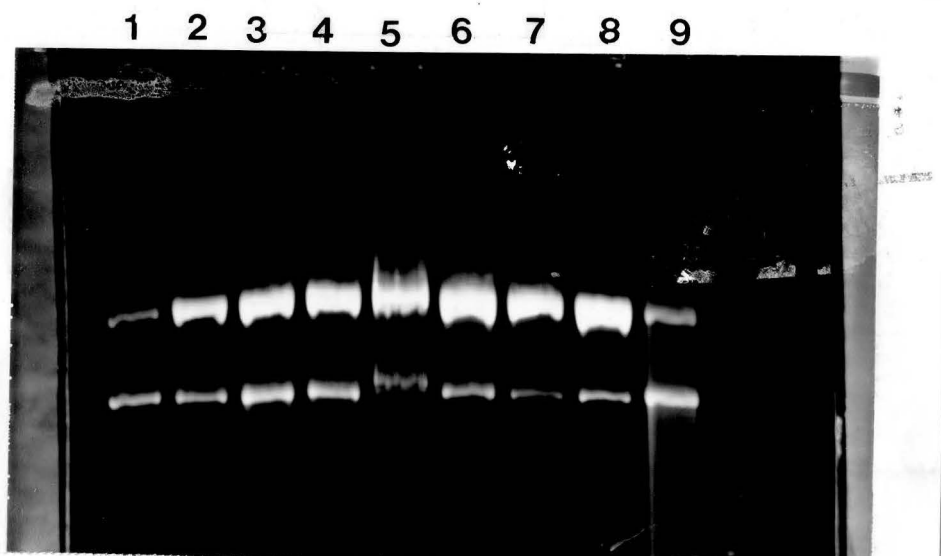


Figure 34. The Catenation Reaction of P. aeruginosa DNA Gyrase.

P. aeruginosa DNA gyrase was incubated with relaxed ϕ X174 DNA as described for the supercoiling reaction in the Methods section. Lane 1 contains relaxed DNA incubated in the absence of DNA gyrase and lane 9 contains supercoiled DNA. Lanes 2-8 contain relaxed DNA incubated with one unit of DNA gyrase.

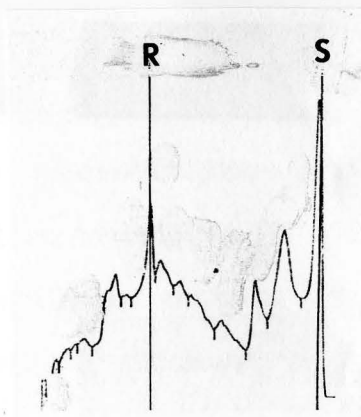
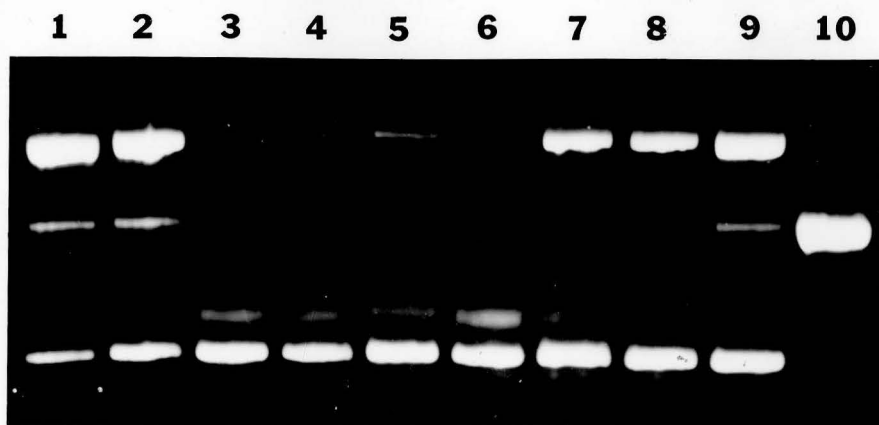


Figure 35. Cleavage of DNA by *P. aeruginosa* DNA Gyrase in the Presence of Pyridonecarboxylic Acid and SDS.

The ϕ X174 DNA used in this reaction is seen in lane 1, the upper band is relaxed, closed circular DNA, the middle band is linear DNA, and the lower band is supercoiled DNA. When this DNA is incubated with *P. aeruginosa* DNA gyrase in the absence of drug, a ladder of bands between the linear and supercoiled which consists of different topoisomers differing by two linking numbers can be seen (lane 2). When the DNA is incubated with pyridonecarboxylic acid followed by incubation in the presence of SDS as described in the text, two bands (which are hard to distinguish in this photograph) consisting of cut linear DNA can be seen (lanes 3-8). Norfloxacin (lanes 3 and 4), nalidixic acid (lane 5), oxolinic acid (lanes 6-9) or novobiocin (lane 9) was used. Proteinase K (lane 5), ATP (lane 7) or AppNp (lane 8) was added to the reaction as described in the text. Linear ϕ X174 DNA is in lane 10. The inset is a densitometric tracing of lane 3. R is the distance migrated by completely relaxed DNA and S is the distance migrated by completely supercoiled DNA.

ATPase REACTION

ATP hydrolysis was examined by measuring the amount of inorganic phosphate released during the supercoiling reaction. This was detected by reading the absorbance at 660 nanometers (A_{660}) of the supercoiling reaction mixture incubated at 37° C for 30 minutes and treated with color reagents as described in the Methods section. The A_{660} of the supercoiling reaction mixture was not significantly different from the A_{660} of the controls lacking DNA gyrase or ATP (Table 13). The A_{660} of the inorganic phosphate standards was directly proportional to the concentration of inorganic phosphate (Figure 36). No ATPase activity was detected in either fresh or frozen DNA gyrase preparations. One unit of M. luteus DNA gyrase (Bethesda Research Laboratories) and incubated in the supercoiling reaction mixture containing 0.5 micrograms of relaxed ϕ X174 DNA exhibited detectable ATPase activity (2×10^{-6} moles of inorganic phosphate released per minute) which was inhibited by 10 micrograms/ml of novobiocin.

Table 13. ATPase Reaction

Experiment	Sample	OD ₆₆₀
<u>P. aeruginosa</u> DNA gyrase:		
1	Supercoiling reaction	3.142 +/- 0.009
	Control, no DNA gyrase	3.224 +/- 0.003
	Control, no ATP	3.284 +/- 0.003
2	Supercoiling reaction	2.821 +/- 0.089
	Control, no DNA gyrase	2.850 +/- 0.021
	Control, no ATP	2.862 +/- 0.013
3	Supercoiling reaction	2.067 +/- 0.062
	Control, no DNA gyrase	2.098 +/- 0.093
	Control, no ATP	2.054 +/- 0.009
4	Supercoiling reaction	2.015 +/- 0.013
	Control, no DNA gyrase	2.098 +/- 0.093
	Control, no ATP	2.017 +/- 0.011
5	Supercoiling reaction	2.954 +/- 0.022
	Control, no DNA gyrase	2.979 +/- 0.018
	Control, no ATP	2.986 +/- 0.052
<u>M. luteus</u> DNA gyrase:		
1	Supercoiling reaction	3.180 +/- 0.024
	Control, no DNA gyrase	2.979 +/- 0.028
	Control, no ATP	2.986 +/- 0.052
	Supercoiling reaction + novobiocin*	3.077 +/- 0.033

*10 micrograms/ml

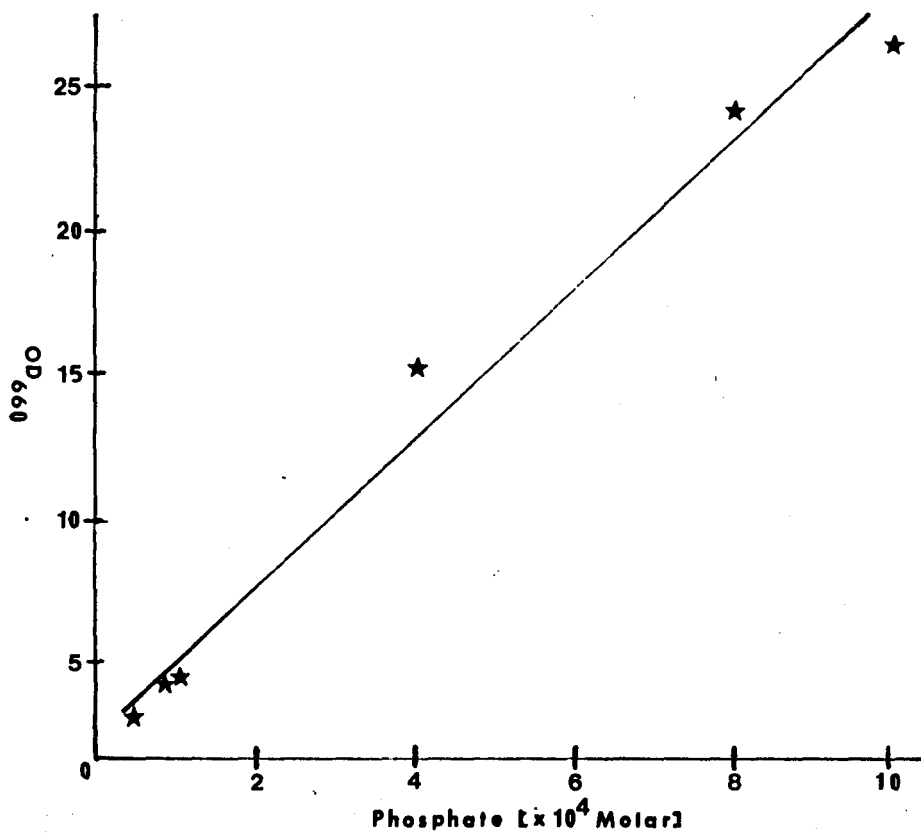


Figure 36. Calibration Curve for the Concentration of Inorganic Phosphate.

DISCUSSION

IN VIVO EFFECTS OF PYRIDONECARBOXYLIC ACIDS ON P. AERUGINOSA

Pseudomonas aeruginosa is more resistant to norfloxacin and nalidixic acid than Escherichia coli (Table 9 and reference 30). These two pyridonecarboxylic acid derivatives affect P. aeruginosa similarly, since spontaneous mutants resistant to norfloxacin, RM166, 167, 168, and 169, are also resistant to nalidixic acid, and a nalidixic acid resistant mutant, RM4000, is also resistant to norfloxacin (Table 9). This suggests that the two derivatives have the same mechanism of transport into the cell, they have the same target of action, or both.

Since pyridonecarboxylic acids inhibit E. coli DNA gyrase and a nalidixic acid-sensitive DNA gyrase has been purified from P. aeruginosa (56), the site of action of these drugs is most likely DNA gyrase bound to the chromosome. DNA gyrase is required for DNA replication (36) and pyridonecarboxylic acids inhibit DNA synthesis (22,25). Therefore, it would be expected that these drugs would be most potent when DNA replication is most active and when the activities of DNA gyrase are clearly needed in early to mid-log phase of an logarithmically growing culture, and less potent when there is little if any DNA replication in late log phase. Treatment of a culture of P. aeruginosa in early log phase with norfloxacin causes a significant inhibition of growth (Figure 4). Treatment of this culture in late log phase causes much less inhibition of growth (Figure 5).

P. aeruginosa cultures treated with bactericidal concentrations of pyridonecarboxylic acids exhibit lethal unbalanced growth. This is a deregulation of the normal growth cycle where cells that are no longer viable undergo cell division and filamentation. Filamentation is an elongation of the cell which keeps the surface to volume ratio constant. This allows the extra chromosomes synthesized in the absence of cell division to be distributed along the cell membrane. Both PA0303 and RM8 exhibit cell division after 24 hours of treatment with norfloxacin or nalidixic acid and RM8 filaments when treated with nalidixic acid (Figure 8). This lethal unbalanced growth may result from the prevention of the completion of a round of chromosome replication by pyridonecarboxylic acids since this completion has been suggested to trigger the initiation of septation which precedes cell division (15). Pyridonecarboxylic acids may prevent the induction of a cell division regulating signal by preventing the completion of a round of chromosome replication.

Alternatively, lethal unbalanced growth may result from pyridonecarboxylic acids effect on gene expression (reviewed in 18). Treatment with pyridonecarboxylic acids causes a decrease in the synthesis of outer membrane proteins in comparison to the synthesis of inner membrane proteins (28). Since a sudden exponential increase in the synthesis of outer membrane proteins has been observed just before cell division (4), pyridonecarboxylic acids could deregulate the cells growth cycle by inhibiting the expression of outer membrane proteins. Also, these drugs may alter the expression of a protein which regulates cell division and thus allowing some of the cells to divide even though the division would not result in two viable cells. Therefore, the

mechanism of drug induced cell death may be the inhibition of the expression of specific genes which are vital to the cell.

The bactericidal activity of pyridonecarboxylic acids in E. coli has been shown to require competent RNA and protein synthesis (76,84). Simultaneous treatment of P. aeruginosa with chloramphenicol or rifamycin, inhibitor of protein and RNA syntheses, respectively, along with pyridonecarboxylic acid alleviates the bactericidal effect of the pyridonecarboxylic acid (Figures 6A and 7A). This protection suggests that there may be a gene responsible for killing whose expression is induced upon drug treatment and therefore, by preventing the expression of this gene, chloramphenicol prevents killing.

RECOVERY SYNTHESIS

In a logarithmically growing culture, the rate of DNA synthesis as followed by pulse labeling DNA increases logarithmically (Figure 11). Pyridonecarboxylic acid treatment causes an immediate inhibition of the rate of DNA synthesis. Within 5 minutes, however, the rate of DNA synthesis is again increasing logarithmically. I will term this second exponential rate of DNA synthesis "recovery synthesis." Recovery synthesis is observed at concentrations below the MBC and not at the MBC.

Pyridonecarboxylic acids may act by binding to DNA at sites complexed with DNA gyrase thereby creating complexes that block the movement of the replication fork (19). Also, these drugs inhibit the activities of DNA gyrase which is part of the enzyme system required for replication (36). Therefore, in order for recovery synthesis to occur,

a repair system to remove the hypothesized drug-DNA gyrase complex and repair the DNA may be needed. Recovery synthesis may also require the synthesis of new DNA gyrase molecules to replace those inactivated by drug.

If recovery synthesis results from an inducible repair system, then simultaneous treatment with drug and chloramphenicol, an inhibitor of protein synthesis, will prevent the synthesis of the necessary proteins and recovery synthesis should not be observed. Simultaneous treatment with pyridonecarboxylic acid and chloramphenicol does prevent recovery synthesis in PA0303 (Figure 13A). In this case, the initial rapid rate of inhibition of DNA synthesis is followed by a slower rate of inhibition. When these cultures are treated with chloramphenicol alone, the rate of DNA synthesis also decreases. This suggests that the enzymes required for the exponential rate of DNA synthesis must be continually synthesized.

The fact that protein synthesis is required for both killing and recovery synthesis suggests that the induced genes which are responsible for these two phenomena may be under the same control or in fact may be the same gene. Perhaps it is the DNA gyrase gene itself. Since DNA gyrase is required for DNA replication, it follows that it would also be required for recovery synthesis because DNA synthesis occurs during both recovery synthesis and DNA replication. Synthesis of new DNA gyrase molecules in a pyridonecarboxylic acid treated cell may be lethal since this would result in more drug-DNA gyrase complexes on the chromosome which will inhibit replication. Also, the synthesis of new DNA gyrase molecules may alter the superhelical density of the chromosome and

result in a greater effect on gene expression which could be lethal to the cell.

THE MECHANISM OF RECOVERY SYNTHESIS

What happens inside a P. aeruginosa cell in the five minutes it takes to overcome the inhibiting effects of pyridonecarboxylic acids and start recovery synthesis? How are the drug-DNA gyrase complexes removed from the DNA? Is more DNA gyrase synthesized, or can the old DNA gyrase molecules be freed from the pyridonecarboxylic acids?

In order to answer some of these questions, the effects of pyridonecarboxylic acids on P. aeruginosa mutants deficient in recombination (a Rec^- phenotype) were also studied; PA02002, a Rec^- mutant of PA02001, which is less resistant to nalidixic acid than its parent and RM8, a Rec^- mutant of PA0303, which is more resistant to norfloxacin and nalidixic acid than its parent. If a recombinational DNA repair system were used to remove drug-DNA gyrase complexes, then recovery synthesis would not be observed in these mutants. These mutants are of interest to study also because they are deficient in lysogeny establishment of phage (a Les^- phenotype) and DNA gyrase is required for lysogenic establishment of prophage lambda into the E. coli chromosome (57).

As expected, PA02002 (Rec^-) does not exhibit recovery synthesis (Figure 14B and D). After a five minute initial phase of rapid inhibition, the rate of DNA synthesis decreases at a slower rate. Biphasic inhibition kinetics is also observed when PA0303 is treated simultaneously with pyridonecarboxylic acid and chloramphenicol (Figure

13A): an initial rapid rate of inhibition is followed by a slower rate of inhibition.

Why are there two distinct rates of inhibition in the absence of recovery synthesis as in the case of PA02002 and chloramphenicol-treated PA0303? If DNA gyrase binds to DNA approximately every 100 kilobase pairs (17), then the replication forks would stop whenever they reached a drug-DNA gyrase complex that had not yet been removed and monophasic inhibition kinetics would be expected. But there is evidence that DNA gyrase binds at or near the replication forks in addition to binding approximately every 100 kilobasepairs and this could explain the biphasic inhibition kinetics (17). The initial rapid rate of inhibition may be due to drug-DNA gyrase complexes occurring at the replication fork. The slower rate of inhibition may be the result of replication forks which escaped the drug proceeding until they reached a drug-DNA gyrase complex on the DNA or until the terminus of replication.

From the hypothesis that the initial inhibition is due to drug-DNA gyrase complexes occurring at the replication fork, it would follow that one can titrate replication forks on DNA with the drug-DNA gyrase complexes. If this is true, then one would expect that for a given bacterial strain, the degree of inhibition should be the same for both norfloxacin and nalidixic acid provided the concentrations of both drugs is sufficiently high (e.g., four times the MIC). As seen in Figures 11 and 12, at a concentration of four times the MIC, nalidixic acid initially inhibits DNA synthesis to a greater extent than norfloxacin.

The MIC's for norfloxacin and nalidixic acid are very different. This may be due to the fact that the drugs' effects on other processes besides DNA synthesis are involved in the inhibition of growth. The

concentration at which RNA and protein synthesis are inhibited most likely also play a role in the minimal concentration required to inhibit growth because the concentration at which RNA and protein synthesis are inhibited is greater than the concentration at which DNA synthesis is inhibited (76). The inhibition of protein synthesis may play a role in the rate of DNA synthesis because enzymes involved in DNA synthesis need to be made. Also, since release of RNA from the chromosome requires translation in a prokaryotic cell (48), drug-inhibited translation may block replication. Since nalidixic acid and norfloxacin inhibit protein synthesis to different extents at the concentration of four times the MIC (Figure 9), the same extent of DNA synthesis inhibition is not expected for this concentration of each drug. Since nalidixic acid inhibits protein synthesis to a greater extent than norfloxacin, it would be expected to have a greater effect on DNA replication than norfloxacin. Norfloxacin would be expected to have a greater initial inhibitory effect on DNA replication if protein synthesis was inhibited simultaneously with norfloxacin treatment. This is observed by comparing the initial inhibitions in Figure 11 and 13. This data is summarized in Table 14.

The biphasic inhibition kinetics can also be explained another way. Perhaps the initial rapid rate of inhibition is due to the inactivation of DNA gyrase on the chromosome causing an immediate stop to the existing replication forks. DNA synthesis during the second phase is limited to DNA stretches unbound by drug-DNA gyrase molecules and can only utilize the activity of the DNA gyrase complexes left unaffected by the drug. The creation of new replication forks is probably required for this second phase. In the case of the Rec⁻ mutant, recovery

Table 14. Initial Inhibitions (percent)

Strain	Nalidixic acid	Norfloxacin	Norfloxacin and Chloramphenicol
PA 0303	80	50	60
RM8	75	30	40
PA02001	85	30	ND
PA02002	80	40	ND

This data is summarized from Figures 11, 12, 13, and 14.
ND = Not determined.

synthesis does not occur because the drug-DNA gyrase complexes are not being removed from the chromosome. In the case of PA0303 treated simultaneously with chloramphenicol and pyridonecarboxylic acid, recovery synthesis does not occur because new DNA gyrase molecules can not be synthesized.

RM8: A SPECIAL CASE

The other Rec⁻ mutant RM8 surprisingly exhibited recovery synthesis in the presence of norfloxacin (Figure 11B). It also exhibited recovery synthesis in the presence of nalidixic acid, but this recovery synthesis could not be sustained (Figure 12B). Since nalidixic acid inhibits protein synthesis about 99%, it probably does not allow the synthesis of large enough quantities of the required enzymes to support recovery synthesis. Since norfloxacin inhibits protein synthesis only about 50%, it allows the synthesis of enough of the required enzymes to support recovery synthesis.

If recombination is required for recovery synthesis, then why does recovery synthesis occur in RM8 if it is deficient in recombination, while the other Rec⁻ strain (PA02002) does not exhibit recovery synthesis? It is very likely that some of the enzymes involved in recombination are also actively involved in recovery synthesis as well and if RM8 is deficient in these enzymes, then it should also be deficient in recovery synthesis. The recombinational deficiency in RM8 may not be due to lacking or inactive recombinational enzymes. Perhaps RM8 is different from the wild type cell because it constitutively synthesizes a low amount of the necessary enzymes which may be enough

for some recovery synthesis to occur but not enough for recombination, whereas its parent, PA0303, only synthesizes these enzymes upon induction by pyridonecarboxylic acids (Figures 11A, 12A, and 13A). To test this, RM8 was treated simultaneously with chloramphenicol and norfloxacin. In this case, recovery synthesis occurred but could not be sustained (Figure 13B). The DNA synthesis curve looked very similar to the DNA synthesis curve of nalidixic acid alone (Figure 12B). By inhibiting protein synthesis at the same time as treatment with norfloxacin, the effects of nalidixic acid were mimicked. This supports the previously mentioned data that nalidixic acid inhibits protein synthesis 99%, while norfloxacin inhibits protein synthesis only about 50% (Figure 9).

If inhibition of protein synthesis prevents recovery synthesis, then why does recovery synthesis occur in PA0303 treated with nalidixic acid if nalidixic acid inhibits protein synthesis 99%? Chloramphenicol inhibits initiation of protein synthesis while allowing elongation of previously initiated amino acid chains to continue. It is not known how nalidixic acid inhibits protein synthesis. Nalidixic acid may inhibit protein synthesis by inhibiting elongation of amino acid chains since the full inhibitory effect of this drug is not felt until 10 minutes after treatment (Figure 9). Under this hypothesis, the induced proteins will be initiated and elongation of enough proteins to perform recovery synthesis might occur in the presence of nalidixic acid. Small concentrations of these proteins may be sufficient to perform recovery synthesis, since the low levels of these proteins postulated to be present in RM8 are sufficient for recovery synthesis but not sufficient to make this strain recombinationally proficient.

Thus, PA02001 and PA0303 exhibit recovery synthesis because they synthesize the required enzymes upon induction by drug. The required enzymes include at least some of the enzymes that are involved in recombination. PA02002 may not exhibit recovery synthesis because its recombination enzymes are inoperative or lacking. RM8 may synthesize recombination enzymes at levels too low for this strain to be considered recombinationally proficient, but in the presence of sufficient protein synthesis, enough of these enzymes may be made to allow some recovery synthesis to occur.

The enzymes involved in both recombination and recovery synthesis most likely would contain exonuclease and polymerase activities if the recovery mechanism is to cut out pieces of DNA bound by drug-DNA gyrase complexes. Alternatively, the enzymes may contain supercoiling activity that may cause the complex to fall off the chromosome. This may be true, since gyrase binds less well to supercoiled DNA than to relaxed.

The bactericidal activity of pyridonecarboxylic acids in RM8 is not alleviated by inhibiting protein or RNA synthesis simultaneously with drug treatment (Figures 6B and 7B, respectively). This suggests that the protein(s) responsible for killing is present before drug treatment. The observation that RM8 naturally has a high percentage of dead cells (56) supports this hypothesis. Since PA0303 synthesizes the protein responsible for killing only upon induction (Figures 6A and 7A), the observation that killing in RM8 is not alleviated by chloramphenicol supports the hypothesis that RM8's mutation is constitutive.

RM8 filaments when treated with nalidixic acid but not when treated with norfloxacin (Figure 8D and F). This is surprising, since nalidixic acid inhibits protein synthesis to a greater extent than does

norfloxacin (Figure 9). This supports the hypothesis that filamentation is caused by a more complex mechanism than the mere inhibition of replication in the presence of continued RNA and protein synthesis.

INDUCTION OF DNA REPAIR

Treatment of E. coli with nalidixic acid has been shown to induce the synthesis of the RecA protein (25,26), which controls the expression of and/or is involved in all of the dark DNA repair pathways known to exist in E. coli (82). Nalidixic acid treatment also induces a mutagenic repair pathway in E. coli that will repair UV-damaged phage DNA (69). This is detected by infecting nalidixic acid-treated cells with UV-damaged phage and determining how many phage survive. If a DNA repair pathway has been induced by the drug treatment, then the number of PFU's in the treated culture will be exponentially greater than the number of PFU's in the control untreated culture. The DNA repair pathway induced in E. coli is mutagenic, because the number of plaques with altered phenotypes found among the reactivated plaques is in proportion to the extent of reactivation (69).

Similar results would be expected when these experiments are repeated with P. aeruginosa, since P. aeruginosa possesses a recA protein which restores Rec^+ phenotypes to Rec^- E. coli cells (42). Neither norfloxacin nor nalidixic acid induced increased repair or mutagenesis of UV-damaged phage D3 in PA0303 or RM8 (Figure 16). This is not completely unexpected, however, since ultraviolet irradiation does not induce repair or mutagenesis of UV-damaged phage D3 in these strains either (72). Speculation on the method used to remove drug-DNA

gyrase complexes from the chromosome in P. aeruginosa must await further knowledge of the P. aeruginosa DNA repair pathways.

P. AERUGINOSA DNA GYRASE AND TOPO II'

Even though pyridonecarboxylic acids bind to DNA instead of DNA gyrase (70), their ultimate effect is to inhibit the activities of the A subunit and DNA gyrase (22). Therefore, in order to further study the mechanism of action of pyridonecarboxylic acids in P. aeruginosa, I isolated DNA gyrase from strain PA0303 and tested the in vitro effects of these drugs on the purified preparation.

P. aeruginosa DNA gyrase has been isolated previously by Miller and Scurlock (56), who used a series of three columns for the purification. After minimal success with this method, I attempted to adapt the method of Staudenbauer et al. (76). Using a novobiocin-Sepharous affinity column, I was able to improve the specific activity from 15,000 units/mg protein to 37,000 units/mg protein.

P. aeruginosa DNA gyrase protein is similar to the E. coli DNA gyrase protein in that it consists of two subunits with molecular weights of approximately 95,000 and 105,000 (Figure 17). Since the molecular weight of the holoenzyme is $3.6 \pm .3 \times 10^5$ (50), it most likely consists of two molecules of each of the two subunits. Two lower molecular weight proteins are also present in the purified preparation at lower concentrations. I suspect that these other proteins may be proteolytic products of the B subunit similar to the E. coli v subunit of topo II' for the following reasons:

1. No ATPase activity was detected in the DNA gyrase preparation (Table 13), and E. coli topo II' has no ATPase activity.
2. ATP does not alter the DNA cleavage pattern within the limits of detection available in the system used (Figure 35). ATP is known to alter the cleavage pattern of DNA gyrase but not of topo II' in E. coli.
3. After freezing or after 24 hours of storage at 4⁰ C, the DNA gyrase preparation loses its supercoiling activity and only cleaves the DNA with a double-stranded cut without completing the reaction (Figures 19 and 22). In E. coli, DNA gyrase cleaves less than one percent of the DNA in a supercoiling reaction mixture (77), and topo II' cleaves all of the effected DNA molecules (6).
4. In attempting to observe inhibition of the supercoiling reaction, supercoiling was observed in samples containing pyridonecarboxylic acid concentrations as high as the MBC, while no supercoiling activity was observed in the absence or in the presence of low concentrations of these drugs. This indicates that the relaxing activity of topo II' is greater than the supercoiling activity of DNA gyrase in my preparation and that this relaxing activity is more sensitive to drug than the supercoiling activity. At concentrations as high as the MBC, topo II' is inhibited enough to allow the supercoiling activity of DNA gyrase to be detected.

There may be contaminating proteases in my preparation that cleaves the B subunit into a v-like subunit or the B subunit may be less stable than the v-like subunit perhaps due to improper renaturation after urea treatment. However the B subunit is cleaved, it appears that it is

cleaved in the portion of the protein that contains the ATPase activity leaving the portion of the protein that interacts with the A subunit intact. If it could no longer interact with the A subunit, then the DNA cleavage reaction most likely would not be observed since the A subunit of E. coli DNA gyrase does not cleave DNA in the absence of the B or v subunit and site specific cleavage is observed in my preparation (Figures 20, 22, and 35).

The activities of E. coli and P. aeruginosa DNA gyrase are also similar. P. aeruginosa DNA gyrase supercoil relaxed closed circular DNA in an ATP-dependent reaction (Figures 19 and 21). It relaxes negatively supercoiled DNA in the absence but not in the presence of ATP (Figure 24). Also, incubating the enzyme with DNA and norfloxacin, nalidixic acid, or oxolinic acid followed by incubation in the presence of SDS, results in cleavage of DNA (Figure 35, lanes 3, 5, and 6, respectively). Cleavage occurs in the presence and absence of ATP (lane 7) and AppNp (a nonhydrolyzable analog of ATP, lane 8), and does not occur if pyridonecarboxylic acid is absent or if novobiocin (a B subunit inhibitor) is used instead (lane 10). P. aeruginosa DNA gyrase also catentates closed circular DNA in the presence of spermidine and ATP (Figure 34). P. aeruginosa DNA gyrase is different from E. coli DNA gyrase in that it has no detectable ATPase activity. This may not be real however since the supercoiling activity is very unstable and E. coli DNA gyrase isolated using the same procedure has similar problems with supercoiling in my system (Figure 18). In fact, the two DNA gyrases may not be different at all.

Since pyridonecarboxylic acids are inhibitors of the A subunit of DNA gyrase (10,22,24) and inhibition of P. aeruginosa DNA gyrase

catalyzed supercoiling by nalidixic acid and novobiocin has already been shown (56), the relaxation activity was examined in detail. The K_m for relaxation is $1.1 \pm 0.5 \times 10^{-9}$ Molar (M). This relaxation activity was inhibited by pyridonecarboxylic acids, but not novobiocin (Figure 17). The more potent derivative, norfloxacin, has a smaller K_i (2.0×10^{-3} M) than the less potent oxolinic acid ($K_i = 3.7 \times 10^{-3}$ M). Nalidixic acid, which is the least potent, has the largest K_i which is 10.8×10^{-3} M. This indicates that the mechanism of action of pyridonecarboxylic acids involves their inhibition of DNA gyrase, since the more potent derivatives inhibit DNA gyrase to a greater extent. Also, the more potent inhibitors of the supercoiling reaction have a higher affinity for DNA (70).

The five fold difference in K_i 's does not completely explain the 100 fold difference in the MIC's and MBC's. The greater inhibition of protein synthesis by nalidixic acid over norfloxacin may also contribute to the lower potency of nalidixic acid as compared to norfloxacin because inhibition of protein synthesis overcomes the bactericidal activity of these drugs. The difference in MIC's and MBC's may also be due to different efficiencies of transportation into the cell, differences in metabolism of the drugs, and possible toxic effects of the metabolic products. No matter how these drugs inhibit DNA gyrase, either indirectly by binding to DNA or by inhibiting DNA gyrase directly, the more potent inhibitors have a greater bactericidal activity.

MECHANISM OF INHIBITION OF DNA GYRASE

Pyridonecarboxylic acids act by binding to DNA (70). Therefore, by increasing the concentration of DNA for a certain drug concentration at a set volume, the ratio of drug bound per unit length of DNA will be decreased. This will result in a lesser extent of inhibition of subunit A activity at the higher substrate (DNA) concentrations. As can be seen in Figures 27, 28, and 29, the relaxation reactions carried out at higher substrate concentrations have greater velocities (less inhibition, smaller $1/v$ values) for the same concentrations of pyridonecarboxylic acids.

In order to understand the mechanism of inhibition of DNA gyrase by pyridonecarboxylic acids, the question of whether or not the drug ever comes in actual physical contact with the enzyme must first be answered. In other words, do these drugs inhibit DNA gyrase indirectly by interacting with DNA or directly by forming a complex with DNA gyrase and DNA? The binding experiments of Shen et al. (64) indicate that the drug does not bind directly to E. coli DNA gyrase. However, cleavage of DNA by DNA gyrase in the presence of SDS and drug, but not in the absence of drug, suggests that the drug does eventually come in physical contact with the enzyme. The drug most likely traps a reaction intermediate in which the enzyme is covalently attached to the DNA. Also, oxolinic acid inhibits the specific binding of DNA gyrase to ori, but does not inhibit the arbitrary binding spread throughout the rest of the DNA molecule (50). The data mentioned above suggest that the inhibition of DNA gyrase by pyridonecarboxylic acids results from the

initial binding of drug to DNA followed by physical contact of DNA gyrase with the drug-DNA complex.

Since pyridonecarboxylic acids bind to the substrate (DNA) and not the enzyme (DNA gyrase), it is unlikely that these drugs exert noncompetitive or mixed inhibition on the relaxation reaction, because noncompetitive inhibitors interfere with the catalytic properties of the enzyme while having no effect on the binding of the substrate, and mixed inhibitors bind both to the free enzyme and the enzyme substrate complex. Also, the inhibition exerted by noncompetitive inhibitors is independent of the ratio of inhibitor concentration to substrate concentration and increasing the substrate concentration causes a decrease in the extent of inhibition for a given pyridonecarboxylic acid concentration (Figures 27, 28, and 29). Pyridonecarboxylic acids may not exert uncompetitive inhibition on the relaxation reaction, because uncompetitive inhibitors bind only to the enzyme-substrate complex, and not to the free enzyme. Since pyridonecarboxylic acids bind to substrate in the absence of enzyme, their effects (if any) on the association of enzyme and substrate would not be considered in uncompetitive inhibition. The most likely type of inhibition exerted by these drugs would be the competitive type, because competitive inhibitors exert their effect before the association of the enzyme and substrate. Also, in competitive inhibition, the degree of inhibition depends on the ratio of inhibitor concentration to substrate concentration and, as mentioned previously, decreasing this ratio causes a decrease in the extent of inhibition.

I propose three models for the inhibition of DNA gyrase by pyridonecarboxylic acids. In the first model, DNA gyrase may supercoil

or relax DNA at any site where it is bound, and the drug will prevent this "twisting" whenever it is in contact with the enzyme (Figure 37A). This inhibition would be uncompetitive and would only occur if drug-bound DNA sites had no effect on the affinity and pattern of binding of DNA gyrase to DNA. Alternatively, DNA gyrase may only be able to supercoil or relax specific "twistable" sites on the DNA, and this is the basis of the second and third models. The site specific cleavage of DNA by DNA gyrase and the discovery of a full binding mode and an incomplete binding mode of DNA gyrase to DNA (34A) supports this assumption (22,59,74). In the absence of drug, DNA gyrase may translocate along the DNA molecule until it reaches a site that is twistable. In the second model, drug-bound sites on the DNA may block this translocation and prevent the enzyme from reaching a twistable site (Figure 37B). In the third model, DNA gyrase may have a higher affinity for drug-bound sites than it does for twistable sites. The presence of enough drug bound sites will prevent the binding of DNA gyrase to twistable sites (Figure 37C). In the second and third models, pyridonecarboxylic acids would effect the association of enzyme and substrate; thus, the inhibition would be competitive.

Cornish-Bowden plots of the inhibition of the relaxation reaction by pyridonecarboxylic acids indicate competitive inhibition at high substrate concentrations and uncompetitive inhibition at low. These results are in agreement with the third model. At high substrate concentrations, there will be less drug bound per unit length of DNA. Thus, not all of the twistable sites will be bound by drug and drug-bound DNA sites will compete with unbound twistable sites for DNA gyrase. A DNA gyrase molecule which is trapped at a drug-bound DNA site

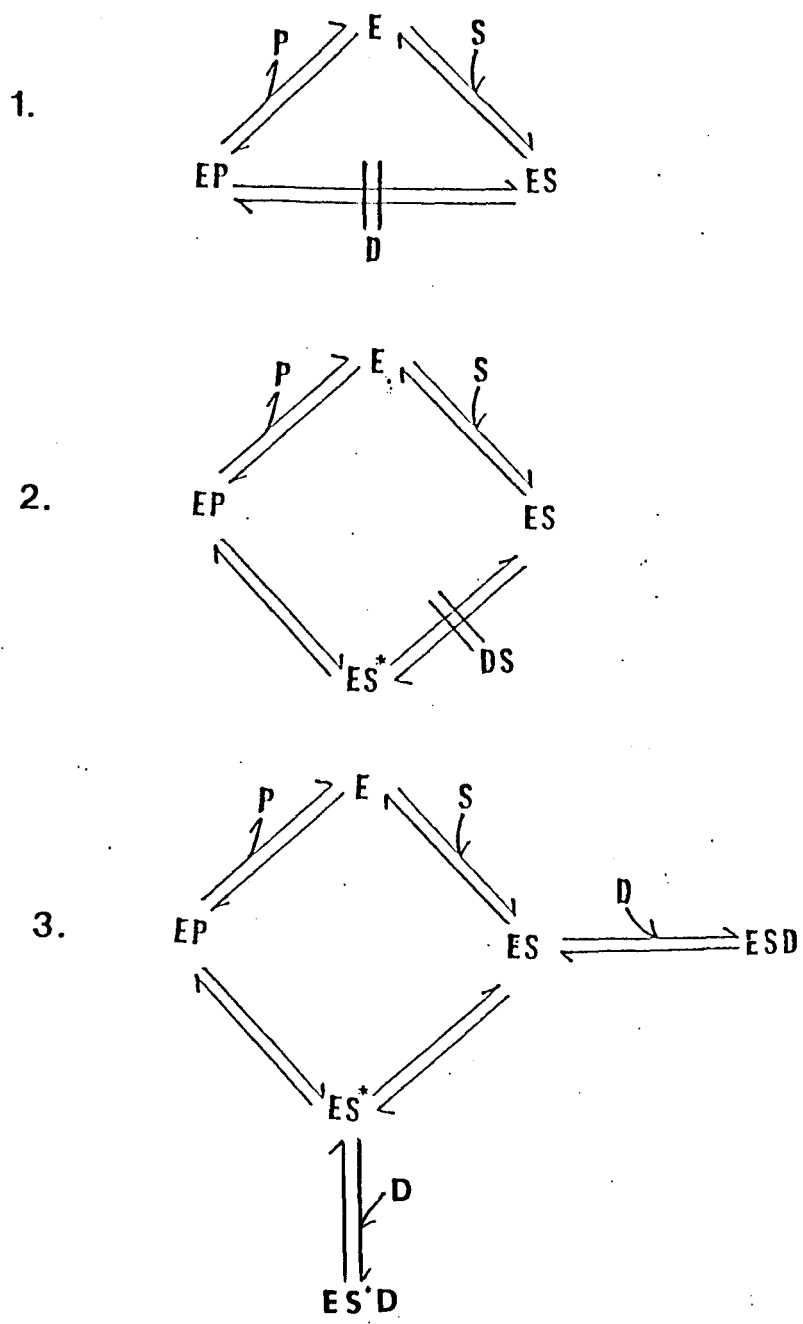


Figure 37. Three Models of Pyridonecarboxylic Acid Inhibition of the Pseudomonas aeruginosa DNA Gyrase Relaxation Reaction.

E = Pseudomonas aeruginosa DNA gyrase, S = supercoiled DNA, S* = "twistable site," D = pyridonecarboxylic acid. Double lines across the arrows represent an inhibition of the reaction.

which is not a twistable site is essentially inactivated. If one considers the drug-bound DNA and not the drug alone as the inhibitor, this type of inhibition is competitive. At low substrate concentrations, more drug will be bound per unit of length of DNA. At the substrate concentration when all of the twistable sites are bound by drug, the inhibition becomes uncompetitive. In uncompetitive inhibition, the inhibitor, which in this case is considered as the drug alone, binds to the enzyme substrate complex and not the free enzyme.

In summary, the model for the inhibition of the DNA gyrase catalyzed relaxation reaction by pyridonecarboxylic acids is as follows. This drug will bind to DNA forming a drug-DNA complex that competes with unbound twistable DNA sites for DNA gyrase, thus resulting in a competitive type inhibition. As the drug concentration is increased (or the substrate concentration decreased), all of the twistable DNA sites will be bound by drug and the inhibition will become uncompetitive.

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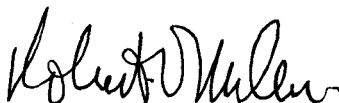
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The final copies have been examined by the director of the dissertation and the signature which appears below verified the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

31 March 1986

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