ANTIBIOTIC MECHANISMS

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Penicillin was first shown to inhibit bacterial growth some 40 years ago. Following its subsequent success in bacterial chemotherapy, massive searches turned up hundreds of natural products, mostly of microbial origin, that also inhibit bacterial growth. The vast majority of these antibiotics do not possess the requisite selective toxicity that will permit their use in chemotherapy. The small number that do possess the properties essential to widespread clinical use was sufficient, however, to usher in a new era of medicine.

Even though the first efforts were necessarily directed at the practical goals of using antibiotics to combat infections, attention was also directed toward the mechanisms whereby antibiotics exerted their selective toxicity. Spurred partly by the desire to understand better the selective toxicity of antibiotics, and partly by practical considerations such as how to design even better antibiotics, studies began early to elucidate the molecular mechanisms of antibiotic action. The understanding of cellular biochemistry was too limited to place the action of any antibiotic at the molecular level and it was apparent early that antibiotics could serve as powerful tools in elucidating metabolic pathways and reactions. Not only the clinically useful antibiotics but also those that were too toxic for clinical use found widespread use in fundamental cellular research. Progress in the areas of bacterial cell wall structure and function, membrane structure and function, ribosome structure and function, and synthesis of nucleic acid and protein has depended greatly upon the use of antibiotics as tools. The process continues and we can expect even more areas of cellular physiology to be illuminated through the use of antibiotics.

There have been periodic reviews of antibiotic mechanisms (1–7) as well as symposia (8, 9), and a book has been devoted to the subject (10). Yearly conferences on antibiotics are sponsored by the American Society for Microbiology and the proceedings are published under the title, *Antimicrobial Agents and Chemotherapy*.

In the space allotted, the intended purpose of this review is a consideration of the work that has allowed the action of many antibiotics to be placed on the molecular level, either in terms of specific proteins or other

cellular structures, or in more general terms such as the area of primary action. Much remains to be done. Because of space limitations, structural formulas of antibiotics, which can be found in Gottlieb & Shaw (10), will be omitted, as will consideration of the polyene macrolides which were recently discussed in these Reviews (11). Additional information on antibiotics covered in this review, as well as other antibiotics not covered, may be found in Gottlieb & Shaw (10).

Antibiotics Affecting DNA Synthesis

Mitomycins.—The mitomycins and porfiromycins are a group of related antibiotics (12). Although they are actively bactericidal, they are such powerful cytotoxic agents that their use in bacterial chemotherapy is precluded. They have been intensively investigated as anti-tumor agents, especially in Japan. Although all evidence is not yet in, it would appear that mitomycins will have very limited use in anti-tumor therapy because of their general cytotoxic effects (13).

The first mitomycins were shown to inhibit selectively DNA synthesis shortly after their discovery (14). Other biological effects of the mitomycins were mutagenic action, induction of lysogenic phages, breakdown of DNA, and chromosomal fragmentation (see 12). Szybalski and co-workers have provided a rational explanation for these effects by demonstrating that the mitomycins are bifunctional alkylating agents of DNA (15–17). In vivo the mitomycins form covalent bonds between complementary strands of DNA. The cross-linking could be demonstrated by the behavior of DNA, extracted from mitomycin-treated cells, in cesium sulfate equilibrium density gradient centrifugation and in thermal denaturation experiments. In vitro, mitomycins do not react with DNA unless they are activated by reducing agents such as sodium borohydride. In vivo activation requires NADPH. Mitomycin not only alkylates DNA in vitro but also RNA, proteins, and carbohydrates (18). Guanine was alkylated to a greater extent than the other bases in nucleic acid. From transfer-RNA that had been alkylated with labeled mitomycin, two products were obtained and were tentatively identified as monoguanyl mitomycin and diguanyl mitomycin (19). Isolation of quantities of the alkylated products sufficient for rigorous chemical characterization has not yet been achieved. The exact positions on either the DNA or the mitomycin that are involved in bond formation are still unknown.

Szybalski (12) considers that the primary mode of action of the mitomycins can be explained in terms of their alkylation of DNA, whether it be mono or dialkylation. Alkylation of other cellular components such as carbohydrates or proteins may also occur and may contribute to secondary effects. Cessation of DNA synthesis, which is the lethal event, results from cross-linking of the DNA by dialkylation. Separation of complementary strands in replication is then impossible. Breakdown of DNA is probably related to the excision and repair process, and fragmentation of chromo-

somes is in turn the result of DNA breakdown. Mutagenic effects are the result of alkylation of DNA and induction of phages is related to cessation of DNA synthesis.

Antibiotics Affecting RNA Synthesis

Actinomycins.—Many members of this class of antibiotics have been produced by means of fermentation (20) and there is a certain amount of confusion in actinomycin nomenclature (20, 21). Chemically, all the actinomycins contain a 2-amino-4,5-dimethyl-phenoxazin-3-one-1,8-dicarboxylate chromophore to which are attached two neutral cyclic pentapeptides. The sole differences between the natural actinomycins are the amino acids that make up the cyclic peptides. The structure of actinomycin C_1 (= D = X_1 = I_1 = IV) has been confirmed by total synthesis (22). Although the use of actinomycins in bacterial chemotherapy is precluded because of their cytotoxicity, they have found limited use in tumor therapy (23).

Slotnick (24) found that inducible enzyme synthesis was prevented by actinomycin and that both RNA and protein synthesis ceased after the addition of the antibiotic. Kirk (25) noted that in *Staphylococcus aureus* the cessation of RNA synthesis was immediate and that protein synthesis ceased after a lag period. Kirk also observed that the action of actinomycin was antagonized by DNA and that, in solution, the DNA and actinomycin formed a complex. In mammalian cells actinomycin selectively inhibited RNA synthesis while DNA and protein synthesis was less affected (26). The replication of an RNA virus (Mengo) was not impaired by actinomycin whereas the replication of a DNA virus (vaccinia) was. With in vitro systems the synthesis of RNA that was directed by DNA was shown to be inhibited by actinomycin (27–29). The inhibitory action could be reversed by the addition of DNA but not by the addition of the polymerase (28, 29), thereby confining the activity of actinomycin to the DNA template rather than the polymerase itself. DNA-directed DNA polymerase was also inhibited by actinomycin but at much higher concentrations than were required for inhibition of DNA-directed RNA polymerase (28). As a result of its selective action against the synthesis of DNA-directed RNA, actinomycin has been widely used as a test of whether transcription of DNA is required in various metabolic reactions, and also to block transcription while other reactions are being studied.

The binding of actinomycin to DNA observed by Kirk (25) and Kersten (30) was obviously related to its mode of action, and considerable study has been devoted to delineating the nature of the binding. The complexes that actinomycin forms with DNA are stable to electrophoresis, dialysis, ultracentrifugation, and gel filtration chromatography. Studies with naturally occurring DNA as well as synthetic DNA duplexes provided information that deoxyguanosine residues were essential for the binding of actinomycin (29). The 2-amino group of deoxyguanosine appeared necessary for binding as did the 2-amino group of the phenoxazone chromophore. A synthetic

deoxynucleotide copolymer of 2,6-diaminopurine and thymine also binds actinomycin, whereas purines lacking a 2-amino group do not (31). In fact, natural DNA and synthetic polydeoxynucleotides that contain deoxyguanosine do bind actinomycin with the greatest efficiency and affinity (32). However, the presence of deoxyguanosine is neither a necessary nor a sufficient condition for binding actinomycin as evidenced by the fact that poly dI binds actinomycin almost as tightly as does salmon sperm DNA (32). Also, the double stranded poly d (A-T-C)· poly d (G-A-T) does not bind actinomycin at all, although the sequence isomer poly d (T-A-C) · poly d (G-T-A) binds actinomycin firmly (33).

The fact that the actinomycins have a planar phenoxazone chromophore suggested that the action of the actinomycins might be analogous to the action of the acridine dyes. The intercalation model of Lerman (34) is widely accepted as providing the most plausible explanation of the mutagenic action of the acridine dyes. Early evidence appeared to exclude intercalation as the mode of binding of actinomycin to DNA (35–37). A model was proposed in which actinomycin was visualized as binding in the minor groove of the helical DNA structure (35). Hydrogen bonding functions were assigned to the 2-amino group of actinomycin and the 2-amino group of deoxyguanosine. The inhibition of DNA-directed RNA polymerase, but not DNA-directed DNA polymerase, was explained on the basis that the RNA polymerase operated in the minor groove and was sterically hindered in its action. Further details concerning this model may be found in recent reviews (38, 39).

The second model is the one that involves intercalation of the phenoxazone chromophore between the base pairs of the DNA helix. Evidence for this model has been provided by spectroscopic, hydrodynamic, and kinetic measurements (40) and by the observation that actinomycin promotes unwinding of the super coils of the double stranded replicative form of ϕ X174 DNA (41) and cyclic λ DNA (42). The most recent evidence for intercalation is the X-ray crystallographic examination of an actinomycin-deoxyguanosine (1:2) crystalline complex (43). A combination of π-complex formation between deoxyguanosine and the chromophore, hydrogen bonding, and hydrophobic interactions stabilizes the complex.

The reason for the selective inhibition of DNA-direct RNA-polymerase by the intercalation model is not completely clear but an answer is suggested by Müller & Crothers (40). The cyclic peptides, after intercalation of the chromophore, undergo conformational changes that make dissociation of the DNA-actinomycin complex very slow. Derivatives of actinomycin in which the peptide chains are removed bind to and dissociate from DNA very rapidly. These compounds are biologically inactive. DNA-directed DNA polymerase can force dissociation of the actinomycin-DNA complex by virtue of the driving force of chain separation in semiconservative replication. DNA-directed RNA polymerase has no such driving force and is unable to force dissociation of the complex. Transcription

would cease due to steric hindrance of the polymerase by the complex. The intercalation model is on a sounder experimental basis than the simple external hydrogen-bonded one and it seems likely to become the accepted model.

Rifamycins.—The original rifamycins proved to be quite unstable, and most studies have been carried out with stable semi-synthetic rifamycin derivatives, particularly rifamycin SV (44) and rifampicin (45). Rifampicin and other semi-synthetic derivatives will be considered together as rifamycins. They have proven to be clinically useful, especially against tuberculosis (46).

Early studies (47, 48) indicated an inhibitory effect of rifamycin on the synthesis of both protein and nucleic acid. Hartmann et al (49) showed that the inhibition of nucleic acid synthesis was the primary effect of rifamycin. The incorporation of ^{14}C-uracil into *Staphylococcus aureus* was inhibited but the poly U directed synthesis of polyphenylalanine by *Escherichia coli* enzymes was unaffected by rifamycin. Rifamycin inhibited the DNA-directed synthesis of RNA whether the template was calf thymus DNA or synthetic polydeoxynucleotides and, in contrast to actinomycin, inhibition was independent of the base composition of the DNA template. DNA-directed DNA synthesis was not inhibited by rifamycin. Also, in contrast to actinomycin, the inhibitory effect of rifamycin could be diminished by addition of increasing amounts of RNA polymerase (50), indicating that the polymerase itself, rather than the DNA template, was the site of action. If rifamycin were added after polycondensation of nucleotides was initiated there was no immediate cessation of RNA synthesis (51), whereas if rifamycin were added to the mixture prior to the addition of the DNA template there was marked inhibition of RNA synthesis. Wehrli et al (50) made the important observation that whereas rifamycin will inhibit the DNA-directed RNA polymerase from *E. coli,* it has only minimal effect on the same enzyme from rat liver nuclei. On the other hand the DNA-directed RNA polymerase from rat liver mitochondria is inhibited by rifamycin (52).

Evidence has been obtained that rifamycins interact directly with DNA-directed RNA polymerase (53–55). The RNA polymerase from a rifamycin-sensitive mutant formed a complex with rifamycin that was stable to both gel filtration chromatography and sucrose gradient centrifugation (53). The extent of inhibition of RNA polymerse by rifamycin was directly proportional to the amount of complex formed. The corresponding enzyme from a rifamycin-resistant mutant failed to form complexes with rifamycin. Rifamycin reacts only with the free polymerase and once the binary complex of RNA and polymerase is formed the effect of rifamycin is minimized (55), and inhibition does not occur until transcription of that segment of DNA is complete. Sigma factor, a site recognition protein for bacterial DNA-directed RNA synthesis (56), is not involved in rifamycin action (54, 55).

Of considerable interest are the recent studies showing the presence of a RNA-directed DNA polymerase in oncogenic RNA viruses (57, 58). This enzyme, purified from acute leukemic lymphoblasts, was inhibited slightly by high concentrations of rifamycin but was markedly inhibited by N-demethylrifampicin (59).

Antibiotics Affecting Protein Synthesis

Translation is the process whereby the information coded in mRNA is converted into protein. This process occurs on the ribonucleoprotein particles, the ribosomes. The mechanisms are as yet imperfectly understood but the broad outlines are now clear. A discussion of protein synthesis is beyond the scope of this exercise and information may be found in current articles (60–62).

In general, however, the functional unit of protein synthesis is the 70S ribosome, which consists of 2 subunits: 30S and 50S. The mRNA attaches to the 30S subunit, which consists of a 16S RNA molecule and approximately 20 proteins. The anticodon of aminoacyl-tRNA is matched to the codon on the mRNA on the 30S subunit. The 50S subunit consists of 2 RNA molecules, 23S and 5S, and at least 34 proteins. The aminoacyl end of tRNA is bound to the 50S subunit where peptide bond formation occurs. The peptide transferase, the bond forming enzyme(s), is one (or more) of the proteins making up the 50S subunit.

The process of protein synthesis may be divided into 3 stages: initiation, elongation, and termination. In the initiation stage, a 30S subunit combines with mRNA and fmet-tRNA to form an initial complex. A 50S subunit then is added to complete the 70S ribosome. Various initiation factors and GTP are involved in the process. The fmet-tRNA is initially bound in what is referred to as the A site (aminoacyl site). In a process that is not understood, GTP is hydrolyzed and the fmet-tRNA is translocated to the P site (peptidyl site). In the elongation stage, the aminoacyl-tRNA called for by the next codon on the mRNA is bound in the empty A site. The fmet is transferred to the amino group of the amino acid on the tRNA occupying the A site. Again translocation occurs, with the fmet aminoacyl-tRNA being placed in the P site, displacing the discharged methionyl-tRNA. The empty A site is now ready to receive the next aminoacyl-tRNA. The process is repeated until the message is read and the protein completed. Various elongation factors in bacteria are T_s (S_1), G (S_2) and Tu (S_3). The T factors are concerned with binding the aminoacyl-tRNA to the ribosome with GTP being split to GDP and P_i in the process. The G factor is concerned with the process of translocation after peptide bond formation has occurred, and GTP is hydrolyzed to GDP and P_i in this process. Termination of the peptide chain occurs when a terminating codon is reached and the completed chain is released from the ribosome. Various termination factors are involved in the release of the completed protein. The 50S and 30S subunits dissociate and join a pool of free subunits before recombining with a new messenger (see also 63).

Many of the clinically useful antibiotics have their primary mode of action at some stage of protein synthesis. Several of these interact directly with the ribosomes, and reviews have been devoted to this subject (64–66).

Puromycin.—Puromycin is a nucleoside antibiotic containing N,N-dimethyladenine, 3′-amino-3′-deoxyribose, and O-methyltyrosine. Information on puromycin and other nucleoside antibiotics may be found in the book by Suhadolnik (67).

Yarmolinsky & De La Haba (68) observed that puromycin was a structural analog of the amino acid bearing adenosyl end of tRNA and showed that, with in vitro systems, puromycin inhibited the incorporation of labeled amino acids into trichloroacetic insoluble material via a mechanism that was not clear at the time. The 2′-structural isomer of puromycin was not inhibitory to protein synthesis (69), indicating that the amino acid was esterified to the 3′-hydroxyl of the terminal adenosine of tRNA. Allen & Zamecnik (70), using a hemoglobin synthesizing rabbit reticulocyte system, found that puromycin caused the premature release of peptides from ribosomes and that ^{14}C-puromycin, labeled in the O-methyltyrosine moiety, was covalently bonded to growing peptide chains in a ratio of approximately one labeled molecule per amino terminal valine. Nathans (71) showed that the entire puromycin molecule was being incorporated into the growing chain at the COOH-terminal end, thereby terminating the peptide. Subsequent to these basic discoveries there have been a number of papers devoted to defining the puromycin reaction (72–76) which has become a powerful model of peptide bond formation. Model building studies indicate that puromycin interacts directly with the terminus of the peptidyl-tRNA rather than by displacing the terminus of the aminoacyl-tRNA (77).

Chloramphenicol.—The early history of chloramphenicol has been reviewed by Brock (78). More recent reviews are those of Vasquez (79) and Hahn (80). Because of its relatively simple structure, chemical synthesis replaced fermentation as a means of producing this clinically useful antibiotic. The synthetic procedures allowed great latitude in the synthesis of closely related compounds that were prepared in efforts to elucidate structure-activity relationships of chloramphenicol. Of several thousand compounds synthesized none proved to have more activity than the natural D (−) *threo* isomer (81).

Gale & Folkes (82) were the first to show that chloramphenicol inhibited protein synthesis in bacteria, without a concomitant inhibition of either DNA or RNA synthesis. In fact, there was an apparent increase in the synthesis of RNA. Much effort has been expended in efforts to elucidate the nature of the RNA that accumulates in the presence of chloramphenicol (see 79). The current view is that the accumulated RNA represents ribosomal precursors of normal ribosomes and perhaps some mRNA (83). In neither case, rRNA or mRNA, does the accumulation represent a primary action of chloramphenicol.

The primary effect of chloramphenicol is the inhibition of protein synthesis. Bacterial cell-free amino acid incorporating systems are inhibited by chloramphenicol at some stage beyond amino acid activation (84). Rendi & Ochoa (85) confirmed that inhibition of ^{14}C-amino acid incorporation into ribosomal bound label was specific for the D(−)*threo* isomer of chloramphenicol and that a similar system derived from rat liver was essentially unaffected by the antibiotic. Early evidence with cell-free ribosomal systems was interpreted to mean that chloramphenicol inhibited the attachment of mRNA to ribosomes (85–87), but as studies progressed this possibility was eliminated (88–90).

^{14}C-chloramphenicol is bound to the intact 70S ribosome and to the separate 50S subunit but to no other component of the bacterial cell (91). Approximately one chloramphenicol molecule is bound per ribosome (90, 92). The high specificity of binding to the 50S subunit focused attention on the reactions of protein synthesis that have been assigned to this subunit. The possibilities of interference with chain initiation or termination were ruled out (89, 90), but chloramphenicol inhibited the puromycin reaction (93, 94), thus implicating the elongation reaction and possibly the peptide transferase itself.

Present methodology does not permit a direct solution to the question of whether the peptide transferase is the actual site of inhibition of chloramphenicol, and the problem has been attacked obliquely as understanding of the ribosomal structure unfolds. Pestka (95) investigated the binding of the terminal oligonucleotide of ^{14}C-phenylalanine-tRNA which had been digested with ribonuclease T_1. This fragment binds to 70S ribosomes without requiring mRNA. The binding of the oligonucleotide bearing phenylalanine on its adenosyl terminus is markedly inhibited by chloramphenicol. The overall binding of phenylalanyl-tRNA is not affected. Pestka suggests that chloramphenicol interferes with the binding of the aminoacyl end of tRNA and terminates protein synthesis at that position. Yukioka & Morisawa (96) found that both GTP and G factor overcome the inhibition of polyphenylalanine syntheses by chloramphenicol in a competitive manner. The significance of this observation is not yet clear.

Streptomycin and aminoglycosides.—Several clinically useful, basic antibiotics containing glycosidic bonds have been loosely termed aminoglycosides. Included in this category are streptomycin, gentamycin, kanamycin, and the neomycins. Inasmuch as most mechanisms studies have been with streptomycin it will be used as a prototype of all the others.

Streptomycin is bactericidal to a wide variety of bacteria and it has found its principal clinical use in the treatment of tuberculosis. Early studies on the mechanism of action of streptomycin were according to the fashion of the day, and in addition to the expected development of resistance there also developed the unusual phenomena of streptomycin dependence.

Early reviews are those of Eagle & Saz (1) and Gale (3). A more recent review is that of Brock (97).

Attention was focused on inhibition of protein synthesis by the results of Erdös & Ullmann (98, 99) who found that in cell-free extracts of *Mycobacterium friburgensis* streptomycin inhibited incorporation of tyrosine into bound form. Streptomycin failed to inhibit protein synthesis in a cell-free system from a resistant strain, indicating the resistance lesion was in the protein synthesizing machinery itself. Shortly thereafter, Spotts & Stanier (100) proposed a unitary theory to account for the genetic observations that streptomycin sensitivity, resistance, and dependence all mapped at the same locus. The unitary theory proposed that there was an alteration in one specific protein, which would prove to be a ribosomal protein responsible for binding mRNA to the ribosome. Speyer et al (101) showed that the synthesis of polyphenylalanine in cell-free systems of *E. coli* was inhibited by streptomycin and the activity was confined to the ribosome. The activity was further localized in the 30S subunit of the ribosome by using hybrid ribosomes from sensitive and resistant organisms (102, 103). This subunit binds mRNA, but the attachment of mRNA to the 30S subunit was not inhibited by streptomycin (101). More recently it has been shown that one specific 30S subunit protein is responsible for streptomycin sensitivity, resistance, and dependence (104, 105). A function for this protein has not yet been determined. Curiously enough this protein is not the one responsible for binding streptomycin to the 30S subunit. At least two other proteins are involved (106). One molecule of ^{14}C-streptomycin is bound per 70S ribosome and specifically to the 30S subunit (106, 107). Even though the protein responsible for streptomycin sensitivity does not bind mRNA, the major postulate of Spotts & Stanier is correct.

A possible explanation for streptomycin action came from the observation that, in cell-free systems with synthetic homopolymers as messengers, streptomycin caused a misreading of the genetic code by inserting wrong amino acids in the resulting product of translation (108). Such misreading in vivo would flood the cell with useless proteins which might be related to the lethal action (see 109, 110). However, as basic understanding of ribosomes and cell-free systems employing synthetic polynucleotides as messengers increased, it became clear that the fidelity of reading of homopolymers could be disturbed by many agents other than aminoglycoside antibiotics (see 111). As the complexity of the synthetic polynucleotide used as messenger increased, the degree of miscoding diminished (112), giving further support to the suspicion that miscoding was an artifact of the cell-free system employed. It has been difficult to show that in vivo streptomycin causes misreading of the genetic code. Gorini (110) isolated some conditionally streptomycin-dependent auxotrophic mutants of *E. coli* that are deficient in ornithine transcarbamylase. In the presence of streptomycin they make sufficient quantities of this enzyme to survive. Gorini attributes this to an in

vivo misreading of the code by streptomycin which corrects for the defect in this one specific protein. However, if streptomycin is responsible for a general misreading of the code it is quite difficult to rationalize how these mutants manage to make the remainder of their proteins in a functional state.

Luzzatto et al (113, 114) observed in a fragile mutant of *E. coli* that streptomycin interrupted the ribosome cycle and the bulk of the ribosomes accumulated as monosomes, which are composed of 70S ribosomes and mRNA. They interpreted the accumulation of monosomes to mean the formation of aberrant initiation complexes with streptomycin in a manner that was irreversible under physiological conditions. The formation of the aberrant complexes would require the completion of initiated chains so that the subunits of the ribosome would enter the ribosome pool before recombination with mRNA to form monosomes.

Modolell & Davis (115, 116) working with a cell-free system of *E. coli,* but using a natural messenger, phage MS2 RNA, which contains the initiating codon AUG, found that streptomycin would completely inhibit polypeptide synthesis at low Mg^{2+} concentration. The termination of peptide synthesis was abrupt and complete. Over a period of time the polysomes were converted to monosomes, in agreement with the finding of Luzzatto et al. The abrupt cessation of chain elongation was not in accord with the interpretation of Luzzatto et al and, to account for the accumulation of 70S monosomes, Modolell & Davis proposed that streptomycin interacted with the A site, causing instantaneous cessation of chain elongation. There followed a slow alteration of ribosome structure so that peptidyl-tRNA was released while the 70S ribosome remained attached to the mRNA. Immediately after cessation of chain elongation, the incomplete peptide could be released rapidly by puromycin, indicating that the peptidyl-tRNA was in the P site.

The effects of streptomycin on the initial steps of mRNA translation have been carefully investigated (117, 118) using fmet-tRNA and messengers containing an AUG codon. Streptomycin did not interfere with the formation of the initiation complex. In the presence of GTP and streptomycin, fmet-tRNA is slowly released without bond formation. Puromycin rapidly releases fmet as fmet-puromycin. In the presence of the nonhydrolyzable GTP analog, GMPPCP, the initiation complex is formed but fmet-tRNA is not released by puromycin and the complex is not labelized by streptomycin.

Obviously, the exact mechanism of streptomycin cannot yet be placed on the molecular level but the site of its action has been restricted to a narrow area of the 30S subunit of the ribosome.

Tetracyclines.—This group includes tetracycline, chlortetracycline, oxytetracycline, and demethylchlortetracycline. All have found widespread clinical use as broad spectrum antibiotics. Reviews on the mechanism of action of tetracyclines are those of Franklin (119) and Laskin (120).

Even though inhibition of protein synthesis by tetracyclines was demon-

strated shortly after their discovery, a multitude of other effects was also observed and it was not possible to establish which were primary effects and which were secondary effects. Much of the early tetracycline literature is confused and citations may be found purporting to show that tetracyclines inhibit the synthesis of DNA, RNA, protein, and cell wall. Other reports placed the effect of tetracyclines as interfering with electron transport and oxidative phosphorylation and still others as interfering with vitamin K metabolism (see 119, 120).

Many of the early reports dealt with the fact that the tetracyclines are excellent chelating agents for divalent metals and the mode of action of these antibiotics was thought to be related to this property (see 1). The exact role, if any, that chelation plays in the mechanism of action of tetracyclines is still not clear.

Hash et al (121) established that any inhibition of DNA, RNA, or cell wall synthesis was secondary to the inhibition of protein synthesis. Work with cell-free systems showed that incorporation of amino acid into ribosomal bound protein was inhibited by tetracycline somewhere beyond the activation stage (122). The poly U directed synthesis of polyphenylalanine was inhibited by tetracyclines (123–125). The binding of mRNA to the ribosome was not impaired (124) whereas the binding of aminoacyl-tRNA was (124, 125). Using N-Acphe-tRNA, to exclude the possibility of peptide bond formation, it was found that the binding was inhibited approximately 50% and it was postulated that one of the two binding sites of aminoacyl-tRNA on the ribosome was blocked by tetracycline (124). ^{3}H-tetracycline was found to bind specifically to the 30S subunit of *E. coli* ribosomes (126) and it thus appeared that the tetracyclines were specifically interfering with the attachment of one of the aminoacyl-tRNAs at the site of mRNA attachment on the 30S subunit. Other binding data for ^{3}H-tetracycline indicate that the antibiotic binds to both the 30S and 50S subunits (127), and the relation of the observed binding to the mechanism of action is not clear. Magnesium ions enhance the fluorescence of tetracycline that is bound to ribosomes, indicating a role for chelation (128).

Resistance to tetracyclines has developed as it has for other antibiotics, but for the most part the resistance has been the result of alteration in permeability rather than changes in the ribosomes. Therefore it has not been possible to carry out studies analogous to those on streptomycin to localize the subunit responsible for the primary effect of tetracycline.

Tetracycline inhibits the binding of lysyl-tRNA to ribosomes but does not impair the binding of polylysyl-tRNA (129). The binding of the polylysyl-tRNA apparently occurs in the P site whereas the binding of lysyl-tRNA occurs in the A site. This finding was confirmed by Sarkar & Thach (130) who found that the binding of fmet-tRNA, in the presence of messengers containing an AUG codon, was inhibited markedly by tetracycline, either in the presence of GTP or its nonhydrolyzable analog GMPPCP. They showed that the nonenzymatic binding of phe-tRNA to ribosomes in

high Mg^{2+} was inhibited 50% in the presence of tetracycline, which was in agreement with Saurez & Nathans. The phenylalanine that is bound is completely released by puromycin. Conversely, when the phe-tRNA is bound in the absence of tetracycline and GTP only 50% of the phenylalanine is releasable by puromycin. These results establish that tetracycline is impairing the binding of aminoacyl-tRNA to the ribosomes in the A site.

Erythromycin and the macrolides.—Erythromycin is the best known member of a family of antibiotics called the macrolides. This term is applied to compounds that contain as part of their structure a macrocyclic lactone and various sugars, either amino sugars, nonnitrogeneous sugars, or both. There have been more than 40 macrolides described and some of the better known ones are oleandomycin, spiramycin, and tylosin. Erythromycin will be taken as a prototype of all the macrolides, but in such a large group of compounds, individual members may prove to have different sites of action. Information on other macrolides may be found in Gottlieb & Shaw (10).

Because of their structural complexity, the macrolides do not lend themselves readily to either synthesis or chemical modification. Studies on structure-activity relationships of macrolides have lagged behind those of other antibiotics as have studies on the mode of action of these compounds.

There were scattered reports on erythromycin action during the fifties that indicated this antibiotic inhibited protein synthesis without a concommitant inhibition of nucleic acid synthesis (see 131). In the poly U system, the synthesis of polyphenylalanine is inhibited (132, 133) beyond the synthesis of aminoacyl-tRNA (133, 134). ^{14}C- or ^{3}H-erythromycin binds to ribosomes (135, 136) and specifically to the 50S subunit (136). The 50S subunit was also implicated as the site of action from results of using hybrid ribosomes from sensitive and resistant mutants in cell-free systems (137), in studies similar to those used for streptomycin. Mao & Wiegand (134) tested 8 macrolide antibiotics and found there was no inhibition of mRNA attachment to ribosomes but there was variable inhibition of the attachment of aminoacyl-tRNA to the ribosomal complex.

Erythromycin prevents the binding of ^{14}C-chloramphenicol to the 50S subunit of the 70S ribosome (see 79), but the effects of these two antibiotics are additive, and do not act on the same common locus of the ribosome. Erythromycin-resistant mutants of *Bacillus subtilis* isolated from the erythromycin-sensitive and chloramphenicol-sensitive parent still retain their sensitivity of chloramphenicol, and in cell-free systems erythromycin does not inhibit the binding of chloramphenicol to erythromycin-resistant ribosomes (138). In the synthesis of polylysine with poly A as messenger, erythromycin prevents the formation of polylysine but small lysine di-, tri-, and tetrapeptides are synthesized (139). This phenomenon is reminiscent of the action of puromycin and its significance is not yet clear. But this property is sufficient to distinguish its action from that of chloramphenicol or

lincomycin, neither of which causes the release of those short lysine peptides (140).

The fact that short lysine peptides can be synthesized in the presence of erythromycin would seem to indicate that the peptidyl transferase itself is not inhibited. This point has been investigated by Mao & Robishaw (141). They found that di-, tri-, and, on some occasions, tetralysine accumulated in the presence of either erythromycin or oleandomycin. Spiramycin and tylosin allowed the synthesis of dilysine but not trilysine. Niddamycin and carbomycin inhibited the synthesis of all lysine peptides. Neither the G- and T-dependent hydrolysis of GTP nor the enzymatic binding of aminoacyl-tRNA was affected by the macrolides. They propose that all macrolides inhibit peptide bond synthesis. To account for lysine peptide accumulation they propose that the synthesis of early peptide bonds is less sensitive to the macrolides than the later peptide bonds. Thus, less potent macrolides allow accumulation of short peptides. The question of whether the macrolides exert their inhibitory action by virtue of binding to the peptidyl transferase or other ribosomal proteins is unresolved.

Lincomycin.—By the time that lincomycin was described in 1962, techniques had been developed to the point that it was possible to identify the general site of action without the confusion that characterized studies of antibiotics discovered earlier. With suitably labeled precursors and fractionation techniques, Josten & Allen (142) could show that, in *S. aureus* H, protein synthesis was completely inhibited in a period of time in which neither cell wall, DNA, nor RNA synthesis was affected.

Chang et al (143), in efforts to pinpoint the site of lincomycin within the translation mechanism, investigated the possibility of using hybrid ribosomes from lincomycin-sensitive and -resistant mutants to localize the ribosomal subunit in a manner analogous to that used for streptomycin. Ribosomes from lincomycin-resistant mutants proved to be sensitive to the antibiotic, indicating that the basis for resistance was due to permeability rather than to alterations in the ribosome. Use was therefore made of heterologous hybrid ribosomes by utilizing ribosomal subunits from the naturally sensitive gram-positive *Bacillus stearothermophilus* and the naturally resistant gram-negative *E. coli*. The 50S subunit proved to be the site of inhibition. The synthesis of polyphenylalanine in the poly U system was inhibited, mRNA attachment was not affected and the binding of ^{14}C-Phe-tRNA was inhibited. Like erythromycin, lincomycin inhibits the binding of chloramphenicol to the 50S subunit of the ribosome (see 79). In a comparative study of lincomycin and erythromycin, Igarashi et al (144) concluded that lincomycin inhibits the peptidyl transferase of the 50S subunit whereas erythromycin inhibited translocation from the A site to the P site.

Lincomycin, erythromycin, and chloramphenicol interact in some manner with sites on the 50S subunit so as to stop protein synthesis. The pro-

tein(s) of the 50S subunit that are responsible will have to await the separation and identification of the proteins that make up the 50S subunit.

Antibiotics Affecting Cell Wall Synthesis

A rigid cell wall is one distinct anatomical structure that is possessed by bacterial but not by mammalian cells. As such, it represents a logical target for chemotherapeutic attack and several clinically useful antibiotics owe their efficacy to interference with cell wall synthesis.

In general, the structural element of the bacterial cell wall, the murein, is a modified chitin. It consists of alternate N-acetylglucosamine and N-acetylmuramic acid (3-O-lactyl-N-acetylglucosamine) residues linked β-1,4. To the lactyl group of muramic acid is attached a tetrapeptide. There is species variation in the composition of this peptide but a diaminoacid is usually present and the peptide terminates in D-alanine. A 3-dimensional structure is obtained by cross-linking the diamino acid in one strand with the terminal D-alanine in a second strand. There is also species variation in the nature of the cross-links. In some cases the cross-links are direct and in other cases there are additional intervening amino acids or peptides.

The 3-dimensional murein gives the cell its structural integrity, and disruption of this structure with bacteriolytic enzymes or with antibiotics leads to osmotic instability and lysis. If external osmotic pressures are sufficient to balance internal osmotic pressures, the cell may survive as a protoplast or spheroplast (145). Associated with the cell wall are other supporting structures each as proteins, lipopolysaccharides, and teichoic acids. These have no structural role, and inhibition of cell wall synthesis by antibiotics is restricted to the synthesis of the murein.

The synthesis of the murein may be divided into three phases: (*A*) synthesis of the required nucleotide intermediates, namely, UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide, the pentapeptide in all cases studied terminating in D-alanyl-D-alanine, (*B*) assembly and modification of the disaccharide intermediate, N-acetylglucosaminyl-N-acetylmuramyl-pentapeptide, and its subsequent incorporation into endogenous murein, and (*C*) the cross-linking of the peptide chains by transpeptidation, with the concomitant release of D-alanine, yielding the tetrapeptide found in the completed murein. Much of the work on penicillin has been done with *S. aureus* for which the pentapeptide attached to the muramyl-nucleotide has the sequence L-ala-D-γ-glu-L-lys-D-ala-D-ala. The synthesis of the disaccharide intermediate involves lipids, and modifications at this stage include the amidation of γ-glutamyl residues and addition of other amino acids that are involved in the final cross-linking step. The addition of these amino acids requires an aminoacyl-tRNA in a rather unusual step. Reviews (146, 147) should be consulted for further details of cell wall structure and synthesis.

Cycloserine.—Cycloserine, or oxamycin, was the first antibiotic whose mechanism of action was placed at the molecular level. Its action is in inter-

ference with the first phase of cell wall synthesis, the assembly of UDP-N-acctylmuramyl-pentapcptide. In *S. aureus,* oxamycin causes the accumulation of a uridine nucleotide containing the peptide sequence L-ala-D-γ-glu-L-lys (148). The terminal D-ala-D-alanine is added to the nucleotide as the dipeptide. The dipeptide is synthesized from D-alanine, which, in turn, is obtained from L-alanine by alanine racemase. Cycloserine competitively inhibits these two reactions, the racemase and the dipeptidyl synthetase, sequentially (149). As it is true competitive inhibition, its antibacterial action may be overcome by increasing the concentration of D-alanine, and resistant mutants have been isolated that overcome the inhibition of cycloserine by increased production of D-alanine (150).

Bacitracin.—The bacitracins are cyclic polypeptides of bacterial origin. Inhibition of cell wall synthesis was implicated when these compounds were found to cause the accumulation of UDP-N-acetylmuramyl-pentapeptide (151). The bacitracins are also chelating agents but the significance of this property to antibacterial activity is not clear (see 152).

In phase two of murein biosynthesis, UDP-N-acetylmuramyl-pentapeptide reacts with a C_{55}-isoprenol phosphate (153) to liberate UMP and to yield N-acetylmuramyl-pentapeptide-lipid pyrophosphate. N-acetylglucosamine is added via UDP-N-acetylglucosamine to yield a disaccharide-lipid pyrophosphate. Murein polymerase is responsible for incorporating the completed disaccharide into an endogenous linear strand with the concomitant liberation of the C_{55}-isoprenol pyrophosphate. A pyrophosphatase releases inorganic phosphate, regenerating the isoprenol phosphate for the next cycle.

Bacitracin was found to inhibit partially the overall synthesis of linear strands of murein from UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide in cell-free particulate systems (154). Siewert & Strominger (155) were then able to show that bacitracin is a specific inhibitor of the lipid pyrophosphatase and, in the presence of bacitracin, the C_{55}-isoprenol pyrophosphate accumulates. The net effect is to deprive the cell of a necessary intermediate and to cause the cessation of cell wall synthesis. The isolation of the bacitracin-sensitive pyrophosphatase has not been achieved, and it is not yet clear whether the specificity of bacitracin is directed against the lipid pyrophosphate or the pyrophosphatase.

Vancomycin and ristocetin.—These two antibiotics will be considered together because their mode of action is similar, if not identical. While their structures have not been determined, both contain carbohydrate and phenolic moieties (156, 157).

Vancomycin and ristocetin were implicated as inhibitors of cell wall synthesis when it was found that they cause the accumulation of UDP-N-acetylmuramyl-pentapeptide in *S. aureus* (158, 159). From the specificity of inhibition it appeared that cell wall synthesis was the primary site of action

of both compounds. The reactions inhibited are those in the second phase of cell wall synthesis. Anderson et al (160), using a cell-free particulate fraction of *S. aureus* containing the murein polymerase, found the synthesis of linear murein strands from UDP-N-acetylglucosamine and UDP-N-acetylmuramyl-pentapeptide to be severely inhibited by both vancomycin and ristocetin. This finding was confirmed by Struve et al (161). The formation of the N-acetylglucosaminyl-N-acetylmuramyl-pentapeptide-lipid pyrophosphate was inhibited by neither vancomycin nor ristocetin and the evidence seemed to suggest that it was specifically the murein polymerase that was inhibited. That it was the polymerization step rather than modification reactions of the disaccharide-pentapeptide lipid intermediate was further indicated by the results of Matsuhashi et al (162), who investigated the addition of glycine from glycyl-tRNA to the staphylococcal disaccharide intermediate. At growth inhibitory concentrations, neither vancomycin nor ristocetin inhibited the addition of glycine to the disaccharide-pentapeptide-lipid intermediate, but the addition of the disaccharide unit to linear murein strands was completely inhibited. Therefore present evidence places both vancomycin and ristocetin as specific inhibitors of murein polymerase.

Perkins (163) has further delineated the nature of the inhibition by finding that vancomycin forms a stable 1:1 complex with cell wall peptides terminating in D-alanyl-D-alanine. Removal of one alanine residue or changing the configuration of either residue prevents the formation of the complex. Acetyl-D-alanyl-D-alanine was the smallest peptide that would form a complex with vancomycin. As yet there is no direct evidence that the formation of this complex is responsible for the antibacterial activity of the antibiotic, but it seems reasonable to think that it might be. Ristocetin also forms complexes with peptides that terminate in D-alanyl-D-alanine (163) but this antibiotic has not been investigated as extensively as vancomycin in this respect.

The mechanism of the inhibition of the murein polymerase by a vancomycin-disaccharide-lipid pyrophosphate complex is as yet unclear. Perhaps the D-alanyl-D-alanine portion of the intermediate has a recognition site on the polymerase. Steric hindrance by the vancomycin complex could thus prevent recognition and subsequent polymerization of the disaccharide unit into the linear murein strands.

As would be expected from complex formation of peptides terminating in D-alanyl-D-alanine, D-alanine carboxypeptidase, a penicillin sensitive enzyme from *E. coli,* is inhibited by both vancomycin and ristocetin (164). A similar D-alanine carboxypeptidase has been found in *Streptomyces albus* G (165). In contrast to the enzyme found in *E. coli* this enzyme is soluble and is excreted into the medium. It is insensitive to penicillin, as might be expected from the known insensitivity of streptomyces to penicillin. Nonetheless, the streptomyces enzyme is inhibited by both vancomycin and ristocetin. Such inhibition would be expected if the antibiotic interacted with the substrate rather than the enzyme.

Therefore, at present, both vancomycin and ristocetin may be considered to be inhibitors of murein polymerase by virtue of combining physically with the substrate for this enzyme.

Penicillins and cephalosporins.—These two groups of antibiotics will be considered together as their mode of action is the same. Both antibiotics contain a β-lactam structure fused to a heterocyclic ring. The parent compound of penicillin is 6-aminopenicillanic acid and the parent compound of cephalosporin is 7-aminocephalosporanic acid. To these parent compounds various acyl groups may be added synthetically, giving rise to vast numbers of semi-synthetic penicillins and cephalosporins. The β-lactam groups of these compounds function as acylating agents.

As the first and probably still the most clinically important antibiotic, penicillin has provided the impetus that led to sorting out the reactions involved in cell wall synthesis. Early observations (see 166) were that penicillin is more effective against gram-positive cells than gram-negative cells and that penicillin causes the production of aberrant cell forms. The first important chemical evidence for the action of penicillin was that of Park & Johnson (167) who found that uridine nucleotides accumulated in cells treated with penicillin. These uridine nucleotides proved to be precursors of the bacterial cell wall murein (168) and one of them was UDP-N-acetyl-muramyl-pentapeptide, although the identity of muramic acid was not known at the time. By 1957 the structure of muramic acid was known, and from analyses of isolated cell walls, Park & Strominger (169) were able to make the correlation between the composition of the nucleotide and the composition of the cell wall murein and could predict with confidence that penicillin interferred with cell wall biosynthesis. Visual confirmation of penicillin action was obtained by electron microscopy where the cell walls became progressively thinner in the presence of penicillin (170).

By 1965, more sophisticated analyses of cell walls revealed that whereas the Park nucleotide from *S. aureus* contained a pentapeptide with one L-alanine and two D-alanine residues, the final cell wall murein contained muramic acid to which was attached a tetrapeptide containing one L-alanine and one D-alanine residue. One D-alanine residue had been lost in the synthesis. The action of penicillin was then confined to the last phase of cell wall synthesis. Both Wise & Park (171) and Tipper & Strominger (172) established the final cross-linking reaction as the site of action of penicillin. The bond energy of the D-alanyl-D-alanine is conserved by the formation of a new bond between the carboxyl group of the penultimate D-alanine and the free amino group of either the diamino acid or the peptide bridge in an adjacent chain. The terminal D-alanine is released. In the presence of penicillin, uncross-linked murein strands accumulate. Tipper & Strominger proposed that penicillin is a structural analog of the D-alanyl-D-alanine portion of the pentapeptide (172) rather than being a structural analog of either the L-alanyl-D-glutamyl portion (171) or N-acetylmuramic acid (173). Pen-

icillin presumably covalently acylates the transpeptidase, effectively inactivating it. This transpeptidase is probably the "penicillin-binding component" which was observed years ago (174).

In the case of gram-negative cells Izaki et al (175) showed that in cell-free systems the transpeptidase of *Escherichia coli* was sensitive to the semi-synthetic penicillin, ampicillin, at the same concentration as that required to give 50% inhibition of growth. On the other hand 10 times the concentration of penicillin G was required for growth inhibition as was required for inhibition of the transpeptidase. These results appear to suggest some sort of permeability barrier between the sensitive enzyme on the cell's surface and penicillin G. A second enzyme, D-alanine carboxypeptidase, was found in *E. coli* that was also sensitive to penicillin. This enzyme removes the terminal alanine from the D-alanyl-D-alanine portion of the molecule and is inhibited by one-tenth the concentration of penicillin required to inhibit the transpeptidase. Inhibition of this enzyme does not appear to be a lethal event and its significance is yet to be assessed.

Cephalosporins also inhibit the murein transpeptidase (175), and the binding of penicillin and cephalosporin correlates well with their growth inhibitory concentrations (176).

Conclusions

The quest for understanding of how antibiotics exert their effect at the molecular level has revealed several modes of action. The mechanisms include competitive inhibition (cycloserine), covalent attachment to enzymes (penicillins and cephalosporins), covalent attachment to templates and substrates (mitomycin and puromycin), and physical binding to templates and substrates (actinomycin, vancomycin, and ristocetin). At this time, those antibiotics that inhibit the translational mechanism of protein synthesis can be placed only at the ribosomal level. Further categorizing will have to await the dissection of the ribosome.

Most antibiotics that are useful in bacterial chemotherapy have as their mode of action inhibition of either cell wall or protein synthesis. An exception is rifamycin which, as we have seen, has its action directed against RNA polymerase rather than the nucleic acid template.

The rationale for the clinical success of antibiotics that inhibit cell wall synthesis has long been understood: there are no analogous structures in mammalian cells. It has been more difficult to understand the therapeutic effectiveness of antibiotics that inhibit protein synthesis because the mechanisms of protein synthesis in bacterial and mammalian cells were presumed to be similar. Various explanations for the efficacy of these antibiotics have been advanced: permeability, growth rates of respective cells, cell volume and various concentrating mechanisms. It is now known that there are fundamental differences between bacterial and mammalian ribosomes: bacteria have 70S ribosomes and mammalian cells have 80S ribosomes. Mitochondrial and chloroplast ribosomes, also, are of the 70S variety and possibly

arose as symbionts in the past. They are inhibited by the same antibiotics that inhibit bacterial protein synthesis. Those antibiotics that have clinical utility are those whose activity is directed against 70S ribosomes. It seems quite likely that the effects of the various translation inhibitors observed in mammalian cells are due to inhibition of protein synthesis by mitochondrial ribosomes.

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