

SPECIFICITY IN THE MECHANISM OF ACTION OF ANTIBIOTIC INHIBITORS OF PROTEIN AND NUCLEIC ACID SYNTHESIS

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ABSTRACT

Antibiotics that interfere with protein synthesis have been distinguished by their effect on procaryotic or eucaryotic cells and extracts, or both; by their binding or action on the smaller or larger ribosome subunit; and by their action on the P- or A-site of the ribosomal-mRNA complex. Sparsomycin, chloramphenicol, gougerotin and ampicillin all inhibit peptide bond formation and interact with the larger ribosomal subunit. Pactamycin, streptomycin and the tetracyclines bind to the 30S ribosomal subunit from bacteria; however pactamycin also interacts with the smaller (40S) ribosomal subunit from mammalian cells. At low concentrations (10^{-6} M) pactamycin inhibits primarily initiation of protein synthesis. On the other hand fusidic acid and thiostrepton inhibit translocation and interfere with the function of the larger ribosomal subunit. Also G factor has recently been implicated in fusidic acid action.

Antibiotics interfere with nucleic acid synthesis and function either by complexing with DNA or by inactivating or competing with the other components involved in nucleic acid replication reactions. Antibiotics which complex with DNA include actinomycin, chromomycin, mithramycin, olivomycin, the anthracyclines and mitomycin. Antibiotics which directly inactivate the RNA polymerase include rifamycin, streptovaricin and streptolydigin. Another inhibitor, kanchanomycin, appears to combine features of the antibiotics which activate the DNA template and those which inactivate the RNA polymerase.

In the antibiotics, nature has provided man with exquisitely potent and specific agents that have proved useful as drugs in the treatment of disease and as tools in the study of biological processes. Some antibiotics have been employed clinically for their antitumour and immunosuppressive properties, others for their effectiveness in infections of bacterial or viral origin. Although certain antibiotics work only on procaryotes (cells lacking a nucleus, such as bacteria), others are toxic only to eucaryotes (cells with a nucleus) or to both types of organisms. This discussion will be limited to a consideration of the mechanisms of action of only a few of the antibiotics that

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interfere with protein or nucleic acid synthesis. Emphasis will be primarily on agents which have been worked on in our laboratory, but we shall also consider briefly some other antibiotics which illustrate specific types of inhibitory actions. It is expected that an understanding of their mechanisms of action will provide a rational basis for their use in clinical situations and in physiological studies. Furthermore, such information will hopefully lead to the design of new agents by man that fill his needs even better than those provided by nature.

INHIBITORS OF PROTEIN SYNTHESIS

Since protein synthesis is a multistep process involving many reactants and complex structures such as ribosomes, it is not surprising that there are antibiotics that interfere specifically with this process at one level or another. Rather than attempt to provide detailed information on the many such antibiotics that have been studied in recent years, attention will be devoted primarily to consideration of two antibiotics, sparsomycin and pactamycin, which have been worked on in this laboratory. Information on their modes of action, however, will be related to those of other antibiotics, many of which are of value clinically.

A simplified view of our state of knowledge (for a detailed summary of current information see reference 1) of the ribosomal phase of polypeptide synthesis in *Escherichia coli* is shown in *Figure 1*. This process can be divided

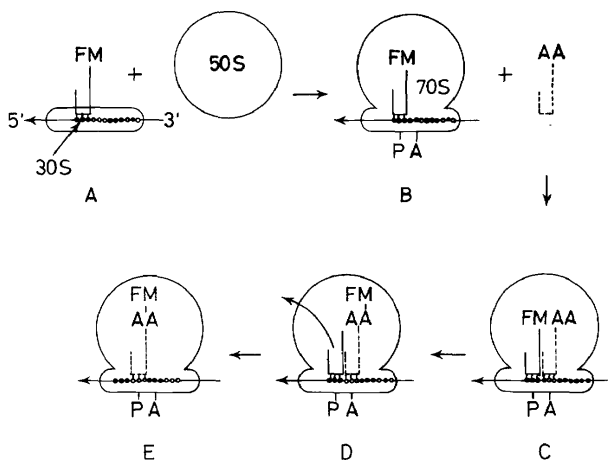


Figure 1. Schematic representation of polypeptide chain initiation and elongation on bacterial ribosomes. See text for description

into the following steps: *Initiation*: (A) addition of *N*-formylmethionyl (FM)-tRNA to the mRNA-30S ribosome subunit complex, involving protein factors and promoted by GTP. (B) addition of the 50S ribosome subunit to the complex (note that FM-tRNA is in the P (peptidyl)-site. *Reading*: (C) arrival of an amino acyl (AA)-tRNA at the A(aminoacyl) site

MECHANISM OF ACTION OF ANTIBIOTICS

(specifically dictated by the nucleotide triplet on the mRNA). *Formation of peptide bond*: (D) peptide transfer (catalysed by the peptidyl transferase located on the 50S ribosome subunit) of FM from the P-site to the aminoacyl-tRNA in the A-site. *Translocation*: (E) the tRNA of the initiator is ejected from the P-site; the dipeptidyl-tRNA (FM-AA-tRNA) is moved to the P-site and the mRNA moves relative to the ribosome to expose a new triplet in the A-site (requires G factor—transferase II in mammalian systems—and GTP is hydrolysed). The cycle can now repeat until—*Termination*: peptide is released when the mRNA terminator codon is read. It should be noted, although it is not shown in *Figure 1*, that at any one time during active protein synthesis there are several ribosomes simultaneously moving along a single mRNA molecule, forming the polyribosome.

In its essential features, this scheme is also valid for mammalian cells, except that the cytoplasmic ribosomes of mammalian cells are characterized by sedimentation coefficients of 80S and are composed of two subunits of 40S and 60S (mitochondrial ribosomes, however, have sedimentation properties like bacterial ribosomes and have similar antibiotic specificity), and that the initiator methionyl-tRNA is probably not formylated as is the bacterial one (although it is capable of being formylated)².

Antibiotics that interfere with protein synthesis have been distinguished by their effect on procaryotic or eucaryotic cells and extracts, or both; by their binding or action on the smaller or larger ribosome subunit; and by their action on the P- or A-site of the ribosome-mRNA complex. Unless otherwise specified, when mammalian protein synthesis is referred to, it is limited to that taking place in the cytoplasmic ribosomal system, not that occurring in the mitochondria. Pactamycin (*Figure 2*)³ at low concentrations

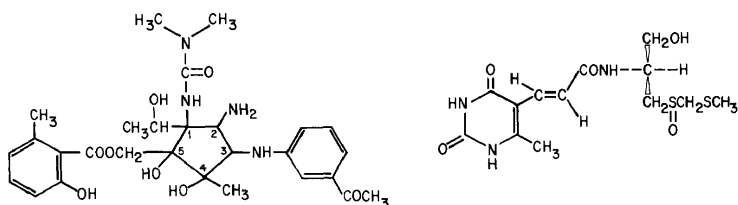


Figure 2. Structures of pactamycin (left) and sparsomycin (right)

affects primarily the initiation of polypeptide synthesis on ribosomes, whereas sparsomycin (*Figure 2*)⁴ inhibits the formation of peptide bonds in the elongation step. Both antibiotics are active on eucaryotes and procaryotes but pactamycin works on the smaller and sparsomycin on the larger ribosome subunit.

Sparsomycin and inhibitors of peptide-bond formation

There are several model systems that can be used for the study of peptide-bond formation independent of the other steps involved in polypeptide synthesis. One that has proved to be of value employs another antibiotic, puromycin, which is an analogue of the aminoacyladenine end of tRNA

(Figure 3). The nascent peptide chain in the P-site on the *Esch. coli* ribosome is transferred in a reaction (catalysed by the ribosome-bound peptidyl transferase to the amino group of the puromycin molecule⁵. This reaction, which leads to the release of the nascent peptide from the ribosomes as

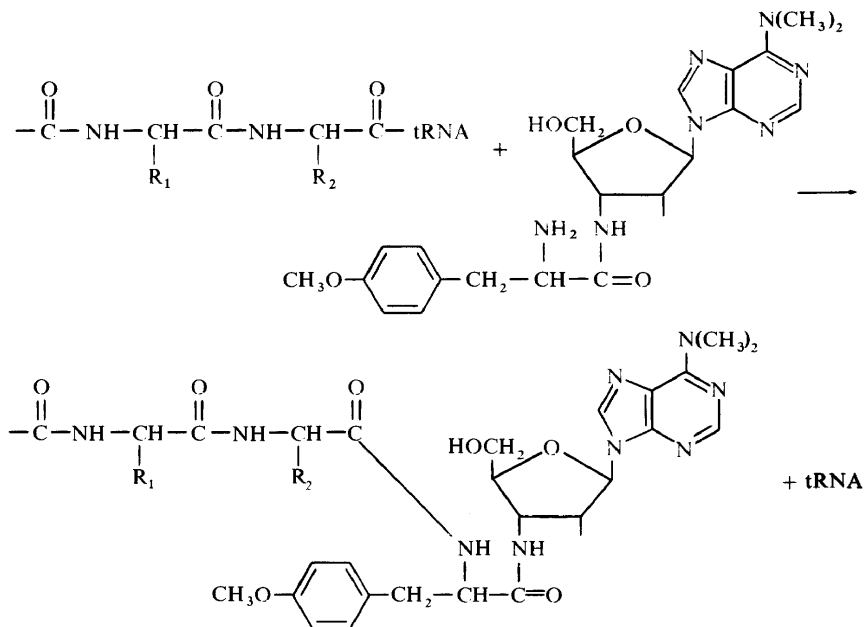


Figure 3. Mechanism of puromycin action. The structure of peptidyl-tRNA is shown in the upper left and that for puromycin in the upper right section⁵

peptidylpuromycin, is readily blocked by sparsomycin^{6,7}, which functions as a competitive inhibitor of the puromycin⁶ (Figure 4). Other antibiotics such as chloramphenicol and gougerotin, which act on peptide chain elongation, are less effective but also inhibit this reaction. Similarly, sparsomycin prevents the synthesis of dipeptide, such as *N*-acetylphenylalanyl-phenylalanine or diphenylalanine, in a ribosome system where only short peptides are formed^{8,9}. Sparsomycin interferes with the function of the peptidyl transferase and does not block the formation of the complex active in polypeptide synthesis^{10,11}. This is also shown in experiments in which isolated 50S bacterial ribosomal subunits are prevented from carrying out peptide-bond formation by sparsomycin¹². In this reaction an *N*-substituted aminoacyl or peptidyl moiety attached to a short fragment of tRNA can be transferred to puromycin in the absence of mRNA, GTP or soluble enzymes. In fact, sparsomycin appears to 'freeze' the initiator-tRNA or the peptidyl-tRNA in the P-site on the ribosome^{13,14}. As shown in Figures 5 and 6, low concentrations of sparsomycin stimulate the binding of initiator-(or peptidyl-) tRNA to ribosomes, in which form it cannot be released by puromycin¹³. This action is likely connected with another effect of sparsomycin wherein the

MECHANISM OF ACTION OF ANTIBIOTICS

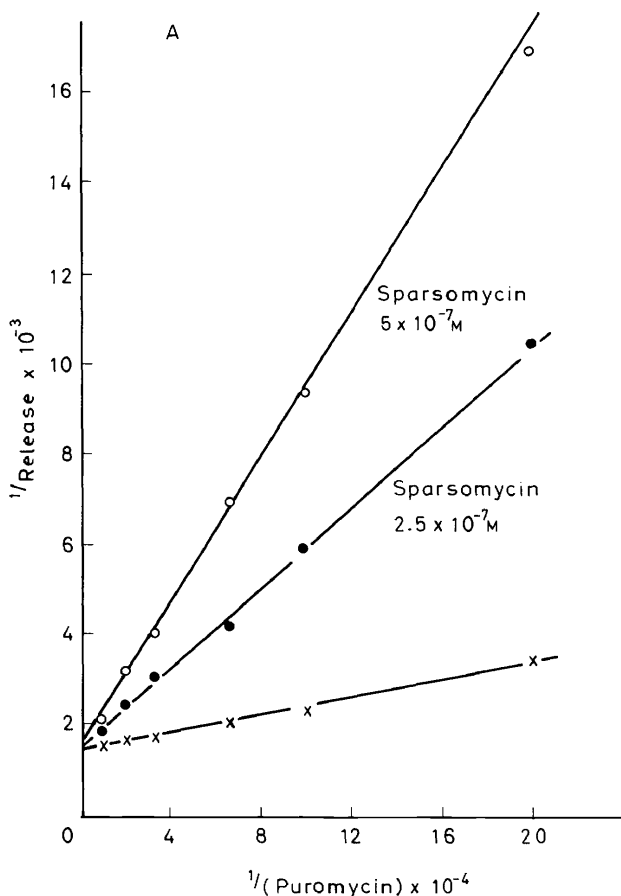


Figure 4. Double reciprocal plot of sparsomycin inhibition of the puromycin reaction⁶

aminoacyl moiety on the tRNA in the A-site on the ribosome is not able to interact properly with the peptidyl transferase¹⁵. Similar effects have been found for several other antibiotics, such as chloramphenicol, gougerotin and ampicillin. Lincomycin, streptogramin A and certain of the macrolides appear to block binding reactions at both P- and A-sites on the ribosome¹⁴⁻¹⁶; all of these agents also act on the bacterial 50S ribosome subunit.

Sparsomycin differs from chloramphenicol and the other antibiotics useful in clinical infections in being a highly effective inhibitor of mammalian as well as bacterial protein synthesis. In this respect, sparsomycin is similar to the nucleoside antibiotics, puromycin and gougerotin. Examination of its structure (Figure 2), in fact, reveals that sparsomycin possesses a pseudouridine-like moiety that is attached to a peptide-like grouping. A molecular model of this compound resembles a pyrimidine nucleoside bearing a peptidic moiety. A molecular model of pactamycin also has

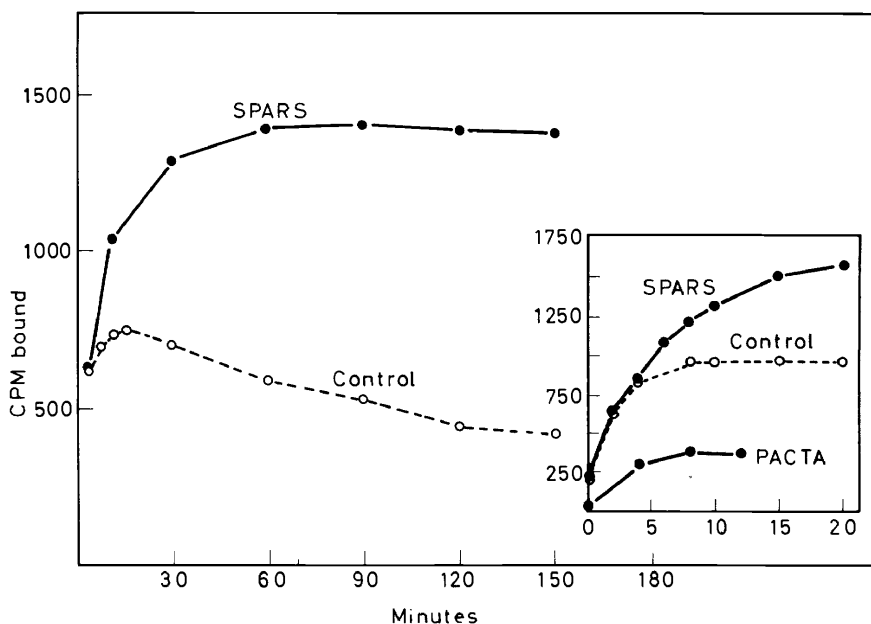


Figure 5. Sparsomycin stimulation of polyuridylylate-promoted binding of (^{14}C) *N*-acetyl-L-phenylalanyl-tRNA to ribosomes. Sparsomycin (10^{-5} M) or pactamycin (10^{-5} M) were added at the start of the reaction as indicated. See reference 13 for experimental details

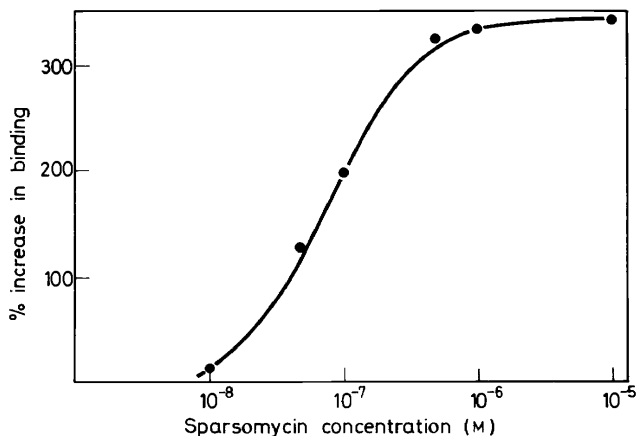


Figure 6. Relation of sparsomycin concentration to the stimulation of (^{14}C) *N*-acetyl-L-phenylalanyl-tRNA binding to ribosomes. Adapted from reference 13

MECHANISM OF ACTION OF ANTIBIOTICS

features in common with the nucleoside antibiotics. It is not surprising that antibiotics that closely resemble normal chemical intermediates in polypeptide formation should be effective in both procaryotes and eucaryotes.

Pactamycin and initiation

Pactamycin, like the aminoglycosides (for example, streptomycin) and the tetracyclines, binds to the smaller (30S) ribosomal subunit from bacteria¹⁷, but also interacts with the smaller (40S) ribosome subunit from mammalian cells¹⁸. At 0°C, pactamycin binds to the rabbit reticulocyte 40S ribosomal subunit, as well as to the 80S ribosome, but not to mRNA bearing-polyribosomes (*Figure 7A*). The binding to 80S ribosomes is presumably by way of the smaller subunit, since there is no binding to the larger subunit.

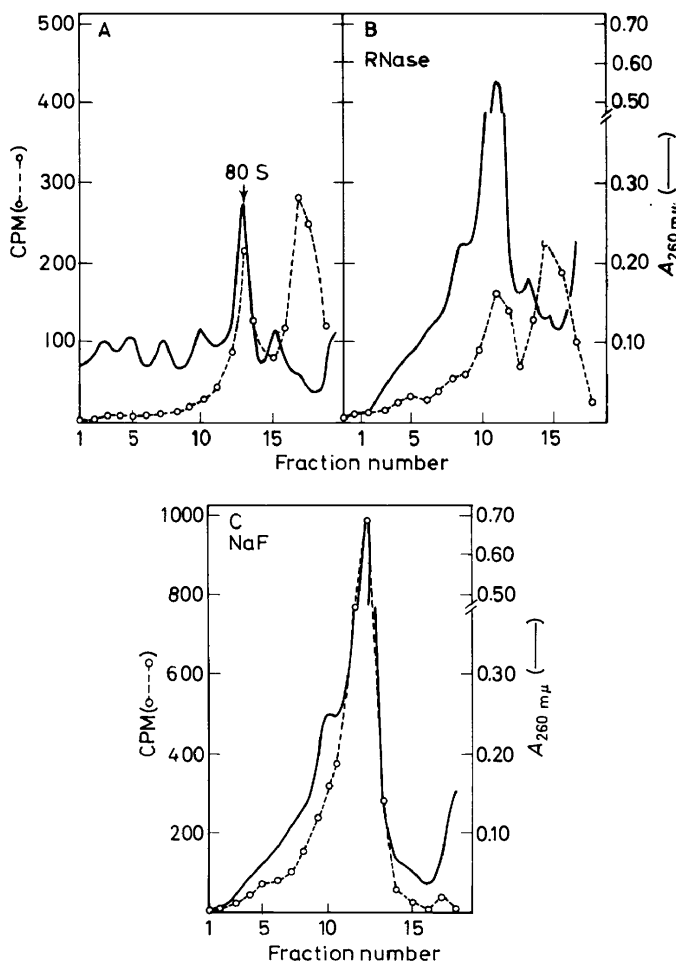


Figure 7. Binding of (³H) pactamycin to reticulocyte ribosomes as analysed by sucrose density gradient centrifugation (from right to left).¹⁸

The binding of pactamycin to ribosomes appears to be prevented by the presence of mRNA on the ribosomes, since the production (by RNase treatment) of more single ribosomes bearing a fragment of mRNA (*Figure 7B*) does not increase pactamycin binding to the 80S ribosomes. On the other hand, 80S ribosomes which are produced by NaF treatment of reticulocytes (by blocking initiation) and which, for the most part, are free of mRNA bind pactamycin readily (*Figure 7C*). Pactamycin is highly effective in inhibiting protein synthesis in cells and extracts from both types of cells¹⁹⁻²³. This inhibition can be overcome by adding more ribosomes. In the ribosomal system from *E. coli*, pactamycin interferes with the binding of initiator-tRNA to ribosomes (*Figure 5*) and alters the structure of the initiation complex, resulting in its decreased stability at low magnesium concentrations^{17, 23}. If antibiotic is added after formation is in progress, its inhibitory effect on protein synthesis is less. Pactamycin does not affect peptide-bond formation as such, since the puromycin reaction with prebound peptide or its equivalent, FM-tRNA, in the P-site is not inhibited by this agent.

Similar effects of pactamycin have been found in our laboratory by M. Stewart-Blair and I. Yanowitz using rabbit reticulocytes or their cell-free lysates. Protein synthesis by lysates from reticulocytes is inhibited over 90 per cent by 10^{-6} M pactamycin (*Figure 8*). As was found by Colombo

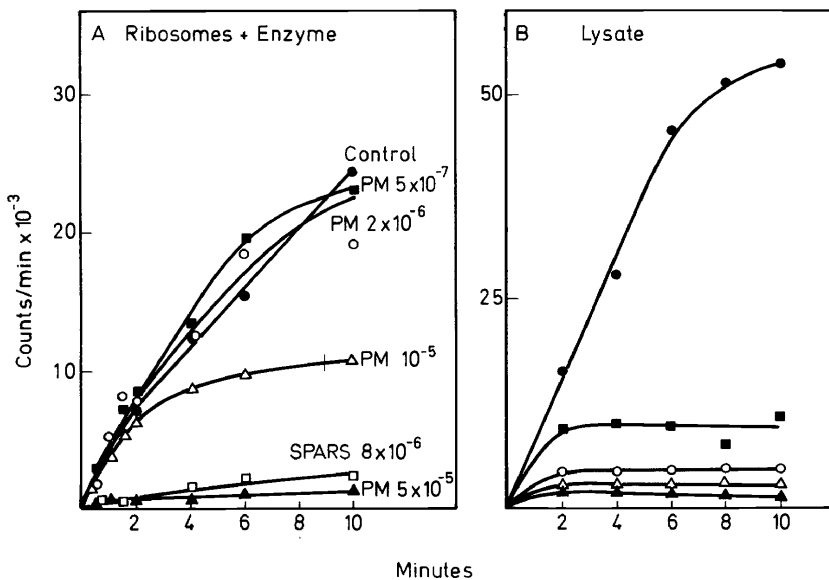


Figure 8. Effect of pactamycin on protein synthesis in fractionated (A) and whole lysates (B) from rabbit reticulocytes. NH₂-terminal (¹⁴C) valine analysis of the synthesized globin revealed that less than 10 per cent of the globin made in (A) is due to the formation of new chains, whereas over 70 per cent of that made in (B) is synthesized *de novo*. PM, pactamycin; SPARS, sparsomycin

*et al.*⁹ with intact reticulocytes, in lysates that are able to initiate new chains a low level of pactamycin (about 10^{-6} M) leads to the rapid breakdown of polyribosomes to single 80S ribosomes with the release of completed

MECHANISM OF ACTION OF ANTIBIOTICS

haemoglobin chains (*Figure 9*). At higher concentrations of pactamycin (10^{-5} M), polyribosome breakdown is incomplete and haemoglobin release is impaired. On the other hand, reticulocyte ribosomes that can only complete nascent haemoglobin chains are minimally affected by 10^{-6} M pactamycin (*Figure 8*). These data suggest that at low concentrations of pactamycin initiation of protein synthesis is affected much more than elongation or

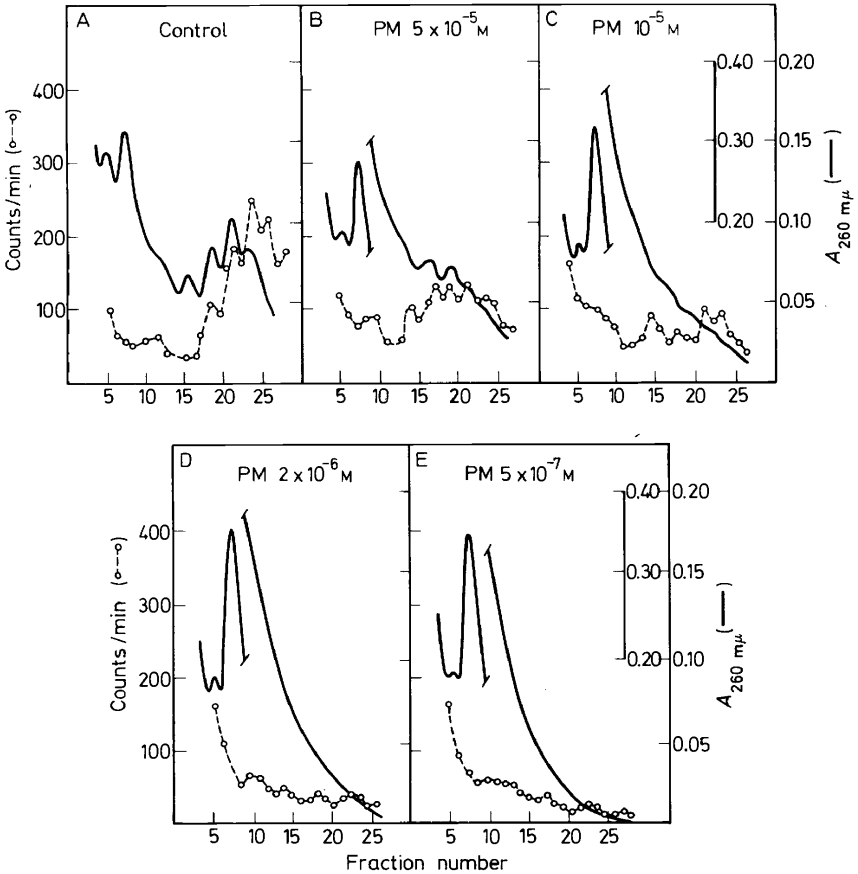


Figure 9. Effect of pactamycin concentration in the breakdown of polyribosomes and release of peptide in rabbit reticulocyte lysate. Polyribosomes prelabelled with (14 C) amino acids in lysates were then incubated for 2.25 min with different pactamycin concentrations. An aliquot of the reaction was analysed by sucrose density gradient centrifugation (from left to right; the main single peak at $A_{260m\mu}$ is at 80S)

termination, but at the higher concentrations elongation may be affected as well. By interfering primarily with the initiation step, pactamycin prevents the formation of new polyribosomes while allowing for normal read-off of mRNA and run-off of ribosomes by existing ones. Antibiotic inhibitors of peptide-chain elongation, such as sparsomycin, cycloheximide or fusidic

acid, prevent the pactamycin-induced polysome breakdown. It is possible that the 80S ribosomes that accumulate in the presence of pactamycin represent abnormal initiation complexes that are unable to proceed down the mRNA chain. Such an effect has been described for streptomycin in bacterial systems²⁴. On the other hand, it is also possible that the 80S ribosomes are 'run-off' ribosomes lacking mRNA and that initiation is inhibited at an earlier stage in complex formation.

In some ways, pactamycin acts like streptomycin²⁴⁻²⁷ and tetracycline²⁴, both of which have effects on initiation as well as on elongation of polypeptides in bacterial systems. Unlike streptomycin, however, pactamycin at any concentration does not induce misreading of the mRNA. The tetracyclines appear to interfere with the binding of aminoacyl-tRNA to the A-site on the ribosome, although at somewhat higher concentrations, binding of the initiator-tRNA can also be blocked²⁸.

Translocation inhibitors

Fusidic acid, a steroid antibiotic, and diphtheria toxin are of considerable interest for their selective actions on the translocation process. Fusidic acid blocks the GTPase activity associated with the translocation protein factor (the bacterial G factor or the mammalian transferase II) and bacterial²⁹ or mammalian³⁰ ribosomes. The bacterial 50S ribosomal subunit, as well as the G factor, has recently been implicated in fusidic acid action³¹. Fusidic acid inhibition of GTP hydrolysis has been shown to be due to its prevention of the dissociation of a ribosome-translocation factor-GDP complex which occurs as an intermediate during GTP hydrolysis^{32, 33}. On the other hand, thiostrepton³⁴, a peptide antibiotic which inactivates the ribosome³⁵, prevents translocation³⁶ by blocking the formation of the ternary complex containing either GTP or GDP^{37, 38}. The function of the 50S ribosomal subunit appears to be interfered with³⁷. Diphtheria toxin, which is a potent inhibitor of protein synthesis in eucaryotic cells and extracts, specifically inactivates transferase II by catalysing the attachment of the adenosine diphosphate ribose moiety of nicotinamide-adenine dinucleotide to transferase II by covalent linkage³⁹. Erythromycin, which binds to the 50S ribosomal subunit⁴⁰, has been found to inhibit translocation on bacterial ribosomes^{21, 41}. Cycloheximide also inhibits translocation on mammalian ribosomes, presumably by inactivating transferase II⁴²; this action appears to involve the larger ribosomal subunit⁴³. Thus, all four antibiotics affecting translocation in procaryotes or eucaryotes, or both, involve the larger ribosomal subunit.

Antibiotic effects in eucaryotic cells

Of the above-mentioned antibiotics, sparsomycin, pactamycin and fusidic acid act on eucaryotes as well as on procaryotes. Cycloheximide, which has no effect on bacteria, inhibits initiation⁴⁴ as well as elongation (resulting in polyribosome 'freezing') in animal cells. Anisomycin is a specific antibiotic inhibitor of peptide-bond formation in eucaryotes^{45, 46}.

As was pointed out earlier, the ribosomes present in the mitochondria or chloroplasts of eucaryotes are of the procaryotic type and their synthesis of protein is sensitive to antibiotics such as chloramphenicol⁴⁷. In fact, such an

effect has been used to explain some of the toxic effects of this antibiotic in man. Levels of chloramphenicol that are found clinically (20 µg per millilitre) have been shown to inhibit the synthesis of mitochondrial proteins in yeast and HeLa cells resulting in the decreased formation of mitochondrial membrane-bound cytochromes *a*, *a*₂, *b* and *c*₁, but not *c* (it is not yet certain whether this is a direct or indirect effect). At very high serum levels of chloramphenicol (100 µg per millilitre), which are reached in newborn infants, especially premature ones, with immature excretory mechanisms, there is generalized tissue toxicity probably due to a direct effect on mitochondrial respiration⁴⁷. This so-called 'grey syndrome' is characterized by cardiovascular collapse and often death⁴⁸.

Although mammalian protein synthesis is generally quite resistant to chloramphenicol, antibody synthesis has been found to be somewhat more sensitive to this antibiotic⁴⁹. A class of spleen ribosomes that are membrane-bound (as opposed to those free in the cytoplasm) has been found to be inhibited by chloramphenicol⁵⁰. It is not yet clear whether these two phenomena are related.

Antibiotic competition

It was pointed out that antibiotics can be classified according to which ribosome subunit is the site of action. Thus, clinically useful agents like chloramphenicol, lincomycin, the macrolide antibiotics such as erythromycin and oleandomycin, and sparsomycin work on the bacterial 50S ribosome subunit, whereas the tetracyclines, pactamycin, and the aminoglycoside antibiotics, such as streptomycin, neomycin and kanamycin, are 30S-ribosome antibiotics. Since the inhibitors of the 50S subunit compete with one another for binding⁵¹ so that only one antibiotic molecule per ribosome can be bound, the simultaneous use of two antibiotics against an organism resistant to one of the antibiotics may result in no antibacterial action⁵². In fact, such a situation has been reported⁵³. Erythromycin can act as an antagonist of lincomycin in erythromycin-resistant cells of *Staphylococcus aureus*. Since erythromycin resistance can be due to a decreased ability of the ribosome to bind erythromycin^{40, 54-56} the observed antagonism may result from the weak binding of erythromycin, which may still be able to prevent lincomycin binding to the ribosome. Similarly, the simultaneous use of two 50S inhibitors in treating an infection due to an organism sensitive to both may result in effects due only to the antibiotic that is more strongly bound⁵².

INHIBITORS OF NUCLEIC ACID SYNTHESIS AND FUNCTION

Since DNA plays a central role in vital processes such as chromosome duplication, cell division, enzyme induction, hormone action, cell nuclear function and nucleotide polymerization reactions, there has been considerable interest in the past decade in antibiotics that interfere with DNA function either by complexing with it or by inactivating or competing with the other components involved in nucleic acid replication reactions. In this discussion we shall consider several antibiotics which are typical of the different mechanisms of action without attempting to cover more than a few of the many agents which have been or are being actively investigated; one large

group, the nucleoside antibiotics, has been recently extensively reviewed elsewhere⁵⁷ and will not be considered here.

Antibiotics complexing with DNA

Actinomycin: The actinomycins are extremely toxic peptide-containing antibiotics (Figure 10) that form complexes with DNA *in vivo* and *in vitro*. Complex formation with DNA accounts for the ability of the actinomycins to interfere with nucleic acid synthesis and DNA function, and this effect is responsible for most, if not all, of the biological properties of these antibiotics^{58, 59}. Actinomycin selectively blocks the DNA-directed synthesis of RNA in procaryotic and eucaryotic cells. All cellular RNA fractions are inhibited; although at concentrations of the antibiotic that do not completely prevent RNA formation certain classes of RNA (in particular ribosomal RNA) are inhibited more than others. Actinomycin has been shown to act by blocking RNA chain elongation rather than chain initiation^{60, 61}. Inhibition can be overcome by adding more DNA to the reaction but not by adding more RNA polymerase or nucleoside triphosphates. DNA synthesis by intact cells or by the isolated DNA polymerase is inhibited by actinomycin but considerably higher levels of the antibiotic are needed than for comparable inhibition of RNA synthesis, indicating that different mechanisms are involved. In fact, inhibition of the DNA polymerase is found only at concentrations of actinomycin which are high enough to stabilize the helical DNA structure against strand separation, normally required for DNA replication⁶².

Complex formation between DNA and actinomycin can be measured by changes in the visible spectrum of the antibiotic; by equilibrium dialysis with radioactive antibiotic; by the decrease in the buoyant density and increase in the melting temperature of the DNA; and by the inhibition of the template activity of the DNA in enzymic RNA synthesis. Actinomycin interacts with DNA, but not with single stranded DNA, RNA or RNA-DNA hybrids⁶³, to form stable but reversible complexes. A considerable body of evidence has been accumulated to show that complex formation requires that the DNA be helical and possess the 2-amino function of guanine residues^{58, 59, 64}. Although there are two classes of binding sites in DNA, the one involving the G-C (guanine-cytosine) base pair is the strongly binding one and is responsible for the biological activity of actinomycin. Thus actinomycin does not form stable complexes with DNA's such as synthetic $d(A-T)_n$ or $d(I-C)_n$ (the strictly alternating deoxyadenylate-deoxythymidylate and deoxyinosinate-deoxycytidylate copolymers, respectively) which lack the 2-amino function of guanine, and these DNA's function as templates for the RNA polymerase with complete immunity to actinomycin. Naturally occurring and most synthetic DNA's containing even small amounts of guanine react with the antibiotic. On the other hand, the binding of actinomycin is less than expected if it were to be strictly proportional to the amount of guanine, especially in the middle and higher ranges of guanine contents in DNA⁶⁵. These data suggest that other factors may also determine actinomycin binding, such as steric hindrance by the bound antibiotic or local distortions in the DNA at the site of antibiotic binding, which prevent the complexing of an adjacent actinomycin molecule. It is also possible that the binding site

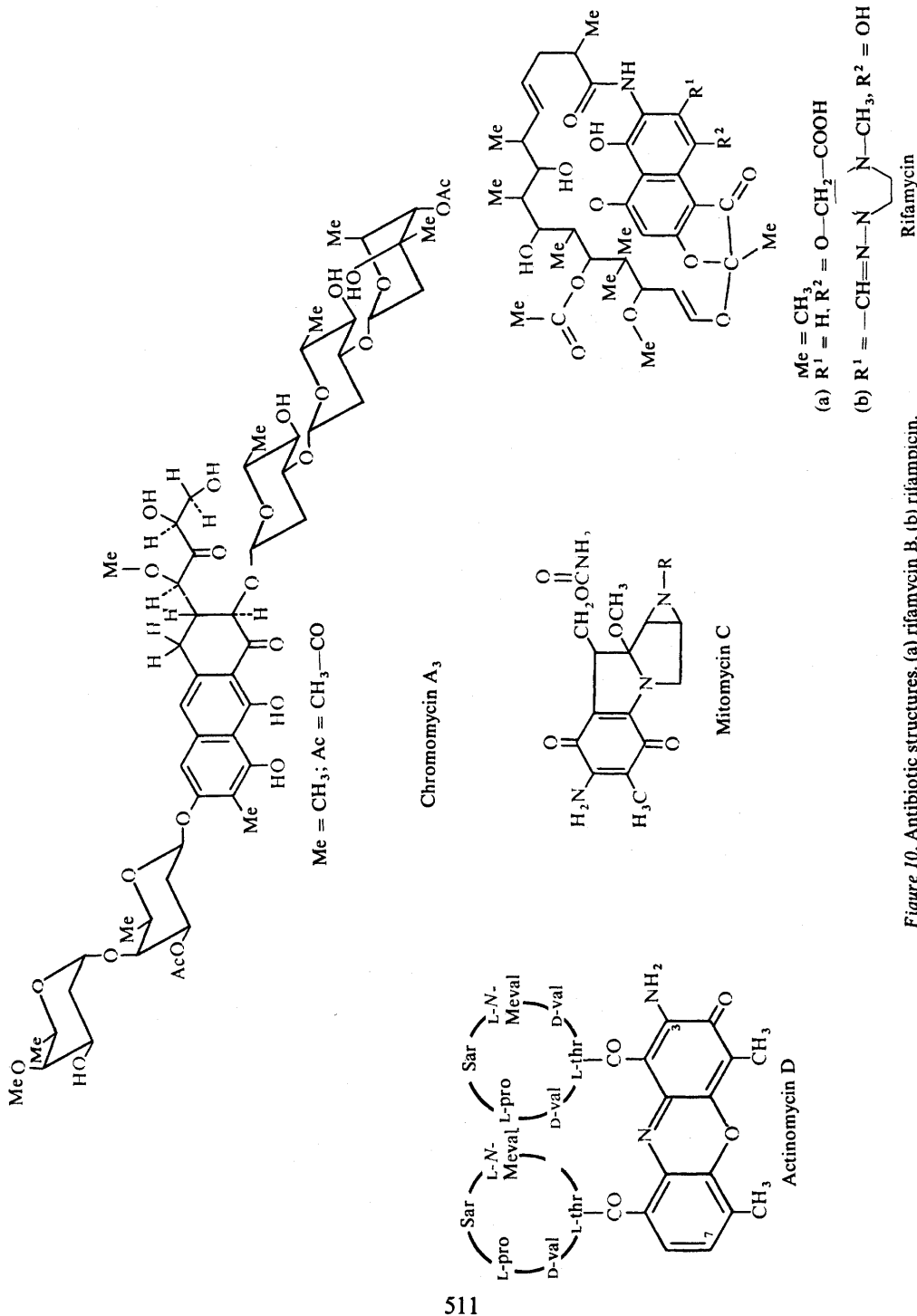


Figure 10. Antibiotic structures. (a) rifamycin B, (b) rifampicin.

on the DNA may involve more than one base pair, one of which is G-C; however, the possibility that adjacent guanines on the *same* strand are required to create the strong binding site has been excluded⁶⁶.

The most direct demonstration that the presence of the 2-amino function of a purine in DNA is necessary for actinomycin binding comes from experiments with synthetic DNA's with identical primary structures except for the presence or absence of the purine 2-amino group (*Figure 11*)⁶⁴.

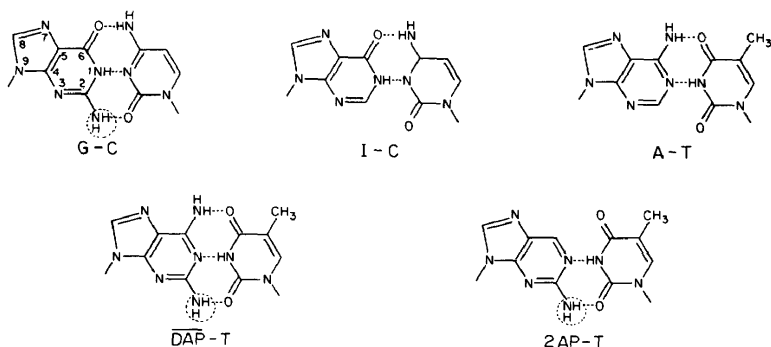


Figure 11. Structure of hydrogen-bonded purine-pyrimidine base pairs. G-C, guanine-cytosine; I-C, hypoxanthine-cytosine; A-T, adenine-thymine; DAP-T, 2,6-diaminopurine, 2AP-T, 2-aminopurine-thymine

Synthetic DNA's possessing 2,6-diaminopurine instead of 6-aminopurine (adenine) as the only purine in the DNA were shown to form stable complexes with actinomycin and their template activity with the RNA polymerase was sensitive to the antibiotic. On the other hand, the analogous DNA possessing adenine, which lacks the 2-amino group, did not interact with DNA at all. Recently, however, there have been reports on two synthetic DNA's, one lacking a purine with a 2-amino group and the other possessing guanine which appear to bind and not bind, respectively, to actinomycin⁶⁷. The results have been interpreted to indicate that while guanine in DNA usually is responsible for creating a structure in the DNA for actinomycin binding, this need not always be so. Since the three-dimensional structure of the polydeoxyribonucleotide is crucial as a determinant for actinomycin binding, the significance of these isolated findings for complex formation between actinomycin and natural DNA must await elucidation of their structures. Furthermore, a recent detailed analysis of the transcription process supports the role of G-C base pairs in actinomycin binding and action⁶¹. A study of the rates of incorporation of the precursor nucleotides in the RNA polymerase reaction showed a selective effect of actinomycin on the utilization of CTP and GTP (not ATP and UTP). In fact, since the rate of utilization of CTP (coded by guanine in DNA) was inhibited twice as effectively as that of GTP (coded by cytosine in DNA), it appeared that the antibiotic interacts with DNA in an unsymmetrical way with respect to guanine and cytosine in the DNA.

Two basically different types of models have been proposed to describe

the molecular nature of the actinomycin binding site on DNA: (1) the outside binding model⁶⁸ and (2) the intercalation model⁶⁹. In the outside binding model actinomycin is considered to be located in the smaller (minor) of the two grooves of helical DNA (*Figure 12*) with which it can form up to seven

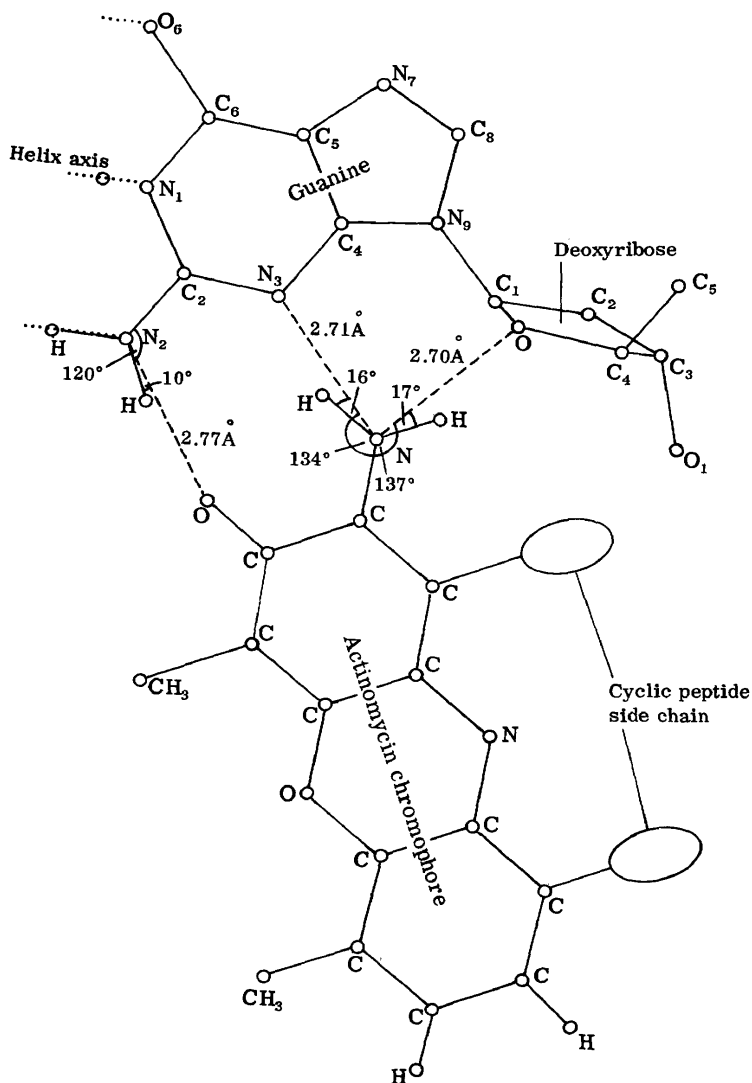


Figure 12. Hydrogen-bonding between deoxyguanosine in DNA and actinomycin D as proposed by Hamilton *et al.*⁶⁸

hydrogen bonds⁶⁸. According to the model, one hydrogen bond is formed between the quinoidal oxygen of actinomycin and the 2-amino group (which projects into the minor groove of DNA) of guanine, and the 3-amino group

of the actinomycin chromophore forms one hydrogen bond each with the ring nitrogen 3 and the ribose-ring oxygen of the deoxyguanosine. The cyclic peptides of actinomycin are packed into the minor groove of the DNA helix. The lactones presumably stabilize the peptide chains in a conformation permitting the formation of four additional hydrogen bonds between the four peptide-NH groups of actinomycin and the phosphodiester oxygens of the DNA strand opposite to that containing the guanine, which interacts with the actinomycin chromophore. Recently a variant of this type of model has been proposed in which the cyclic peptides lie *trans* to one another in the minor groove of DNA⁷⁰. In this model the cyclic peptides occupy a region in the DNA minor groove equivalent to about 6 base pairs, thus excluding, by steric hindrance, the binding of another actinomycin molecule within this distance. Such a proposal is consistent with binding data that show that a maximum of about one actinomycin molecule is bound per 6 G-C base pairs in DNA's rich in G-C.

The outside binding model accounts for the structures in DNA on which complex formation depends. Thus, only guanine can furnish the 2-amino hydrogen in the DNA minor groove for which the actinomycin quinoidal oxygen can serve as acceptor. Furthermore, the model depends critically on the relative positions of the DNA constituents as they are disposed in helical DNA in its native conformation and thus is in accord with the fact that actinomycin binds poorly, if at all, to single-stranded DNA, and does not bind to DNA-RNA hybrids or double-stranded RNA, which are thought to exist in other conformations.

In the second type of model the actinomycin phenoxazone chromophore is postulated to be intercalated between adjacent base pairs in the DNA⁶⁹. Müller and Crothers found that actinomycin increased the viscosity and decreased the sedimentation coefficient of low molecular weight DNA but had opposite effects on DNA of high molecular weight. They interpreted these results to indicate that the length of the DNA is increased by complex formation as would be found with intercalation. Presumably actinomycin induces a sort of crosslinking of high molecular weight DNA (which is flexible enough to coil back on itself) by increasing the tendency of parts of the DNA to interact with each other. Furthermore, substitution of bulky groups on the 7 position of the actinomycin chromophore markedly slowed down the combination of actinomycin with DNA. This would not have been predicted by the outside binding model in which actinomycin lies on the outside of the DNA molecule, since the 7 position on the actinomycin chromophore would project away from the helix. These workers proposed that the actinomycin chromophore intercalates between the base pairs of DNA adjacent to any G-C pair. The chromophore is inserted from the minor groove, into which the peptide rings project. The specificity for guanine is attributed to electronic interactions in the π -complex formed in the intercalated structure; the actinomycin chromophore ring nitrogen would fall directly under the 2-amino group of the purine. In the most stable form of the actinomycin-DNA complex, the peptide rings undergo conformational changes that adapt their structure to interact specifically with the DNA backbone, one ring interacting with each strand of the double helix in the minor groove. The slow reversal of the peptide ring conformation

is viewed as accounting for the slow dissociation of actinomycin from DNA and as being the basis for the high order of effectiveness of actinomycin on the RNA polymerase reaction as compared with simpler analogues of the antibiotic. It should be noted that in both of the proposed model types the peptide rings of actinomycin lie in the minor groove of the DNA where the path of the advancing RNA polymerase might be blocked. Müller and Crothers suggest that the selective resistance of the DNA polymerase reaction to actinomycin is due to the local denaturation immediately ahead of the enzyme that causes the antibiotic to dissociate away much faster. Consistent with the intercalation model are experiments⁷¹ in which actinomycin has been found to lead to the uncoiling of the replicative form (closed circular duplex DNA) of bacteriophage $\phi \times 174$ in a manner similar to that of ethidium bromide, an intercalating dye, although other explanations may also be possible.

Recently, a new model combining features of the two previous models has been proposed for the actinomycin-DNA complex based on x-ray data obtained from a crystalline complex containing actinomycin and deoxyguanosine⁷². In this model the phenoxazone ring system of actinomycin intercalates between adjacent G-C base pairs of DNA, where the guanine moieties are on *opposite* DNA strands, and the 2-amino groups of the guanines interact with both cyclic peptides through specific hydrogen bonds. As in the other models, the cyclic peptides lie in the minor groove of helical DNA. There is much to suggest that this model may be the correct one.

The actinomycin-deoxyguanosine complex contains one actinomycin, two deoxyguanosines, and ten water molecules. Both the actinomycin molecule and the complex formed with the two deoxyguanosine molecules have twofold symmetry (*Figures 13 and 14*). The actinomycin chromophore

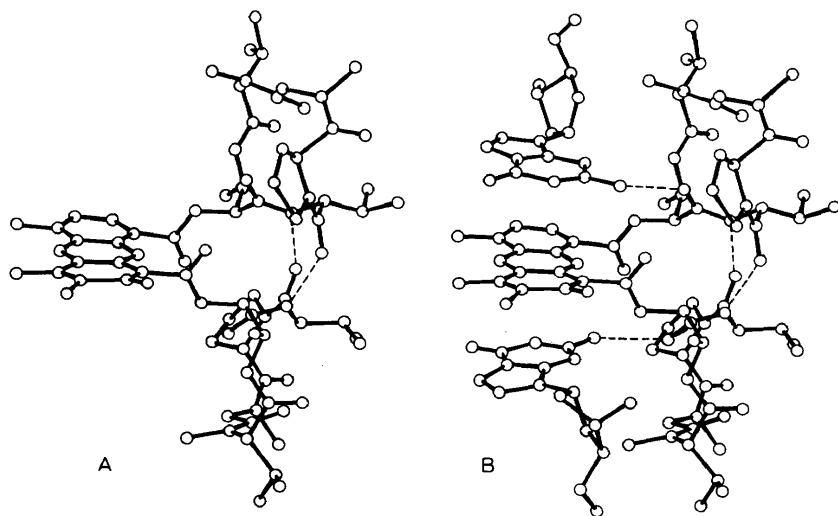


Figure 13. Computer drawn illustration of the actinomycin molecule (A) and the actinomycin-deoxyguanosine complex (B) viewed from a sidewise direction. Modified after reference 72

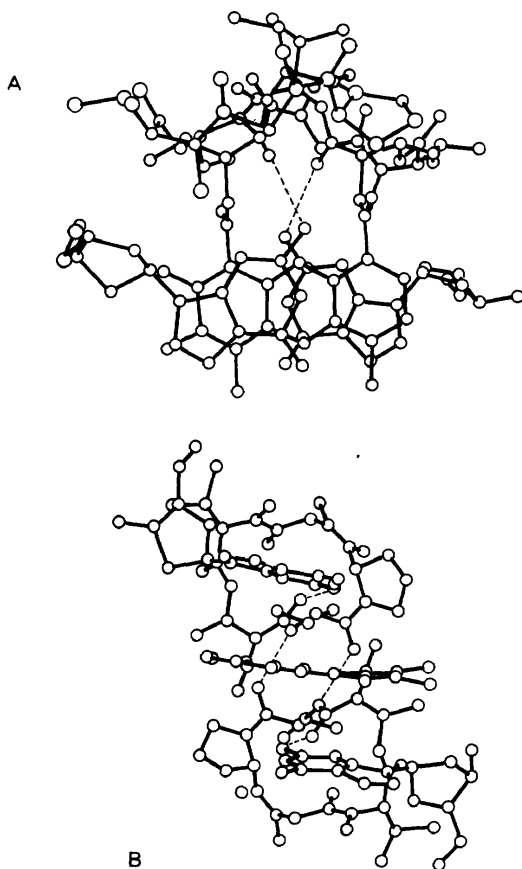


Figure 14. Computer drawn illustration of the actinomycin-deoxyguanosine complex viewed perpendicular to the plane of the chromophore (A) and down its approximate dyad axis (B). Modified after reference 72

extends outside and perpendicular to the peptide rings. In the complex the two deoxyguanosine molecules interact with the two cyclic peptide residues by a strong hydrogen bond between the 2-amino group of guanine and the carbonyl oxygen of the L-threonine residue and stack on alternate sides of the phenoxazone ring system. A weaker hydrogen bond connects the guanine N (3) ring nitrogen with the NH group on this same L-threonine residue. The sugar residues of both deoxyguanosine molecules are in close steric juxtaposition with the isopropyl groups of the L-methyl valine residues, and such hydrophobic interactions, as well as the stacking of guanine and phenoxazone rings, provides stability to the complex; but it is the hydrogen-bonding which plays a key role in the association and explains the requirement for guanine in the binding of actinomycin with DNA.

In the proposed actinomycin-DNA model both sugar residues on deoxyguanosine and deoxycytidine of the same strand are rotated so as to

reduce the helix twist from 36° in the B form of DNA to about 15° and this provides adequate space for intercalation of the actinomycin chromophore between the base pairs. This results in close steric juxtaposition of the 2-amino group on the chromophore residue to phosphate and the deoxycytidine furanose ring oxygen, with likely hydrogen bonds between them. This model predicts that poly(G-C)_n (strictly alternating deoxyguanylate-deoxycytidylate) which contains the sequence G₃'P₅'C should bind actinomycin best, although other sequences containing guanine can bind actinomycin but with lower affinity and efficiency. This is supported by the binding data of Wells and Larson⁶⁷ (although possibly not by those of Gellert *et al.*⁶⁵).

Chromomycin A₃, mithramycin and olivomycin: Like actinomycin, chromomycin A₃ (Figure 10) and the other two closely related antibiotics selectively inhibit the RNA polymerase reaction and form reversible complexes with helical DNA which contains the 2-amino group of guanine (or 2,6-diaminopurine)^{64, 73, 74}, but in addition stoichiometric quantities of divalent cation are required for interaction⁷³. It seems likely that the metal binds first to the antibiotic chromophore by way of its oxygen containing groups and that the antibiotic-Mg²⁺ complex binds to DNA. The chelation step appears not to be rate limiting and the bulky sugar side chains of the antibiotic appear not to influence the rate of association with DNA, although the rate of complex dissociation increases with a decrease in the size of the chains⁷⁵. While the latter considerations are important in determining the inhibitory activity of these agents against the RNA and DNA polymerases, their guanine specificity resides in the chromophore part of the molecule. The number of binding sites on DNA for chromomycin increases as the G-C content increases up to a limit of approximately 33 moles per cent guanine, where about one molecule of chromomycin is bound for every 4 nucleotide base pairs. This limitation of the number of binding sites in DNA's with higher guanine contents has been attributed to steric factors⁷⁵. As with actinomycin, chromomycin has been found to require the presence of a helical structure in DNA; heat-denatured DNA binds the antibiotic poorly and single-stranded DNA does not bind chromomycin at all. Since chromomycin protects DNA against degradation by nucleases, it has been possible to isolate DNA fragments (of high G-C content) bearing the antibiotic by nuclease treatment. Unlike actinomycin (and intercalating dyes), chromomycin and mithramycin do not cause uncoiling of the DNA double helix, suggesting that these agents do not intercalate into the DNA⁷¹. There has been virtually no work reported, however, in an effort to define the detailed molecular structure in DNA for the binding of this interesting group of antibiotics.

Anthracyclines: Antibiotics (daunomycin—Figure 15, nogalamycin, the cinerubins, the pyrromycins, rhodomycin and the rutilculomycins) which contain a planar tetrahydrotetracenquinone chromophore linked to a sugar are included in the anthracycline group of antibiotics. These agents form relatively stable complexes with DNA (weaker ones with RNA)⁷⁶ and, for the most part, do not exhibit a specific base requirement in the DNA for interaction^{73, 74}, although nogalamycin binding to DNA and activity against

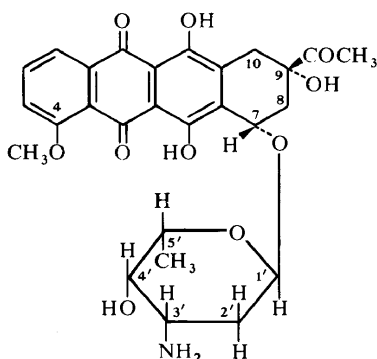


Figure 15. Structure of daunomycin

the RNA polymerase appear to be most effective when the DNA contains sequences of alternating adenine and thymine in the same strand⁷⁷. Both daunomycin and nogalamycin inhibit the RNA and DNA polymerase about equally. The anthracyclines behave similarly to the acridine dyes, increasing the viscosity and decreasing the sedimentation of DNA, but unlike the acridines binding persists at high salt concentrations. These properties plus the finding that daunomycin and nogalamycin cause uncoiling of the supercoiled structure of closed circular duplex DNA⁷¹ have been taken to indicate that the anthracyclines intercalate between the DNA base pairs. An intercalation model for the daunomycin-DNA complex has been recently built and found to be stereochemically satisfactory (W. Fuller, personal communication).

Daunomycin is a potent anti-mitotic agent. Since it is able to prevent cells from entering mitosis, even when added after completion of the cycle of DNA synthesis, it appears that the anti-mitotic action of daunomycin is independent of any effect on the DNA synthetic reaction⁷⁸. While it has not been excluded that the anti-mitotic action is due to inhibition of synthesis of a fraction of messenger RNA essential for mitosis, it seems likely that the DNA to which antibiotic is bound is unable to function properly in the mitotic process.

Mitomycin: Mitomycin C (Figure 10) (and its derivative, porfiromycin) is representative of antibiotics that bind to the DNA template by covalent linkage⁷⁹. Mitomycin C inhibits selectively the synthesis of DNA in susceptible organisms and, secondly, leads to the degradation of DNA in some organisms. The biological effects of mitomycin C and the presence of an aziridine ring in the antibiotic suggest that its mechanism of action is similar to that of the antitumour alkylating agents. Mitomycin C is inert until activated in the cell by a reductive step that un masks the alkylating aziridine ring. The lethal effects of mitomycin C presumably are due to changes in the structure of the DNA that prevent its replication and function. Unlike native DNA, DNA isolated from mitomycin-treated organisms renatures spontaneously after heat denaturation. The spontaneous reversibility of the denaturation process results from mitomycin-induced covalent links between

the complementary strands of the DNA that ensure the return of the native structure of the partially separated strands. In order to crosslink DNA, mitomycin must act as a bifunctional alkylating agent. Actually, only one in every five to 20 mitomycins are involved in the crosslinking of the two DNA strands; the rest are attached to one or the other strand. The latter lesions are more susceptible to repair in the cell than the former. Mitomycin C appears to react with the guanine moiety of the DNA, and when it functions as a crosslinking agent, the 6-oxygen groups of guanines from each strand are viewed as being the sites of covalent linkage with the antibiotic, although some other possibilities (except for the N-7 and C-8 positions⁸⁰) have not been excluded.

Antibiotics complexing with RNA polymerase

Rifamycin, streptovaricin and streptolydigin: These antibiotics⁸¹⁻⁸³ are of particular interest because of their great specificity in combining with and inactivating the RNA polymerase of procaryotes. Their counterpart, as an inhibitor of RNA synthesis in eucaryotes by the extra-nucleolar RNA polymerase, is α -amanitin, the highly toxic sulphur-containing bicyclic polypeptide isolated from the deadly mushroom, *Amanita phalloides*⁸⁴. The rifamycins (*Figure 10*) and the closely related streptovaricin, selectively inhibit the synthesis of all cellular RNA in susceptible bacteria⁸⁵, thus accounting for their bactericidal action⁸⁶. Rifampicin, the active semi-synthetic derivative of rifamycin B, specifically inhibits the bacterial DNA-dependent RNA polymerase⁸⁷⁻⁹⁰ but not that of nuclear origin in eucaryotic cells^{88,89}. The bacterial RNA polymerase is inhibited 50 per cent by 2×10^{-8} M rifampicin, but the DNA polymerase reaction is unaffected by even four magnitudes greater antibiotic concentration⁸⁷. Thus, rifampicin is not only more effective than actinomycin on the RNA synthetic reaction, but is also far more selective. This greater selectivity is accounted for by the fact that rifampicin, unlike actinomycin, does not bind to DNA but attaches to the enzyme itself, forming a stable complex⁹¹ which only slowly exchanges with free rifampicin⁹², but which can be dissociated with 6M guanidine hydrochloride⁹³. One molecule of rifampicin binds per molecular weight of the monomer RNA polymerase 'core'⁹². The macrocyclic ring of the rifamycin molecule (*Figure 10*) determines its binding to the RNA polymerase, while other parts of the molecule affect its permeability into intact bacteria⁹⁴. As expected of an agent which directly inactivates the RNA polymerase, inhibition of *in vitro* RNA synthesis can be overcome by increasing the concentration of the enzyme, but not of the other components of the reaction⁸⁰. Furthermore, bacterial mutants resistant to the antibiotic possess a RNA polymerase which is rifampicin-resistant^{95,96} and does not complex with the antibiotic⁹¹. The mutation involves the β chain of the RNA polymerase 'core'^{97,98}.

Rifampicin is most effective when present at the beginning of RNA synthesis^{89,99} although the antibiotic does not prevent the binding of the RNA polymerase to DNA⁸⁹. Once the complete initiation complex (consisting of enzyme, DNA and purine nucleoside triphosphate) is formed, the antibiotic is less effective, and if it is added after the polymerization of RNA has begun it is without effect. Nevertheless, the antibiotic is still able to

bind to the polymerizing complex¹⁰⁰. Since incubation of enzyme with DNA and Mg^{2+} affords some protection against its inactivation by rifampicin, it is assumed that a very early step in initiation of RNA synthesis is interfered with by the antibiotic^{100, 101}.

The growth of vaccinia virus in mammalian cells is inhibited by very high levels of rifampicin^{102, 103}, much higher than needed for the inhibition of bacterial growth. The precise mechanism of this effect is not yet known with certainty, although rifampicin interferes with the assembly of mature vaccinia virus¹⁰⁴, perhaps by complexing with and inhibiting the newly synthesized viral RNA polymerase^{105, 106}. Whatever the nature of this effect, it appears to be different from that exhibited in procaryotes, since the anti-pox activity resides not in the macrocyclic ring of rifampicin but in the hydrazone side chain^{107, 108}. Of interest are recent reports that rifampicin can suppress Rous sarcoma virus—induced transformation of chick fibroblasts¹⁰⁹ and that demethylrifampicin can inhibit the RNA dependent DNA synthesizing enzyme from human leukaemia cells¹¹⁰.

Streptolydigin¹¹¹ is like the rifamycins and streptovaricin in directly inactivating the bacterial RNA polymerase, but differs in that it blocks RNA synthesis even after it has started^{112, 113}.

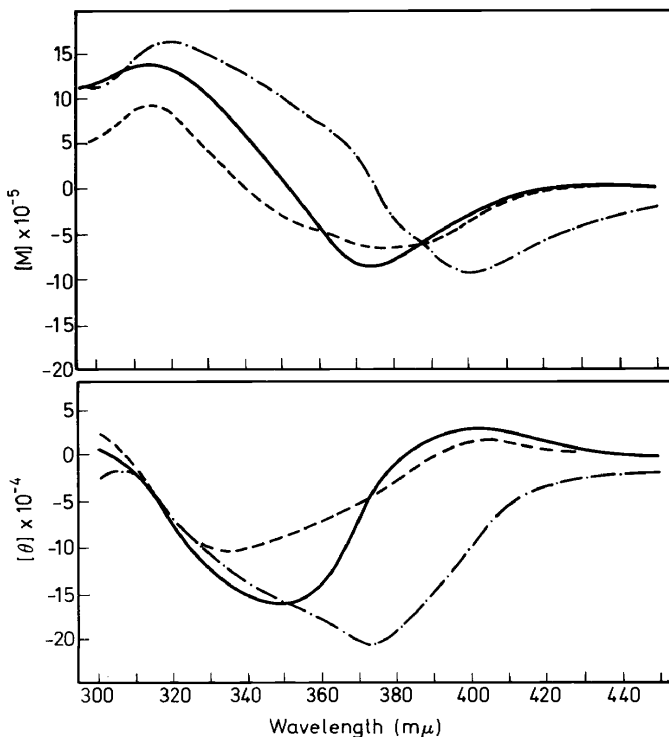


Figure 16. Optical rotatory dispersion (upper) and circular dichroism (lower) spectra of the interaction of kanchanomycin with native calf thymus DNA. (-----) kanchanomycin and Mg^{2+} ; (—) kanchanomycin, DNA and Mg^{2+} without incubation; (-·-·-) same after 20 h at $37^{\circ}C$ ¹¹⁸

MECHANISM OF ACTION OF ANTIBIOTICS

Kanchanomycin: The mechanism of kanchanomycin action appears to combine features of the antibiotics which inactivate the DNA template and those which inactivate the RNA polymerase. Kanchanomycin is a yellow, very sparingly water-soluble antibiotic of unknown structure which is bactericidal and tumouricidal at extremely low concentrations^{114,115}. Kanchanomycin complexes with polynucleotides in the presence of stoichiometric amounts of divalent cation in a two-step, time dependent reaction^{116,117}. An initial complex forms immediately and changes with time to a second more stable complex with different spectral (Figure 16) and chemical properties. The antibiotic appears to combine first with Mg^{2+} and this complex then interacts with DNA or other polynucleotides.

Kanchanomycin inhibits *in vitro* RNA and DNA synthesis in two distinctive ways¹¹⁸. While the inhibition of DNA synthesis by kanchanomycin can be overcome by increasing the concentration of DNA (not DNA polymerase), the inhibition of RNA synthesis is overcome by increasing the RNA polymerase concentration. The latter is not reversed by increasing amounts of DNA to which a fixed amount of antibiotic has been previously bound. In a double reciprocal plot of the kinetics of inhibition kanchanomycin

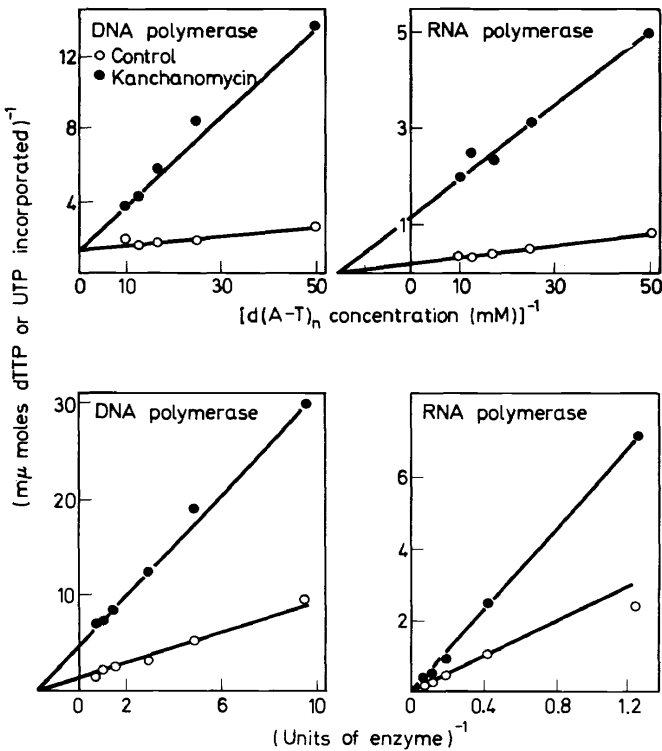


Figure 17. Double reciprocal plots of kinetics of inhibition of DNA and RNA polymerase reactions by kanchanomycin. In the upper drawings the concentration of $d(A-T)_n$ was varied; in the lower drawings the concentration of the DNA or RNA polymerase was varied¹¹⁹

appears to act as a competitive inhibitor of DNA in DNA synthesis, but as a competitive inhibitor of the RNA polymerase in RNA synthesis (*Figure 17*). Thus the inhibition of RNA synthesis by kanchanomycin is not due solely to the binding of the inhibitor to the DNA, but must also involve the inactivation of the RNA polymerase in the complex. It is possible that the enzyme is attracted to sites on the DNA where inhibitor is located and that excess enzyme can go to sites free of inhibitor. On the other hand, in the case of the DNA polymerase it is the template function of the DNA which is altered by the antibiotic.

CONCLUSION

It is within the realm of expectation that agents will eventually be found that block with exquisite specificity each of the steps and components involved in the synthesis of macromolecules in procaryotic or eucaryotic cells. This will hopefully lead to the availability of drugs of high efficacy and low toxicity for use in human disease. Similarly, powerful tools for the dissection of normal physiological processes will be an important by-product of such a search. The relatively recent discoveries of the mechanism of action of rifamycin-like antibiotics and of α -amanitin has given new impetus to this type of endeavour. It is of note, however, that although several of the inhibitors of nucleic acid synthesis that work in eucaryotes (and procaryotes) are clinically useful, the inhibitors of protein synthesis that are effective in eucaryotes have not yet found such use—at least in part because of their considerable toxicity for all human cells. The finding that the sensitivity of several animal viruses to sparsomycin varies with the base composition of the viral nucleic acid¹¹⁹ raises the possibility of the selective use of this antibiotic or others like it in infections due to certain viruses.

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