REVIEW LETTER

USE OF INHIBITORS TO STUDY THE STRUCTURE AND FUNCTION OF NUCLEIC ACIDS AND RIBOSOMES

Recent advances

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1. Introduction

During the past ten years, the molecular mechanisms by which several antibiotics or drugs interfere with cell metabolism have been elucidated. Since then, a new era in research started when it became evident that many of these inhibitors can be used as highly specific reagents for blocking complex cellular processes such as the synthesis of cell wall constituents, the synthesis of nucleic acids and proteins, biological oxidations, ion transport across membranes and gluconeogenesis. These topics have been discussed extensively in 1969 at Madrid [1, 2], Mosbach [3] and Tokyo [4], and in 1970 at Washington [5] and Riga [review 6]. From recent advances in this field, a few which in the author's view are the most impressive examples have been selected to show the contribution of this research to solving problems on the structure and function of nucleic acids and ribosomes.

2. Nucleic acids

2.1. *DNA*

Actinomycin (I)* is probably the best and most thorougly studied antibiotic which has been shown to alter the structure and function of DNA. Hamilton et al. [7] proposed a model in which actinomycin is bound in the minor groove and the purine 2-amino-group of deoxyguanosine is required for actinomycin binding to native DNA. Müller and Crothers [8] suggested from their results a hydrogen-bonded model in which the chromophore of actinomycin intercalates between adjacent base pairs with the peptide rings at the outside of the DNA double helix. Wells and Larson [9] have now studied the binding of actinomycin to 17 different synthetic DNA polymers by a variety of techniques including equilibrium dialysis, *in vitro* transcription and analytical buoyant density centrifugation. The results of these experiments can be briefly summarized: The presence of deoxyguanosine is not necessary for the binding of actinomycin to all kinds of DNA, since poly dI binds actinomycin almost as tightly as does native DNA; the presence of deoxyguanosine is not sufficient for binding since poly d(A–T–C) · poly d(G–A–T) which contains 33% G + C binds virtually no actinomycin. A marked nucleotide sequence preference exists for the binding reaction. By comparing the binding capacity of sequence isomeric DNAs, the isomer which contains both purines and pyrimidines on both complementary strands, binds more actinomycin and binds it more tightly than the isomer containing all purines in one strand and the pyrimidines in the complementary strand. These findings strongly suggest that the apparent specificity of guanine for actinomycin binding to DNA must be a secondary effect

* The numbers identify the structures in the Appendix to this Review letter.

and caused by an influence of this base on a certain steric and electronic environment. Most important for actinomycin association with DNA is the presence of a suitable DNA configuration. The results are not consistent with the model of Hamilton et al. but favour the hydrogen-bonded intercalation model of Müller and Crothers.

Ethidium bromide (II), *daunomycin* (III), *nogalamycin* (IV), *chromomycin* (V) and *mithramycin* (VI): That actinomycin is partially intercalated between adjacent base pairs of the DNA double helix is also suggested by Waring, who compared several DNA complexing antibiotics with respect to their influence on supercoiled circular DNA. Intercalation of a dye between adjacent base pairs of the double helix of DNA requires that the helix becomes partially uncoiled at the point of insertion of a dye molecule between the base pairs. This proposed uncoiling has been shown experimentally by Crawford and Waring [10]. Circular duplex DNA ϕx 174 displays large changes in supercoiling in response to a local uncoiling upon insertion of a dye molecule. During the first phase of drug binding, the number of right-handed supercoils originally present in the circle decreases. At a critical level of binding, the supercoils are just removed so that the DNA molecules behave as untwisted open circles. As further drug binding takes place, left-handed supercoils appear. Since supercoiled circles are more compact than the equivalent open circles, they sediment more rapidly. Therefore, the loss and reappearance of supercoils can be easily observed by the changes in sedimentation coefficients. By using this technique it could be demonstrated that, like ethidium bromide, actinomycin, daunomycin and nogalamycin cause uncoiling of the helix whereas chromomycin and mithramycin do not affect the supercoils [11]. It was concluded that chromomycin and mithramycin do not bind by intercalation as had been proposed from earlier physicochemical binding studies [12]. Among several drugs which are believed to interact with DNA by mechanisms other than intercalation one exception was found, *irehdiamine A* (VII), which caused uncoiling of the double helix. Thus uncoiling of the double helix might occur also by non-intercalative processes. Like several other psysicochemical measurements the variation of the supercoils in closed circular DNA induced by antibiotics or drugs only favour the intercalation model of Lerman, but do not prove it.

Bauer and Vinograd [13] studied the binding isotherms of ethidium bromide to closed circular and nicked circular DNA of the phage SV 40. From the binding isotherms the free energy of superhelix formation of SV 40 closed DNA at 25° in 5.8 M CsCl could be calculated. The differences in the binding isotherms observed for the two SV 40 DNAs are a result of the additional free energy required for superhelix formation in the closed molecule.

Ethidium bromide is an effective mutagen causing respiratory deficiency in yeast. Normally, this mutation occurs spontaneously at an unusually high frequency of approx. 1% per generation. This mutation is carried by a cytoplasmic factor, ρ, generally assumed to be mitochondrial DNA, missing in the petite phenotype (ρ^-). Slonimski et al. [14] could convert a respiratory sufficient culture to more than 98% petites within 3.2 cell doublings by the use of ethidium. This system allows the study of the molecular events associated with the conversion of normal to petite cells. Goldring et al. [15] studied the properties of mitochondrial DNA at various times during the mutation process and observed that ethidium bromide alters the synthesis of DNA and RNA in mitochondria without affecting extramitochondrial nucleic acid synthesis; mitochondrial DNA synthesis is preferentially inhibited. Pre-existing mitochondrial DNA is progressively degraded. The authors suggest that supercoiled molecules of mitochondrial DNA exist and are distorted upon insertion of the dye, leading to an increased nuclease attack which accounts for the degradation. The degradation products might be replicated, but are no longer able to be transcribed and translated to produce functional gene products. In petites formed by prolonged treatment, no mitochondrial DNA could be detected. Zylber et al. [16, 17] also observed that ethidium bromide alters the synthesis of DNA and RNA in mitochondria without affecting extramitochondrial nucleic acid synthesis.

To explain the preferential effect of ethidium bromide on mitochondrial DNA, it is assumed that at low concentrations the drug preferentially binds to mitochondrial DNA because this can exist in a supercoiled form. However, the size and structure of yeast mitochondrial DNA is not yet established.

Hollenberg et al. [18] showed closed circular molecules, 25–27 μm long, in Marmur's laboratory linear mitochondrial DNA molecules of two different sizes, 25–28 μm long and 50–55 μm long, were isolated.

2.2. *tRNA*

Ethidium bromide has also proved to be valuable in studying the structure of tRNA. Phenylalanine specific tRNAs of yeast, wheat and rat liver have in common a fluorescent base called "Y" adjacent to the anticodon loop. Low concentrations of the dye show a strong binding site for $tRNA^{Phe}$ which only binds one ethidium bromide per RNA molecule [19, 20]. Assuming that the drug binds by intercalation, the bound dye probably remains on the macromolecule within the lifetime of the fluorescent state and possibly has a fixed orientation with respect to the macromolecule. The visible absorption of ethidium bromide overlaps with the emission of the "Y" base. Therefore, it can act as an energy-acceptor and cause quenching of the fluorescence of the "Y" base. The amount of quenching depends on the distance between the energy donor and acceptor. Fluorescence quenching of the "Y" base by ethidium bromide is greatly depressed in the presence of Mg^{2+} indicating that the conformation of tRNA is drastically changed by Mg^{2+} so that the distance between the binding site for ethidium bromide and the location of the base increases considerably. These results clearly show an effect of Mg^{2+} on the tertiary structure of tRNA.

3. RNA-polymerase

3.1. *Bacterial enzyme*

In the RNA-polymerase reaction, several sequential processes are involved: (1) association of the DNA template with the enzyme, (2) stabilization of the complex of enzyme and DNA by the nucleotide which forms the 5′-terminus of the RNA chain, (3) initiation of the chain, by formation of the first 5′–3′ linkage, (4) chain elongation, (5) liberation of the synthesized polyribonucleotide chain from the template.

Rifampicin (VIII) which specifically inhibits bacterial RNA-polymerases, has recently been used as a tool to discriminate sigma specific and unspecific active complexes of RNA-polymerase and template [21]. The primary effect of rifampicin is the inhibition of the association of the DNA template with the polymerase. Once this complex is formed, the further steps of RNA synthesis in bacterial systems are insensitive to rifampicin; chain-initiation, formation of the first phosphodiester bond and chain elongation can occur in the presence of the inhibitor. However, the instability of the binary complex between RNA polymerase and template causes a residual inhibition of the RNA polymerase reaction by rifampicin.

The complex between RNA-polymerase and templates which lack sigma specific initiation sites, poly d(AT) or salmon-sperm DNA, are more stable than those which are formed when sigma specific templates, such as T_2 DNA, are used. The less stable complexes lose their resistance to rifampicin rather rapidly. The stable complexes between RNA-polymerase and sigma unspecific templates lose their resistance much more slowly. This now allows one to distinguish sigma specific and unspecific complexes of RNA-polymerases and DNA templates.

3.2. *Eucaryotic enzyme*

α-Amanitin (IX), a toxic peptide, has been used to discriminate between two different types of mammalian RNA-polymerases [22]. It was suggested that RNA polymerase from isolated nuclei contains two types of enzyme, one which appears to synthesize mainly ribosomal RNA and accumulates in the nucleoli and another responsible for the synthesis of DNA-like RNA. This latter activity was found to be mainly extranucleolar. The two types of RNA-polymerases show different sensitivities to α-amanitin. The enzyme preparation of whole nuclei and the soluble enzyme preparation made from nuclei were extensively inhibited by α-amanitin, whereas the polymerase activity of the nucleolar enzyme was only slightly affected by the toxin under the same conditions.

The resistance to α-amanitin or to rifampicin of polymerases of different origins, mammalian, ciliata (tetrahymena) and yeast can be used to get insight into similarities or dissimilarities of these enzymes. It was found that the tetrahymena [23] and the yeast [24] enzymes resemble mammalian RNA-polymerase in sensitivity to α-amanitin and resistance to rifampicin.

4. Ribosomes

4.1. *Peptide bond formation and translocation*

Ribosomes have two sites for functional binding of aminoacyl tRNA. These sites have been called acceptor and donor sites. The donor site (D) is the site where the amino acid of peptidyl tRNA is donated to the incoming aminoacyl tRNA. The site where the incoming aminoacyl tRNA is bound, is called acceptor site (A). Aminoacyl tRNA can be replaced by the antibiotic puromycin.

Kaji et al. [25, 26] have developed an assay which allows one to study partial reactions of protein synthesis such as peptide bond formation and translocation of peptidyl tRNA. Using this assay it is possible to discriminate between antibiotics acting on peptide bond formation or translocation. When the complex of ^{14}C-Phe-tRNA, ribosomes and poly U is prepared in the presence of low Mg^{2+} concentrations, the tRNA is preferentially bound to the donor site, while at higher Mg^{2+} concentrations about equal distribution of ^{14}C-Phe-tRNA to both donor and acceptor sites was observed. At low concentrations of Mg^{2+} 80% of the ^{14}C-Phe-tRNA interacts immediately with puromycin; at high Mg^{2+} concentration a peptide bond must first be formed between the two phenylalanine residues. In a second step the newly formed peptidyl tRNA must be translocated by a translocase and GTP. Using these two different experimental conditions, it was possible to distinguish between the following antibiotics by their effects on peptide bond formation and translocation.

Fusidic acid (X), *erythromycin (XI), chloramphenicol* (XII), *cycloheximide* (XIII) and *lincomycin* (XIV): Fusidic acid was found to be inhibitory to the *puromycin* (XV) reaction only under conditions which involve translocation and peptide bond formation. Erythromycin like fusidic acid specifically inhibits translocation. Cycloheximide affects translocation like fusidic acid [27]. Chloramphenicol was suggested to be a specific inhibitor of peptide bond formation. As expected, chloramphenicol inhibits the puromycin reaction under both conditions to the same extent. Lincomycin behaves like chloramphenicol and, therefore, inhibits peptide bond formation.

4.2. *Ribosomal proteins*

Recent work from several laboratories has shown that there are about 20 different proteins in the 30 S and 34 proteins in the 50 S ribosomal subunit. For review see Traut et al. [28]. Very little is known up to now about the function of the ribosomal proteins, thus it is impossible to distinguish the different proteins by their function. Therefore, antibiotics which interact specifically with ribosomal proteins will become most valuable to get insight into the structural and functional relationship of ribosomal proteins.

Streptomycin (XVI): The first step forward in this field was made by Nomura's group, who identified the 30 S ribosomal protein controlled by the streptomycin-resistant locus in *E. coli.* The ribosomal proteins from both streptomycin-resistant and streptomycin-sensitive strains were fractionated. Reconstitutions were performed by combining fractions from the streptomycin-sensitive strain and mixing them with 16 S RNA under standard conditions for reconstitution. The streptomycin sensitivity of the resultant particle was assayed. It was found that a definite protein called P_{10} is responsible for the streptomycin phenotype [29]. This P_{10} protein seems to be involved as a control factor in the specificity of codon recognition [30].

Pactamycin (XVII), *kanamycin* (XVIII), *neomycin* (XIX), *bryamycin, oleandomycin* (XIa), *erythromycin* and *lincomycin* can probably be used as markers for different ribosomal proteins and thus may help to elucidate the functional role of these proteins. This conclusion is based on the results of Harford and Sueoka [31] who have mapped resistance loci on the genome of *Bacillus subtilis* (W 128) for these inhibitors of protein synthesis. Since different loci for each antibiotic have been obtained one should expect specific binding of the inhibitors to certain ribosomal proteins.

4.3. *Cytoplasmic and mitochondrial ribosomes*

Another fruitful and promising field opened when it became evident that yeast and eucaryotic systems contain two different types of ribosomes, mitochondrial and cytoplasmic ribosomes, which respond differently to certain antibiotics. In yeast, for example, the function of cytoplasmic ribosomes can be totally

inhibited by cycloheximide whereas this inhibitor does not affect mitochondrial ribosomes. On the other hand, chloramphenicol does not prevent cytoplasmic protein synthesis, but inhibits mitochondrial ribosomes specifically. Both inhibitors in combination have been used to investigate the synthesis of mitochondrial proteins in wild type yeast and in the respiration-deficient petite mutant under anaerobic conditions and during adaptation to oxygen [32, 33].

Promitochondria occur in wild type yeast and in the respiratory deficient mutant. Under anaerobic conditions, when cytoplasmic protein synthesis was totally blocked by cycloheximide, promitochondria of the wild type exhibit a chloramphenicol-sensitive protein synthesizing system, whereas the petite mutants failed to show any promitochondrial protein synthesis. During adaptation to oxygen, mitochondria arise via the differentiation of promitochondria, in both the wild type strain and the petite mutants. The mitochondria of the wild type strain differ from the mitochondria of the petite mutants. The mutants lack ribosomal RNA and are unable to synthesize protein. This *in vivo* technique establishes conclusively that the defect of the mutant mitochondria is not a consequence of their respiration deficiency or their increased lability.

References

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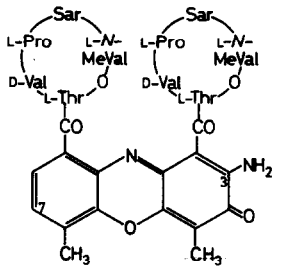
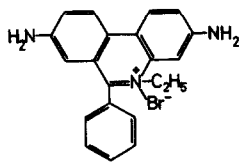
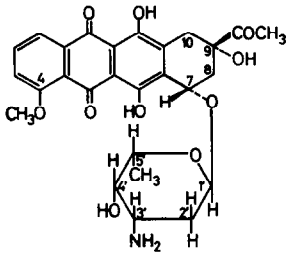
Appendix



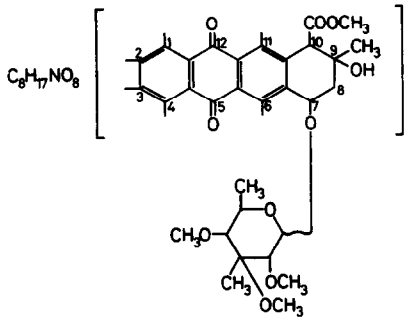
(I) Actinomycin C_1 (D). L-Thr, L-threonine; D-Val, D-valine; L-Pro, L-proline; Sar, sarcosine; L-*N*-MeVal, L-*N*-methylvaline.



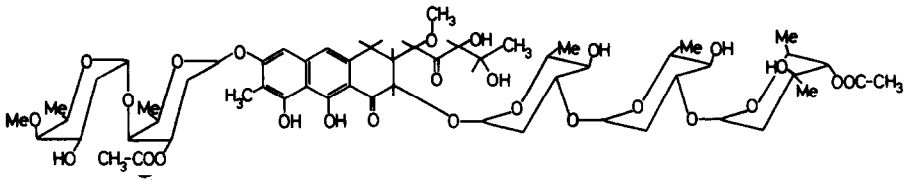
(II) Ethidium bromide.



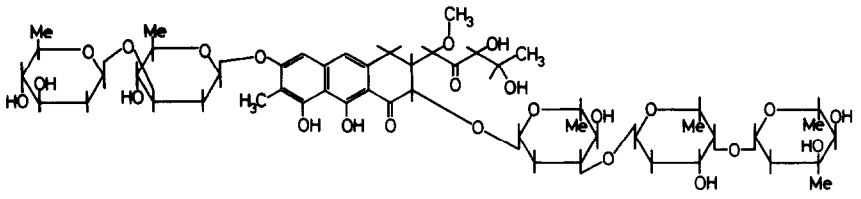
(III) Daunomycin.



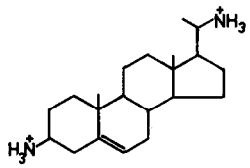
(IV) Nogalamycin.



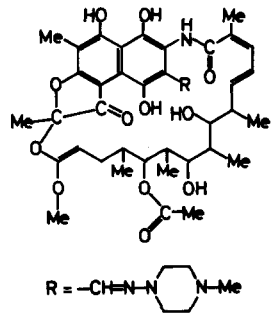
(V) Chromomycin A_3.



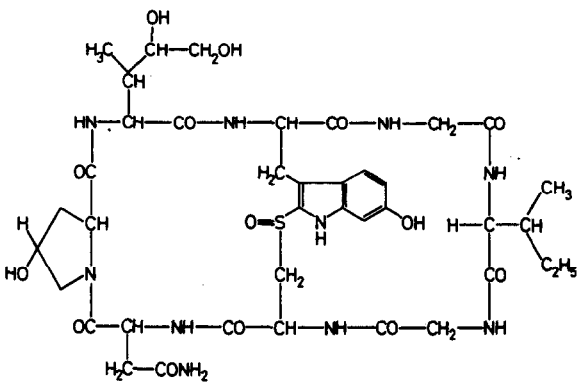
(VI) Mithramycin.



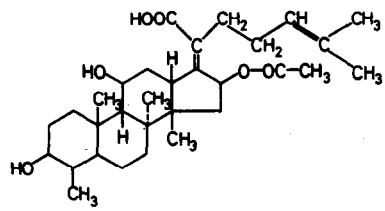
(VII) Irehdiamine A.



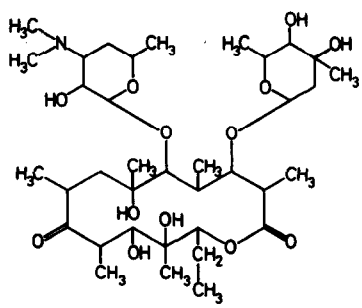
(VIII) Rifampicin.



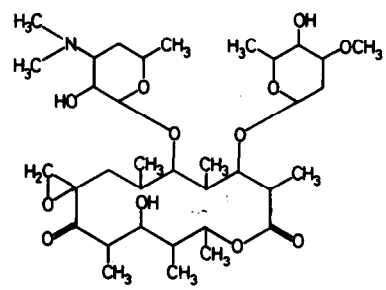
(IX) α-Amanitin.



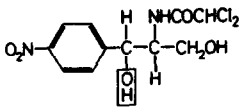
(X) Fusidic acid.



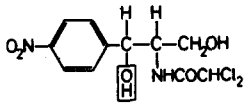
(XI) Erythromycin C.



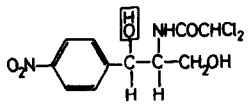
(XIa) Oleandomycin.



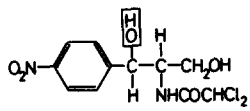
D(−) Threo isomer (Chloramphenicol)



L(+) Erythro isomer

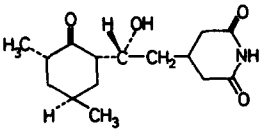


D(−) Erythro isomer

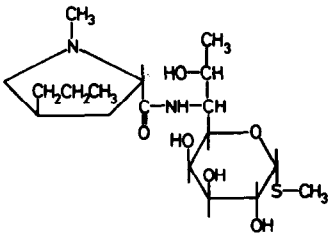


L(+) Threo isomer

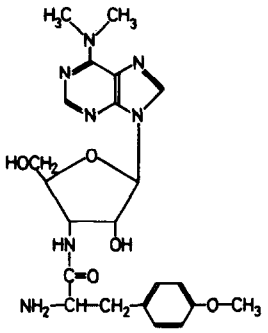
(XII) Chloramphenicol and its stereoisomers.



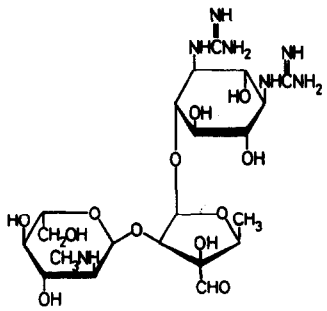
(XIII) Cycloheximide.



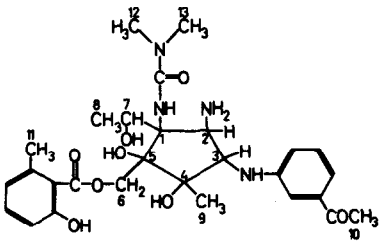
(XIV) Lincomycin.



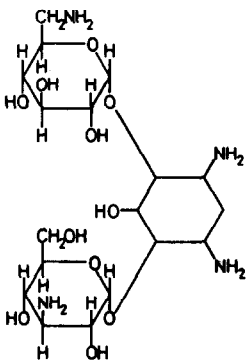
(XV) Puromycin.



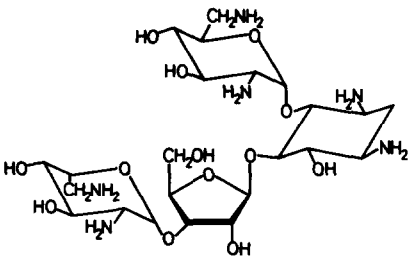
(XVI) Streptomycin.



(XVII) Pactamycin.



(XVIII) Kanamycin A.



(XIX) Neomycin B.