PONTIFICIAE ACADEMIAE SCIENTIARVM SCRIPTA VARIA

70

WORKING GROUP

ON:

MOLECULAR MECHANISMS OF CARCINOGENIC AND ANTITUMOR ACTIVITY

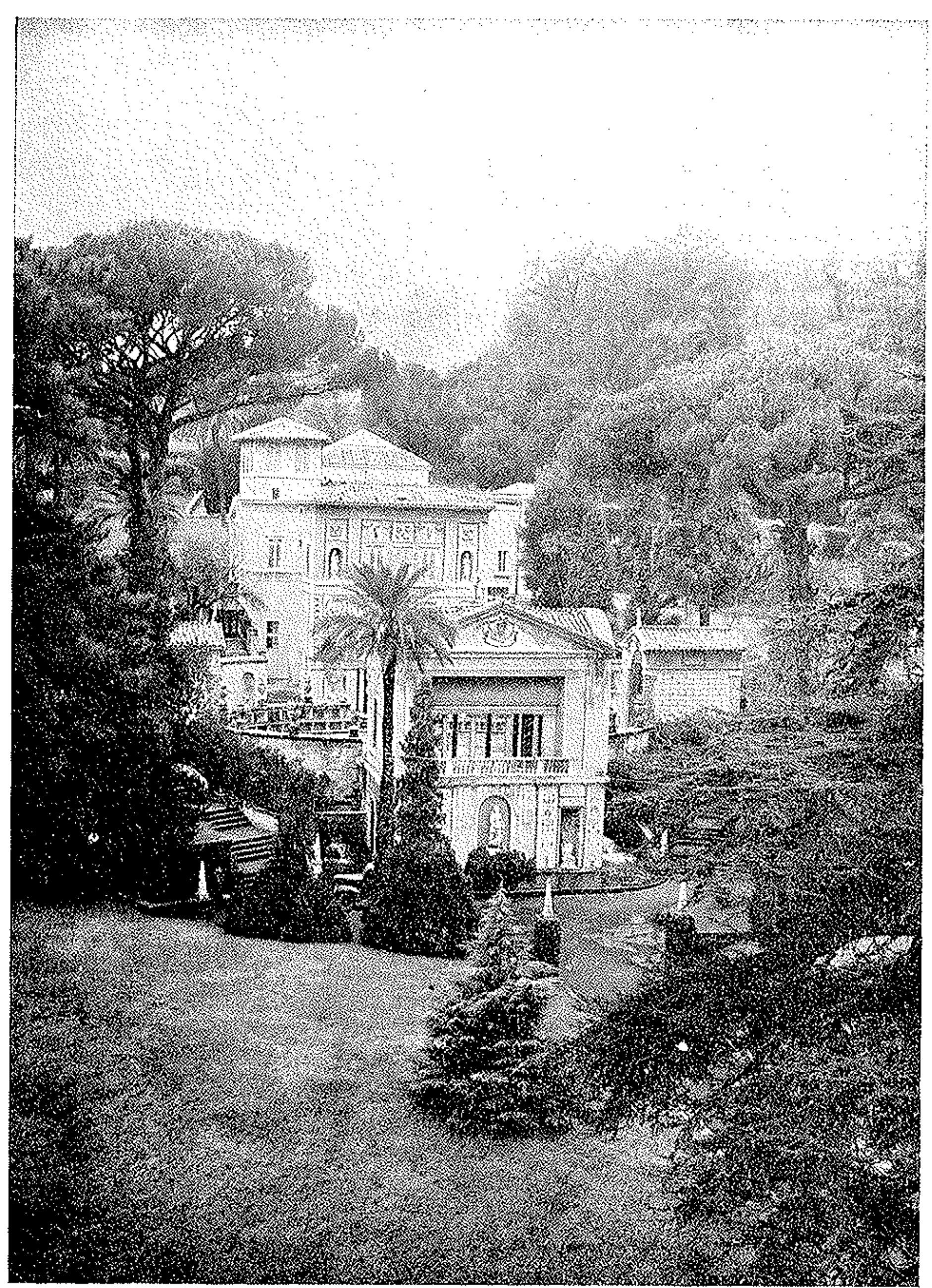
October 21-25, 1986

EDITED BY

CARLOS CHAGAS and BERNARD PULLMAN

EX AEDIBVS ACADEMICIS IN CIVITATE VATICANA

MCMLXXXVII



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FOREWORD

It is a great honour and pleasure for me to introduce the volume of "Scripta Varia" containing the papers presented at the Working Group on "The Molecular Mechanisms of Carcinogenic and Antitumor Activity", held at the Pontifical Academy of Sciences on October 21-25, 1986. I wish to express the deep thanks and appreciation of the Academy to all the distinguished scientists who have accepted our invitation and devoted a part of their valuable time to come to this meeting and to share with us their knowledge, plans, and hopes. I am particularly indebted to my friend Professor Bernard Pullman, Member of the Pontifical Academy, who has carried out brilliantly the heavy task of planning, organizing, and presiding over this Working Group.

Needless to say, the problem of cancer is of major preoccupation to humanity and the Pontifical Academy has on a number of occasions devoted to it some of its discussions. The most striking features of this Working Group are the dimension and the depth of the treatment of the problem, resulting in an unprecedented overview of its numerous and diversified facets. Thus, the presentations and discussions covered both the most fundamental molecular aspects of the mechanism of action of the drugs involved, the physical chemistry of their intracellular interactions, and also their biological and medical effects. They combined the study of both the carcinogenic and antitumor agents which share the common main target DNA, and sometimes the same site on this target. They concentrated significantly on the recent discoveries related to oncogenes and to technical advances in the attempts of establishing a possible control, through appropriate chemicals, of gene expression.

Although many discoveries in the fields of carcinogenesis or cancer chemotherapy have occurred, as in other fields of science, by haphazard

experimentation, the time appears ripe nowadays for new progress based on the detailed comprehension of the mechanisms involved in nucleic acid reactions and gene functioning. This rational approach may be expected to be a source of more economical and more rapid beneficial developments. It is my deep hope and profound wish that the Proceedings of this remarkable meeting will contribute to these developments.

The Holy Father, by receiving the members of the Study Group in a private audience, has manifested his strong interest in the work of this group as one of those whose efforts aim primarily at the alleviation of human sufferings. We thank him respectfully for this honour.

We wish to thank Farmitalia Carlo Erba S.p.A. in Milan, as well as the Institute of Scientific Research and Treatment of the "Bambino Gesù" Hospital in Rome, for their generous financial contribution to the publication of these Proceedings.

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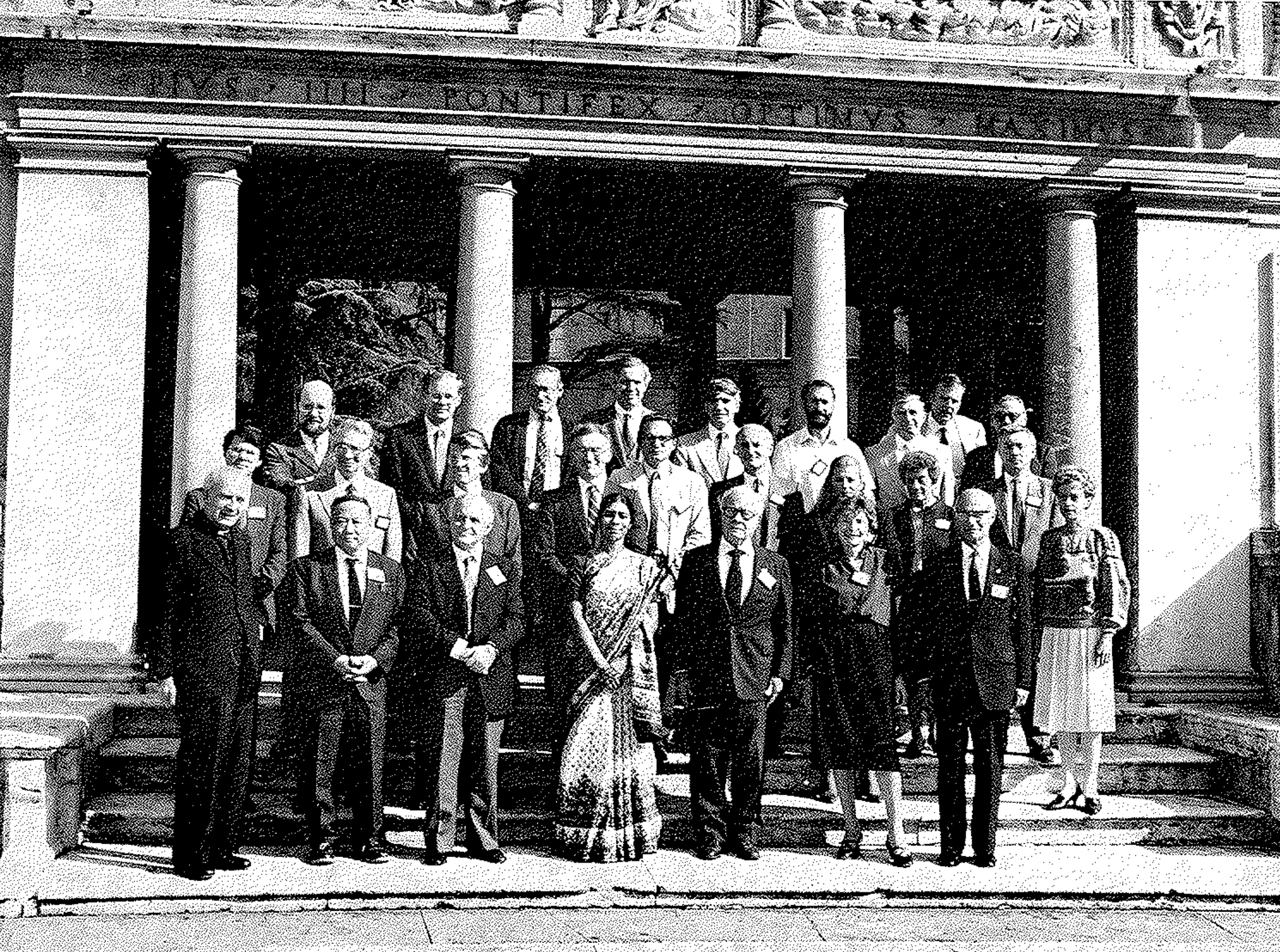

Participants in the Working Group

AUDIENCE OF THE HOLY FATHER

On October 23, 1986, His Holiness John Paul II granted an Audience in the "Sala dei Papi" of the Apostolic Palace in the Vatican to the participants in the Working Groupon "The Molecular Mechanisms of Carcinogenic and Antitumor Activity".

The group, introduced in the Apostolic Palace by the President of the Pontifical Academy of Sciences, His Excellency Prof. Carlos Chagas and accompanied by the Director of the Chancellery, Rev. Father Enrico di Rovasenda and by the Co-Director, Ing. Don Renato Dardozzi, was paternally received by His Holiness, who at the end of the Audience wished to greet personally all the participants.

The President of the Academy, Prof. Carlos Chagas, delivered the following address:

Holy Father,

I would lik to thank You once more for the generous attention with which Your Holiness follows the activities of Your Academy, and also convey the homage of the working group which is at present at a meeting at the Academy, organised by Pontifical Academician, Professor Bernard Pullman. Furthemore, in the name of all of us, I would like to wish You success in the gesture of assembling at Assisi, the religious chiefs of the world for a day of prayer for Peace.

The working group was convened to study the molecular activity of carcinogenic and antitumoral substances. Many discoveries have been made in the field of cancer research, among these it would be enough to cite the isolation of the oncogenes and the progress made in immunological reactions capable of identifying the carcinogenic state.

Progress has been enormous but it accentuated even more the com-

plexity of the problem. The efforts of thousands of scientists, many of whom sacrificed their lives so that this dreadful illness could be overcome, will be rewarded one day. Their zeal will most certainly achieve the cure for those who suffer from a disease the knowledge of which is intimately linked to the fundamental process that characterises cellular activity.

I gratefully thank You, Holy Father, for the interest with which You follow the activities of the Academy, over which I preside and beg You to extend Your Apostolic Benediction to all those who are dedicated to one of the most difficult of sicknesses, which only the tools of science and technology, provided to man by Divine Grace, can overcome.

The Holy Father answered with the following discourse:

Mr. President,
Ladies and Gentlemen,

For the third time in its history, the Pontifical Academy of Sciences directs its attention towards cancer, an illness that destroys the organism of a great number of human beings and is terrifying in the diversity of its forms.

In 1948 a study week was devoted to "The Biological Problems of Cancer". In 1977 another study week researched "The Role of Nonspecific Immunity in the Prevention and Treatment of Cancer". At the present meeting you have chosen the theme "Molecular Mechanisms of Carcinogenic Anti-tumor Activity".

The working group gathered this week in the Pontifical Academy of Sciences is composed of renowned scientists from around the world, who have devoted their activity to investigate, at the most fundamental level, the origins of cancer, the means to cure it and, if possible, the ways to prevent it.

You are coming from the most developed countries, which have the material means of conducting research at such a fundamental level and on such a large scale. The benefits of your work are, however, destined to all the world.

The particular feature of this working group is to combine, in the same exploration and discussion, the mechanism of action of carcinogenic and anti-tumor agents, those which cause the terrible disease and those which help to cure it.

The discussions thus bear on the suffering of man, but also on his efforts to find a remedy for it.

Another striking feature of this working group is that it tries to go into the very fundamentals of the problem by investigating the molecular mechanisms of the events which are responsible for the action of the carcinogenic and anti-tumor agents.

I wish to express my gratitude to the Pontifical Academy for having chosen such an important and urgent theme. I also thank the eminent scientists who have vigorously worked during these days. May your research achieve the results necessary to defeat this terrible scourge which is cancer.

God bless you and your families.

SCIENTIFIC PAPERS

The opinions expressed with absolute freedom during the presentation of the papers and in the subsequent discussions by the participants of the Working Group — although published by the Academy — represent only the points of view of the participants and not those of the Academy.

INTRODUCTORY LECTURE

INTRODUCTORY LECTURE
CARCINOGENS, ANTITUMOR AGENTS AND DNA

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1. Introduction

The idea of grouping together in the same exploration and discussion the molecular mechanisms of action of *carcinogenic* and *antitumor* drugs was inspired by the striking similitudes of some of the fundamental aspects of these mechanisms.

Thus, in the first place, both types of drug have as their main biological target the same essential biomacromolecule: DNA. Moreover carcinogens and antitumor drugs frequently attack selectively or at least preferentially the same type of base and, in a number of cases, the same site on that base. Thus, e.g., the carcinogenic polycyclic aromatic hydrocarbons (of which benz[a]pyrene is an oustanding example) (Jennette *et al.*, 1977; Nakanishi *et al.*, 1977), N-hydroxy-2-naphtylamine (Kadlubar *et al.*, 1980), 1′-hydroxysafrole (Phillips *et al.*, 1981), dehydroretronecine (Robertson, 1982) and the antitumor pyrrolo(1,4)benzodiazepines (e.g., anthramycin) (Hurley and Petrusek, 1979; Petrusek *et al.*, 1981) and saframycins (Lown *et al.*, 1982) all attack the NH_2 group of guanine. Similarly, the carcinogenic N-hydroxy-1-naphtylamine (Kadlubar *et al.*, 1978) and the antitumor mitomycin C (Tomasz *et al.*, 1983) both attack O6 of guanine. Also, the carcinogenic 9-anthryloxirane (Yang and Chang, 1985) and the antitumor agent CC-1065 (Hurley *et al.*, 1984; Reynolds *et al.*, 1985) both fix themselves on N3 of adenine. Similarly, a large number of simple alkylating agents whether carcinogenic (Singer and Grunberger, 1983) or antitumor (Wilman and Connors, 1983) attack

abundantly N7 of guanine. So do also a number of "huge" carcinogens such as aflatoxin B_1 (Croy *et al.*, 1978) or sterigmatocystin (Essigmann *et al.*, 1979) and the antitumor mitomycin C, when acid activated (Verdine and Nakanishi, 1985; Tomasz *et al.*, 1985) or *cis*-$Pt(NH_3)_2Cl_2$) (Robbins, 1973; Wing *et al.*, 1984). C8 of guanine seems to be the only important site of attack by carcinogens, e.g., acetylacetoxyaminofluorene (Poirier *et al.*, 1977; Yamasaki *et al.*, 1977) but which does not seem to be attacked by any antitumor drug.

What is unknown, however, in relation to the above enumerated reactions is whether the indicated *compositional* preference of carcinogenic and antitumor drugs for a certain type of purine or pyrimidine base and a precise site on that base coincides or not with a *positional* preference for the *same* base in a polynucleotide chain. Important recent developments, experimental and theoretical, in the analysis of the dependence upon base sequence of the specificity of interaction with DNA of a series of carcinogenic and antitumor drugs open wide prospects for the possible elucidation of this problem in the near future. Footprinting and affinity cleaving techniques seem particularly promising in this respect. Outstanding examples of such studies concern in the field of carcinogens benz[a]pyrene (Boles and Hogan, 1986), aflatoxin B_1 (Wang and Cerutti, 1980; Groopman *et al.*, 1981; Muench *et al.*, 1983; Furois-Corbin *et al.*, 1984), N-methyl-N-nitrosourea (Briscoe and Cotter, 1984, 1985; Furois-Corbin and Pullman, 1985), N-ethyl-N-nitrosourea (Nehls *et al.*, 1984), various nitrogen mustards (Mattes *et al.*, 1986), N-acetoxy-N-2-acetylaminofluorene (Fuchs, 1983) and in the field of antitumor drugs the sequence specific recognition of DNA by large groove-binding agents of the family of distamycin (Schultz and Dervan, 1984; Youngquist and Dervan, 1985a, b), or by the mixed covalent-groove binding agents anthramycin (Hertzberg *et al.*, 1986) and CC-1065 (Hurley *et al.*, 1984; Reynolds *et al.*, 1985; Needham-Van Devanter *et al.*, 1984; Krueger *et al.*, 1985). As an outcome of these procedures there appears the large possibility of design of sequence-specific DNA-binding new drugs (see e.g., Dervan, 1986).

The same problem appears in the study of the positional base specificity with respect to a number of reactants in tRNA's (Lavery and Pullman, 1984; Furois-Corbin and Pullman, 1985). It may also underlie the point mutation (of a specific guanine into thymine) in the process of activation of the oncogen in T24 human bladder carcinoma cells (Reddy *et al.*, 1982; Tabin *et al.*, 1982).

Another common feature in the mechanism of action of carcinogens and antitumor drugs is the involvement of a large number of these substances in the form of electrophilic agents. In fact, as concerns carcinogens, their active forms (the so-called proximate or ultimate carcinogens) are practically always cationic species, this being true both for carcinogens considered as being active per se (a number of simple alkylating agents) or those which necessitate a metabolic activation (aromatic hydrocarbons, aromatic amines, aflatoxin B_1, etc.) (for a review see Pullman, 1979). The same situation prevails for such important antitumor drugs as proflavine, its derivatives or analogs, the anthracyclines (daunomycin, adriamycin, etc.), mitoxantrone, ellipticine, netropsin, distamycin and analogs, tilorone, irehdiamine, saframycins, mitomycin C etc. This *electrophilic* nature of all these attacking species suggests the probable significance in their interaction with DNA of the nucleophilicity of the reactive centers at this target and points thus to a probably very important role in both cases of electrostatic factors in their mutual affinity (see e.g., Pullman and Pullman, 1980; Pullman B., 1984).

These analogies in the physico-chemical aspects of the mechanism of action of carcinogenic and antitumor drugs do not prevent striking differences from existing also. The most significant such difference could possibly be that while practically *all carcinogens interact with DNA by covalent bond formation* (even if for some of them, e.g., benz[a]pyrene, their mechanism of interaction with DNA may involve an intercalating step) (Geacintov *et al.*, 1981; Meechan *et al.*, 1982), *the antitumor drugs display, to a nearly equivalent degree, three different modes of interaction* with DNA: 1) covalent bond formation, 2) intercalation and 3 non-intercalative groove binding. In distinction to the strong *chemical* interaction represented by the covalent bond formation, shared with carcinogens, the two remaining modes of interaction consist of significantly weaker, although in no way less biologically efficient, physical associations. The distribution of the main antitumor agents among the three categories is illustrated in Table I. Some of them may exhibit a double behavior. Thus, e.g., CC-1065 is at the same time covalently bound to N3 of adenine and interacts physically with a segment of the minor groove of DNA (Hurley *et al.*, 1984; Reynolds *et al.*, 1985). Also while intercalation is considered as being the most significant mechanism for the interaction of the anthracyclines, daunomycin and adriamycin, with DNA (Pigram *et al.*, 1972; Quigley *et al.*, 1980; Arcamone, 1984; Arcamone *et al.*, 1985; Chen *et al.*, 1985), these com-

TABLE I - *DNA - Antitumor Drug Interactions.*

Mode of binding	Representing compounds	Primary target site
1) COVALENT	Bifunctional alkylating agents	N7 (G)
	Pyrrolo-1,4-benzodiazepines: anthramycin	NH_2 (G)
	Mitomycin C	O6 (G)
	Saframycins	NH_2 (G)
	CC-1065	N_3 (G)
		N7 (G)
2) INTERCALATION	Anthracyclines: Daunomycin, Adriamycin	
	Actinomycin D	
	Acridines	
	Mitoxantrone	
	Tilorone	
	Ellipticine	
	Bleomycin	
	Neocarzinostatin	
BISINTERCALATION	Acridines	
	Quinoxaline antibiotics (Echinomycin, Triostin, Tandem)	
3) NON-INTERCALATIVE GROOVE BINDING	Netropsin	
	Distamycin	Minor groove, AT sequences, B-DNA.
	Bisquartenary Ammonium Heterocycles	
	Bisguanylhydrazones	
	Diarylamidines	
	Olivomycin, Mithramycin	Major groove, GC sequences.
4) ?	Streptonigrin	

pounds have also been claimed to form a covalent bond with this biopolymer (Sinha and Chignell, 1979).

With these fundamental analogies and differences as a background for an overall discussion, the study of the mechanism of action of the carcinogenic and antitumor drugs raises a large number of more precise questions and problems, which, I am sure, will come into the forefront during this meeting. Two such problems which appear of predominant

importance to me are: 1) The elucidation of the factors governing the origin of the different observed *specificities* in the interaction of both carcinogens and antitumor drugs with the nucleic acid receptor and the significance of these specificities for their mechanism of action and 2) the nature of the physico-chemical and biological *consequences* of the different types of interaction on the structure, properties and function of the target DNA. In our laboratory we have explored primarily during the recent years the first of these problems and we did it in relation to the three modes of interaction enumerated above, namely the two physical ways of association, non-intercalative groove binding and intercalation, and the chemical mode of association through the formation of a covalent bond. I propose now to present to you some of our essential results in this field.

2. Factors Governing the Specificity of Interaction of non-Intercalating Groove Binding Antitumor Ligands with DNA.

Figure 1 represents some of the most representative and best known compounds of this series. The striking feature of their mechanism of interaction with DNA is a triple specificity of binding to 1) the minor groove, 2) of AT rich sequences, 3) of B-DNA. However, while the experimental evidence on this preference is well substantiated (see e.g., Zimmer, 1975, 1983; Zimmer and Luck, 1984; Zimmer and Wähnert, 1986), the associated interpretations, based on some apparent structural features of the two most studied compounds of the series, netropsin and distamycin A, are generally oversimplified, if not partly mistaken.

Thus the current postulate is that this specificity is due essentially to hydrogen bond formation between the peptidic NH groups of the drugs and the O2 atoms of thymine and/or N3 atoms of adenine of DNA. The charged end groups are considered to be also involved in the interaction, with the phosphate groups of DNA by some, in a less specified way by others.

That the situation is more complicated than this simple picture suggests and that, in particular, the precise role of the hydrogen bonds has to be reconsidered becomes evident from the examination of other molecules studied. Thus, in particular the bisquaternary ammonium heterocycle SN 18071, which has no hydrogen bonding possibilities, binds also to DNA and shows a similar AT minor groove specificity (Braithwaite and Baguley, 1980; Baguley, 1982). The role of the charged end group as factors of specificity is also questionable since it has been shown that a netropsin

Netropsin

Distamycin A (Dist 3)

Distamycin 2

SN 18071

NSC-101327

Fig. 1. Representative non-intercalating groove binding antitumor drugs.

derivative with both ends removed, also complexes to poly(dA)·poly(dT) (Zimmer, 1975).

An indication that the source of the specificity common to all these diverse drugs (and many others) may reside to a large extent in the properties of the minor groove of AT sequences of B-DNA, rather than in special features of the drugs, was suggested by our finding (Pullman and Pullman, 1981) that the grooves were the sites of location of the deepest molecular electrostatic potential in DNA and that for AT sequences the deepest values occur in their minor groove. One could conceive then that provided that this groove could also offer an appropriate steric fit to the drugs involved, the secret of specificity could simply reside in the combination of this fit with a corresponding strong electrostatic interaction.

Due to the complexity of the system, the problem was investigated in a number of consecutive steps, involving increasing degrees of computational refinements (Zakrzewska *et al.*, 1983, 1984, 1986). We shall summarize here briefly some of the essential results.

In the first study of the problem (Zakrzewska *et al.*, 1983) the interaction energy of a large number of compounds of figure 1 with model poly(dA)·poly(dT) and poly(dG)·poly(dC) duplexes in B-DNA conformation (with the phosphates screened by Na^+ ions), was computed taking into account the electrostatic and Lennard-Jones components, following:

$$E_V = \sum_{i,j} (q_i q_j / r_{ij} - B_{ij}/r^6_{ij} + A_{ij}/r^{12}_{ij})$$

where q_i and q_j are Hückel-Del Re type charges reparametrized to reproduce the electrostatic potentials of the nucleic acid subunits calculated with the use of Overlap Multipole Expansions derived from *ab initio* wave functions and where the parameters A_{ij} and B_{ij} depend on the class of the atoms i, j considered, r_{ij} being the distance between them. These parameters have been evaluated by the systematic proposed by Zhurkin *et al.* (1980).

The Lennard-Jones energy term may be considered as a measure of the quality of steric fit between the ligand and the macromolecule because of its strong distance dependence which causes it to become favourable only for very close interactions, but also to rapidly become extremely repulsive if any close contacts are produced. Its significant values signify a good fit.

The conformational adaptability of the drug was allowed for but not that of the macromolecule.

The results presented in table II invariably show, *for all the compounds investigated*, that the greatest values of Ev are obtained for interaction with the minor groove of the AT sequences. This preference is favoured both by the electrostatic and the Lennard-Jones energy terms. This demonstrates that whatever the significance of hydrogen bonds for the stability of the complex, the *formation of these bonds is not necessary either for binding or for the preference for the minor groove of the AT sequences of B-DNA*. It seems that if a relatively good steric fit can be obtained in the minor groove the ligand will be sufficiently stabilized there by the favourable potentials generated by the AT sequences.

When possible, hydrogen bonds between the proton donating sites of the ligand and the proton accepting sites of the macromolecule are, of

TABLE II - *Interaction energies for the formation of DNA-ligand complexes* (i vacuo) (kcal/mole) (Zakrzewska *et. al.*, 1983).

Ligand	DNA			Interaction energy			
	confor-mation	se-quence	groove	Electro-static	Lennard Jones	Ligand conforma-tional energy	Total
Netropsin	B	AT	Min.	−203.5	−42.3	7.6	−238
			Maj.	−104.4	−19.5	15.3	−108
		GC	Min.	−155.3	−34.1	8.2	−181
			Maj.	−138.0	−15.6	17.2	−136
SN 18071	B	AT	Min.	− 86.4	−31.1	9.8	−107
			Maj.	− 49.8	−21.3	9.8	− 61
		GC	Min.	− 72.2	−25.8	9.8	− 88
			Maj.	− 65.6	−21.3	9.8	− 77
Distamycin 3	B	AT	Min.	− 95.9	−48.8	15.3	−129
		GC	Min.	− 77.8	−42.4	18.0	−102
Distamycin 2	B	AT	Min.	− 94.1	−36.7	16.6	−114
		GC	Min.	− 75.6	−30.9	18.5	− 88

course, formed and contribute to the energy of binding. This formation is visualized in figure 2 for netropsin and distamycin, from which it may be inferred that the hydrogen bonds between the drug and the receptor should be stronger at the extremities of the former than in its central part. The results show on the other hand that the charged ends of the ligands are well in the groove and do not exhibit direct interaction with the phosphates.

These interesting features of the interaction were confirmed recently by an X-ray study of the crystal structure of the complex between netropsin and the double helical DNA dodecamer $CGCGAATT^{Br}CGCG$ (Kopka *et al.*, 1985).

The results presented in table II, while satisfactory with respect to the basic problem of specificity, are less so when other aspects of the interaction are considered. In particular the values of the binding energies obtained are very high, of the order of 1-2 hundreds kcal/mole, while the experimental values may be expected to be much smaller. In fact, a recent determination of the enthalpy of binding of netropsin indicates a value of - 9.2—11.2 kcal/mole (Marky *et al.*, 1983, 1985).

The reasons for this discrepancy are easy to determine: they are due to the fact that the previous results were obtained for binding in vacuum while the experimental results refer to situation in solution. The fact that the free space computations account correctly for the observed specificity signifies that it is an *intrinsic property of these associations.* The understanding of the further aspects of the association needs, however, obviously the introduction of the solvent effect.

This refinement has been introduced in a variety of ways (Pullman, 1984; Zakrzewska *et al.*, 1984; Lavery *et al.*, 1986). One of them (Lavery *et al.*, 1986) consisted of modeling the effect of the solvent by the use of a distance dependent dielectric function. This computation (which included the flexibility of DNA and mobile counterions) was performed only for binding to the minor groove of poly(dA)·poly(dT) in the B conformation. The results are shown in table III in which the complexation energy E_C of the ligands studied is given by:

$$E_C = \Delta E^{DNA} + \Delta E^{LIG} + E^{DNA\text{-}LIG}$$

where ΔE^{DNA} is the distorsion energy of DNA, ΔE^{LIG} the distorsion energy of the ligand and $E^{DNA\text{-}LIG}$ the interaction energy between the ligand and DNA.

FIG. 2. Hydrogen bond formation between netropsin or distamycin A and receptor atoms in the minor groove of AT sequences of DNA.

TABLE III - *Summary of the energy terms for the optimal antibiotic complexes with* poly(dA)·poly(dT) *in the B conformation* (all values in kcal/mole), *with the inclusion in the computations of a distance dependent dielectric constant* (Lavery e tal., 1986).

LIGAND	ΔE^{DNA}	$\Delta E^{DNA\text{-}LIG}$	ΔE^{LIG}	E_c
SN-18071	21.9	0.3	− 49.3	− 27.1
NSC-101327	21.5	1.9	− 69.1	− 45.7
Distamycin-2	21.4	6.4	− 70.7	− 42.9
Distamycin-3	21.4	4.9	− 85.2	− 58.9
Netropsin	22.6	5.9	− 87.2	− 58.7

One notes immediately the relatively large but roughly constant DNA distorsion energy. On the other hand, the ligand distorsion energies and DNA-ligand interaction energies show stronger variations. SN 18071 and NSC 101327 which cannot easily bend to follow the shape of the DNA groove and have little chance of forming hydrogen bonds (none in the case of SN 18071) have small ligand distorsion energy, while for the remaining antibiotics, which bend easily and form multiple interactions, this distorsion is considerably greater. The weakest interaction is calculated for SN 18071 which cannot form hydrogen bonds with DNA and cannot distort enough to have its charged terminal rings deep in the minor groove. NSC 101327 is both longer and somewhat more flexible and it forms two hydrogen bonds from its inner peptide groups to an Ade (N3) and a Thy (O2) and this leads to a clear increase in its interaction. Distamycin 2 is still more strongly bound to DNA despite the fact that it has one positive charge less than the preceding ligands. This reflects an increase in flexibility and in hydrogen bonding capacity. In fact, distamycin 2 forms hydrogen bonds from each of its peptide groups and from its terminal propio-amidinium group to successive Thy (O2) atoms. Distamycin 3 binds in a similar way. Netropsin has the highest interaction energy which is connected with its high hydrogen bond forming possibilities, high flexibility and double positive charge.

It is instructive to remark that the ordering of netropsin, NSC 101327 and SN 18071 is in good agreement with recent experimental results on the competitive interactions of these antibiotics with poly(dA)·poly(dT) (Zimmer *et al.*, 1984 . This interaction confirms that hydrogen bonds be-

tween the ligand and DNA, while not indispensable either for the binding or for the specificity, enhance, when available, the interaction energies.

The binding energies as listed in table III, although smaller than those obtained in table II, are still significantly above the plausible experimental values (as can be judged from the known case of netropsin, quoted above). A better agreement between the two sets of numbers, in fact a rather satisfactory one, can be obtained by an explicit introduction of the solvent water, by a mixed discrete-continuum procedure, as carried out in Zakrzewska *et al.* (1984). It involved a discrete representation of water molecules which are directly bound to the entities in interaction, and the addition of the effect of the bulk solvent by a cavity treatment, following the procedure of Halicioglu and Sinanoglu (1969).

The binding energy of the complex in solution E_W is then equated to:

$$E_W = E_V + \Delta H + \Delta C$$

where E_V is the interaction energy in vacuum,

$$\Delta H = H_{comp} - (H_{DNA} + H_{lig})$$

with H_{comp}, H_{DNA} and H_{lig}, respectively, the hydration energies of the complex, of DNA and of the ligand and where:

$$\Delta C = C_{comp} - (C_{DNA} + C_{lig})$$

with C_{comp}, C_{DNA} and C_{lig}, respectively, the cavity energies of the complex, of DNA and of the ligand.

The principal results of this evaluation of the interaction energies in water are summed up in table IV.

It is immediately seen that the new results:

1) confirm the preference of the compounds for binding to the minor groove of AT sequences. The intrinsic preference, visible already in vacuum, remains dominant in water;

2) reduce strongly the absolute values of the interaction energies, bringing, for example, that of netropsin within the range of the experimental determination.

Two more interesting observations may be made:

The first one pertains to the possible binding of these ligands, at least some of them, to some extent, to the GC sequences of B-DNA. This

TABLE IV - *Solvent corrected DNA-ligand binding energies* (kcal/mole (Zakrzewska *et al.*, 1984).

Ligand	DNA confor-mation	DNA se-quence	DNA groove	Interaction energy E_V	ΔH	ΔC	E_W
Netropsin	B	AT	Min.	−238.2	380.7	−159.2	− 16.7
			Maj.	−108.7	322.2	− 98.5	115.0
		GC	Min.	−181.2	408.7	−147.5	80.0
			Maj.	−136.4	336.2	− 80.5	119.3
SN 18071	B	AT	Min.	−107.7	214.6	−127.0	− 20.1
			Maj.	− 61.3	193.0	− 89.0	42.7
		GC	Min.	− 88.2	188.4	− 96.4	3.8
			Maj.	− 77.1	147.8	− 65.1	5.6
Distamycin 3	B	AT	Min.	−129.4	265.5	−163.7	− 27.6
		GC	Min.	−102.2	233.3	−139.3	− 8.2
Distamycin 2	B	AT	Min.	−114.2	212.4	−125.7	− 27.5
		GC	Min.	− 88.0	218.7	− 97.8	32.9
Netropsin	A	AT	Min.	− 77.6	248.9	− 71.3	103.0
			Maj.	− 48.7	327.7	−136.4	142.6
		GC	Min.	− 58.6	152.1	− 64.6	28.9
			Maj.	− 64.6	307.8	−143.6	99.6

problem has recently gained interest with the discovery that while distamycin 2 does not bind to GC sequences, distamycin 3 does so slightly (Zimmer *et al.*, 1984). The theoretical data of table IV agree fairly well with the experimental observations: while the two distamycins have a rather similar affinity for the AT sequences, their E_W for the interaction with the GC sequences is positive (repulsive) for distamycin 2, but negative (attractive), although small, for distamycin 3.

The second remark concerns the specificity of binding of the non intercalating ligands mentioned here to B-DNA. They quite generally do

not bind to A-DNA. The problem was investigated on the example of netropsin, and the corresponding results in table IV show that E_W for the interaction of this ligand with A-DNA is always positive, it means repulsive.

The only disturbing data in table IV is the relatively large value of E_W for SN 18071. It must, however, be realized that the E_W values are small differences between three large contributions and therefore can easily lead to such distorsions.

Similar studies were performed for the related antitumor drugs berenil and stilbamidine (Zakrzewska *et al.*, 1983, 1984; Gresh and Pullman, 1984a), an aliphatic and an aromatic bisguanylhydrazone, methylglyoxalbisguanylhydrazone and parabenzylbisguanylhydrazone (Gresh and Pullman, 1984b) and 4′,6-diamidino-2phenylindole (DAPI) (Gresh, 1985).

3. Factors Governing the Sequence Selective Intercalation of Antitumor Drugs into DNA.

This problem was investigated in our laboratory on the example of representative anthracycline antibiotics, daunomycin (Chen *et al.*, 1985) and adriamycin (Chen *et al.*, 1986a) (Fig. 3), important because 1) they

ADRIAMYCIN R= OH

DAUNOMYCIN R= H

Fig. 3. Daunomycin and adriamycin.

are among the most active antitumor antibiotics known, 2) the intercalative mechanism of their interaction with DNA is well established (Pigram *et al.*, 1972; Quigley *et al.*, 1980; Arcamone, 1984; Valentini *et al.*, 1985; Neidle and Sandersen, 1983) and 3) the assessment of the base sequence preference, if any, for their interaction was the subject of numerous experimental studies carried out with both natural and synthetic polynucleotides with conflicting results. We shall center here our attention on daunomycin (DM) and consider only some of the most representative results. Early (Phillips *et al.*, 1978) thermal denaturation studies, spectrophotometric analysis and inhibition of polymerisation reactions indicated equal binding affinities of DM for poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT) but a significantly greater affinity for these alternating copolymers than for the poly(dA)·poly(dT) homopolymer. Competition dialysis experiments (Chaires *et al.*, 1982) indicated a preference of DM for G-C rich DNA's and were originally interpreted as indicating a preference of DM for the GC base pair. Somewhat later (Chaires, 1983) fluorescence and absorbance study from the same laboratory, carried out with synthetic polynucleotides, established, however, that their intrinsic binding constants with DM decreased in the order: poly(dA-dT)·poly(dA-dT) > poly(dG-dC)·poly(dG-dC) > poly(dG)·poly(dC) > poly(dA)·poly (dT). The apparent conflict between the results found for the synthetic polynucleotides and DNA was tentatively explained by observing that the binding to native DNA sequences must represent an average property weighted by the relative frequency of the various types of binding sites. Now, the results with the synthetic oligonucleotides show that DM binds best to alternating AT sequences but poorly to non-alternating such sequences. In contrast, its affinity for alternating and non-alternating GC pairs is nearly the same. Overall, therefore, — so it was deduced — the binding constants for the two types of AT sites must average to a lower value than the averaged binding constant for GC site, leaving an apparent preference for GC rich DNA's. We shall see later that, however elegant this interpretation is, it may only represent a partial answer to the problem.

Meanwhile, a valuable crystal X-ray diffraction study of a DM-$d(CGTACG)_2$ complex (Quigley *et al.*, 1980) provided the first explicit information about the stereochemistry of the interaction, such as the structural features of DM involved in DNA binding and the conformational changes undergone upon the DNA-oligomer complexation. In this complex *DM intercalates between the CG base pairs* of the double helix, a result

which seems thus in conflict with the previous preferences deduced from the study of the interaction with "regular" oligonucleotides.

Previous theoretical attempts were unable to solve this dilemma. Extensive computations by Newlin *et al.* (1984) on the interaction complexes of DM with tetramer DNA duplexes distinctly indicated a preference of the drug for intercalation between AT base pairs. In these conditions the authors suggested that the observed intercalation between the GC base pairs in the DM-d(CGTACG)$_2$ complex could be the result of crystal packing forces and proposed that it would be interesting to observe whether intercalation would occur between the TA base pairs in d(TACGTA)$_2$. Although they do not state it explicitly, it seems obvious from their work that they would expect this to happen. We shall consider later our answer to this query.

We have investigated the problem of the base sequence specificity of intercalation of daunomycin by considering the six following self-complementary double-stranded hexanucleotides:

I : d(CGTACG)$_2$; II : d(CGATCG)$_2$; III : d(TGATCA)$_2$; IV : d(TATATA)$_2$; V : d(CGCGCG)$_2$; VI : d(TACGTA)$_2$.

of which the first is the one whose complex with DM has been resolved crystallographically. This crystallographic complex will serve as an overall model of the interaction in the sense that, corresponding to the location of a DM molecule in the crystal structure, the intercalation will be considered in all these complexes at a similar site, namely between base pairs 6-1′ and 5-2′ of the double helices (Fig. 4).

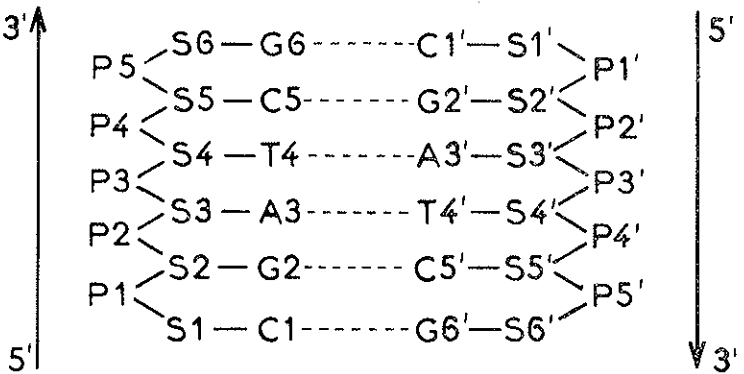

FIG. 4. Notations for the intercalation complexes of daunomycin with DNA.

In all the computations, the conformation of the hexanucleotides was maintained in the conformation found crystallographically in the $d(CGTACG)_2$-DM complex. The conformation of DM was however re-optimized upon binding in each case.

The computations were performed by the SIBFA method (Sum of Interactions Between Fragments computed ab initio) (Gresh *et al.*, 1984, 1985) which takes into account all the principal components of the intra- (for DM) and intermolecular energy of interaction: electrostatic, polarization, dispersion, repulsion and charge transfer, calibrated in a highly refined way for their coherent simultaneous computation. The authors have also introduced a component measuring the energy expense necessary to unstack the base pairs at the intercalation site (for details see Gresh *et al.*, 1984, 1985 and Chen *et al.*, 1985, 1986).

The results of the computations on the DM-DNA complexes are reported in table V, which lists, for all investigated sequences, the intermolecular interaction energy ΔE_{inter}, its components, (electrostatic E_{MTP}, polarization E_{pol}, charge-transfer E_{CT}, dispersion E_{disp} and repulsion E_{rep}), the conformational energy change of DM, ΔE_{conf}, the unstacking energy of the two base pairs of the intercalation site $\Delta E_{unstack}$, the overall energy balance $\delta E = \Delta E_{inter} + \Delta E_{conf} + \Delta E_{unstack}$, and the difference δ of these overall energy balances with respect to the best value of δE taken as energy zero.

The results of table V indicate the following ordering of sequence preference for DM complexation:

$$\underset{II}{d(CGATCG)_2} \geqslant \underset{I}{d(CGTACG)_2} > \underset{III}{d(TGATCA)_2} \geqslant \underset{IV}{d(TATATA)_2} >$$

$$\underset{V}{d(CGCGCG)_2} > \underset{VI}{d(TACGTA)_2}$$

It may be noticed that the values of ΔE_{inter} are paralleled by the corresponding values of E_{MTP} which represent by far the preponderant contribution to the binding.

The most striking observation to be deduced from these results is that while among the two regularly alternating oligonucleotides, $d(TATATA)_2$ and $d(CGCGCG)_2$, a stronger binding is predicted for the former, in agreement with experimental results obtained with poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC), the strongest complexes are, however, computed for the mixed oligonucleotides $d(CGATCG)_2$

TABLE V - *Interaction energies of daunomycin with sequences I-VI* (see text fo definitions). *Energies in kcal/mole.*

Hexanu-cleotide	d(CGT-ACG)$_2$	d(CGA-TCG)$_2$	d(TGA-TCA)$_2$	d(TAT-ATA)$_2$	d(CGC-CGC)$_2$	d(TAC GTA
ΔE_{inter}	−433.5	−433.9	−429.9	−428.8	−416.4	−413
E_{MTP}	−369.2	−369.2	−366.7	−366.8	−353.6	−350
E_{pol}	− 20.8	− 20.3	− 19.9	− 21.5	− 20.9	− 21
E_{CT}	− 14.0	− 14.0	− 13.2	− 11.0	− 15.2	− 13
E_{disp}	− 85.9	− 86.4	− 85.4	− 82.8	− 88.6	− 86
E_{rep}	56.3	56.1	55.2	53.3	61.8	58
ΔE_{conf}	2.6	2.6	2.5	2.6	2.7	2
$\Delta E_{unstack}$	10.5	10.5	10.5	9.5	10.5	9
δE	−420.4	−420.8	−417.2	−416.8	−403.2	−401
δ	0.4	0.0	3.6	4.0	17.6	19

and d(CGTACG)$_2$, containing the intercalation site between two CG base pairs and an adjacent TA base pair. This situation may be related to the increased affinity of the anthracyclines for GC rich DNA's and to the situation in the crystal structure of the DM-d(CGTACG)$_2$ complex. These results provide thus a solution to the *apparent* contradiction between the experimental results obtained with the synthetic polynucleotides as opposed to natural DNAs.

The explanation of this situation is obtained from a detailed analysis, of the intrinsic base sequence preferences expressed by the individual constituents of the anthracycline: the daunosamine side chain, the chromophore ring and its two 9-hydroxy and 9-acetoxy substituents which reveals that the overall sequence preference found is the result of a rather intricate interplay of partial sequence preferences shown by these constituents. Among those no stringent sequence preference is manifested by the chromophore ring or the 9-acetoxy substituent so that the major effects are due to the daunosamine and the 9-hydroxyl group.

The binding preferences of daunosamine rank in the order:

$$\underset{IV}{d(TATATA)_2} > \underset{I}{d(CGTACG)_2} > \underset{II}{d(CGATCG)} > \underset{III}{d(TGATCA)_2} >$$

$$\underset{VI}{d(TACGTA)_2} > \underset{V}{d(CGCGCG)_2}$$

Sequences V and VI are considerably disfavoured with respect to sequences I-IV due essentially to the repulsive interaction of daunosamine with the 2-amino group of G_4 in V and VI.

On the other hand the binding preferences of the 9-hydroxy substituent rank in the order:

$$\underset{\text{II}}{d(CGATCG)_2} > \underset{\text{III}}{d(TGATCA)_2} > \underset{\text{I}}{d(CGTACG)_2} > \underset{\text{V}}{d(CGCGCG)_2} >$$

$$\underset{\text{IV}}{d(TATATA)_2} = \underset{\text{VI}}{d(TACGTA)_2}$$

This substituent manifests thus a definite preference for those sequences which comprise a $G_{2'}$ base as a result of hydrogen bonding interaction involving the hydroxyl oxygen and one of the 2-amino hydrogens of this guanine. This produces an overall preferential affinity of DM for sequences I, II and III and the preference of V over VI.

It becomes thus evident altogether from these studies that the sequence specificity of DM in its interaction with oligo- or polynucleotides cannot be simply described in terms of the two base pairs which compose the intercalation site of the chromophore. Its specification needs to be carried out at the level of *triplets of base pairs*, comprising in addition to the base pairs 6-1′ and 5-2′ of the intercalation site, the neighbouring 4-3′ base pair (Fig. 2). Limiting our considerations to such triplets in the model oligonucleotides studied, and among them to those susceptible to occur in DNA, we can rank these in the following order of decreasing affinity towards DM:

	3′ 5′										
	G_6-$C_{1'}$		G-C		A-T		A-T		G-C		A-T
intercalation→ site	C_5-$G_{2'}$	≫	C-G	>	C-G	>	T-A	≫	C-G	>	T-A
	T_4-$A_{3'}$		A-T		T-A		A-T		G-C		G-C
	5′ 3′										

Similar results have been obtained for the binding of adriamycin to DNA (Chen *et al.*, 1986). The further extension of the computations to a series of known and unknown analogs of daunomycin has enabled the prediction of new compounds (Chen *et al.*, 1986b) whose affinity to DNA is estimated to be significantly greater than that of the parent compounds. Such compounds were conceived as a result of the observation that the crystallographically observed (Quigley *et al.*, 1980) and theoretically computed (Chen *et al.*, 1985) location of the cationic ammonium group

of daunomycin (or adriamycin) was not an optimal one for its binding possibilities in the minor groove of DNA in which it is located. This observation led to a search for anthracycline derivatives in which the positioning of this group would be modified so as to enable it to reach more effectively this most satisfactory location. It appeared that one way of doing so was to interpose one or two methylene groups between the sugar ring and the ammonium group, with the strongest enhancing effect predicted for the former of the two derivatives.

This prediction represents an interesting challenge for experimental studies both on DNA binding and on the antitumor activity of these molecules. Although the correlation between these two latter properties of anthracyclines is not always completely consistent, recent work by Arcamone and collaborators (Valentini *et al.*, 1985) points to a far reaching general overall parallelism between them. These new compounds deserve thus certainly an exploration of their chemotherapeutic activity.

Very recently, the theoretical computations have been extended to a related anthraquinone derivative, with a structure, however, significantly different from that of the anthracyclines: mitoxantrone (Fig. 5) (Chen *et al.*, 1986c). One of the most interesting aspects of the results relating to this compound, as compared to the anthracyclines, concerns the location of the side chains; while the daunosamine side chain of daunomycin is located in the minor groove of DNA, the two side chains of mitoxantrone are both located in its major groove. Theory and, within the available evidence, experiment agree about this situation. They agree also on the sequence specificity of this drug (Chen *et al.*, 1986c; Kotovych *et al.*, 1986).

OH O $NHCH_2CH_2NHCH_2CH_2OH$

OH O $NHCH_2CH_2NHCH_2CH_2OH$

MITOXANTRONE

Fig. 5. Mitoxantrone.

4. Factors Involved in the Sequence Selective Interaction of Covalently Bound Antitumor Drugs with DNA.

This problem or rather an aspect of it was studied in our laboratory on the example of anthramycin (Fig. 6), an antitumor drug of the family of pyrrolo(1,4)benzodiazepines. Following a model proposed by Hurley and colleagues (Hurley and Petrusek, 1979; Petrusek *et al.*, 1984; Hurley and Thurston, 1984; Hurley and Needham-Van Devanter, 1986) this molecule is covalently bound through its carbinolamine carbon atom C11 to the amino group (N2) of guanine and lies in the minor groove of B-DNA, covering three base pairs with the attacked GC pair in the center. Recent developments, using the footprinting analysis with MPE·Fe(II) as the cleaving agent, have linked the DNA sequence specificity of anthramycin and associated drugs to the nature of these base pair triplets (Hertzberg *et al.*, 1986). The highest binding preference is found for triplets with purines on both sides of the central attacked guanine (PuGPu), while the lowest preference is observed for a guanine flanked by two pyrimidines (PyGPy). Intermediate behaviour is observed for guanines placed between a purine and a pyrimidine.

We have undertaken the study of this specificity by considering the role played in it by the interaction of the *already covalently linked drug* with the DNA framework adjacent to the binding guanine site. The energy of the covalent bond and the possible specificity related to its formation are thus not taken into account in our present computations. We only evaluate the component energy term corresponding to what may be denoted as "physical" binding of the drug (Zakrzewska and Pullman, 1986).

Anthramycin

Fig. 6. Anthramycin.

This study involved two stages. The first one consisted of a minimisation of the interaction energy of anthramycin with a $(dG)_7$ $(dC)_7$ duplex in the conformation of B-DNA proposed by Arnott *et al.* (1980). The adduct obtained was very similar to the model advocated by Hurley *et al.* with the anthramycin molecule following the minor groove. It involved, besides the essential covalent aminal linkage, two additional hydrogen bonds, one between HN10 of anthramycin and the O2 of the cytosine paired to the guanine to which the ligand is covalently bound, the second between the phenolic OH of anthramycin and the amino proton of the guanine on the 3′ side of the modified guanine. Interestingly enough the flexible tail of anthramycin does not form any hydrogen bond with the bases on the 5′ side of the modified guanine, despite its hydrogen bond acceptor and donor capacities. This is due to the position of this arm rather on the outside of the DNA minor groove, a consequence of the bulky ring section of the drug.

This optimal adduct possible with a rigid DNA in the B conformation presented, however, a number of important steric contacts between the anthramycin ligand and various DNA atoms resulting in an altogether unsatisfactory situation. In the next step we therefore performed minimisations of the energy of the system described above allowing the nucleic acid fragment to change its conformation, following a procedure developed recently in our laboratory (Lavery *et al.*, 1986a,b). In the new minimised adduct the hydrogen bonds described previously are preserved, but the close contacts previously detected are eliminated. It is found as a general feature that the helicoidal parameters of DNA in this anthramycin adduct bear a notable ressemblance to those of A-DNA, which is principally characterised by a strong shift of the base pairs towards the minor groove, a small displacement between successive base pairs and a strong tilt.

In order to elucidate the factors determining the sequence specificity mentioned previously we performed then a series of calculations in which we examined the energetics of systems with different base pairs flanking the guanine binding site. For this sake we have utilised a 5 base pair fragment in the conformation found in the $(dG)_7$ $(dC)_7$ anthramycin adduct in which we successively replaced the base pairs flanking the central GC one. We subsequently performed energy minimisations, keeping the nucleic acid conformation rigid but allowing rotations around the glycosidic bonds of all the bases, around the aminal linkage with anthramycin as well as around all the flexible bonds within the ligand. Similar calculations

were performed for the oligonucleotides of the corresponding sequences without anthramycin, but in the conformation adopted for the anthramycin adduct.

The detailed results obtained are given in table VI. In the first column of this table we indicate the base pair sequence of the central triplet of the modified guanine chain by three letters describing it in the 5'-3' direction. In this table the first 4 adducts correspond to modified chains composed only of purines, the 8 following ones to sequences in which the attacked guanine is surrounded by one purine and one pyrimidine and the last 4 to adducts in which the attacked guanine is surrounded by two pyrimidines. This classification corresponds to the experimentally observed order of decreasing anthramycin binding (Hurley and Needham-Van Devanter, 1986). In column 2 we give the energy of the anthramycin-DNA adduct E^{ADD}. Columns 3 and 4 present the energy of the sole nucleic acid fragment of the corresponding base pair sequence in the conformation of

TABLE VI - *Base sequence dependence for anthramycin binding to DNA* (all energies in kcal/mole).

Central Triplet	E^{ADD}	E^{DNA}	$E^{B\text{-}DNA}$	E^{Int}	E^{Def}	E^{Stab}
GGG	−293.1	−269.8	−285.3	−23.3	15.6	− 7.8
AGG	−279.7	−256.7	−276.4	−23.0	19.7	− 3.3
GGA	−278.8	−256.4	−276.4	−22.4	20.1	− 2.3
AGA	−269.1	−274.4	−267.5	−21.7	20.1	− 1.6
CGG	−278.3	−259.0	−284.4	−19.3	25.4	6.1
TGG	−275.1	−253.7	−276.2	−21.4	22.5	1.1
CGA	−266.7	−247.9	−275.5	−18.8	27.6	8.8
TGA	−260.0	−239.3	−265.9	−20.7	26.7	6.0
GGC	−283.7	−261.1	−285.0	−22.6	23.9	1.3
GGT	−276.6	−254.2	−276.8	−22.4	22.5	0.1
AGC	−269.9	−248.0	−276.1	−21.9	28.1	6.2
AGT	−262.6	−241.0	−269.1	−21.6	28.1	6.5
CGC	−270.6	−250.8	−284.1	−19.9	33.4	13.5
CGT	−265.7	−244.2	−273.7	−21.5	29.5	8.0
TGC	−264.8	−243.4	−273.7	−21.4	30.3	8.9
TGT	−258.2	−237.0	−267.5	−21.2	30.5	9.3

the anthramycin-DNA adduct (E^{DNA}) and in the minimised B conformation ($E^{B\text{-}DNA}$), respectively. Column 5 contains the difference $E^{Int} = E^{ADD} - E^{DNA}$, representing the interaction energy of anthramycin with the DNA in the modified conformation. This term represents thus the "physical" interaction energy of anthramycin with the nucleic acid. In column 6 we show the energy difference $E^{Def} = E^{DNA} - E^{B\text{-}DNA}$ between the DNA fragment in the conformation which it adopts in the anthramycin adduct and in the optimised B-conformation. This term characterises thus the deformation energy necessary to change the conformation of the nucleic acid to accommodate anthramycin in the minor groove. Finally the last column of table VI gives the stabilisation energy of the adduct E^{Stab} as a sum of the anthramycin-DNA interaction energy and the nucleic acid deformation energy ($E^{Int} + E^{Def}$). It is equivalent, of course, to the difference $E^{ADD} - E^{B\text{-}DNA}$.

Table VII gives a simplified version of the results, presenting the mean values of the different computational data, as relevant to the three types of sequences distinguished experimentally PuGPu, PuGPy+PyGPu and PyGPy. This procedure enables a direct comparison of the theoretical and experimental indications.

The first major conclusion to be drawn from Table VII is that the computed energies of the anthramycin-DNA adducts, E^{ADD}, range in the order corresponding to the experimental observation, namely that they are, as a mean, the greatest for the adducts in which the attacked guanine is surrounded by two purines, the smallest for the adducts in which it is surrounded by two pyrimidines and have intermediate value for the alternating PuGPy+PyGPu sequences. It seems therefore plausible to consider that the physical interactions computed here between the bound

TABLE VII - *Base sequence dependence for anthramycin binding to DNA. Mean values* (kcal/mole).

Central Triplet	E^{ADD}	E^{DNA}	$E^{B\text{-}DNA}$	E^{Int}	E^{Def}	E^{Stab}
Pu G Pu	−280.2	−264.3	−276.4	−22.7	18.9	−3.8
Py G Pu + Pu G Py	−271.6	−250.6	−276.1	−21.0	25.5	4.5
Py G Py	−264.8	−243.9	−274.3	−20.9	30.4	9.5

anthramycin and the neighbouring bases in the modified DNA chain constitute the determinant or at least a major determinant fixing this sequence selectivity.

Remains the interesting question of the factors responsible for this ordering. Informations about those may be obtained by studying the data included in the remaining columns of table VII.

Thus, it may be observed that while $E^{B\text{-}DNA}$ and E^{Int} have practically constant values in the three types of sequences, the remaining energies exhibit significant and relevant variations. Thus the energies of the deformed DNA, E^{DNA}, run parallel to those of E^{ADD}. Consequently, the deformation energies of the DNA, $E^{Def} = E^{DNA} - E^{B\text{-}DNA}$, exhibit a clear difference between the three types of sequences, the smallest being (18.9 kcal/mole) for the chains composed entirely of purine bases, the greatest (30.9 kcal/mole) for the chains in which the attacked guanine is surrounded by two pyrimidines and intermediate (25.6 kcal/mole) for chains in which this guanine is placed between a purine and a pyrimidine.

In these conditions it is therefore understandable that the overall stabilization energies $E^{Stab} = E^{Int} + E^{Def} = E^{ADD} - E^{B\text{-}DNA}$ show clear preference for binding to the PuGPu sequences. It can be noticed that these energies are negative for these sequences (mean value - 3.8 kcal/mole), while they are positive (mean value - 9.9 kcal/mole) for the PyGPy sequences. They are also positive but with a smaller mean value (4.5 kcal/mole) for the mixed PuGPy+PyGPu sequences. We remind here that the positive values obtained for the stabilisation energy of these last adducts do not signify that their formation is not possible since our calculations do not take into account the strong binding contribution due to the formation of the covalent bond between anthramycin and the attacked guanine. They only describe the contribution of the residual "physical" interactions.

The evolution of the adduct stabilization energy obtained agrees very satisfactorily with the sequence specificity observed for anthramycin by Hurley and colleagues. This result leads thus to the significant conclusion that DNA flexibility as a function of base sequence seems, at least in the present case, to be an important factor controlling the specificity of binding of the covalently attached ligand.

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MOLECULAR ASPECTS OF CARCINOGENESIS

THE ROLE OF *ras* ONCOGENES IN CHEMICALLY-INDUCED TUMORS

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Abstract

Altered forms of cellular proto-oncogenes have been implicated in the development of human cancer. Carcinogen-induced animal tumor models, in which some of these oncogenes are reproducibly activated are providing a means of understanding the role of oncogene activation in neoplasia. Recent findings indicate that *ras* oncogenes can be activated during initiation of carcinogenesis as a result of the direct mutagenic action of the carcinogen.

Introduction

Recent advances in cancer research have established the involvement of transforming genes (oncogenes) in the multistep process of carcinogenesis. Over the span of several decades, epidemiological studies have drawn attention to the fact that there is a higher incidence of cancer among individuals exposed to mutagens in the workplace. The effect of many of these carcinogens, both physical and chemical, has been shown to result in direct alteration of the DNA in target cells by induction of point mutations or chromosomal translocations, suggesting a crucial role for genes in carcinogenesis [1, 2]. The observation that human tumors contain oncogenes activated by these mechanisms has led to the speculation that the direct mutagenic effect of the carcinogen occurs at the level of activation

of the proto-oncogene, bestowing upon it the ability to cause phenotypic transformation.

Using the transfection technique involving the ability of NIH/3T3 mouse fibroblasts to accept and express genes from donor DNA, a wide variety of human tumors were shown to contain genes capable of transforming these cells [3]. Transforming genes have been detected in 10-15% of solid tumors and as many as 25% of certain forms of leukemias [4, 5]. Isolation and characterization of these genes revealed that the majority of them belonged to the *ras* gene family, which includes the H-*ras*-1 and K-*ras*-2 loci, identified previously as the transforming principle of the Harvey and Kirsten strains of murine sarcoma virus [6, 7, 8]. The third member, N-*ras* [9, 10] was initially identified as a transforming gene present in a human neuroblastoma cell line and does not appear to have a retroviral counterpart. These results provided the first evidence for the presence of dominant oncogenes in human tumors, analogous to those responsible for the transforming properties of acute transforming retroviruses.

In recent years, we have focused our research efforts on the involvement of oncogenes in carcinogen-induced animal tumors. Carcinogen-induced animal tumors frequently contain activated *ras* oncogenes, and exhibit a certain specificity in terms of the type of tumor that results due to the carcinogen exposure and subsequent activation of endogenous proto-oncogenes [11-15]. These model systems have provided scientists with a powerful tool to study the role of oncogene activation in the multistep process of carcinogenesis.

All three mammalian *ras* genes code for highly related proteins termed p21 consisting of 188 or 189 amino acid residues. They bind guanine nucleotides [16, 17], possess intrinsic GTPase activity [18, 19], have been localized to the inner surface of the plasma membrane [20, 21] and share significant homology with G-proteins [22, 23]. These properties suggest that *ras* proteins may participate in the process of signal transduction across the cellular membrane [24].

Molecular cloning and characterization of *ras* genes from a number of human tumor cell lines as well as from biopsy samples indicated that their tumorigenic properties are due to a simple change in their coding sequences. The *ras* genes were shown to acquire malignant properties by miscoding single point mutations, most commonly localized in codons 12 and 61 [25-28]. Quantitative alterations in the levels of *ras* protein expression due to either increased transcription or gene amplification may

also participate in malignant transformation. Introduction of the normal H-*ras*-1 proto-oncogene under the control of retroviral LTRs or integration of multiple copies of the normal human H-*ras*-1 gene with resultant expression of abnormally high levels of cellular p21, promotes phenotypic and tumorigenic transformation of NIH/3T3 cells [29, 30]. Although so far, there is no evidence for alterations in the transcriptional control of *ras* proto-oncogenes due to mutations in their regulatory elements, amplification of *ras* genes has been observed in murine and human tumors [31-33].

The overall low (10-15%) detection frequency of activated *ras* oncogenes in human tumors has raised questions regarding the significance of the activation of these oncogenes in the development of human cancer [34]. The argument revolves around the central issue of whether oncogene activation is the cause or the consequence of malignant transformation, i.e., a mere manifestation of the genetic disarray characteristic of a tumor cell. The resolution of this argument requires the establishment of a clear link between oncogene activation and tumorigenesis. Animal models with well defined etiological agents, reproducible induction of the same type of tumor and the possibility of experimental manipulation have provided us the means of studying the how and when of oncogene activation in the multistep process of carcinogenesis. In this report, we will review studies using experimental model systems which are helping to define the molecular events involved in the onset and progression of neoplasia.

ras *oncogenes in animal tumors*

Mammary carcinomas are reproducibly induced in female rats by a single injection of NMU at puberty [35]. The intravenous mode of injection of the carcinogen ensures distribution throughout the body and the age at injection provides a target of cells in the breast that are differentiating and proliferating under the influence of the sex hormones. In addition, mammary tumor development is hormone dependent. Ovariectomy prior to, or following NMU administration lowers tumor incidence to negligible levels. NMU is a direct acting carcinogen with a half life of about twenty minutes in the rat [35], which implies that tumor development occurs as a result of a single carcinogenic insult. These unique features make this model particularly suitable for studying the involvement of oncogenes in tumor development.

We have shown that DNAs extracted from sixty-one out of seventy-

one primary NMU-induced mammary carcinomas from three different strains of rats caused the appearance of morphologically altered foci in NIH/3T3 mouse cells due to the presence of transforming H-*ras*-1 oncogenes [11, 36]. In contrast, in the same assay, no transforming activity was observed using DNA from normal breast tissues obtained from age-matched female rats. Molecular cloning and sequence analysis of the H-*ras*-1 oncogene from one of these tumors revealed that this carcinogen-induced tumor contained the same type of activating miscoding mutation previously seen in *ras* oncogenes of human tumors. This observation validates the choice of this system as an adequate model for studying the role of *ras* gene activation in human neoplasia.

We have also utilized the DMBA-induced rat mammary tumor model in our studies [37]. The mammary tumors induced by a single intragastric dose of this indirect-acting carcinogen are similar to those induced by NMU both in terms of histology and hormone responsiveness, but transforming H-*ras*-1 genes were detected in only 25% of the tumors tested, indicating the existence of alternate pathways to tumorigenesis [36].

Reproductible activation of H-, K-, and N-*ras* genes has also been reported in other carcinogen-induced animal models. These results are summarized in Table 1. Activation of the K-*ras*-2 locus has been observed in 40% of rat kidney mesenchymal tumors induced by single dose of DMN(OMe) [15] and 74% of lung tumors that arose following chronic exposure by inhalation to tetranitromethane (TNM) (S. Reynolds and M.W. Anderson, personal communication). Whereas NMU-induced rat mammary tumors contain H-*ras*-1 oncogenes, consistent activation of the *neu* oncogene has been observed in tumors of the peripheral nervous system that arise following transplacental exposure to the same carcinogen (our unpublished observations). This transforming gene, first identified in three neuroblastomas cell lines derived from tumors induced by ENU, codes for a transmembrane receptor protein closely related to the EGF receptor [14, 38].

In mice, H-*ras*-1 oncogenes were found to be reproducibly activated in skin papillomas and carcinomas of Sencar mice initiated by DMBA or dibenz(c,h)acridine (DBACR) painting followed by promotion with TPA [12, 39, 40]. Similarly, H-*ras*-1 oncogenes have been found in mammary carcinomas arising in mice treated with DMBA following implantation of the hyperplastic alveolar nodule line D1/UCD [41]. Mouse thymomas induced by X-rays had transforming K-*ras*-2 oncogenes while the same type of tumor induced by chronic NMU treatment contained activated

TABLE 1 - *Transforming genes in Carcinogen-Induced Animal Tumors.*

Species	Carcinogen	Tumor	Oncogene	Oncogenes/No. Tumors Tested	Reference
Rat	NMU	Mammary ca.	H-*ras*-1	61/70	[11, 36]
	DMBA	Mammary ca.	H-*ras*-1	6/29	[36]
	DMN (OMe)	Renal ca.	K-*ras*-2	10/35	[15]
	Radiation	Skin tumors	K-*ras*-2	6/12	*b*
	TNM	Lung ca.	K-*ras*-2	14/19	*a*
	ENU	Neuroblastomas	*neu*	3/3	[14]
	NMU	Schwannomas	*neu*	10/13	*c*
	MMS	Nasal ca.	?	8/8	[56]
Mouse	DMBA	Skin ca.	H-*ras*-1	33/37	[12, 39]
	DBACR	Skin pap/ca.	H-*ras*-1	4/5	[40]
	DMBA	Mammary ca.	H-*ras*-1	3/4	[41]
	Several	Hepatomas	H-*ras*-1	25/25	[44]
	None	Hepatomas	H-*ras*-1	11/13	[43]
	X-rays	Lymphomas	K-*ras*-2	4/7	[13]
	3-MCA	Fibrosarcomas	K-*ras*-2	2/4	[55]
	TNM	Lung ca.	K-*ras*-2	9/9	*d*
	NMU	Lymphomas	N-*ras*-1	5/6	[13]

a S. Reynolds and M.W. Anderson, personal communication.
b S. Garte, personal communication.
c Our unpublished observations.
d J. Stowers and M.W. Anderson, personal communication.

N-*ras* genes [42]. K-*ras*-2 oncogenes have also been observed in lung tumors induced by TNM (J. Stowers and M.W. Anderson, personal communication). In the B6C3F$_1$ strain of mice, used very extensively for the screening of carcinogenic potential of chemicals, H-*ras*-1 oncogenes have been consistently detected in hepatocellular carcinomas arising spontaneously [43] or after treatment with carcinogens such as N-hydroxy-2-acetylaminofluorene (HOAAF) [44], vinyl carbamate [44], 1′-hydroxy-2′-3′-dehydroestragole (HODE) [44]. The frequency of detection as well as the reproductible activation of *ras* genes in animal tumors strongly support the concept that *ras* oncogenes play a causative role in tumor development.

Mode of activation of ras *oncogene in rat mammary tumors*

Molecular cloning and sequencing of one of the H-*ras*-1 oncogenes isolated from a mammary tumor induced by NMU revealed that the mechanism of malignant activation of this gene was by single point mutation [11]. The activating mutation was identified as a G→A transition in the second nucleotide of codon 12 (Figure 1). In order to define the exact nature of the mutation in each of the H-*ras*-1 oncogenes present in NMU-induced mammary tumors, we used oligonucleotide probes capable of identifying specific point mutations in genomic DNA. As seen in Figure 2, using nonadecamers capable of identifying substitutions in position 35 of the 12th codon of H-*ras*-1 gene, each of 61 NIH/3T3

	+1									
	met	thr	glu	tyr	lys	leu	val	val	val	gly
H-*ras*-1	ATG	ACA	GAA	TAC	AAG	CTT	GTG	GTG	GTG	GGC
NMU-H-*ras*	—	—	—	—	—	—	—	—	—	—

	+31									
	ala	gly	gly	val	gly	lys	ser	ala	leu	thr
H-*ras*-1	GCT	GGA	GGC	GTG	GGA	AAG	AGT	GCC	CTG	ACC
NMU-H-*ras*	—	---A---	—	—	—	—	—	—	—	—
		glu								

	+61									
	ile	gln	leu	ile	gln	asn	his	phe	val	asp
H-*ras*-1	ATC	CAG	CTG	ATC	CAG	AAC	CAT	TTT	GTG	GAC
NMU-H-*ras*	—	—	—	—	—	—	—	—	—	—

	+91						
	glu	tyr	asp	pro	thr	ile	glu
H-*ras*-1	GAG	TAT	GAT	CCC	ACT	ATA	GAG
NMU-H-*ras*	—	—	—	—	—	—	—

FIG. 1. Comparative analysis of the nucleotide sequence of the first exon of rat H-*ras*-1 gene and its transforming allele, the NMU-H-*ras* oncogene. Nucleotide sequence identity in both genes is indicated by (—). The deduced amino acid sequence is also indicated. The single base change of G → A at + 35 resulting in substitution of glycine to glutamic acid in p21 is shown.

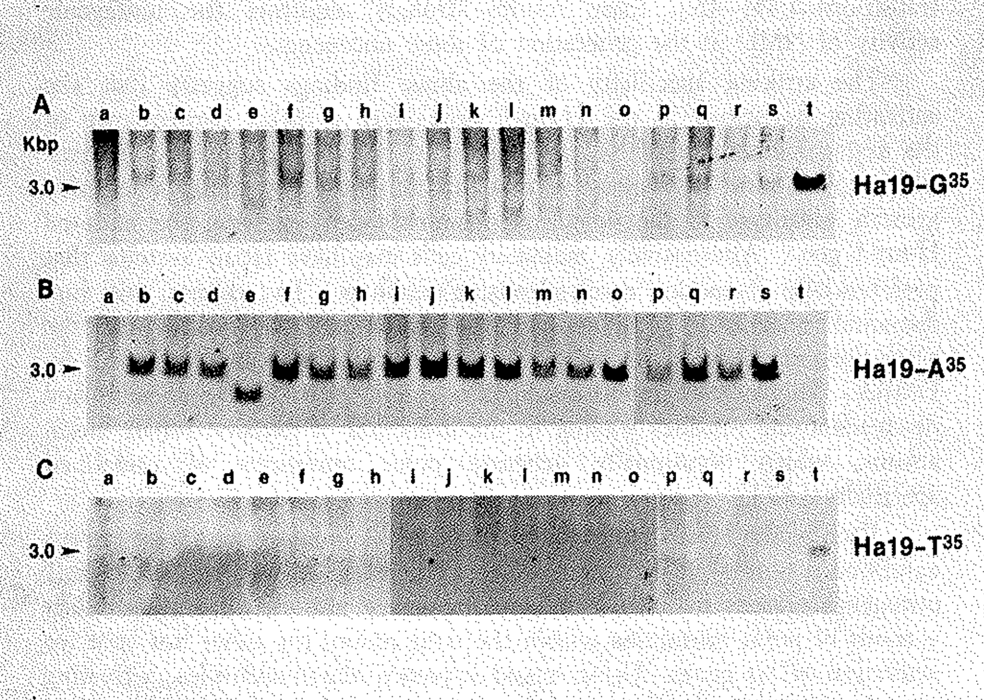

FIG. 2. Use of synthetic oligonucleotide probes to determine the specific point mutation responsible for malignant activation of the H-*ras*-1 locus in NMU-induced mammary carcinomas. DNAs were isolated from NIH/3T3 cells (a), representative NIH/3T3 transformants derived from NMU-induced mammary carcinomas (b to s), and NIH/3T3 cells cotransfected with the normal H-*ras*-1 gene and pSV2-neo and selected for growth in the presence of G418 (t). A) Hybridization with probe $Ha19\text{-}G^{35}$ (5'TGGGCGCTGGAGGCGTGGG 3'); B) Hybridization with probe $Ha19\text{-}A^{35}$ (5'TGGGCGCTG*A*AGGCGTGGG 3'); C) Hybridization with probe $Ha19\text{-}T^{35}$ (5'TGGGCGCTG*T*AGGCGTGGG 3'). Arrowheads indicate the expected 3.0 kpb Hind III DNA fragment of H-*ras*-1, which contains all coding sequences except the first 12 nucleotides.

transformants derived from NMU-induced mammary tumors hybridized to $Ha19\text{-}A^{35}$ but not to $Ha19\text{-}G^{35}$ or $HA19\text{-}T^{35}$, indicating that their H-*ras*-1 oncogene carried identical G→A transitions in positions 35 [36]. These results demonstrate that each of the H-*ras*-1 genes present in NMU-induced mammary carcinomas became activated by the same mutation, a G→A transition in second nucleotide of the critical 12th codon. Taking into account that the preferred mutations induced by NMU are G→A

transitions [45, 46], these results strongly implicate the mutagenic activity of NMU in the generation of these oncogenes.

The striking specificity of the above findings suggests that the activating mutations in the H-*ras*-1 gene were a direct consequence of the mutagenic activity of NMU. One must, however, consider other possible explanations. It is possible that this mutation may confer a selective growth advantage to the neoplastic mammary cells carrying the mutated H-*ras*-1 gene. Alternatively, mammary cells may have repair systems that preferentially introduce deoxyadenosine residues. To address this fundamental question, we examined the mutations responsible for the activation of H-*ras*-1 oncogenes in mammary carcinomas induced by DMBA. This carcinogen forms large adducts with deoxyguanosine and deoxyadenosine residues leading to the induction of excision repair mechanisms, which occasionally generate point mutations of undefined specificity [47].

Each of the H-*ras*-1 oncogenes from DMBA-induced mammary tumors exhibited a normal 12th codon. It was, therefore, plausible that H-*ras*-1 oncogenes present in DMBA-induced mammary tumors were activated as a result of point mutations within the 61st codon, the other hot spot for activation of *ras* genes. Using mixed sequence oligonucleotides, activating mutations were localized in the two adenosine residues of codon 61 (CAA) [36] (Figure 3). These findings rule out the possibility that the G→A mutations present in each of the NMU-induced H-*ras*-1 oncogenes are the result of either positive growth selection or specific repair systems. Instead they indicate that malignant activation of the H-*ras*-1 locus in NMU-induced mammary carcinomas is the result of the direct mutagenic effect of NMU on this locus.

ras *gene activation and initiation of carcinogenesis*

The results reported thus far are highly indicative of an important role for *ras* activation in carcinogenesis. To understand the contribution of *ras* activation to the multistep process of carcinogenesis one must define the stage at which its activation occurs. Transforming H-*ras*-1 genes have been found in papillomas, a preneoplastic stage of mouse skin carcinomas induced by painting the skin with DMBA, followed by chronic application of a tumor promoter [39, 48]. This points to *ras* activation being an early event in skin carcinogenesis. It is difficult to define the stage at which *ras* activation occurs in the NMU-induced mammary tumor because

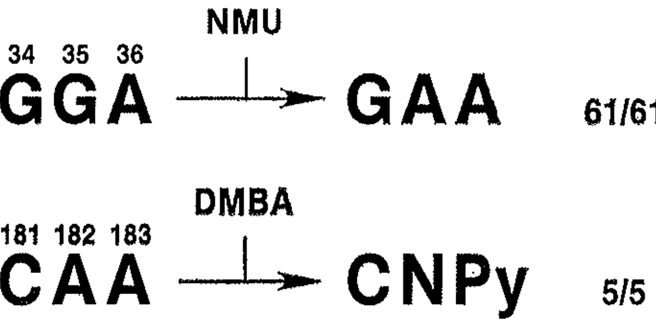

FIG. 3. Mutagenesis of H-*ras*-1 oncogenes in NMU and DMBA-induced mammary carcinomas of rats. 61 H-*ras*-1 oncogenes present in NMU-induced mammary carcinomas acquired their transforming properties by a G → A transition in the second nucleotide (position 35) of codon 12 (GGA). 5 H-*ras*-1 oncogenes present in DMBA-induced mammary carcinomas exhibited normal codon 12. Instead, their activating mutations were localized in either of the two deoxyadenosine residues (position 182 and 183) of codon 61 (CAA). N means any nucleotide other than A, and Py means a pyrimidine (C or T) residue.

tumor induction does not proceed through identifiable preneoplastic stages. However, this model has other properties that made it possible to study whether *ras* oncogenes play a role in initiation of carcinogenesis. NMU is a potent alkylating agent that preferentially induces G→A mutations due to the generation of O^6-methyl-guanine adducts [45, 46]. Our results using oligonucleotide technique have shown that each of the 61 NMU-induced H-*ras*-1 genes carried the same activating G^{35}→A mutation [36]. These findings strongly support the concept that these mutations are the result of NMU-directed methylation of the O^6 position of G^{35} in the critical codon 12. This direct acting carcinogen is very labile and must exert its mutagenic activity within hours after administration. Considering these two facts together, our results imply that the activating G→A mutation in the H-*ras*-1 gene must occur during initiation of carcinogenesis in this system.

ras *genes as direct targets of carcinogens*

The mutagenic action of most carcinogens is mediated via the formation of adducts with DNA bases [45]. Some of these adducts are highly mutagenic due to their miscoding properties [46, 49], while others

lead to mutations due to generation of apurinic sites or because of the limited fidelity of repair polymerases [50]. Although it was well recognized that a small number of these had the potential to trigger neoplastic transformation, identification of the genes in which these critical mutations occur proved elusive for many years.

The opportunity to correlate the known mutagenic effect of carcinogens with the activating point mutations in *ras* oncogenes arose following the detection of reproducibly activated oncogenes in chemically-induced animal tumors. The detection of a G→A transition in the H-*ras*-1 gene cloned from NMU-induced mammary tumors was a provocative finding in that one of the preferred mutations induced by NMU had occurred in an oncogene, resulting in its malignant activation. The O^6-methyl-guanosine adducts generated by the methylating activity of NMU are highly mutagenic as they are not removed efficiently by excision repair, mispair with thymidine residues and lead to G→A transitions [46, 49]. In contrast, DMBA forms large adducts with adenine and guanine residues whose repair rarely leads to generation of G→A transitions [45]. These observations led to the proposal that NMU is directly responsible for the malignant activation of H-*ras*-1 oncogenes in this animal tumor system [11, 36].

That oncogenes may be direct targets of chemical carcinogens is corroborated by the results obtained in other model systems (Table 2). Induction of mouse skin carcinomas by DMBA [39, 40] and mouse mammary carcinomas induced by DMBA [41], specifically involve activation of H-*ras*-1 oncogenes by an A→T transition in the second base of codon 61. In spontaneous hepatomas arising in aged $B6C3F_1$ male mice H-*ras*-1 oncogenes are activated by random mutations in the first two nucleotides of codon 61 (S. Reynolds and M.W. Anderson, personal communication). However, when these tumors are induced by exposure to carcinogen, specific mutational activations of the H-*ras*-1 oncogenes were observed, depending on the carcinogen used in the induction of hepatomas [44]. Thus, hepatomas induced by a single dose of HO-AAF contained H-*ras*-1 oncogenes activated by C→A transversions in their 61st codon. The observed lesions arising from the formation of N-(deoxyguanosin-8-yl)-2-aminofluorene, the major DNA adducts of HO-AAF in mouse liver, are G-C→T-A transversions [51, 52]. On the other hand, in hepatocellular tumors initiated by vinyl carbamate, H-*ras*-1 oncogenes were frequently activated by A→T transversions in the A^{182} residue [44]. These results

TABLE 2 - *Activation of H*-ras *Oncogenes by Specific Mutations in Carcinogen-Induced Animal Tumors.*

Species	Type of Tumor	Carcinogen	Activation Mutation *	Incidence	Ref.
Rat	Mammary ca.	NMU	$G^{35} \rightarrow A$	61/61	[11, 36]
	Mammary ca.	DMBA	$A^{182}/A^{183} \rightarrow N$	5/5	[11, 36]
	Lung ca.	TNM	$G^{35} \rightarrow A$	14/14	*a*
Mouse	Skin papil./ca.	DMBA	$A^{182} \rightarrow T$	33/34	[39]
	Skin ca.	DMBA	$A^{182} \rightarrow T$	3/3	[40]
	Skin papil./ca.	DBACR	$A^{182} \rightarrow T$	4/4	[40]
	Mammary ca.	DMBA	$A^{182} \rightarrow T$	3/3	[41]
	Hepatoca.	HODE	$A^{182} \rightarrow T/G$	10/10	[44]
	Hepatoca.	HO-AFF	$C^{181} \rightarrow A$	7/7	[43]
	Hepatoca.	VC	$A^{182} \rightarrow T$	6/7	[43]
	Hepatoca.	None	$C^{181}/A^{182} \rightarrow T/G$	10/10	*a*
	Lung ca.	TNM	$G^{35} \rightarrow A$	9/9	*b*

* Residue 35 corresponds to the second nucleotide of codon 12. Residues 181-183 correspond to codon 61.

a S.W. Reynolds and M.W. Anderson, personal communication.

b J. Stowers and M.W. Anderson, personal communication.

strongly support the concept that H-*ras*-1 genes are the direct targets of initiating carcinogens.

Secondary events in multi-step carcinogenesis

The latency period of carcinogen induced tumors usually varies from a few weeks to several months, depending upon several factors including mutagenicity of the carcinogen, dose of carcinogen, and the species used for its induction. It is obvious that multiple steps are involved between the time of the infliction of genetic lesions by the carcinogen and the commencement of unrestrained growth characteristic of a tumor [53, 54]. Studies by Balmain and co-workers using the mouse skin model have provided a direct demonstration of secondary events involved in carcinogenesis following *ras* gene activation during initiation [12, 39, 48]. Transforming H-*ras*-1 genes were present in the preneoplastic papil-

lomas induced by painting the skin with DMBA followed by promotion with phorbol esters, a procedure that, among other things, stimulates cellular proliferation. Most of these skin papillomas regress, but a select few progress to form carcinomas. Thus, in this system, one is able to distinguish at least three steps: initiating genetic lesions by the carcinogen, cellular proliferation induced by the tumor promoter, and an additional genetic lesion that commits a proportion of the papillomas to form carcinomas.

In the rat mammary tumor model, it is not possible to define the intermediate steps between initiation by the carcinogen and the appearance of a malignancy, since tumor development does not proceed through well-defined, morphologically distinguishable, preneoplastic stages. Even so, there are at least two events that we can perceive as necessary for mammary carcinogenesis in this system: 1) activation of H-*ras*-1 during initiation and 2) cellular proliferation under the influence of the sex hormones during puberty. The proliferative state of the developing mammary gland at the time of the carcinogenic insult has been shown to play a fundamental role in mammary tumor development. Our recent results indicate that the two events, *ras* activation and mammary gland development do not have to occur concomitantly (unpublished observations). Newborn female rats treated with a single dose of NMU develop mammary carcinomas 2-3 months following sexual maturity. Most of these tumors carry H-*ras*-1 oncogenes, each activated by G→A transition diagnostic of NMU-induced mutagenesis. Interfering with sexual maturation by treatment with anti-estrogens followed by ovariectomy completely prevents tumor development (unpublished observations). These results suggest that whereas H-*ras*-1 oncogenes might be activated early in life, hormone-mediated proliferation and/or differentiation is essential for the manifestation of its malignant properties.

Role of normal development in activation of specific oncogenes by carcinogens

Our observation that there is exclusive induction of mammary tumors in rats treated with NMU at puberty is at odds with the fact that the carcinogen is administered intravenously and must therefore have access to all the organs of the body. Additionally, the only transforming gene observed in the NIH/3T3 gene transfer assay in each of these tumors is the H-*ras*-1 gene which is activated in each case by a G→A mutation. It is

possible that NMU can exclusively activate only H-*ras*-1 genes. On the other hand, other oncogenes may become activated in various organs, but the phenotypic expression of their malignant properties is possible only under controlled physiological conditions. Since the mammary gland is undergoing differentiation and cell division at sexual maturity, the developmental stage of this organ might be playing a crucial role in determination of specific oncogene targets of carcinogens. It is probable that the H-*ras*-1 gene plays an important role in the normal development of the mammary gland. Thus, in the presence of activated *ras* genes, the developmental stage of the organ with the accompanying cellular proliferation provides the physiological conditions necessary for the phenotypic expression of the transformed phenotype.

In order to determine if normal developmental factors influence oncogene activation, we changed the timing of the carcinogenic insult from puberty to the late stages of embryonal development, when several organs are undergoing active differentiation and development. NMU was given to pregnant rats at the 17th or 18th day of gestation. About 30% of the offspring that survive the transplacental treatment developed tumors of various types including those of neuroectodermal, epidermal, mesenchymal, and occasionally tumors of embryonic origin. DNAs extracted from these tumors were tested for transforming activity in the NIH/3T3 assays. Four different oncogenes were found to be activated in the tumors tested. For instance, tumors of the peripheral nervous system contained *neu* oncogenes, kidney mesenchymal tumors contained activated K-*ras*-2 genes, the three mammary tumors that arose in this protocol contained H-*ras*-1 oncogenes, and a neurofibrosarcoma contained a transforming gene distinct from 21 known oncogenes (unpublished observations). These results clearly ruled out the possibility that NMU could only activate H-*ras*-1 genes, and suggest that specific oncogenes can be activated in certain tumors depending on their developmental origin.

Each of the three mammary carcinomas were found to carry H-*ras*-1 oncogenes, adding further support to the concept that the nature of the target cell plays a fundamental role in determining the type of oncogene that participates in tumor development. Interestingly, the H-*ras*-1 gene present in these breast carcinomas became activated by G→A transitions, suggesting that their activation occurred as a result of direct mutagenic effect of NMU in the fetal stage (unpublished observations). If this is the case, it offers the opportunity to design experimental protocols in

which initiation events involving *ras* oncogene activation in mammary carcinomas can be dissected from endogenous hormonal promotion occurring during sexual maturation and mandatory for the development of these tumors. In summary, chemical carcinogens can target multiple proto-oncogenes. However, it appears that phenotypic expression of the malignant properties of the activated oncogene appears to be, in a large part, determined by the developmental stage of the harboring cell.

Concluding Remarks

There is substantial evidence, direct as well as indirect, that oncogenes are involved in the genesis of cancer. The combined use of gene transfer and molecular cloning techniques has allowed the discovery in the past five years of the existence of dominant transforming genes in human and animal tumors and how they differ from their normal counterparts. Of particular importance have been studies using animal models with relevance to human cancer, which are providing insights into how oncogenes are mutationally activated and at what stage in the multi-step process of carcinogenesis their activation occurs. The reproducibility of activation of *ras* oncogenes by specific point mutations in these animal tumor systems strongly argues against oncogene activation being a random event. In fact, this reproducibility casts them in a causative role in carcinogenesis. These systems, in which it is possible to dissect various stages of neoplastic development, offer us the tools to be able to study the nature of the cellular factors that govern the phenotypic expression of activated oncogenes, and the role of the activated oncogene in the malignant progression of mammary tumors.

Although great strides have been taken in understanding the relevance of oncogenes in tumor development in carcinogen-induced animal tumors, and in neoplastic transformation of cells in tissue culture, attempts to extrapolate these findings to human cancer must be made with caution. In most human cancers, the etiology is not known. It is possible that human tumors could be induced by chronic exposure to subcarcinogenic doses of a variety of carcinogens. Such exposures might cause the activating lesion in the oncogene; however, the expression of the neoplastic phenotype will depend on the existence of highly defined physiological conditions in the host, which in most cases may not occur during one's lifetime. Moreover, oncogenes could also be involved in later stages of tumor develop-

ment and activation may be the result of an error in the replication machinery.

The concerted efforts directed towards understanding the significance of activated oncogenes in human cancer is being supplemented by the recent efforts towards the development of fast and reliable diagnostic tests [57, 58, 59]. This data may help us find an association between activation of oncogene, etiology and pathology of human cancer, offering new strategies for early diagnosis and perhaps, treatment of cancer.

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MOLECULAR MECHANISMS OF ONCOGENESIS BY POLYOMAVIRUSES

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ABSTRACT

The mouse polyoma virus and the simian virus 40 are oncogenic DNA viruses which have been studied extensively at the molecular level. In the case of polyoma, the oncogenic potential requires the cooperation of at least two viral early genes, one which controls immortalization and the other which controls transformation of primary rodent embryo cells in culture. In SV40 a single early gene appears to be important in transformation, but the dual localization of its product in the nucleus and in the plasma membrane also suggest a cooperative transformation model. Recently the DNA sequence of a third oncogenic polyomavirus, the hamster papovavirus has been described. This virus has the same coding potential as the mouse polyomavirus in its early region.

The genomes of the polyomaviruses are contained in small supercoiled circular DNA molecules in which three regions have been recognized: an early region and a late region, each of which behaves as a transcription unit, and which are separated at their 5′ ends by a non-coding region which contains the promoters for the early and late regions, enhancer sequences and the origin of DNA replication. The only viral functions which are required for cellular transformation are the early functions (Tooze, 1981).

The mouse polyoma virus and the simian virus 40 (SV40) have many similarities, but they profoundly differ in the organization of their early regions. While two alternate splicings of the unique SV40 early transcript produce two mRNA's which are translated into a large tumor antigen (L-T) and a small tumor antigen (s-t), a third alternate splicing enables the polyoma early region to code for an intermediate size antigen, the middle tumor antigent (M-T) (Tooze, 1981).

All polyomaviruses isolated from primates (the human viruses BK and JC, the African green monkey lymphotropic papovavirus, LPV) have the same coding capacity as SV40 and produce two early antigens. The mouse polyoma virus was, up to a recent time, the only papovavirus with three early antigens. The cloned DNA from a hamster papovavirus (HaPV), isolated by Graffi *et al.* (1968), has recently been sequenced (Delmas *et al.*, 1985). From a consideration of the open reading frames and of possible splicing sites, it appears that HaPV has a putative early region which could also encode for three early proteins.

Cellular transformation by polyoma virus

The complexity of the early region of the mouse polyoma virus made it difficult to appreciate precisely the contribution of each early protein to cellular transformation. The production of recombinant plasmids, separately coding for each of them (Treisman *et al.*, 1981; Zhu *et al.*, 1984; Rassoulzadegan *et al.*, 1982, 1983) and their use in transformation experiments have finally provided information on the interaction of each of the three tumor antigens with cells in the transformation process.

Land *et al.* (1983), in their classical approach to cellular transformation through the use of two cooperating oncogenes, have proposed to divide some of the known oncogenes into class I oncogenes, exemplified by the T24-Ha-*ras* gene, and class II oncogenes, exemplified by v-*myc*. Although it is now clear that the distinction between transforming class I oncogenes and immortalizing class II oncogenes can only be made in particular experimental conditions, it remains established that class I oncogenes are more efficient than class II oncogenes at inducing morphological transformation and tumorigenicity in already established cell lines, while class II oncogenes are more efficient at inducing the *in vitro* establishment of primary cell cultures (Land *et al.*, 1986).

With these limitations in mind, polyoma L-T behaves as a class II oncogene, and can promote the immortalization of primary rodent embryo

cells (Tyndall *et al.*, 1981; Rassoulzadegan *et al.*, 1983). Like the *c-myc* protein, polyoma L-T is a nuclear protein, which is able to modulate the expression of both viral and cellular genes, a function which it has acquired through evolution to provide for the needs of viral DNA replication in non-proliferating cells.

Polyoma M-T, on the other hand, has clearly the ability to induce the morphological transformation of already established cell lines (e.g., Fisher rat FR3T3 or F111, mouse NIH3T3 or BALB/C 3T3) and therefore is a class I oncogene. Although the phosphorylation of protein tyrosine residues is observed in polyoma T antigen immunoprecipitates (Eckhart *et al.*, 1979; Smith *et al.*, 1979; Schaffhausen and Benjamin, 1979), polyoma M-T has no protein kinase activity of its own, but it forms a tight complex with the product of the c-*src* gene (Courtneidge and Smith, 1983) and stimulates its kinase activity about 20-fold (Bolen *et al.*, 1984). The ability of M-T to participate in cellular transformation is clearly linked to its ability to bind to $pp60^{c-src}$ (Markland *et al.*, 1986a; Markland *et al.*, 1986b). The formation of the $pp60^{c-src}$-M-T complex could also be regulated through phosphorylation of M-T by protein kinase C (Matthews and Benjamin, 1986).

The formation of the $pp60^{c-src}$-M-T complex is, in part, dependent upon the existence of a hydrophobic sequence located six residues away from the C-terminal end of M-T (Markland *et al.*, 1986a). Although the hydrophobic sequence is required for membrane localization of M-T, it probably takes part more directly in the transforming function of M-T, because some amino acid substitutions in the hydrophobic sequence which do not prevent membrane association, suppress M-T transforming ability (Markland *et al.*, 1986a). Two tyrosine residues, Tyr 315 and Tyr250, also appear to play a role in the transforming function of M-T (Carmichael *et al.*, 1984; Courtneidge *et al.*, 1984; Markland *et al.*, 1986b). The extent of the transformation response to polyoma M.T in rat F111 cells transfected with a recombinant plasmid in which M-T transcription was driven by the mouse mammary tumor virus LTR could be modulated by addition of dexamethasone to the medium. A direct correlation between the amount of $pp60^{c-src}$-M-T complex formed and the extent of *in vitro* transformation has been observed (Raptis *et al.*, 1985). Nevertheless, in order to confer tumorigenicity to F111 rat cells, a low level of total M-T accumulation is required, after transformation with a murine retrovirus shuttle vector system constitutively expressing polyoma M-T (Jat *et al.*, 1986).

Cooperation between polyomavirus L-T and M-T is sufficient to impart a fully transformed phenotype to primary rodent cells, (Asselin *et al.*, 1986), although it has been claimed that expression of the third tumor antigen, s-t, is also required (Cuzin *et al.*, 1984). Actually the continuous expression of s-t modifies the growth characteristics of NIH3T3 cells and increases their saturation density (Cherington *et al.*, 1986). From experiments performed with SV40, it is also known that the transient expression of s-t disrupts the cellular cytoskeleton organization. This effect is eliminated by mutations which affect Cys_{111} in the sequence $Cys_{111}X$ CysXX $CysX_{21}$ CysX CysXX cys (Bikel *et al.*, 1986). Therefore, the s-t protein can modify cellular functions which could play a role in cellular transformation. A recombinant which encodes polyoma M-T and s-t but not L-T, can indeed elicit a fully transformed phenotype when introduced into primary rat embryo fibroblasts (Asselin *et al.*, 1986). In other words, according to Asselin *et al.* (1986) only two of the three viral proteins are required for the polyomavirus transformation of primary cells. One is the M-T protein whereas the second one can be either s-t or L-T. As a matter of fact, previous *in vivo* experiments had shown that both the M-T and the s-t genes are able to cooperate in the induction of tumors in newborn rats (Asselin *et al.*, 1984). Nevertheless the s-t protein from either polyoma (Rassoulzadegan *et al.*, 1983) or SV40 (Sompayrac and Danna, 1985) is not able to immortalize primary cells. Therefore the s-t gene can cooperate with the class I M-T gene to transform cells, without behaving as a typical class II oncogene.

It has been shown that the class I oncogene T24-Ha-*ras* alone can induce the tumorigenic conversion of rat embryo fibroblasts when it is expressed at high levels (Spandidos and Wilkie, 1984) and under conditions which prevent interactions between transformed and normal cells (Land *et al.*, 1986). To our knowledge no similar observation has been made as yet with polyoma M-T when the target for transformation is a primary rodent embryo cell. On the contrary, primary chicken embryo fibroblasts can be transformed into foci forming anchorage-independent cells by polyoma M-T alone (Kaplan *et al.*, 1985; Kornbluth *et al.*, 1986). A variant of Rous sarcoma virus that encodes polyoma M-T in place of v-*src* induces hemangiomas and hemangiosarcomas when inoculated into 1-week-old chickens (Kornbluth *et al.*, 1986).

Cellular transformation by SV40

The simian virus 40, which codes for two early antigens only, is less efficient than polyomavirus as a transforming agent (Perbal and Rassoulzadegan, 1980) and this low transforming capacity probably correlates with the absence of the M-T gene.

The ability of SV40 L-T to act as an immortalizing class II oncogene has been well established (Petit *et al.*, 1983). A fraction of SV40 L-T in SV40-transformed cells forms a complex with the cellular protein, p53 (Lane and Crawford, 1979; Linzer and Levine, 1979; McCormick and Harlow, 1980). As a result, the half-life of the p53 protein is considerably extended and the steady-state level of the p53 protein in transformed cells is increased well above the level observed in non-transformed control cells (Oren *et al.*, 1981; Oren and Levine, 1983). Because the p53 protein appears to play a role in the cell cycle Go-Gl/S transition (Mercer *et al.*, 1982; Reich and Levine, 1984) and because the p53 gene can cooperate with T24-Ha-*ras* in primary rodent cell transformation Eliyahu *et al.*, 1984; Parada *et al.*, 1984), it could be suggested that SV40 L-T owes, at least in part, its immortalizing ability to its stabilizing effect on p53. But immortalization of primary rodent cells can be obtained with SV40 mutants which express N-terminal sequences of L-T only (Sompayrac and Danna, 1984; Gardes and Feunteun, unpublished observations). These truncated forms of L-T are not able to form a complex with p53 (Sompayrac *et al.*, 1983). On the other hand, the F8dl mutant of Sompayrac and Danna, which immortalizes Fisher rat embryo primary cells at 20% efficiency of the wild type virus and fully transforms rat F111 cells and mouse BALB/c 3T3 A31 cells as efficiently as the wild type (Sompayrac and Danna, 1984), does not induce foci on Fisher rat embryo fibroblasts (Sompayrac and Danna, 1985). Michalovitz *et al.* (1986) have recently shown that the p53 protein, expressed from an efficient expression vector, can cooperate with the F8dl SV40 mutant in the focus assay performed with primary rat embryo cells. Therefore increased levels of p53, obtained either through over-expression of the p53 gene or through SV40 L-T complex formation might be more important to impart transformed properties to primary cells than to immortalize them. It should be recalled in this respect that, in adenovirus transformation, p53 forms a complex with a transforming E1B protein and not with an immortalizing E1A protein (Van der Eb *et al.*, 1977; Shiroki *et al.*, 1979). As to polyomavirus L-T, it immortalizes primary cells without forming a complex with p53 (Dilworth *et al.*, 1984).

It has recently been suggested that SV40 L-T gene is similar to the establishment class II genes *myc* and adenovirus E1A and does not share acute transforming properties with polyomavirus M-T (Jat *et al.*, 1986). This suggestion stemmed from the observation that F111 cells expressing SV40 L-T after infection with a murine retrovirus recombinant constructed by inserting the appropriate DNA segment into p Zip Neo SV(x)1 and selection for G418 resistance, were not tumorigenic. Nevertheless these cells displayed a high plating efficiency in soft agar, i.e., possessed a fully transformed phenotype according to *in vitro* criteria. The level of accumulation of the non-selected L-T gene was estimated at about one-fifth the level observed in SV40-transformed monkey Cos M_6 cells taken as controls. Because it has been previously shown that the extent of transformation by polyoma M-T itself is directly correlated with the level of accumulation of the protein and that threshold concentrations for various transformation properties exist (Raptis *et al.*, 1985), one may question the validity of the suggestion made by Jat *et al.* (1986) in the absence of data on cells expressing higher levels of L-T. In any case, SV40 L-T did convert F111 cells to anchorage independence and therefore behaved in this respect as a transforming class I gene.

Although the majority of SV40 L-T molecules (90-95%) is associated with the cell nucleus, a minor fraction is located at the plasma membrane and both N- and C-terminal sequences are exposed at the cell surface (Deppert and Walter, 1982; Gooding and O'Connell, 1983; Klockmann *et al.*, 1984). It has been suggested that nuclear L-T is more directly involved in cellular immortalization, while membrane L-T could play the role of a class I oncogene (Jarvis *et al.*, 1986; Paucha *et al.*, 1986). Actually, mutants of the L-T gene, in which the transport of L-T to the nucleus is impaired, still efficiently induce the formation of foci on established rodent cell lines, but are much less efficient at inducing foci on primary rodent embryo cells (Lanford *et al.*, 1985; Kalderon *et al.*, 1984; Fisher *et al.*, 1985). The same is true for a set of mutants in which the specific binding of L-T to SV40 DNA is lost and which encode mutated L-T's localizing both to the cytoplasm and to the cell nucleus (Welsh *et al.*, 1986). These observations are in agreement with the above hypothesis but a direct test of the immortalizing ability of the L-T nuclear transport mutant SV40 (cT)-3 (Lanford and Butel, 1984) has now been performed by Vass-Marengo *et al.* (1986) who have found that an SV40 (cT)-3 recombinant plasmid immortalized primary rat embryo fibroblasts as efficiently as did the wild type DNA, when cotransfected in the presence

of pSV2neo. Nevertheless, these observations do not rule out the possibility that enough mutant T antigen accumulates in the nucleus to induce immortalization without reaching the level required for positive nuclear anti-L-T immunofluorescence. Actually Lanford and Butel (1984) have observed that the SV40 (cT)-3 mutant replicates on Cos-1 cells, although, in these cells, the effect on nuclear anti L-T-immunofluorescence is *trans*-dominant. Therefore, SV40 (cT)-3-infected Cos-1 cells, which are negative in nuclear anti-L-T-immunofluorescence do accumulate enough L-T to permit the initiation of viral DNA replication.

On the other hand, the molecular basis for the transport to and the association with the plasma membrane of the minor L-T membrane fraction has not been exactly defined (Jarvis *et al.*, 1986) and mutants in which association with the plasma membrane would be impaired and the nuclear transport would be conserved are not available at the moment.

The nuclear transport mutant SV40 (cT)-3 produces an L-T protein which still associates with p53 and the intracellular partitioning of p53 in cells expressing SV40 (cT)-3 L-T mimics that of L-T itself (Lanford *et al.*, 1985). An alternative explanation of the nuclear transport mutant's lack of transforming ability towards primary embryo cells could be based on the inappropriate intracellular localization of the p53-L-T complex.

But because recent observations have shown that the exact influence on cell behavior of class I and class II oncogenes can vary as a function both of the target cell and of the level of accumulation of the oncogene product, the data on SV40-nuclear transport and localization mutants could also be explained on the basis of different threshold levels of nuclear L-T required for transforming established or primary cells.

A new member of the polyomavirus family: the hamster papovirus HaPV

Although the hamster papovirus has been isolated from skin epitheliomas spontaneously arising in a colony of Syrian hamsters by Graffi and collaborators in 1968 (Graffi *et al.*, 1968), the exact relationship of this virus to other papoviruses remained unknown until recently, because of a lack of an efficient *in vitro* replication system. The morphology of the virions and the size of the DNA suggest that it belongs to the polyomavirus family, despite the fact that its interaction with keratinized cells may be reminiscent of the papillomavirus pathology. Moreover both virus and DNA preparations are able to induce lymphomas

and leukemias when injected subcutaneously into newborn hamsters (Graffi *et al.*, 1970).

After cloning of HaPV DNA, the complete sequence has been established by Delmas *et al.* (1985). From a consideration of the open reading frames and potential splicing sites, a genomic structure has been proposed which includes a putative early region, coding for three early proteins. Although a formal identification of each of these three proteins has not been accomplished as yet, the predicted aminoacid sequences display convincing similarities with the predicted aminoacid sequences of polyomavirus proteins. In particular, aminoacid sequences typical of polyoma virus M-T, such as the C-terminal hydrophobic sequence responsible for membrane association (Markland *et al.*, 1986a) and the acidic sequence next to Tyr315 of M-T are found in equivalent positions, with limited aminoacid substitutions in the putative HaPV M-T proteins (figure 1). Upon transfection, cloned HaPV DNA is able to immortalize primary rat embryo fibroblasts and to induce the formation of foci on rat F111 cells. The viral DNA is integrated in the cellular genome (Delmas *et al.*, 1985).

Further work is in progress to characterize the early proteins and to define their respective roles in transformation.

Conclusions

Despite the fact that polyoma viruses have simple genomes, and contrary to some acute oncogenic retroviruses, like the Rous sarcoma virus, it appears that their oncogenic properties are dependent upon the expression of more than one gene.

In the case of the mouse polyoma virus, the M-T antigen is always required for full transformation and tumorigenesis. Although the interaction of M-T with other cellular proteins is not excluded, it is clear that the formation of a complex with $pp60^{c-src}$ plays an essential role in cell transformation. Polyoma M-T therefore activates the product of the cellular proto-oncogene, c-*src*, with consequences in terms of enzymatic specific activity and target choice which could be related to those which result from activation of $pp60^{c-src}$ by retroviral transduction.

In both polyoma and SV40, one step towards full cellular transformation involves an interaction between the cell nucleus and the viral L-T antigen. This interaction enables primary rodent embryo cells to grow continuously in culture. In both SV40 and polyoma, the totality

AMINOACID HOMOLOGIES BETWEEN POLYOMA M-T AND THE PUTATIVE HAPV M-T

	MIDDLE-T HYDROPHOBIC DOMAIN		
	390	400 410	
POLYOMA (421)	RRLGR	TLLLVTFLAALLGICLMLFILI	KR ...
	370	380 390	
HAPV (401)	*RNRLRR*	*LVLMIFLAALGGFFLTLFFLI*	*KR ...*
	MIDDLE-T TYROSINE PHOSPHORYLATION		
		309 315	
POLYOMA		EEEEEEY	
		312 318	
HAPV		*EEEEPQY*	

Fig. 1

of the L-T protein is not required. The N-terminal sequences are sufficient to ensure cellular immortalization (Rassoulzadegan *et al.*, 1983; Sompayrac and Danna, 1985). L-T functions, which are absolutely required for viral DNA replication and viral transcription regulation, can be dispensed with. This is true of the ATP-binding and ATP-ase activities associated with the C-terminal half of L-T (Cole *et al.*, 1986) as well as of the binding of L-T to specific DNA sequences located near or at the viral DNA replication origin (Gluzman and Ahrens, 1982). It is likely that the immortalizing ability of L-T is directly related to the effect that this protein exerts on cellular DNA replication in lytic infections (Tooze, 1981) and one may suggest that L-T bypasses cellular signals which are involved in the control of cellular proliferation.

Although SV40 does not code for three viral tumor antigens, it is likely that SV40 L-T is a multifunctional protein which operates at two different levels in cellular transformation. Besides its involvement in cellular immortalization, SV40 L-T may take part in transformation through its interaction with the plasma membrane and/or the cellular p53 protein.

In other words, it appears that full transformation by polyoma viruses is brought about by viral proteins with at least two different types of targets within cells.

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CRYSTALLOGRAPHIC AND MOLECULAR MODELLING STUDIES ON CARCINOGEN-DNA INTERACTIONS

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1. Introduction

The identification of DNA as a critical macromolecular target for carcinogetic polycyclic aromatic hydrocarbons (PAHs) (Brookes and Lawley, 1964), and the subsequent identification of a diol epoxide as the ultimate carcinogenic metabolite for benzo(a)pyrene (Sims *et al.*, 1974), have been the foundation for much of the major increases in our understanding of the molecular basis of early events in cancer processes. In this chapter, we shall concentrate on studies of these processes at the detailed molecular structural level, and of relationships between the biological actions of PAHs and these structural results.

A) *Metabolism and Reactivity*

Different polycyclic hydrocarbons can show orders of magnitude difference in carcinogenic activity. Examination of the hydrocarbon chemical structures alone has not been able to satisfactorily explain these differences, even when electronic factors were invoked. The identification of specific regions in a polycyclic hydrocarbon (Fig. 1) that have particular electronic properties, especially a reactive K-region, has enabled carcinogenic ranking orders to be correctly predicted in some instances; there are however many exceptions to such rules, which suggest that the hydrocarbons themselves are not ultimate carcinogenic agents.

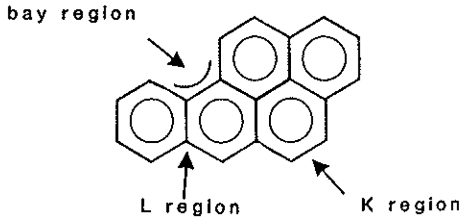

FIG. 1. The reactive regions in a polycyclic aromatic hydrocarbon.

The central role of metabolic activation was first realised following the finding that upon applying benzo(a)pyrene and other polycyclics to mouse skin, a small fraction becomes covalently bound, and that only the extent of binding to cellular DNA correlates with carcinogenic potency (Brookes and Lawley, 1964). Initially, it was believed that K-region epoxides were the responsible compound; subsequent studies showed that the reactive compounds are diol epoxides, with the epoxide group in the bay region of the hydrocarbon (Harvey, 1981; Sims *et al.*, 1974; Newbold and Brookes, 1976). In the case of benzo(a)pyrene, initial metabolism is to the 7,8-epoxide, then to the 7,8-diol, and finally to the 7,8-diol-9,10-epoxide (BPDE). These processes (Fig. 2) are at least initially catalysed by cytochrome-P-450 enzymes (the mixed-function oxygenases), with ring-opening of the 7,8-epoxide catalysed by the epoxide hydrolase enzyme. This produces the highly reactive electrophile diol epoxide which can then form a carbonium ion. This in turn can directly react with cellular macromolecules such as DNA, become involved in detoxification pathways, or be hydrolysed to the 7,8,9,10-tetrol. Bay-region reactivities have received quantum-mechanical rationalisations based on estimates of the relative increase in delocatisation energy on proceeding from a diol epoxide to the reactive cation (Lehr *et al.*, 1985), which indicate that the stability of this cation can correlate with carcinogenicity, and that non-bay-region cations would be both less stable and less reactive. This, the so-called "bay-region" theory, works well for a given hydrocarbon (especially for benzo(a)pyrene, on which much of these theoretical studies have been performed), but rather less well for comparisons between hydrocarbons. This is unsurprising since the theory takes no account of other major factors involved in biological response. Particularily relevant to this review are interactions with DNA; stereochemical and electronic aspects of these would need to be taken into account for a more complete bay-region theory.

In particular, there is now increasing evidence that the critical carbonium ion is generated from the diol epoxide when the latter is bound to DNA in an initial recognition mode (reviewed in Geacintov, 1986).

B) *Stereochemical Aspects*

The enzymatic metabolism of polycyclics is highly stereospecific. For benzo(a)pyrene, (BP), the pathway indicated in Fig. 2 involves the (+)-7,8-epoxide initially being formed, then *trans* ring-opening to the (−)-7,8-diol. The principal product of further oxidation is the (+)-*anti* isomer of the 7,8-diol 9,10-epoxide (Thakker *et al.*, 1971). This is one of four possible stereoisomers (Fig. 3). Much biological work has been performed on *anti* and *syn* forms. In general, the (±) *anti* is the more biologically active by far, compared to the (±) *syn* in terms of tumour-initiating and mutagenic properties (Newbold and Brookes, 1976; Kapitulnik *et al.*, 1978) and the (+)-*anti* enantiomer has much higher tumorigenicity than the (−) form. It is generally accepted that this (+) form is the ultimate metabolite of benzo(a)pyrene. The stereochemistry of its substituents can be described as 7β, 8α, 9α, 10α (Yagi *et al.*, 1977).

The *syn* isomers have been predicted to be more reactive than the *anti* ones, on the basis of stereochemical considerations (Hulbert, 1975); this has been borne out by studies on the rate of nucleophilic attack on the epoxide ring in solution (Yang *et al.*, 1977; Whalen *et al.*, 1978) *in vitro*. The *syn* isomers are also mutagenic, to a greater extent than the *anti* in *Salmonella typhimurium* (Malaveille *et al.*, 1977), although the reverse is true in mammalian cells. The *syn* isomers are only very weakly carcinogenic (Buening *et al.*, 1978).

Numerous studies on polycyclic aromatic hydrocarbons such as benz(a)anthracene (BA); 7,12-dimethylbenz(a)anthracene and chrysene have similarly shown the critical importance of metabolic activation and stereochemistry of resulting metabolites in relation to biological activity (Searle *et al.*, 1984; Harvey, 1981). X-ray crystallographic studies have been reported on some of these metabolites as well as on a number of benzo(a)pyrene derivatives, and are discussed below. By contrast, *in vitro* studies of interactions with DNA have almost exclusively concentrated on benzo(a)pyrene metabolites, as the archetypal chemical carcinogens. This has meant that an understanding at the DNA interaction level, of differences in behaviour between polycyclics with widely varying carcinogenicities has yet to be achieved.

FIG. 2. Pathways of metabolism and DNA binding for benzo(a)pyrene.

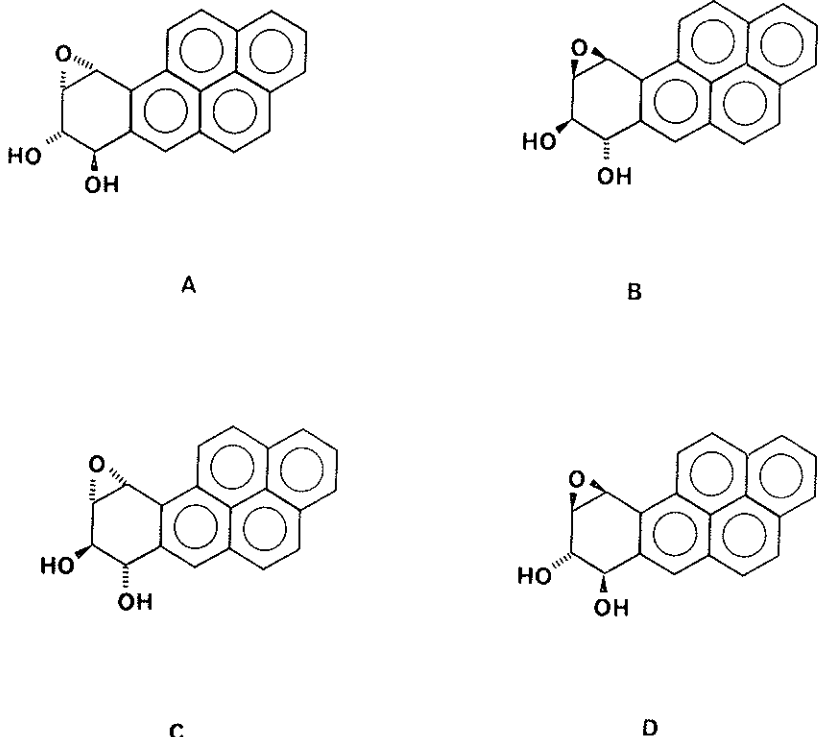

Fig. 3. The stereoisomers of benzo(a)pyrene 7,8-diol-9,10-epoxide. Reproduced by permission of Cancer Research, Temple U., Phila., Pa.

2. Crystallographic Studies

A) *Overview*

In principle, X-ray crystallography is capable of providing a uniquely detailed picture of the three-dimensional arrangements of atoms in molecules. This picture, it is important to bear in mind, is both time-averaged over the lengthy period (typically several days) of an X-ray crystallographic data collection experiment, and spatially-averaged over the thermal motions of the atoms involved. Conformations observed by crystallography are necessarily low-energy ones, though not necessarily global minima. This is largely on account of such factors as hydrogen-bonding to solvent and hydrophobic interactions with neighbouring

molecules in the crystalline state. It is nevertheless possible to obtain dynamic information about molecular motions from analysis of the thermal parameter data obtainable from accurate and highly-refined X-ray studies. Such analyses have been made for several short oligonucleotide sequences (Holbrook and Kim, 1984), and for an intercalation complex between proflavine and the self-complementary dinucleoside CpG (Aggarwal and Neidle, 1985). These studies have shown the importance of coupled motions of groups within nucleic acids, a conclusion reinforced by the few molecular dynamics calculations performed to date on oligonucleotides (Singh *et al.*, 1985; Seibel *et al.*, 1985).

X-ray crystallographic analyses have now been reported on some 10-12 distinct oligonucleotide sequences (for example, the dodecamer dCGCGAATTCGCG (Dickerson and Drew, 1981) and octamers dGGTATACC (Shakked *et al.*, 1983), dGGCCGGCC (Wang *et al.*, 1985) and dGGGGCCCC (McCall *et al.*, 1985)). Even though these as yet represent only a small fraction of the possible combinations of sequence, it is clear that a picture of sequence-dependent structural properties is emerging (Dickerson, 1983), which is beginning to be paralleled by NMR nuclear Overhauser effect structural assignments in solution (Clore and Gronenborn, 1985). There is also a significant body of X-ray crystallographic data on non-covalent complexes between low-molecular weight drugs and oligonucleotides (Neidle and Berman, 1983; Shieh *et al.*, 1980; Aggarwal *et al.*, 1985; Quigley *et al.*, 1980; Jain, Tsai and Sobell, 1977).

Equivalent data on the crystal structures of covalent complexes between polycyclic aromatic hydrocarbons and oligonucleotides is at present entirely lacking. This is doubtless due, at least in part, to the experimental difficulties involved in producing sufficient quantities of oligonucleotide that have appropriate modifications. It is only within the past few years that synthetic methods have evolved sufficiently for large quantities ($>$ 10 mg) of ultra-pure oligomers to be made relatively widely available, albeit at high cost. The problems of purification from the low-yield reactions between the polycyclics and nucleobases, to produce equivalent quantities of adducts, have not as yet been fully overcome. NMR studies on other than mononucleotide adducts, have similarly not been able to proceed.

This lack of direct structural data has resulted in a plethora of molecular modelling studies. These have ranged from the use of hand-built models, to quantum-chemical and molecular-mechanical calculations. Although these are more indirect approaches to understanding the problems

of structure-activity relationships for carcinogen polycyclic aromatic hydrocarbons, they do have the advantage of potentially providing information on dynamic, electronic and stereochemical aspects of the interactions, as well as being able to define their energetics. However, these advantages are critically dependent on, for molecular mechanics (the only feasible quantitative approach for large molecule assemblies), the development and utilisation of reliable force-fields and parameterisations. This goal has as yet only been partially realisable for nucleic acids themselves.

The approach developed in the author's laboratory for studying carcinogen-DNA interactions, has been to combine the results of X-ray crystallographic studies on the metabolites of PAHs with those on nucleic acids, by means of molecular modelling techniques. This paper describes progress to date, in relation to parallel studies by other workers; it is only recently that it has been possible to relate such analyses to chemical, biochemical and biological data; we emphasise the relevance of such correlations since these, together with predictive ability in respect of new experiments and their results, are probably the most important criteria by which the results of modelling studies can be finally judged.

B) *The Structures*

This section reviews metabolites of polycyclic aromatic hydrocarbons, their adducts with nucleic acid fragment, and model systems for them. Table 1 summarises these former structures. The structures of the hydrocarbons themselves are not detailed here as their relevance to carcinogenic processes is only indirect by comparison with their activated metabolites.

The first three structures in Table 1 are all non-K-region-hydroxylated. In all cases, the vicinal hydroxyl groups are *trans* to each other. Whereas they are diaxial in the bay-region 1,2-dihydroxy-benz(a)anthracene, they are diequatorial in the non-bay-region 10,11-dihydroxy-benz(a)anthracene 7,8-dihydroxy-benzo(a)pyrene. Parallel NMR studies in solution have shown that this bay-region conformer is the sole one present, whereas the non-bay-region structures show axial↔equatorial equilibria in solution. This difference is primarily due to steric interactions in the bay region; the proximity of the hydrogen atom at the 12 position to the hydroxy group attached to the Cl atom forces it to be axial with consequent hindrance to ring repucker and axial-equatorial interconversion. A recent *ab initio* quantum chemistry calculation (Beland *et al.*, 1984) has confirmed that

TABLE 1 - *X-ray Crystallographic Analyses of Metabolites of Polycyclic Aromatic Hydrocarbons.*

		Reference
(a)	*Diols and Tetrols*	
(±)	-1,2-Dihydroxy-BA	Zacharias *et al.*, 1979.
(±)	-10,11-Dihydroxy-BA	*Idem, Ibid.*
(±)	-7,8-Dihydroxy-BP	Neidle *et al.*, 1981a.
(±)	-5,5-Dihydroxy-7,12-DMBA	Zacharias *et al.*, 1977.
(±)	-8,9,10,11-Tetrahydroxy-BA	Neidle *et al.*, 1982.
(±)	-7,8,9,10-Tetrahydroxy-BP	Neidle *et al.*, 1981b.
(b)	*Epoxides and Diolepoxides*	
(±)	5,6-Epoxide of DMBA	Glusker *et al.*, 1976.
(±)	9,10-Epoxide of Phenanthrene	*Idem, Ibid.*
(±)	4,5-Epoxide of BP	Glusker *et al.*, 1976.
(±)	3,4-Epoxide of Cyclopenta(cd)pyrene	Kuroda and Neidle, 1983.
(±)	-*Anti*-7,8-dihydroxy-9,10-epoxy-BP	Neidle *et al.*, 1980.
(±)	-*Syn*-7,8-dihydroxy-9,10-epoxy-BP	Neidle and Cutbush, 1983.
	Anti-3,4-dihydroxy-1,2-epoxy - napthalene	Klein and Stevens, 1984a.
	Syn-3,4-dimethoxy-1,2-epoxy-naphthalene	Klein and Stevens, 1984b.
(±)	*Syn*-2-methyl-3,4-epoxy-1-naphthol	Glusker *et al.*, 1982.

Abbreviations: BP: benzo(a)pyrene
BA: benzo(a)anthracene
DMBA: dimethylbenz(a)anthracene

the diequatorial conformation is the most stable one for bay-region vicinal diols of polycyclic aromatic hydrocarbons.

A recurrent observation in these structures is the introduction of olefinic bond character adjacent to the hydroxyl groups, compared to the parent hydrocarbons. Thus, further hydroxylation or ring epoxidation could be expected to occur at these electron rich regions. This is indeed the case, as observed, for example, in the case of benzo(a)pyrene→7,8-diol-BP→7,8-diol-9,10-epoxy-BP. The C9-C10 distance in the 7,8-diol of benzo(a)pyrene, of 1.32 Å is 0.036 Å (i.e., 9 esds) shorter than in the parent hydrocarbon. Several of the benzo(a)pyrene and benz(a)anthracene diols (and possibly their other hydroxylated metabolites), show charac-

teristic K-region bond shortening, as do the parent hydrocarbons. It has been established in the case of benzo(a)pyrene, that the K-region 4,5-epoxide is a primary metabolite, and may be produced in detoxification processes (Harvey, 1981). Hydroxylations to produce phenols at various positions also occur during metabolic activations, although few of them have been fully characterised. The crystallographic results probably indicate that carcinogenic polycyclic hydrocarbons are sensitive to geometric changes produced by partial ring saturation and hydroxylation, with resultant stereoelectronic changes remote from the initial sites of attack, although at the present time, the relatively small body of crystallographic data does not enable this issue to be fully resolved.

The crystal structures have been determined of the hydroxylated ring-opened tetrols that may be obtained directly from hydrolysis of the reactive *anti* "ultimate carcinogen" diol epoxides of both benzo(a)pyrene and benz(a)anthracene. Their stereochemistries are directly relevant to those when covalently bound to the N2 atom of guanine in DNA. In both cases, the racemic structures have the same relative orientations of four hydroxyl group substituents, and in particular the *trans* orientations of the two hydroxyl groups replacing the epoxides confirm the *trans* ring-opening of this ring. There is evidence from a number of studies that this involves an intermediate carbonium ion. The relative orientations of the hydroxyl groups in the benzo(a)pyrene tetrol (Neidle *et al.*, 1982) are 7α, 8β, 9β, 10α, which is equivalent to the 8α, 9β, 10β, 11α, in the benzo(a)anthracene tetrol (Neidle *et al.*, 1981). However, the conformational properties of the two structures are dissimilar, with the former having 7(e),8(e),9(a),10(a), and the latter 8(a),9(a),10(e),11(e). Solution evidence from proton NMR indicates that the benz(a)anthracene tetrol is an approximate 1:1 mixture of two conformers, one of which is the crystallographically-observed one, and the other being the e,e,a,a. The crystallographically-observed conformation for the benzo(a)pyrene tetrol (Fig. 4) would be expected to be preferred in solution, since the axial hydroxyl group at C10 minimises steric repulsions between it and the hydrogen atom attached to C11. This conformation is in accord with NMR and chemical assignments. The saturated rings in these polycyclics adopt distorted half-chair puckers. Table 2 shows that quantitatively the puckers in these rings of the benzo(a)pyrene and benzo(a)anthracene tetrols are rather similar.

Table 3 details the epoxide ring geometries found in these structures. Average C1-O and C2-O bond lengths are 1.456 Å and 1.467 Å respectively. These are significantly longer than values found from a survey of the

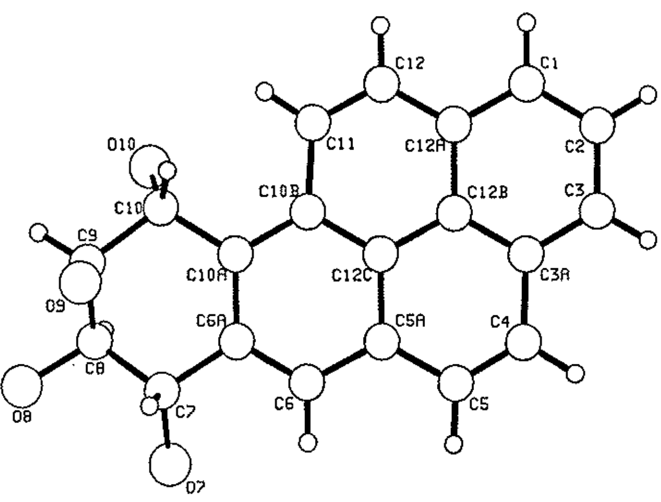

FIG. 4. The crystal structure of benzo(a)pyrene 7,8,9,10-tetrol.

TABLE 2 - *Ring torsion angles, in degrees, in various metabolites of PAHs, as determined by X-ray crystallography.*

Structure	α	β	γ	δ	ε	μ
BP 7,8-diol	−9.1	−14.9	1.6	33.7	−54.2	44.1
BP 7,8,9,10-tetrol	−3.7	14.7	−14.4	65.9	−53.8	22.8
anti BP 7,8-diol 9,10-epoxide	6.6	−19.4	− 1.9	34.0	−44.2	24.8
syn BP 7,8-diol 9,10-epoxide	4.9	−22.6	− 2.5	45.3	−59.2	37.0
BA 8,9,10,11-tetrol	7.7	15.8	−49.0	61.6	−38.5	4.2

The ring torsion angles are designated as shown.

ε μ
δ α
γ β

TABLE 3 - *Geometry of the epoxides of polycyclic aromatic hydrocarbons, from X-ray crystallographic analyses. Bond lengths are in Å, and angles in degrees.*

	C1-O	C2-O	C1-O-C2
(±) 5-6-Epoxide of DMBA	1.445 (3)	1.457 (3)	60.4 (2)
(±) 9,10-Epoxide of phenanthrene	1.461 (6)	1.459 (6)	60.6 (4)
(±) 4,5-Epoxide of BP	1.478 (5)	1.481 (5)	60.3 (2)
(±) 3,4-Epoxide of cyclopenta (cd)pyrene	1.448(10)	1.496 (8)	61.3 (4)
(±) *Anti*-7,8-dihydroxy-9,10 epoxide of BP	1.460(15)	1.446(15)	59.2(15)
(±) *Syn*-7,8-dihydroxy-9,10-epoxide of BP	1.507(12)	1.474(12)	61.0(10)
Anti-3,4-dihydroxy-1,2-epoxy-naphthalene	1.412 (8)	1.468 (7)	60.6 (4)
Syn-3,4-dimethoxy-1,2-epoxy-naphthalene	1.432 (3)	1.448 (4)	60.9 (2)
(±) -*Syn*-2-methyl-34-epoxy-1-naphthol	1.466 (4)	1.473 (3)	60.0 (2)

Atomic labelling is as shown.

O
C1—C2

In the cases of epoxides not in the bay-region, the arrangement is as shown.

O
2
1

Cambridge Crystallographic Data base, and may be a reflection of the differences in electronic character in the proximities of the epoxide rings in these diverse structures. Asymmetry of the C-O bond lengths is overall indicated in the Table, although the high esds in some structures makes individual comparisons of dubious significance.

The consistent trend in the two *anti* and *syn* benzo(a)pyrene structures of bond length C1-O being longer than C2-O, when taken together, is noteworthy, especially since it is in accord with the established epoxide ring-opening pattern in this series. This involves carbonium ion formation on the more electrophilic atom, and the epoxide oxygen atom being retained on C9, in due course as a hydroxyl group. This C-O bond length asymmetry is in the opposite sense to that found in the epoxides of naphthalene (Klein and Stevens, 1984a,b; Glusker *et al.*, 1982), which do not possess a bay-region. The notion that bay-region-induced steric forces are

responsible for the benzo(a)pyrene epoxide asymmetry is not in accord with force-field calculations (Silverman, 1983) which found both C-O bond lengths to be 1.430 Å in length. It may however be significant that the crucial bay-region H10...H11 non-bonded hydrogen...hydrogen distance was calculated to be 0.46 Å longer than the short 1.696 Å found in the *anti* diol epoxide (Neidle *et al.*, 1980), suggestive of considerable extra strain in the actual molecular structure.

The conformational properties of epoxides of polycyclic aromatic hydrocarbons, especially of benzo(a)pyrene have long been a topic of interest and controversy. The *anti* 7,8-diol-9,10-epoxide (Neidle *et al.*, 1980) crystal structure (Fig. 5) shows, in the solid state, that the two hydroxyl groups are *trans* to each other, and are both equatorial. The epoxide group thus has an axial conformation. This e,e,a, arrangement had previously been predicted on the basis of non-bonded contacts (Zacharias *et al.*, 1979; Yeh *et al.*, 1978). It is also consistent with several theoretical studies on the relative stabilities of different conformers which all indicate that the crystallographically-observed one has the lowest energy, as well as with NMR data (Yagi *et al.*, 1975). The overwhelming evidence of the observed conformation being the lowest energy one, is important for subsequent computer modelling studies on its interactions with DNA.

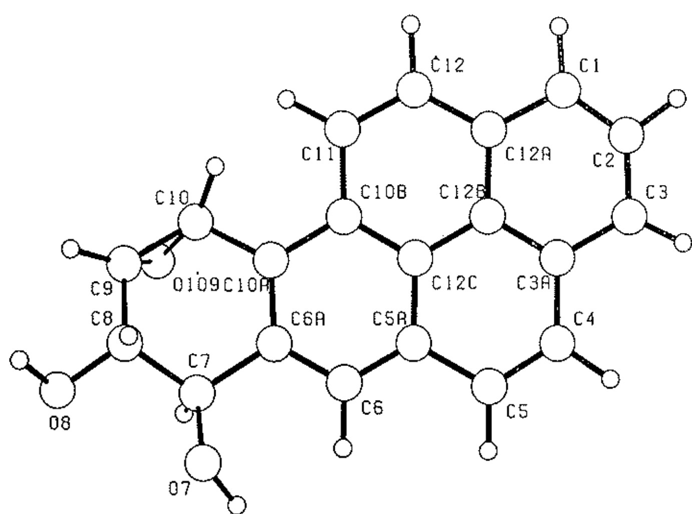

FIG. 5. The crystal structure of *anti*-benzo(a)pyrene 7,8-diol-9,10-epoxide.

The (±) *syn* isomer of benzo(a)pyrene 7,8-diol-9,10-epoxide has high reactivity (see below). It has been suggested (Hulbert, 1975) that this is due to a conformation that could promote nucleophilic attack on the epoxide ring, and theoretical studies have generally found that the preferred conformation would have an a,a,e conformation, such that there is an intramolecular hydrogen bond between the hydroxy group on C7, and the epoxide group, by means of calculations of the relative stabilities of the two *syn* conformers. The X-ray crystallographic analysis of the (±)-*syn* benzo(a)pyrene diol epoxide itself (Neidle and Cutbush, 1983), however, shows the alternative e,e,a conformation (Fig. 6). Thus, the proposed intramolecular hydrogen bond cannot be formed. The apparently severe steric interactions between the hydrogen atom on C8, and the epoxide oxygen atom, are in actuality relieved by a combination of cyclohexene ring pucker changes and movement of the epoxide oxygen atom, so that the H8...O distance is 2.55 Å. This is quantitatively illustrated in Table 2, which shows that the ring torsion angles differ most in the region of the epoxide group, compared to the *anti* isomer. On the other hand, the crystal structures of two *syn* model compounds, both naphthalene derivatives (Klein and Stevens, 1984; Glusker *et al.*, 1982), consistently show the a,a,e conformation with the latter study finding the expected intramolecular

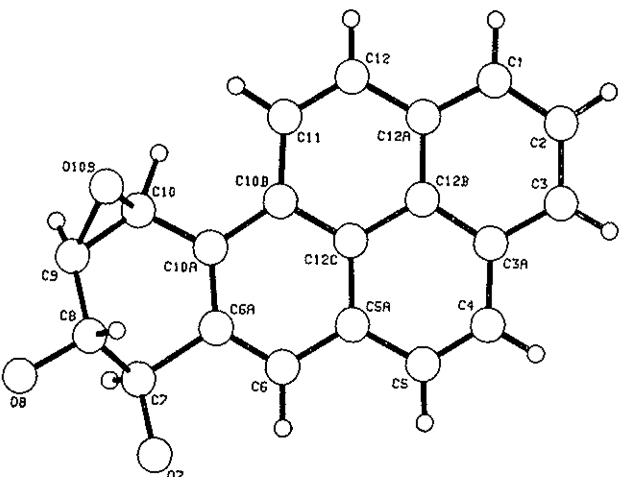

Fig. 6. The crystal structure of *syn* benzo(a)pyrene 7,8-diol-9,10-epoxide.

hydrogen bond. This difference may be a reflection of the lack of a bay-region in these model compounds. Further experimentally-derived structures and more exact theoretical calculations should be useful in illuminating this issue. In particular, the known deficiencies of several approximate quantum chemistry procedures need to be overcome, preferably by reliable *ab initio* methods. The increasing availability of supercomputers should make this task a realistic one.

The difference in reactivity between the *syn* and *anti* benzo(a)pyrene diol epoxides, has been the subject of a number of studies, with water being used as a model nucleophile; solvolysis at C10 produces tetrols with stereochemistry analogous to the DNA adduct at N2 of guanine. An early study (Whalen *et al.*, 1977) showed that the *syn* isomer is some 30 times more reactive to spontaneous hydrolysis, which was rationised in terms of intramolecular hydrogen bonding providing anchimeric assistance to increased negative charge on the epoxide group. This was supported by solution NMR assignments of the epoxides, derived from the stereochemistries of their hydrolysis products (Whalen *et al.*, 1978). A more recent study of the kinetics and product distribution of hydrolysis (Sayer *et al.*, 1982) has concluded that the stereochemistry of the intermediate *syn* carbonium ion corresponds to the e,e,a conformer. Further analysis (Sayer *et al.*, 1984) in terms of alignment of the epoxide C-O bonds with the orbitals of the aromatic ring of the diol epoxides has indicated that the reactivity of the *syn* isomer is due to this alignment, as seen in the crystal structure (Neidle and Cutbush, 1983). Hydrogen bonding effects were not invoked. It was further suggested (Sayer *et al.*, 1984) that the *anti* conformers are less reactive by virtue of their non-aligned conformation; however, detailed examination of the crystal structures of the two BP diolepoxides shows that neither have their epoxide group orthogonal to their aromatic chromophore. For the (±) *anti*, the angle involved is 83°, and 118° for the (±) *syn*.

To date no X-ray crystallographic structural studies have been reported on oligonucleotides alkylated by carcinogenic compounds, such as benzo(a)pyrene 7,8-diol-9,10-epoxide. Eight structures of PAH-alkylated nucleosides have been reported (Carrell *et al.*, 1981; Stezowski *et al.*, 1984). However, since the alkylation site of these compounds is either at N6 of adenine or O6 of guanine, they are not directly related to the active adducts of benzo(a)pyrene, and so will not be further discussed here.

3. Molecular Modelling Studies

A) *Experimental Background*

Electrophilic mutagens and carcinogens generally form covalent bonds to nucleophilic sites on DNA. The N7 site of guanine possesses relatively high nucleophilic strength compared to hydroxyl groups and amino groups. Electrophiles reacting through a SN2 mechanism usually prefer such higher nucleophilic sites. By contrast, compounds reacting mainly via a SN1 mechanism are usually less selective for nucleophilic strength and may cause alkylation at hydroxy and amino groups. It is believed that the greater the positive charge density at the reaction site, the more extensive is the reaction at an exocyclic oxygen as compared to exocyclic nitrogen atoms (Moschel *et al.*, 1979). However, the application of these simple rules has little significance to the reaction of more complicated compounds such as PAHs. DNA adducts formed with benzo(a)pyrene diol epoxide (BPDE) *in vivo* and *in vitro* have been characterised by a number of workers (for example, Jeffrey *et al.*, 1976; Koreeda *et al.*, 1976). The extent of binding of (+)-*anti* BPDE is approximately 90% at N2 of guanine, 5% at N6 of adenine, 1% at N4 of cytosine, with 4% unidentified. There is some reaction with the N7 position of guanine (Osborne *et al.*, 1978, 1981; King *et al.*, 1979); however, the N7 adduct is labile and difficult to detect. O6 of guanine and N6 of adenine derivatives are thought (Osborne *et al.*, 1981) to arise mainly from the (−)-*anti* BPDE (59% at N2 of guanine, 21% of guanine, 18% to N6 of adenine). However, the relative binding to DNA of the (+)- and (−)-isomers is 10:1 (Meehan and Straub, 1979). This stereoselectivity presumably arises at least in part from the steric factors which generally dominate interactions between ligands and DNA. The level of binding to N2 of guanine is correlated to the mutagenic activity in mammalian cells, when the (+)- and (−)-*anti* isomers are compared.

The study of the molecular features of the interactions of polycyclic aromatic hydrocarbons continues to be controversial. The nature of the molecular geometries of covalently bound complexes remains to be definitely established in the absence of either X-ray crystallographic or NMR data. A number of physico-chemical techniques have been used in the case of the best-studied such complex, that of the stable guanine-N2 adduct formed with *anti*-benzo(a)pyrene, 7,8-diol-9,10-epoxide. These techniques (summarised in Neidle and Kuroda, 1987), do not provide fine-structure detail, but nevertheless have enabled the orientation

of the benzo(a)pyrene chromophore to be determined with respect to the DNA helix axis. Data from electric dichroism (Geacintov *et al.*, 1978), optical and electric fluorescence measurements (Prusik *et al.*, 1979; Ridler and Jennings, 1984) and optically-detected magnetic resonance studies (Lefkowitz *et al.*, 1979), have been consistently interpreted in terms of an external-groove binding model with the benzo(a)pyrene molecule inclined at 35-50° to the helix axis and accessible to solvent. On the other hand, the finding of benzo(a)pyrene diol epoxide-induced unwinding of closed circular SV40 DNA (Drinkwater *et al.*, 1978), a property characteristic of the physical intercalators such as ethidium or the acridines, has been interpreted in terms of the bound complex having an intercalated structure. This model has received controversial support from studies of benzo(a)pyrene 7,8-diol-9,10-epoxide complexed to sonicated and restriction fragments of DNA (Hogan *et al.*, 1981), who further suggest that the nucleic acid is kinked at the site of intercalation. The interpretation of these results has been critically examined (Geacintov, 1985); it is pointed out that the experimental data is more consistent with an externally-bound chromophore rather than an intercalated one.

Non-covalent intercalation itself is now increasingly accepted as a major mechanism of interaction between DNA and benzo(a)pyrene-7,8-diol-9,10-epoxide, and is the initial recognition event for these species prior to both covalent interaction, and detoxifying hydrolysis of the epoxide to tetrols (Geacintov, 1986; Geacintov *et al.*, 1984a,b). Kinetic evidence indicates that, in cellular conditions, the level of final covalent adduct formation is directly proportional to the fraction of non-covalently-bound intercalated diol epoxide. The tetrol hydrolysis product is also capable of intercalation. Thus, the experimental finding of local helix unwinding may be a consequence of the necessarily large ratio of tetrol to covalent adduct.

B) *Molecular Modelling of Non-covalent Intercalative Binding*

Two detailed studies of BPDE intercalated into DNA have been reported (Miller *et al.*, 1985; Subbiah *et al.*, 1983). In both, short lengths of oligonucleotide sequence have been used; the former study has specified nucleotide conformations purely from molecular modelling whereas that of Subbiah *et al.* has taken as a starting-point the geometry of the intercalated dinucleoside dCpG as found in the crystal structure of its proflavine complex (Shieh *et al.*, 1980), as well as the crystal

structure of the BPDE itself (Neidle *et al.*, 1980). This pyrimidine-3′,5′-purine sequence is a favoured one for non-covalent intercalation (reviewed in Neidle and Abraham, 1984). In this study, the optimal low-energy positions for both (+) and (−) *anti*-BPDE were established, using an interactive molecular graphics technique combined with energy calculations that took account of the partial charges at individual atomic centres. Since intercalation is a directional process, BPDE entry to the binding site was studied via both major and minor grooves of the DNA mini-helix. The hydroxyl and epoxide substituents of the BPDEs, being non-coplanar with the benzo(a)pyrene chromophore, inhibit part of the BPDEs from stacking with the base pairs, and thus in all low-energy arrangements, the epoxide ring protrudes into a groove of the DNA. It is notable that the low-energy position found for (+)-BPDE in the DNA minor groove, has the epoxide oxygen atom in attractive close contact (2.82 Å) with an exocyclic amino N2 atom of a guanine base (Fig. 7). In the major groove, close contacts were found between the epoxide oxygen atom and the N4 amino substituent of a cytosine. Although the (−)-BPDE enantiomer can also make a close minor-groove N2 guanine contact, the overall arrangement is of significantly higher energy than that for the (+) one. These stereo-selective differences have been interpreted as having relevance to the initial interaction of BPDEs with DNA prior to covalent adduct formation. It may be significant that the computed lowest-energy minor-groove position has the reactive atoms in close proximity. However, the final adduct geometry has a *trans* arrangement about the 9,10 bond of BPDE, which would involve a complex conformational arrangement subsequent

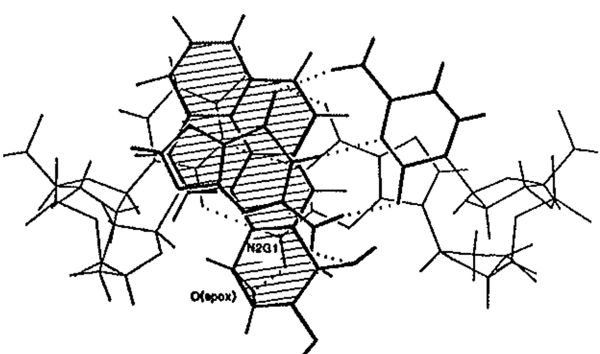

FIG. 7. Minimum-energy structure of a non-covalent intercalative adduct of (+)-*anti* BPDE, with dCpG, as found by computer modelling (Subbiah *et al.*, 1983).

to initial intercalation. This problem has been addressed in terms of covalently intercalated models with kinking of the DNA (Miller *et al.*, 1985; Taylor *et al.*, 1983).

A recent re-examination of non-covalent intercalation (Pearl and Neidle, 1986) has employed novel modelling approaches to focus attention on low-energy major-groove complexes with (+) and (−) *anti*-BPDE. This study has been prompted by the finding (Lobanenkov *et al.*, in press) that guanine-specific BPDE-induced strand cleavage occurs predominantly at pyrimidine-3′,5′-guanine sites. This strand cleavage has earlier been associated with N7 guanine interaction (Osborne *et al.*, 1981), which readily results in depurination. The modelling study shows that the global energy minimum in the major groove has the epoxide oxygen atom of (+)-*anti*-BPDE in close proximity to N7 (Table 4; Fig. 8), which is interpretable in terms of a pre-reaction position.

The biological relevance of BPDE-induced depurination to the carcinogenic activity of benzo(a)pyrene metabolites is controversial and still very unclear (Osborne and Merrifield, 1985). It may however be relevant that base substitutions have been found in C-Ha-ras oncogenes following treatment with (±)*anti*-BPDE at sites such as the CCGG one between codons 11 and 12 (Marshall *et al.*, 1984).

TABLE 4 - *Intercalation Energy Minima for (+)-anti-BPDE*[c].

E_{inter} (kcal)	u (Å)	v (Å)	Θ (°)	D_{C-N} (Å)[a]
−69.7	−1.001	−0.571	315.6	3.00[b]
−68.6	−0.193	−0.584	319.3	2.46[b]
−68.2	−0.104	−0.103	322.8	2.47[b]
−65.9	0.002	0.031	202.5	5.67
−65.6	1.017	−0.782	44.4	6.86
−65.5	−0.295	0.271	105.6	6.63
−64.4	0.552	0.096	210.3	5.92
−62.1	−2.505	0.824	131.1	5.51

[a] This distance is defined between N7 of guanine and 0109 of BPDE.
[b] These distances represent atoms within van der Waals contact.
[c] For definitions of u, v, Θ, see Pearl and Neidle, 1986.

Fig. 8a, b. Two computer-drawn views (using the MIDAS program of Langridge *et al.*), showing the minimum-energy major-groove (+)-BPDE intercalation complex. Figure 8b shows the solvent-accessible surface of the complex.

C) *Molecular Modelling of Covalent Binding*

The numerous studies of this topic have entirely focussed attention on the N2 guanine adduct with *anti*-BPDE. This is in part a reflection of its likely importance as a critical lesion to DNA that results in ultimate carcinogenesis. It is also a resultant of the severe difficulties attendant upon the development of detailed plausible molecular models in the absence of fine-structural experimental data.

An early, non-quantitative model (Beland, 1978) suggested that the necessarily minor-groove N2 attachment implied some degree of steric clash, although this factor has not been examined in detail until very recently. A subsequent model (Kadlubar, 1980), although still entirely quantitative, attempted to resolve this problem by suggesting an *anti* to *syn* glycosidic angle change for the BPDE-bound guanine residue, thus placing the carcinogen in the much wider major groove of B-DNA. The resulting mis-pairing was considered to be a plausible rationalisation for the traversional mutagenesis of benzo(a)pyrene.

Detailed studies of the conformational properties of BPDE adducts with various short-sequence models for DNA (Hingerty and Broyde, 1983, 1985); Lavery and Pullman, 1979) have indicated the large potential range of both classical and non-classical DNA structures that might be accessible to a BPDE-DNA adduct. However, the relevance of modelling studies on such very short sequences to the situation pertaining to a rather longer one with very different conformational constraints, remains unclear.

A study has been made of the N2 adduct with DNA kept in a standard nucleic acid conformation such that the benzo(a)pyrene chromophore is in the minor groove, in accord with the physico-chemical data discussed in an earlier section (Aggarwal *et al.*, 1983). Since the standard B-DNA minor groove is in reality too narrow to accommodate the non-planar BPDE (Table 5), A-form DNA was used instead. Energetically-feasible structures for the (+) and (−) adducts were readily found. The assumption of A-DNA being relevant has the advantage that it incorporates a measure of helix unwinding, in accord with the data of Drinkwater *et al.*, (1978). However, the differentiation of geometry between (+) and (−) adducts suggested by this modelling does not appear to be in accord with the biological data on their repair, which generally does not find a significant difference between them (Brookes and Osborne, 1982).

The question of the relevance of the A-form of DNA to its structure in solution is also an important one. Recent NMR data (Clore *et al.*, 1985) on oligonucleotide structure suggests that the B-form is the dominant

TABLE 5 - *Groove dimensions in nucleic acids, in Å.*

	Minor		Major	
	depth	width	depth	width
A helix*	2.8	11.0	13.5	2.7
B helix*	7.5	5.7	8.5	11.7
Dodecamer**:				
at central AATT	—	3.2	—	12.7
at end of AATT	—	7.0	—	10.6
d(GGATGGGAG)***		8.3-9.7		9.3

* In classic monopolynucleotide duplex helices (Arnott, 1981).
** Dickerson *et al.*, 1985. Groove depths are not given in this study.
*** McCall *et al.*, 1986.

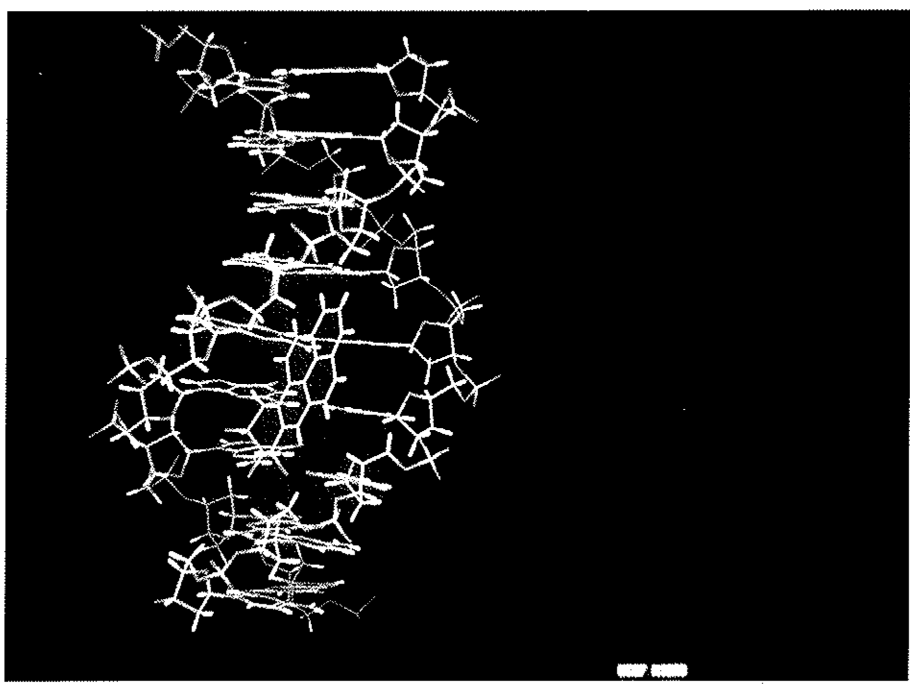

FIG. 9. Computer-drawn figure of (+)-anti-BPDE covalently bound to N2 of guanine in modified B-DNA.

one in solution. This, coupled with the finding of BPDE adduct hot-spots in oligo dG · oligo dC sequences of DNA with the α-globin gene (Boles and Hogan, 1986), has led to the development of a new BPDE-DNA model (Neidle *et al.*, to be published). This is based on standard B-DNA, in which the minor groove around and in the vicinity of the bound BPDE has been systematically widened by up to 1 Å, by means of small concerted changes in nucleotide backbone conformation. Such a widening has been postulated for GC-rich regions in natural DNA (Drew and Travers, 1984), (Figure 9). An interesting feature of this model is that in order to avoid repulsive close contacts around the hydroxyl groups, it is necessary to rotate the benzo(a)pyrene moiety about the C2-N2 bond (Figure 10) by

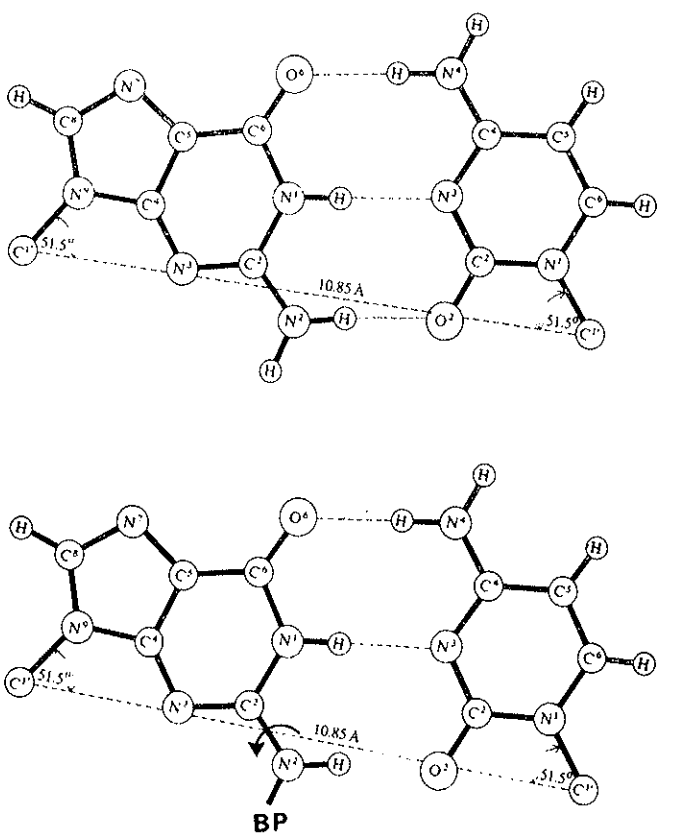

Fig. 10 Guanine-cytosine base pairing without and with a BPDE adduct.

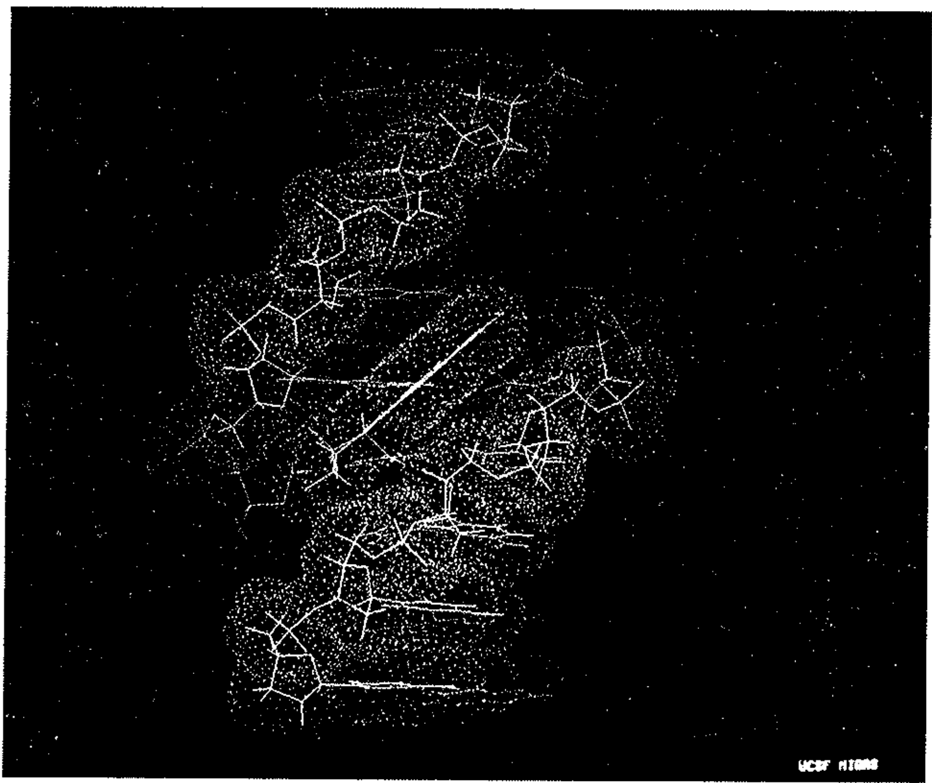

FIG. 11. Computer-drawn van der Waals representation of benzo(a)pyrene covalently bound to guanine, and lying in the minor groove.

35-40°. This change is at a modest energy cost that is more than offset by the resultant loss of steric hindrance. It orients the benzo(a)pyrene along the minor groove (Figure 11), and also results in a loss of one Watson-Crick hydrogen bond in the affected CG base pair. This in itself may naturally lead to transversion mutations following replication, and resultant mis-pairing of the modified guanine with, for example, adenine (Figure 12). These possibilities are currently being further studied.

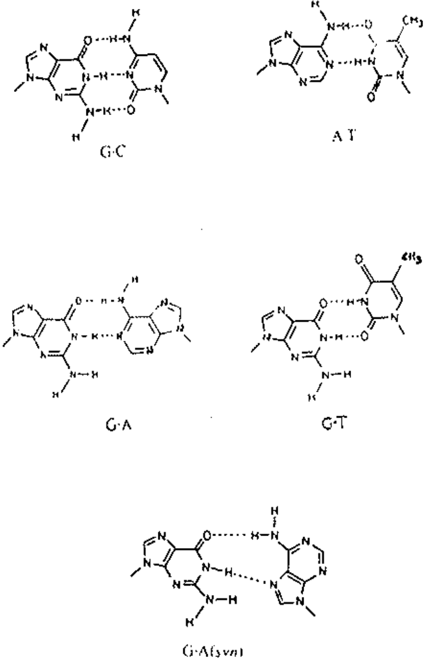

FIG. 12. Various types of base and mis-pairing in DNA relevant to transversion mutations.

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THE MOLECULAR MECHANISM OF CARCINOGENESIS OF POLYCYCLIC HYDROCARBONS

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Abstract

Carcinogenic polycyclic hydrocarbons are ubiquitous environmental pollutants. Current evidence indicates that their mechanism of cancer induction involves covalent interaction of reactive epoxide metabolites with nucleic acids. This topic is reviewed and the detailed molecular mechanism of covalent binding of the active intermediate to DNA is discussed. Evidence is presented that there are significant differences in the extents of binding to DNA and the types of adducts formed by the active epoxide metabolite of carcinogenic hydrocarbons and the analogous derivatives of noncarcinogenic hydrocarbons.

I. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants, and some PAHs are highly potent carcinogens. Hydrocarbons are products of incomplete combustion of organic matter, and significant levels are produced in automobile exhaust, refuse burning, smoke stack effluents, and tobacco smoke. As a consequence, human populations are commonly exposed to carcinogenic PAHs on a daily basis through the air they breathe, the food they eat, and the water they drink. There is substantial reason to suspect that PAHs may play an important role in the causation of human cancers.

The prime objective of research in chemical carcinogenesis is elucidation of the molecular mechanism. How do relatively simple molecules, such as PAHs, trigger the induction of tumors in mammalian tissues? This is one of the major scientific problems of our time. Aside from its intellectual challenge, there is the potential that the knowledge gained could be exploited to aid humanity. This article will review the state of current knowledge concerning the mechanism of PAH carcinogenesis with emphasis on the covalent interaction of reactive hydrocarbon metabolites with nucleic acids.

II. Historical Developments

The earliest evidence implicating PAHs in human cancer were the observations by Percival Pott in 1775 on scrotal cancer in chimney sweeps which he linked to their exposure to soot (Pott, 1963). The foundation for experimental carcinogenesis research was laid much later, in 1915, by Japanese investigators (Yamigawa and Ichikawa, 1915) who demonstrated that the application of coal tar to the ears of rabbits elicited a carcinogenic response. However, it was not until the 1930s that Kenneway and Cook and their colleagues succeeded in identifying the hydrocarbon benzo[a]pyrene as the major biologically active component of coal tar. They started with two tons of coal tar pitch from which they were able to isolate a few grams of benzo[a]pyrene. A key property of the active fraction was its characteristic fluorescence. The complete story, which is one of the classics of scientific discovery, has been reviewed (Kenneway, 1955).

The discovery that carcinogenic activity is a property of specific PAH molecules stimulated extensive investigations to determine the molecular structural requirements for biological activity. From these studies there emerged certain generalizations. Maximum activity requires 4-6 fused aromatic rings, a molecular K-region (typified by the 5,6-bond of benz[a]anthracene), and methyl groups at specific molecular sites, particularly in L-regions (Fig. 1). Benz[a]anthracene (BA), which contains both a K-region and an unsubstituted L-region, exhibits only weak activity, while its dimethyl analog, DMBA, is one of the most potent carcinogens known. 3-Methylcholanthrene (3-MC) which bears a meso methylene group is also highly carcinogenic. Dibenz[ah]anthracene (DBA) is weakly active, while the activity of its dimethyl analog exceeds that of even DMBA (Di Giovanni *et al.*, 1983).

L-region

K-region

Benz[a]anthracene
(BA)

Benzo[a]pyrene
(BP)

Dibenz[ah]anthracene
(DBA)

CH_3

CH_3

7,12-Dimethylbenz[a]anthracene
(DMBA)

H_3C

3-Methylcholanthrene
(3-MC)

CH_3

CH_3

Dimethyldibenz[ah]anthracene

Fig. 1

These structure-activity relationships were the basis of various electronic theories which attempted to explain biological activity in terms of the chemical reactivities and molecular properties of the PAH molecules. Implicit in these concepts was the assumption that the carcinogenic molecules acted directly on a cellular receptor, presumably at the K-region. The terms K- and L-region were introduced by the Pullmans (Pullman and Pullman, 1955), who contributed importantly to the development of these ideas. These theoretical concepts strongly influenced thinking about the mechanism of carcinogenesis until the early 1970s, when it began to be realized that PAHs were not directly active, but required metabolic activation.

The essential role of metabolism was most convincingly suggested by experiments conducted by Brookes and Lawley, 1964. They applied a series of tritium-labelled PAHs to the skins of mice and observed that the extents of covalent binding to DNA correlated rather closely with the relative PAH carcinogenicities. Direct chemical characterization of the bound metabolites was impractical due to the tiny amounts of substances involved. While it was well known that metabolism of PAHs affords a variety of oxidized products, including phenols, dihydrodiols, quinones, and their conjugates, these known metabolites were found to be only weakly carcinogenic or noncarcinogenic. This suggested that the active species might be an intermediate too chemically reactive or unstable to be isolated. The most likely candidate, on the basis of the information then available, was an arene oxide metabolite.

Intermediates of this type, typified by benzo[a]pyrene 4,5-oxide, had been suggested earlier (Boyland, 1950) to be the primary metabolites of PAHs which give rise to the metabolites commonly observed (Fig. 2). Methods for the synthesis of the K-region arene oxide derivatives of the carcinogenic PAHs BP and DMBA were developed subsequently in our laboratories (Goh and Harvey, 1973; Harvey *et al.*, 1975). However, the results of nucleic acid binding experiments with these synthetic arene oxides indicated that the DNA adducts were different chromatographically from those formed by metabolism of the parent PAHs in cells (Baird *et al.*, 1975; and Blobstein *et al.*, 1975). This negative result served the useful purpose of shifting attention away from the K-region to other molecular sites.

III. Identification of Active Metabolites

The modern era of PAH carcinogenesis research began in 1974 with a proposal by Peter Sims that the active metabolite of BP was a diol epoxide derivative formed by microsomal oxidation of its 7,8-dihydrodiol (Sims *et al.*, 1974) (Fig. 2). Methods for the synthesis of the two isomeric forms (Fig. 3) of this diol epoxide, *anti*- and *syn*-BPDE, were developed in our laboratories (Beland and Harvey, 1976; Harvey and Fu, 1978) and by Yagi *et al.*, 1975. The *anti* isomer has the epoxide oxygen atom on opposite face to the benzylic OH group, whereas the *syn* isomer has these groups on the same face. Both isomers exist as pairs of optically active (+) and (−) enantiomers.

Nucleic acid binding studies conducted in collaboration with Weinstein and Brookes and their associates provided strong evidence that (+)-*anti*-BPDE is the principal biological form of BP which binds to the DNA of mammalian cells *in vivo*. Thus, reaction of (±)-*anti*- and (±)-*syn*-BPDE with DNA gave alkylated DNA products which were then degraded to the nucleoside level and chromatographed. The major adduct formed by *anti*-

Benzo[a]pyrene 4,5-oxide

CH_3
CH_3
DMBA 5,6-oxide

HO OH
BP 7,8-dihydrodiol

rat liver microsomes
O_2

HO OH
BP 7,8-diol-9,10-epoxide

Fig. 2

(+)-Anti-BPDE (-)-Anti-BPDE

(-)-Syn-BPDE (+)-Syn-BPDE

FIG. 3

BPDE was found to be identical in all respects with the major product of metabolism and DNA binding of BP in mammalian cells, including human lung tissue explants (Weinstein *et al.*, 1976; King *et al.*, 1976). The molecular structures of the adducts, including their absolute stereochemistries, were completely elucidated by NMR, mass spectral analysis, and other physical analytical techniques (Jeffrey *et al.*, 1977; Jeffrey *et al.*, 1976; Nakanishi *et al.*, 1977) (Fig. 4). The major adduct which accounted for about 80-85% of the alkylated product, was shown to be a guanosine derivative covalently linked between the 2-NH_2 group and the 10-position of the (+)-enantiomer of *anti*-BPDE. Minor nucleic acid-bound products arising from reactions of *anti*-BPDE on 6-NH_2-dA, 7-N-dG, and other base sites were also characterized (Jennette *et al.*, 1977; Osborne *et al.*, 1981). These findings, confirmed subsequently in numerous laboratories, established that (+)-*anti*-BPDE is the principal reactive metabolite of BP which binds covalently to DNA in cells.

Mutagenicity studies provided further support for the hypothesis that diol epoxide metabolites are the ultimate active forms of carcinogenic PAHs. Both the *anti* and *syn* isomers of BPDE showed exceptional potency as mutagens in a variety of bacterial and mammalian cell lines

(Newbold and Brookes, 1976; Huberman *et al.*, 1976; Wislocki *et al.*, 1976). *Anti*-BPDE was generally more active as a mutagen than *syn*-BPDE, and (+)-*anti*-BPDE was more potent than (−)-*anti*-BPDE in this respect. *Anti*-BPDE was also more active than *syn*-BPDE in the induction of malignant transformation of mouse fibroblasts (Marquardt and Baker, 1977). On the other hand, *anti*-BPDE was found to be only a weak carcinogen on mouse skin and *syn*-BPDE was essentially inactive (Levin *et al.*, 1978). This low activity is apparently a consequence of the facility of secondary reactions with proteins and other cellular nucleophiles. However, in the more sensitive mouse lung system (+)-*anti*-BPDE was found to be considerably more tumorigenic than BP or the other isomers of BPDE (Buening *et al.*, 1978). These findings, coupled with the results of the nucleic acid binding studies, provide the strongest evidence that (+)-*anti*-BPDE is the active carcinogenic form of BP.

Analogous diol epoxide metabolites have been implicated subsequently as the active forms of other PAHs (Reviews: Sims and Grover, 1981; Conney, 1982). The weakly active PAHs* (Fig. 5) benz[a]anthracene (Wood *et al.*, 1983; Levin *et al.*, 1984), chrysene (Wood *et al.*, 1982; Chang *et al.*, 1983; Glatt *et al.*, 1986), benzo[e]pyrene, benzo[c]phenanthrene (Wood *et al.*, 1984; Levin *et al.*, 1986, and dibenz[ah]anthracene have been studied more intensively than the more highly potent PAHs. The reason is simply that the dihydrodiol and diol epoxide derivatives of the inactive or weakly active PAHs were more synthetically accessible because they lack methyl groups. The major active isomer has been identified in all cases as the bay region *anti*-diol epoxide, i.e., the epoxide ring is located in a bay region. In most cases the evidence is much less complete than in the case of benzo[a]pyrene and rests primarily on comparison of the relative mutagenicities and tumorigenicities of the isomeric dihydrodiols or the corresponding diol epoxides obtained through synthesis. Since these are weak carcinogens, the level of DNA binding is low, and the DNA adducts have been only partially characterized. There is evidence in the cases of chrysene (Glatt *et al.*, 1986) and benz[a]anthracene (Sims and Grover, 1981) for the involvement of a triol epoxide metabolite and a nonbay region diol epoxide metabolite, respectively, as minor active forms.

Similar studies have been conducted, or are currently being conducted,

* References are given only for more recent publications not cited in the reviews (Sims and Grover, 1981; Conney, 1982).

with the more potent carcinogenic PAHs (Fig. 6) 7-methylbenz[a]anthracene, 7,12-dimethylbenz[a]anthracene (DiGiovanni *et al.*, 1986; Dipple *et al.*, 1984; Dipple *et al.*, 1983; Moschel *et al.*, 1983; Sawicki *et al.*, 1983), 9-fluoro- and 10-fluoro-DMBA (DiGiovanni *et al.*, 1986), 5-methylchrysene (Melikian *et al.*, 1985), 3-methylcholanthrene (Osborne *et al.*, 1986), dibenz[ah]pyrene, dibenzo[ai]pyrene, and dibenzo[ae]fluoranthene (Perin-Roussel *et al.*, 1985). The major active isomer has been identified, at least tentatively, as the bay region *anti*-diol epoxide in all cases except 3-methylcholanthrene. In the latter case, the *anti*-diol epoxide is only a minor DNA-bound metabolite; the principal active form has been

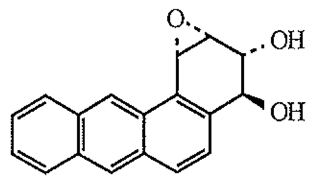
$2\text{-NH}_2\text{-dG}$ $6\text{-NH}_2\text{-dA}$

7-N-dG 6-O-dG

FIG. 4

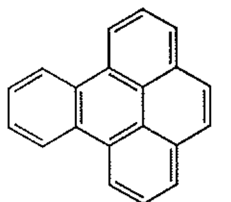
MAJOR

MINOR

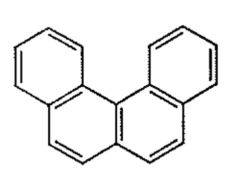


Benz[a]anthracene

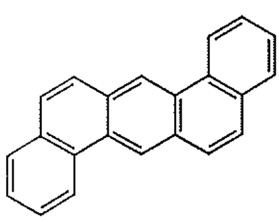
Chrysene



Benzo[e]pyrene



Benzo[c]phenanthrene

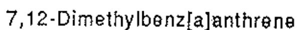


Dibenz[ah]anthracene

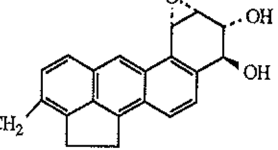
FIG. 5

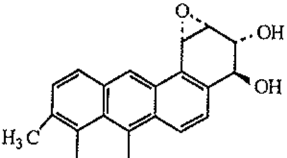
7=Methylbenz[a]anthracene

R = H, F



5-Methylchrysene





3-Methylcholanthrene

Dibenz[ah]pyrene

Dibenz[ai]pyrene

Dibenzo[ae]fluoranthene

FIG. 6

tentatively identified as a related triol epoxide bearing an additional hydroxyl, probably on the methyl group (Osborne *et al.*, 1986). A non-bay region diol epoxide derivative has also been identified as a DNA-bound metabolite of dibenz[ae]fluoranthene (Perin-Roussel *et al.*, 1985). Synthesis of all of the diol epoxide derivatives in Figs. 5 and 6 with the exceptions of the last 3 PAHs in Fig. 6 have been accomplished in our laboratories.

IV. Covalent Binding to DNA

A. *Sites of Covalent Binding*

In most cases, the site of covalent binding to DNA has not been determined with complete certainty, but the available experimental data suggests that 2-NH_2-dG is generally the principal site of attack of all diol epoxide derivatives, independent of carcinogenic activity. Although attention has focused on the major DNA adducts formed, it must be emphasized that numerous minor DNA-bound adducts are also formed and there is no certainty that the major adducts are the most important biologically.

The full range of minor DNA-bound adducts which are actually formed in cells has been investigated only for three PAHs, benzo[a]pyrene (BP), 5-methylchrysene (5-MC), and 7,12-dimethylbenz[a]anthracene (DMBA), and these studies are not entirely complete. In the case of BP, the stereochemistry of metabolism has been investigated in detail (Conney, 1982), and it has been shown that (+)-*anti*-BPDE is the predominant isomer produced metabolically (90%), but minor amounts of (−)-*anti*-BPDE and (+) and (−)-*syn*-BPDE are also formed (Fig. 7). All four isomers are capable of reacting with nucleic acids. (+)-*Anti*-BPDE exhibits a strong preference for reaction on the 2-NH_2 group of guanosine in DNA, yielding ~90% 2N-dG adducts (Osborne *et al.*, 1981) along with lesser amounts of 6N-dA and other products. Analogous reaction of (−)-*anti*-BPDE is less regiospecific, yielding only ~50% of the 2N-dG product along with substantial amounts of 6N-dA, 60-dG, and 7N-dG adducts. The fact that the (−) enantiomer is formed to much lower extent metabolically, reacts much less efficiently with DNA (Meehan and Straub, 1979), and is much less mutagenic (Wood *et al.*, 1977) and carcinogenic (Buening *et al.*, 1978; Slaga *et al.*, 1979) than the (+) enantiomer argues against these minor products being important in either mutagenesis or carcinogenesis.

Reactions of *syn*-BPDE with DNA have been less thoroughly studied. This is due in part to the greater hydrolytic instability of this isomer coupled with its lesser extent of reaction with DNA (Shahbaz *et al.*, 1986). In a typical set of experiments, *syn*-BPDE in solution with DNA underwent 30-40% hydrolysis to tetraols within 20 sec. under conditions where

Fig. 7

hydrolysis of *anti*-BPDE was negligible; moreover, the extent of covalent binding was ~2% for the *syn* isomer compared with 9-10% for the *anti* isomer.

Evidence that the *syn* diol epoxide isomers of some PAHs may play an important role in carcinogenesis is suggested by recent experiments in the DMBA series. Dipple and coworkers (Sawicki *et al.*, 1983) demonstrated that metabolism of DMBA in mouse embryo cells gave both *syn* and *anti* DMBA diol epoxide adducts with DNA (Fig. 8) The stereochemical assignments, made initially on the basis of relative chromatographic retention times, have subsequently been confirmed using the authentic *syn* diol epoxide of DMBA synthesized in our laboratories (Lee and Harvey, 1986). Surprisingly, the major *syn* diol epoxide adduct and one of the

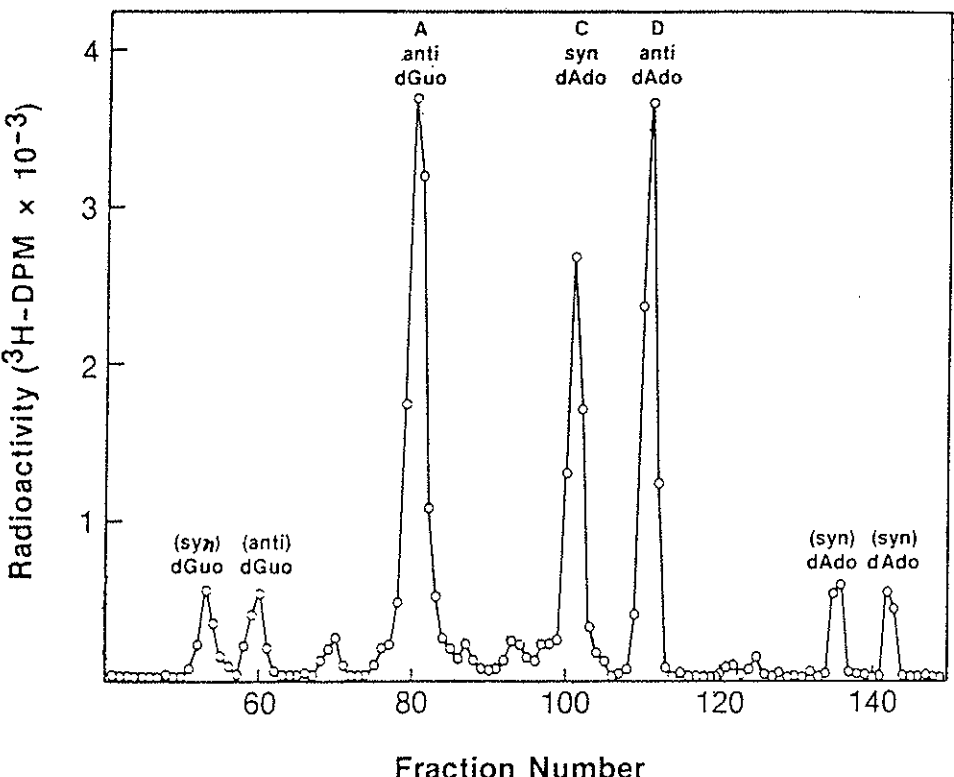


FIG. 8. Reprinted from Dipple *et al.*, 1983, Figure 3, with permission of Elsevier Science Publisher, Amsterdam, The Netherlands.

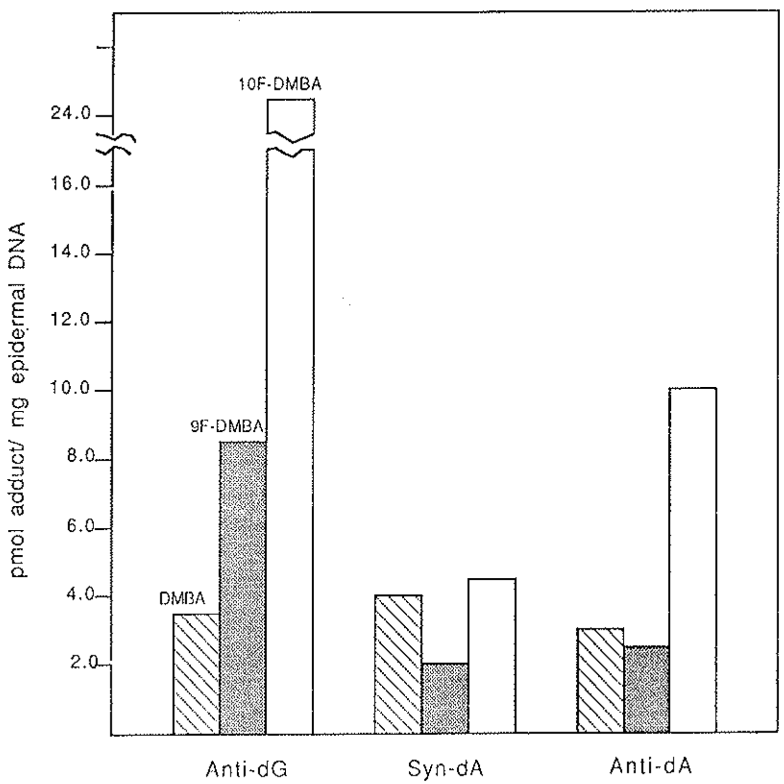


FIG. 9. Reprinted from Di Giovanni *et al.*, 1986. Figure 3, with permission of American Assoc. Cancer Research, Temple Univ. School of Medicine, Philadelphia, Pa.

two major *anti* diol epoxide adducts were bound to deoxyadenosine rather than deoxyguanosine (Dipple *et al.*, 1983).

In a related study (DiGiovanni *et al.*, 1986), the epidermal DNA adducts formed by metabolism of DMBA and its 9- and 10-fluoro derivatives in mouse skin were compared (Fig. 9). 9-F-DMBA is equipotent with DMBA as a carcinogen in this tissue, whereas 10-F-DMBA is more active than the parent hydrocarbon. An excellent correlation was found between the formation of an adduct, tentatively identified as the major *anti*-diol epoxide-d-Ad adduct, and the relative skin tumorigenicities of the parent PAHs. These results suggest that covalent binding of active carcinogen metabolites to adenine residues may be more important than binding to guanine sites. More experiments with a wider range of compounds will be required to determine the generality of this relationship.

B. *Mechanism of Covalent Binding of* anti-*BPDE to DNA and the Structures of the Adducts.*

Another important question concerns the molecular mechanism of interaction of reactive PAH metabolites with nucleic acids. Studies of the reaction of *anti*-BPDE with native calf thymus DNA using UV absorption and fluorescence and linear dichroism techniques have established that the major pathway (90-95%) is not covalent adduct formation, but hydrolysis of BPDE to tetraols (Geacintov, 1985; Geacintov *et al.*, 1984; Geacintov *et al.*, 1982). Moreover, hydrolysis is catalyzed by DNA which increases the rate some 80X.

Current evidence is consistent with a mechanism (Fig. 10) entailing initial rapid intercalation of the diol epoxide between the base pairs of the nucleic acid. Stop flow kinetic experiments indicate that intercalation is complete within 5 msec (Geacintov *et al.*, 1982; Geacintov *et al.*, 1981). The intercalation complex, which is detectable spectroscopically, undergoes rate-determining protonation to yield an intercalated triol carbonium ion intermediate which decomposes to products via two pathways, hydrolysis and covalent adduct formation. The major path is hydrolysis, and the resulting tetraols remain associated with DNA in a noncovalent intercalation complex. To simplify spectral analysis, they may be removed by equilibrium dialysis. The minor, more biologically important path, leads to covalent adduct formation. Although the overall rate of reaction is dependent upon pH, temperature, ionic strength, and other factors, the ratio of the two paths leading to products is independent of these variables (Geacintov, 1985; Geacintov *et al.*, 1984).

This mechanism is also consistent with the findings of theoretical molecular modeling studies (Subbiah *et al.*, 1983; Miller *et al.*, 1985). These studies confirm that an intercalated intermediate structure which leads to covalent bonding of the type observed is energetically favorable.

Spectroscopic experiments indicate that interaction of *anti*-BPDE with DNA affords two types of covalent adducts (Fig. 11). The minor product (Site I) is characterized by a red shift ($\sim$10 nm) in the absorption maxima and a negative ΔA linear dichroism spectrum. These properties are consistent with an intercalation complex in which the planar pyrene chromophore is oriented approximately parallel to the DNA bases. The major product (Site II) exhibits a smaller red shift ($\sim$2-3 nm) in the absorption spectrum and a positive ΔA spectrum. In this conformation the plane of the pyrene ring system is aligned approximately parallel to

Mechanism of Diol Epoxide-DNA Interaction

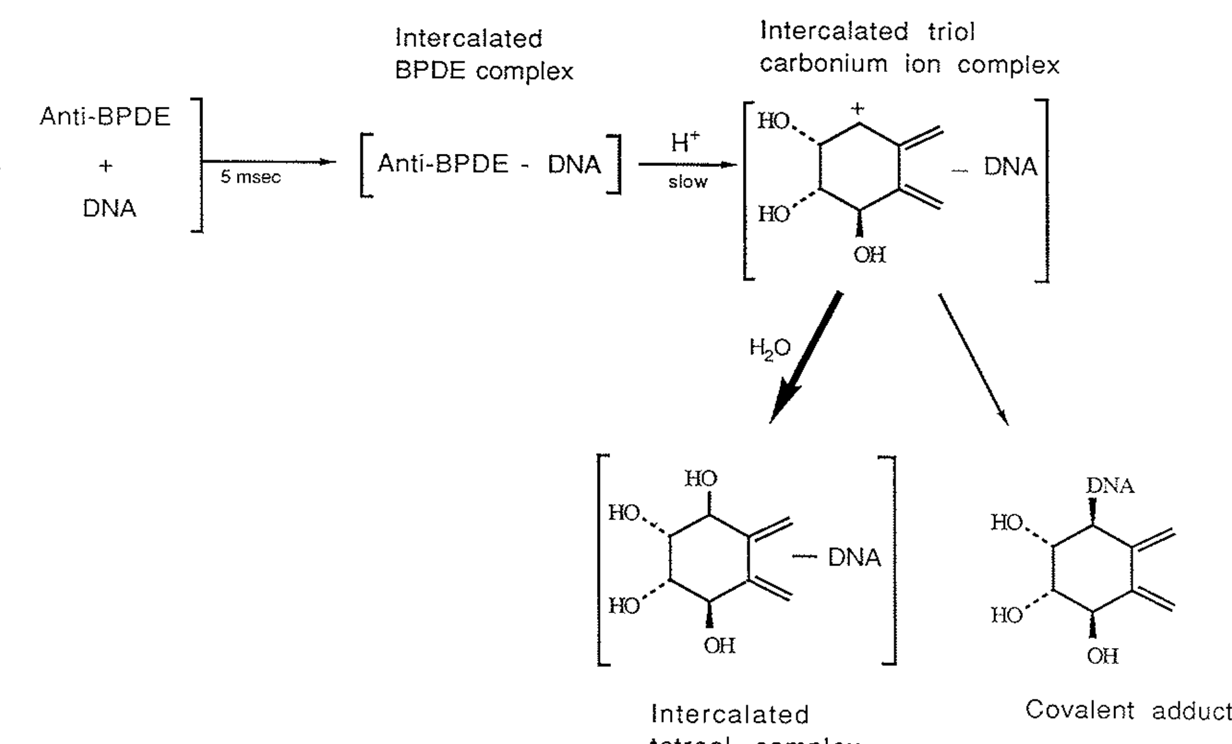


Fig. 10

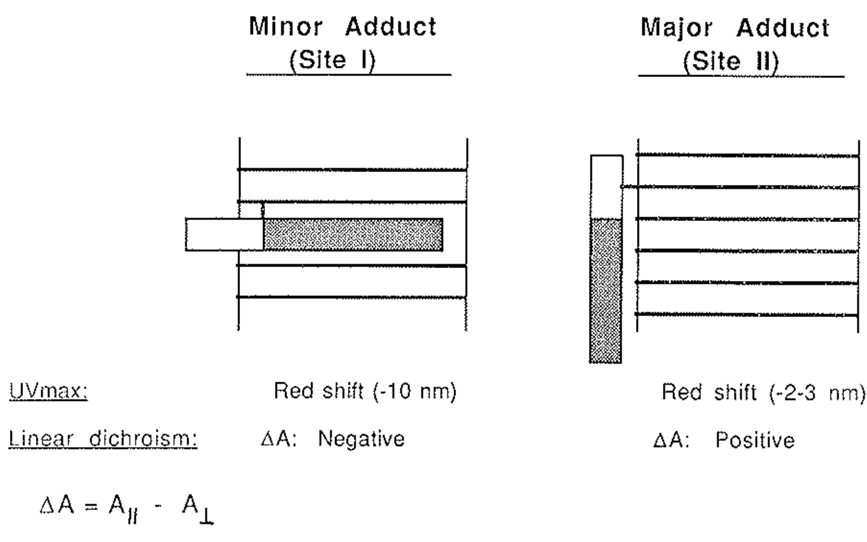


FIG. 11

the DNA helix, and the hydrocarbon molecule is believed to lie in the minor groove of the DNA helix. The site II products may reasonably be assumed to derive from the predominant 2-NH_2-dG adducts, while the Site I products are most likely derived from the dA and other minor adducts. Analogous experiments with the *syn*-BPDE yield predominantly Site I intercalated adducts, as evidenced by a negative ΔA spectrum (Undeman *et al.*, 1983; Geacintov, 1985).

If covalent binding of *anti*-BPDE to DNA takes place in an intercalated intermediate and the resulting major adduct is externally bound to DNA, as represented by the mechanistic model proposed, a re-orientation entailing partial unwinding and rewinding of the DNA helix is required (Miller *et al.*, 1985).

While the evidence is entirely consistent with the proposed mechanism (Fig. 10), an alternative mechanistic pathway (Fig. 12) in which intercalation leads to hydrolysis with formation of tetraols and covalent binding takes place on the exterior of the helix without involvement of intercalation cannot be excluded (Geacintov, 1985; Harvey *et al.*, 1985; Meehan and Bond, 1984). In order to obtain more direct evidence on this question,

Alternative Mechanism of Diol Epoxide-DNA Interaction

BPDE + DNA ⟶ [BPDE - DNA] $\xrightarrow{H^+}$ [H^+BPDE - DNA] $\xrightarrow{H_2O}$ [BP tetraol - DNA]

BPDE + DNA ⟶ BPDE - DNA covalent adducts

Fig. 12

we investigated the effects of substitution of alkyl groups of increasing size in the 1-position of benzo[a]pyrene (Fig. 13) on biological activity (Harvey *et al.*, 1985). These experiments were based on the rationale that groups attached to the aromatic ring system that increase molecular thickness may be expected to sterically interfere with the intercalation of the corresponding diol epoxides. If intercalation is required for covalent binding, the level of adduct formation may be expected to decrease resulting in diminished biological activity. The 1-position was chosen for substitution because this site is remote from the benzo ring, and least likely to interfere with enzymatic activation in the latter molecular region.

Mutation assays were carried out with a series of 1-alkyl-substituted BPs in both bacterial and mammalian cell systems (Harvey *et al.*, 1985). In hamster V79 cells an inverse relationship was observed between mutagenic activity and alkyl group size (Fig. 13). 1-Methyl-BP exhibited highest activity, and there was a rapid dropoff in activity as alkyl group size increased to Et, i-Pr, and t-Bu. In three bacterial strains, *Salmonella typhimurium* TA 100 and TA 98 and *Escherichia coli* WP2uvrA (pKM101), none of the 1-alkyl-BPs were mutagenic in the absence of S9 liver microsomes. In the presence of microsomes, all three cell lines exhibited mutagenic activity with a marked decrease in activity with substitution of alkyl groups larger than methyl. The results of tumor initiation studies with female SENCAR mice correlated well with the mutagenicity data, exhibiting a parallel inverse relationship with alkyl group size (Fig. 13). These findings are consistent with the hypothesis that intercalation is essentially involved in the mechanism of PAH carcinogenesis.

R	Mutagenicity[a] (mutants/ 10^5 survivors)	Mutagenicity[a] % survivors	Tumorigenicity[b] % mice with papillomas	Tumorigenicity[b] papillomas/ mouse
H	139	62	90	3.8
CH_3	288	14	100	4.7
CH_2CH_3	30	77	73	0.9
$CH(CH_3)_2$	5	93	52	0.5
$C(CH_3)_3$	4	96	57	0.7
Control	2	96	10	0.1

[a]Assay in V79 Chinese hamster cells with a feeder layer of X-irradiated BHK21 Syrian hamster cells for metabolic activation.

[b]Female SENCAR mice (30/group) were initiated by skin application of 200 nmol of the hydrocarbon and promoted by topical application of 3.4 nmol of TPA twice weekly.

Fig. 13

Further support for this idea is provided by metabolism studies of the 1-alkyl-BPs with rat liver micromes (Harvey *et al.*, 1985). These studies were carried out to determine whether alkyl substitution in the 1-position interferes with enzymatic activation in the benzo ring of these PAHs (Fig. 14). The findings showed that the 7,8-dihydrodiol, the metabolic precursor of the corresponding diol epoxide, was indeed a major metabolite in all cases, independent of alkyl group size. In order to verify that the 7,8-dihydrodiols of alkyl-substituted BPs were capable of conversion to the corresponding diol epoxides by microsomal enzymes, the 7,8-dihydrodiol of 1-isopropyl-BP and the corresponding diol epoxide were synthesized in our laboratory (unpublished studies), and it was demonstrated that microsomal metabolism of the dihydrodiol converted it to a tetraol identical spectroscopically with the tetraol obtained by hydrolysis of the synthetic diol epoxide. These findings confirm that the

R = Me, Et, i-Pr, t-Bu

Microsomes

Microsomes

H_2O

R = i-Pr

R = i-Pr

FIG. 14

dihydrodiol is fully capable of undergoing metabolic activation to a diol epoxide. Therefore, the weak activity of 1-isopropyl-BP as a mutagen and a tumorigen is not due to resistance to metabolic activation, but is consistent with the hypothesis that intercalation of active diol epoxide metabolites between the base pairs of nucleic acids is critically involved in the mechanism of their covalent binding to DNA.

C. *Covalent Binding of Other PAH Diol Epoxides to DNA and the Structures of the Adducts in Relation to Bioactivity.*

In order to gain insight into which features of the mechanism of interaction of diol epoxide metabolites with DNA are essential and which are irrelevant to ultimate tumor induction, our studies of these complex reactions have been extended to other PAHs, both carcinogenic and non-carcinogenic. Some of the important questions to be answered include: (1) Do the diol epoxide derivatives of relatively potent carcinogenic PAHs, such as BP, DMBA, and 5-methylchrysene, react to greater extent with DNA than those formed by the structurally analogous diol epoxide derivatives of weakly carcinogenic PAHs, such as benzo[e]pyrene, benz[a]anthracene, or chrysene? (2) Do the diol epoxide derivatives of the more active carcinogens react differently with DNA (e.g., do they form higher percentages of dA adducts, or do they alkylate specifically certain regions, such as regulatory genes which control oncogenes)? (3) Do the structures of the adducts formed with DNA by the diol epoxide metabolites of various PAHs differ in any significant way (e.g., Site I or Site II) which uniquely relates to their carcinogenicity?

While there is insufficient information to provide definitive answers to these questions at the present time, significant progress has been made, particularly with respect to question (3) concerning differences in the structures of the DNA adducts. Therefore, this discussion will focus mainly on that topic. However, I would like to comment briefly on question (1), concerning the role of the relative reactivities of PAH diol epoxides. According to the "bay region hypothesis" (Lehr *et al.*, 1985) diol epoxide derivatives in this molecular region of PAHs are distinguished by exceptional reactivity which is theoretically predicted by MO calculations, and a correlation between predicted reactivity and carcinogenic activity is proposed. While it is now reasonably well established experimentally that bay region diol epoxides are the principal active metabolic forms of most alternant PAHs investigated to date, there is no

direct evidence for a correlation between predicted reactivity and the extent of alkylation of DNA or any other cellular receptor. Indirect evidence on this question has been obtained from studies of the inhibition of infectivity of ∅X174 viral DNA in *E. coli* spheroplasts (Hsu *et al.*, 1979; Harvey *et al.*, 1980) by PAH epoxides and diol epoxides. Although *anti*-BPDE and the diol epoxide derivatives of other potent carcinogenic PAHs exhibited significant inhibitory activity, no satisfactory correlation between calculated reactivity and inhibition of viral infectivity was found. It is unlikely that any simple correlation between a theoretically calculated parameter and carcinogenic activity will be found because of the multiplicity of the steps involved in metabolic activation and detoxification of potentially carcinogenic PAHs and the likely importance of steric and other molecular structural factors in the mechanism of DNA interaction and subsequent steps leading ultimately to tumor induction. The importance of bay regions may not be related to any special reactivity of epoxides in this region, but rather be more a consequence of steric inhibition of detoxification by epoxide hydrase in this sterically crowded molecular region (Harvey, 1981; ibid., 1982).

In order to gain information on the effects of molecular structural differences on PAH diol epoxide DNA interaction and the structures of the adducts formed, we have investigated the reactions of a series of PAH diol epoxides with DNA. The PAH compounds selected for study include derivatives of both carcinogenic and noncarcinogenic PAHs (Fig. 15). In order to minimize molecular structural differences between active and inactive compounds, relatively close structural analogs have been chosen. The compounds studied fit three groups, related structurally to benzo[a]pyrene, benz[a]anthracene, and chrysene. The BP analogs, 9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPE) and 1-oxiranylbenzo[a]-pyrene (1-OP), are of interest because they lack the hydroxyl groups of *anti*-BPDE and both are good mutagens (Wood *et al.*, 1976; Newbold *et al.*, 1979), but are apparently nontumorigenic (Levin *et al.*, 1977; Slaga, private communication). In the benz[a]anthracene series, the parent PAH is only very weakly tumorigenic, while 3-methylcholanthrene and DMBA are highly potent carcinogens. In the chrysene series, the parent PAH is essentially noncarcinogenic, whereas its 5-methyl analog is equipotent with BP (Hecht *et al.*, 1976). The bay region diol epoxide isomer I has been implicated as its active form (Melikian *et al.*, 1984; Hecht *et al.*, 1985). 5-Methylchrysene and DMBA are examples of the "bay region methyl effect" whereby introduction of methyl groups into nonbenzo

Benzo[a]pyrene Series

Benz[a]anthracene Series

Chrysene Series

I

II

FIG. 15

ring bay region positions often dramatically enhances carcinogenic activity (Di Giovanni *et al.*, 1983) (Fig. 16).

Physical chemical studies on the reactivity and adduct formation of 1-oxyranylpyrene (1-OP) with DNA have been conducted (Kim *et al.*, 1984). In the absence of DNA, 1-OP undergoes acid-catalyzed hydrolysis which is an order of magnitude slower than that of *anti*-BPDE (Fig. 17). Hydrolysis of 1-OP is catalyzed by DNA in solution via rapid formation of a physically bound intercalation complex. The noncovalent binding constant for complexation ($K = 4000\ M^{-1}$) is the same for both 1-OP and *anti*-BPDE. This suggests that π-electron interaction between the pyrenyl moiety and the nucleic acid bases is the dominant factor in the formation of physical complexes and that the hydroxyl groups of the diol epoxide do not play a significant role in determining the binding constant. The percentage of covalent adducts formed by 1-OP and *anti*-BPDE is also identical. The most striking difference between the reactions of 1-OP and *anti*-BPDE with DNA is in the structures of the adducts formed. In contrast to the *anti*-BPDE-DNA adduct which exhibits a positive linear dichroism (LD) signal, characteristic of external Site II binding, the 1-OP-DNA adduct exhibits a negative LD for which the angle of orientation of the pyrenyl chromophore with respect to the DNA helix is calculated to be $\theta = 70 \pm 3°$, indicative of a Site I complex having a conformation similar, but not identical to, those of the intercalation complexes of dyes, such as proflavine. Similar spectroscopic studies of the covalent adducts of BPE with DNA show predominantly Site I adduct structure along with a minor amount of Site II adducts (Geacintov *et al.*, 1982). The major adduct shows a negative LD signal with a value of $\theta = 68°$ similar to that of the 1-OP-DNA complex, indicative of a similar structure.

The low tumorigenicity of 1-OP and BPE may be a consequence of the difference in the conformation of their DNA adducts compared with that of *anti*-BPDE. However, it cannot be excluded that their low activity may derive from differences in the specific types of nucleoside adducts formed. While the structures of nucleoside adducts formed by 1-OP are unknown, the major adducts formed by BPE have been shown to arise from *cis* and *trans* addition of the 2-NH_2 group of guanine to the epoxide ring (Kinoshita *et al.*, 1982). Minor products, tentatively identified as deoxyadenosine derivatives, were also detected. The lack of stereospecificity of addition to the epoxide ring contrasts with the previously observed highly stereospecific *trans* addition to *anti*-BPDE. It appears that the

Examples of the "Bay Region Methyl Effect" on Carcinogenesis

Inactive or Weakly Active

Highly Active

BR methyl

CH_3

CH_3

CH_3

CH_3

CH_3

CH_3

CH_3

O

H_3C

O

Fig. 16

	1-OP	anti-BPDE	BPE
Binding Constant (K)(M^{-1})	4000 ± 300	4100 ± 400	
Rate constant, hydrolysis,free (k_h)(s^{-1})	0.46×10^{-3}	3.4×10^{-3}	
Rate constant, covalent binding (k_3)(s^{-1})	5.8×10^{-3}	15×10^{-3}	
Covalent binding,%(f_{cov})	8 ± 1	8 ± 1	
θ (deg)	65 ± 3	< 35	68 ± 4

FIG. 17

hydroxyl groups of the diol epoxide play a major role in determining the stereospecificity of adduct formation.

In the benz[a]anthracene series, studies of the covalent binding of *trans*-9,10-dihydroxy-*anti*-7,8-epoxy-7,8,9,10-tetrahydro-3-methylcholanthrene (*anti*-3-MCDE) to double-stranded DNA recently have been completed (Kim *et al.*, 1986). The mechanism of reaction and the structures of the products were found to be similar to those of *anti*-BPDE with some notable differences (Fig. 18). In the case of *anti*-3-MCDE, the physical association constant (K) is four times lower and the overall rate of reaction (k_3) is about five times smaller than in the case of *anti*-BPDE. On the other hand, the level of covalent binding (f_{cov}) of *anti*-3-MCDE is more than twice as high (18 ± 2%) as that of *anti*-BPDE (8 ± 1%). The structure of the covalent adduct was shown by the use of linear dichroism techniques to be similar to that previously observed for the highly tumorigenic (+)-*anti*-BPDE. The average tilt of the long axis of the aromatic ring system relative to the axis of the helix is $\theta = 48 \pm 5°$. Since 3-MC

and BP are both potent carcinogens, these findings suggest that differences in biological activity may be related to differences in the extents of covalent binding or the conformations of the various PAH diol epoxide adducts.

Similar studies with the analogous diol epoxide derivatives of benz[a]anthracene, *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydrobenz[a]anthracene (*anti*-BADE) and its *syn* isomer (*syn*-BADE), support a similar mechanism of reaction with DNA, but indicate differences in adduct structures (Carberry *et al.*, manuscript submitted for publication). In the case of *anti*-BADE (Fig. 19), the physical association constant (K), the overall rate of reaction (k_3), and the extent of covalent binding (f_{cov}) closely resemble those for *anti*-3-MCDE. In contrast, *syn*-BADE reacts less efficiently than the *anti* isomer with DNA, like *syn*-BPDE, yielding a lower proportion of covalent adducts (8% vs 23%). The values of k_3 and K for *syn*-BADE are also both lower than those for *anti*-BPDE.

	anti-3-MCDE	anti-BPDE
Binding Constant (K)(M^{-1})	900 ± 200	4100 ± 400
Rate constant, hydrolysis,free (k_h)(s^{-1})	5.7×10^{-4}	3.4×10^{-3}
Rate constant, covalent binding (k_3)(s^{-1})	4.3×10^{-3}	15×10^{-3}
Covalent binding,%(f_{cov})	18 ± 3	8 ± 1
θ (deg)	48 ± 5	< 35

FIG. 18

	anti-BADE	syn-BADE	anti-3-MCDE	anti-BPDE
Binding Constant $(K)(M^{-1})$	4000 ± 300	600 ± 50	4400 ± 600	4100 ± 4
Rate constant, hydrolysis,free $(k_h)(s^{-1})$	1.6×10^{-4}	1.1×10^{-3}	6.9×10^{-4}	3.4 x 10
Rate constant, covalent binding $(k_3)(s^{-1})$	20×10^{-3}	9×10^{-3}	24×10^{-3}	15 x 10
Covalent binding,%	23 ± 1	8 ± 1	23 ± 3	8 ± 1

Fig. 19

Spectral analysis indicates that the *syn*-BADE adduct is homogeneous with the long axis of the chromophore tilted toward the DNA helical axis, and thus is a Site II adduct, resembling the *anti*-3-MCDE and *anti*-BPDE adducts. This is opposite to that observed for *syn*-BPDE for which a negative dichroism signal is observed (Geacintov, 1985), indicative of a Site I adduct. On the other hand, *anti*-BADE gives rise to two distinct adducts both of which are externally bound. One is a Site II adduct oriented approximately parallel to the DNA helix, the other is oriented more perpendicular to the DNA helix, but is not intercalated, as shown by the absence of a 10 nm or larger shift indicative of an intercalated species. The significance of these findings with respect to carcinogenicity is not clear. The relatively high level of covalent binding of *anti*-BADE and the formation of a Site II adduct resembles the findings for potent carcinogens. On the other hand, metabolism studies indicate that microsomal metabolism of benz[a]anthracene affords relatively low levels of the 3,4-dihydrodiol, the metabolic precursor of the corresponding diol epoxides. The weak carcinogenicity of benz[a]anthracene appears to be due more to its relatively low level of metabolic activation in the appropriate molecular region than to differences in adduct structure compared with more potent carcinogens.

DNA binding studies with the DMBA diol epoxides (Fig. 20) are currently in progress. However, these investigations have been seriously hampered by the chemical instability of the synthetic *anti* and *syn* diol epoxides (Lee and Harvey, 1986) as well as by their facility of photo-oxidation to yield transannular epidioxides. The structures of the products of spontaneous decomposition are not known. Although the *syn* isomer is more stable than the *anti* form, both isomers decompose relatively rapidly in solution, even in the absence of DNA. For this practical reason, we chose to study first the reactions of the more stable 5-methylchrysene diol epoxides as examples of the important bay-region methyl carcinogens. 5-methylchrysene is unusual in that it contains two bay regions only one of which has a methyl group.

The mechanism of reaction of *trans*-1,2-dihydroxy-*anti*-3,4-epoxy-

Fig. 20

1,2,3,4-tetrahydro-5-methylchrysene (*anti*-5-MeCDE-I) with DNA was found to be similar to that of *anti*-BPDE (Kim *et al.*, 1985) (Fig. 21). The rate of hydrolysis of *anti*-5-MeCDE-I is $k = 1.0 \times 10^{-4}$/s which is 35 times smaller than that of *anti*-BPDE under similar conditions. As in the case of *anti*-BPDE, the rate of reaction is accelerated by DNA and proceeds via initial rapid formation of a physical intercalation complex. The values of K and k_3 are 450 ± 100/M and 1.8×10^{-3}/s, respectively, in 0.1 M NaCl solution. The value of K is one-tenth that observed for *anti*-BPDE under similar conditions. This difference is attributed to the smaller aromatic ring system of *anti*-5-MeCDE-I.

The experimental results (Kim *et al.*, 1986) for the alternative bay-region diol epoxide isomer, *anti*-MeCDE-II, yield values for the rate constants k_h (5.3×10^{-4}/s) and k_3 (2×10^{-2}) which are consistent with the theoretically predicted greater reactivity of this isomer (Silverman *et al.*, 1982, 1985; Adams and Kaminsky, 1982). On the other hand, the values of K and f_{cov} for *anti*-5-MeCDE-II are approximately half those for *anti*-5-MeCDE-I. The greater extent of covalent binding of the bay-region methyl isomer, *anti*-5-MeCDE-I, than the alternative isomer which lacks a methyl group in this region, is likely a consequence of the fact that intercalation is more favorable for *anti*-5-MeCDE-I, as shown by its larger binding constant. Thus it appears that reactivity is a less important determinant of the extent of covalent binding than the facility of formation of an appropriate intercalation complex.

Both *anti*-5-MeCDE-I and -II have been shown to bind predominantly to the exocyclic 2-amino group of guanine (Melikian *et al.*, 1984). The structures of the adducts formed with DNA by both isomers were shown by the techniques employed in previous cases to be nonintercalated and externally bound with the long axis of the aromatic ring system tilted toward the average orientation of the DNA helix (Kim *et al.*, 1986). The formation of adducts of this type, designated as Site II, was previously observed for the potent carcinogens *anti*-BPDE and *anti*-3-MCDE and is consistent with the high tumorigenic potency of 5-methylchrysene.

V. Summary and Conclusions

Considerable progress has been made in recent years in elucidating the mechanism of carcinogenesis of PAHs at the molecular level. Bay region diol epoxide metabolites have been identified as the active species.

	anti-5-MeCDE-I		anti-5-MCDE-II	anti-BPDE
	No NaCl	0.1 M NaCl	No NaCl	0.1 M NaCl
Binding Constant (K)(M^{-1})	2800 ± 300	450 ± 100	1500 ± 200	4100 ± 400
Rate constant, hydrolysis,free (k_h)(s^{-1})	1.0×10^{-4}	1.0×10^{-4}	5.3×10^{-4}	3.4×10^{-3}
Rate constant, covalent binding (k_3)(s^{-1})	8.7×10^{-3}	1.8×10^{-3}	2×10^{-2}	15×10^{-3}
Covalent binding (f_{cov})(%)	7 ± 1	6 ± 1	2-3	8 ± 1
Angle of binding (O)	46 ± 6		45 ± 7	< 35

FIG. 21

These reactive intermediates have been shown to be highly mutagenic and tumorigenic and to bind covalently to nucleic acids, *in vivo* and *in vitro*. The mechanism of covalent binding and the structures of the adducts formed with DNA by the bay region diol derivatives of benzo[a]pyrene, benz[a]anthracene, benzo[e]pyrene, 3-methylcholanthrene, and 5-methylchrysene, as well as the structural analogs 1-oxiranylpyrene and 9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, have been investigated in detail. The findings are consistent with a mechanism involving initial rapid reaction with DNA (Fig. 10) to form a physical intercalation complex, followed by slow, rate-determining proton addition to yield an intercalated triol carbonium ion complex which decomposes to products via hydrolysis to tetraols, which is the major path, and covalent adduct formation. While the major adducts appear to arise from the (+)-*anti*-diol epoxide isomers by covalent bond formation at the exocyclic amino group of deoxyguanosine, minor adducts arising from other diol epoxide isomers and attack at other base sites are also detected. The structures of the DNA adducts formed by the most potent carcinogenic PAHs, benzo[a]pyrene, 3-methylcholanthrene, and 5-methylchrysene, are similar, having the aromatic moiety externally bound with the long axis of the aromatic chromophore tilted toward the DNA helix.

While it is too early to reach any definitive conclusions concerning the significance of these findings with respect to the biological consequences of DNA alkylation leading to tumor induction, some speculation may be permitted. The relative rates of reaction of diol epoxides appears less important than the levels of covalent binding and the structures of the adducts formed. The levels of covalent binding are determined not only by inherent reactivity, as suggested by the "bay region hypothesis", but by steric and electronic factors favoring intercalation, as well as by the orientation of the carbocation intermediate with respect to an appropriate base site for covalent bond formation in the intercalation complex.

The significance of the tendency of the DNA adducts of more potent carcinogens to be oriented more parallel than perpendicular or intercalated within the DNA helix may be that this orientation permits some degree of replication past the site of alkylation damage, leading to mutations.

Many problems remain to be solved. These include determination of the specific sites on DNA alkylation of which leads to tumor induction, perhaps by turning on an oncogene.

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STRUCTURAL AND FUNCTIONAL PROPERTIES OF GENOMIC DNA CONTRIBUTING TO THE NON-RANDOM FORMATION AND REPAIR OF CARCINOGEN-DNA ADDUCTS

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Abstract

This report deals with some of the structural and functional properties of genomic DNA that contribute to the non-random formation of carcinogen-DNA adducts (in particular the well-defined DNA alkylation products resulting from exposure to N-nitroso carcinogens) and to their differential removal and repair by cellular enzymes. Of the molecular variables influencing the relative local frequencies (distributions) of specific carcinogen adducts in genomic DNA, we discuss (i) nucleotide sequence, (ii) effects of different nucleotides flanking a target nucleotide, (iii) the nuclear organization of DNA, including the degree of packaging of DNA and the conformation of chromatin in a transcribable *versus* non-transcribable state, and (iv) the localization of DNA with respect to the "nuclear matrix". A further contributing factor may be the toposelective action of DNA repair enzymes due to the differential accessibility of specific gene sequences.

I. Introduction

With very few exceptions, the interaction of chemical carcinogens (and cytotoxic agents used in cancer therapy) with target cells results in structural alterations of genomic DNA (see, e.g., Rajewsky, 1980; Singer

and Grunberger, 1983; Hemminki and Ludlum, 1984). Under *in vivo* conditions, the ultimate, DNA-reactive derivatives of chemical carcinogens are generated either by enzymatic "bioactivation" (see, e.g., Miller and Miller, 1979; Gelboin, 1980; Pelkonen and Nebert, 1982) or by spontaneous heterolytic decomposition of the parent compounds, as in the case of "directly acting" monofunctional alkylating agents; see Rajewsky, 1980; Singer and Grunberger, 1983).

Among the various classes of carcinogenic chemicals, the alkylating N-nitroso compounds (Preussman and Stewart, 1984) have probably been most completely characterized with regard to their reaction products in DNA (see Pegg, 1977; Rajewsky, 1980; Singer and Grunberger, 1983; Saffhill *et al.*, 1985). These agents give rise to highly reactive electrophiles, leading to the covalent binding of alkyl groups to electron-rich 0 and N atoms at about a dozen molecular sites in DNA (besides nucleophilic centers in other cellular macromolecules). Depending on the nature of their nucleophilic substitution reactions (see, e.g., Wiessler, 1986), individual N-nitroso compounds are characterized by their specific relative frequencies of alkylation at different nucleophilic centers. Among the reaction products of N-nitroso carcinogens in genomic DNA, 0^6-alkyl-2'-deoxyguanosine (0^6-alkyldGuo), and the minor alkylation product 0^4-alkyl-2'-deoxythymidine (0^4-alkyldThd), have received particular attention. This is due to the pronounced propensity of 0^6-alkyldGuo and 0^4-alkyldThd for causing point mutations (transitions) by miscoding, and to the correlation of the carcinogenic effects of N-nitroso compounds with the formation of these DNA lesions and their (unrepaired) persistence through DNA replication in proliferation-competent target cells (Loveless, 1969; Goth and Rajewsky, 1974; Rajewsky *et al.*, 1977; Rajewsky, 1980, 1983; Singer and Grunberger, 1983; Müller and Rajewsky, 1983; Swenberg *et al.*, 1984; Saffhill *et al.*, 1985; Essigmann *et al.*, 1986).

Both the detection and quantitation of low levels of specific alkylation products in DNA have been greatly facilitated by the recent development of sensitive immunoanalytical techniques using high-affinity monoclonal antibodies (Mab) specific for defined alkyl-deoxynucleosides (Rajewsky *et al.*, 1980, 1985; Adamkiewicz *et al.*, 1982). Carcinogen-exposed DNA hydrolyzed enzymatically to mono-deoxynucleosides may be analyzed by competitive radioimmunoassay (RIA) or enzyme immunoassays (EIA), and immuno-slot-blot techniques (Nehls *et al.*, 1984a) may be applied to quantitate specific alkylation products in DNA bound to nitrocellulose filters. Furthermore, specific DNA alkylation products may now be

visualized in the nuclei of individual cells from carcinogen-exposed tissues or cell cultures by immunocytological analysis (ICA), using electronically intensified, direct immunofluorescence (Adamkiewicz *et al.*, 1985), or may be localized (as Mab binding sites) in individual DNA molecules (of known or unknown sequence) by immuno-electron microscopy (IEM) (Nehls *et al.*, 1984b).

In the present report, we shall repeatedly refer to the potent N-nitroso carcinogen N-ethyl-N-nitrosourea (EtNU) and its reaction products in DNA, as a model for the discussion of some of the molecular parameters affecting the formation, distribution, and repair of carcinogen-induced structural modifications in genomic DNA. The relative amounts of different ethylation products formed by EtNU in cellular DNA are as follows (Rajewsky *et al.*, 1977; Singer *et al.*, 1978; Rajewsky, 1983): (i) *ethylation on 0 atoms* (~ 80% of all DNA ethylation products): 0^6-ethyldeoxyguanosine (0^6-EtdGuo, ~ 10%), 0^2-ethyldeoxythymidine (0^2-EtdThd, ~ 7%), 0^2-ethyldeoxycytidine (0^2-EtdCyd, ~ 4%), 0^4-ethyldeoxythymidine (0^4-EtdThd, ~ 2%), and ethylphosphotriesters (~ 56%); (ii) *ethylation on N atoms*: 7-ethyldeoxyguanosine (7-EtdGuo, ~ 14%), 3-ethyldeoxyadenosine (3-EtdAdo, ~ 5%), 7-ethyldeoxyadenosine (7-EtdAdo, ~ 0.4%), 1-ethyldeoxyadenosine (1-EtdAdo, ~ 0.3%), 3-ethyldeoxycytidine (3-EtdCyd, ~ 0.2%), 3-ethyldeoxyguanosine (3-EtdGuo, ~ 0.1%), and 3-ethyldeoxythymidine (3-EtdThd, ~ 0.1%).

II. Molecular Parameters Affecting the Formation, Distribution and Repair (Persistence) of Specific Carcinogen-Adducts in Genomic DNA.

Both the formation of carcinogen adducts in genomic DNA and their enzymatic removal and repair do not occur at random, as reflected, for example, by the non-random distribution of specific alkylation products (e.g., 0^6-EtdGuo; Nehls *et al.*, 1984b) observed in the DNA of cells exposed to N-nitroso carcinogens *in vivo*, and by the preferential repair of UV-induced pyrimidine dimers in distinct regions of the genome (Bohr *et al.*, 1985, 1986). Nucleotide sequence, the nature of the nucleotides flanking the target nucleotide, ionic strength, the helical conformation of DNA, and the packaging of DNA in nuclear chromatin (with its structural correlates of transcriptionally active *versus* non-transcribed genes), all contribute to the toposelective formation of carcinogen-DNA adducts, and probably to a large extent to their differential

accessibility and rate of removal and repair by cellular enzymes. Correlations between the formation and persistence of specific carcinogen adducts in defined nucleotide positions of mammalian genes on the one hand, and the occurrence of mutational "hot spots" on the other, are a subject of current research.

1. *Nucleotide Sequence and Effects of Neighboring Bases and Ionic Strength.*

The work of the Pullmans as well as of other groups (see the respective reports in this volume) has shown, that the binding to DNA of the ultimate electrophilic reactants of carcinogenic chemicals largely depends on the electrostatic potentials and on the steric accessibility of nucleophilic atoms. The resulting preferential binding of specific agents to defined bases, and the sequence specificity and dependence on neighboring bases, are dealt with extensively in a number of contributions to this volume. Therefore, only a few selected examples will be mentioned which mainly concern the covalent attachment to DNA bases of alkyl groups generated from N-nitroso compounds.

The influence of neighboring bases on the relative extent of alkylation of guanine and adenine in synthetic polydeoxynucleotides has been investigated by Briscoe and Cotter (1985). Hartley *et al.* (1986) have analyzed the sequence selectivity of 7-guanine alkylation by chloroethylating agents. The differential effects of neighboring bases on the degree of alkylation in the 0^6- and 7-positions of guanine have been explored on a theoretical basis by Furois-Corbin and Pullman (1985; see also the contribution by A. Pullman to this volume). Lobanenkov *et al.* (1986) have recently studied the effects of neighboring bases on guanine-specific DNA cleavage following *in vitro* exposure of double-stranded DNA fragments of known sequence to the anti-diol epoxide of benzo(a)pyrene. This work has shown that strand scission at deoxyguanosine (dGuo) residues (based on guanine-specific adduct formation resulting in the generation of alkali-labile apurinic sites) is sequence-specific. A strong preference was found for the central dGuo residues in 5′-CGG-3′, 5′-TGG-3′ 5′-TGT-3′, and 5′-CGT-3′ sequences, whereas no cleavage at internal dGuo residues was observed in 5′-GGA-3′ and 5′-GGC-3′ sequences, and only very few cases of scission were found in the sequences 5′-GGG-3′ and 5′-GGT-3′. Similarly, *in vitro* studies by Muench *et al.* (1983) on aflatoxin B1-exposed DNA sequences have shown that

neighboring bases can affect the reactivity of specific nucleophilic centers towards this carcinogen. In our group, we have recently investigated the influence of bases flanking dGuo in synthetic double-stranded DNA polymers in regard to the formation of 0^6-EtdGuo after exposure to EtNU *in vitro*. As shown in *Table 1*, dGuo, and to a similar extent dCyd, flanking dGuo provide favorable conditions for ethylation of the 0^6 atom of guanine by EtNU, whereas dAdo, m^5dCyd and dThd, in this order, exert a negative influence. The degree of alkylation at atoms susceptible to electrophilic attack is also dependent on ionic strength, i.e., on the shielding of nucleophilic sites in the major and minor groove, and in the base-pairing region of the DNA double helix, by monovalent (Zakrzewska *et al.*, 1981; Nehls and Rajewsky, 1985a) and divalent ions (Marushige and Marushige, 1983).

2. *Nuclear Organization of DNA*

A) *General Aspects*

Most of eukaryotic DNA is transcriptionally inactive and encompasses regions of the genome organized in densely packed chromatin fibers with a contour diameter of ~ 30 nm and a DNA "packing ratio" (relative to native DNA) of ~ 25 nm (Finch and Klug, 1976; Renz *et al.*,

TABLE 1

Polymer	0^6-EtdGuo/dGuo (x 10^5)
poly (dG) x (dC)	88.4 ± 17.8
poly (dG - dC) x (dG - dC)	51.8 ± 0.4
poly (dA - dG) x (dC - dT)	33.8 ± 3.8
poly (dG - m^5dC) x (dG - m^5dC)	27.0 ± 0.1
poly (dA - dC) x (dG - dT)	9.6 ± 0.4

0^6-EtdGuo/dGuo molar ratios in synthetic DNA polymers exposed to N-ethyl-N-nitrosourea (EtNU) *in vitro*.

Experimental conditions: 1 mg of EtNU/ml; 25 mM MOPS, 60 mM NaCl, 0.5 mM EDTA, pH 7.4; 37° C, 20 min. After complete enzymatic hydrolysis of the DNA polymers (Müller and Rajewsky, 1980), dGuo and 0^6-EtdGuo concentrations were determined by HPLC and competitive RIA using anti-(0^6-EtdGuo) Mab ER-6 (Rajewsky *et al.*, 1980), respectively (P. Nehls and M. F. Rajewsky, in preparation).

1977). The detailed structure of this chromatin fiber is still a matter of discussion (see, e.g., Butler, 1983; Zentgraf and Franke, 1984; Felsenfeld and McGhee, 1986; Williams *et al.*, 1986). The conformation of the 30 nm-fiber is known to be stabilized by histone H1 as well as by mono- or divalent ions (Renz *et al.*, 1977; Thoma *et al.*, 1979). Removal of histone H1 or decreased ionic strength leads to a relaxation of the condensed 30 nm-fibers into extended polynucleosome filaments (contour diameter, ~ 11 nm). These fibers represent the elementary structure of chromatin and are characterized by an alternating arrangement of nucleosomes and internucleosomal DNA ("beads on a string") and a DNA packing ratio of 6-7 (for comparison, DNA packing ratio in mitotic chromosomes, ~ 10^4). Nucleosomes consist of 146 bp of DNA wrapped in 1 3/4 left-handed turns around a core of 2 of each of the histones H2A, H2B, H3, and H4 (Kornberg, 1977; McGhee and Felsenfeld, 1980; Mirzabekov, 1980; Richmond *et al.*, 1984). Internucleosomal DNA, varying in length between 20 bp and 100 bp in a cell type- and species-dependent manner, is the site of interaction with histone H1.

Further packaging of DNA in the interphase nucleus is achieved by the organization of chromatin fibers in the form of large looped "domains", ranging in size from ~ 30,000 bp to ~ 100,000 bp (Cook and Brazell, 1975; Benyajati and Worcel, 1976; Laemmli *et al.*, 1978; Igo-Kemenes *et al.*, 1982). These loops are attached to subnuclear structures termed "nuclear matrix", which morphologically appears as a nuclear lamina-pore complex and an internal network of proteins and other components (Berezney, 1984; Lewis *et al.*, 1984; Gasser and Laemmli, 1986). The recent detection of specific DNA sequences apparently involved in the anchorage of chromatin loops in the nuclear matrix, is a further indication of the highly heterogeneous organization of nuclear DNA (Gasser and Laemmli, 1986; Cockerill and Garard, 1986). Interestingly, nuclear matrix-associated DNA sequences seem to contain topoisomerase II binding sites (Cockerill and Garard, 1986). This enzyme, which represents a major constituent of interphase nuclei and mitotic chromosomes (Berrios *et al.*, 1985; Earnshaw and Heck, 1985), may thus also be involved in the control of torsional stress at the bases of chromatin loops.

The structural characteristics of the small part ($< 20\%$) of chromatin containing transcriptionally active genes (i.e., the transcribable conformation of chromatin) are largely unknown. However, some properties of chromatin permit us to distinguish transcriptionally inactive from active

conformations, e.g., the preferential accessibility of transcribable chromatin to cleavage by endonucleolytic enzymes (Weintraub and Groudine, 1976; Garel and Axel, 1976; Bloom and Anderson, 1978). It has been proposed that this nuclease sensitivity might be due to the association of distinct nuclear proteins (HMG 14 and 17) with nucleosomes (Weisbrod and Weintraub, 1979). An alternative explanation may be provided by the observation that looped domains of chromatin containing transcriptionally active (or replicating) DNA, are under torsional stress (Weintraub, 1983; Selleck *et al.*, 1984; Han *et al.*, 1984; Kmiec and Worcel, 1985). This torsional stress is probably introduced by the action of topoisomerase II (Glikin *et al.*, 1984; North, 1985). As in the case of other genes, "DNase I-hypersensitive sites" have recently been found in several regions 5′ to the rat serum albumin gene, and it has been suggested that the binding of factors (proteins) to these sites might be required for both cell type-specific transcription and the control of transcription rate (Babiss *et al.*, 1986; and references cited therein). Furthermore, there is evidence indicating that both the transcriptional activity of genes and their nuclease sensitivity are correlated with an undermethylation of DNA (Keshet *et al.*, 1986; for review see also Riggs and Jones, 1983; Jones, 1986). Although a role of 5-cytosine methylation in the control of transcriptional activity is not obvious for all eukaryotic genes (Bird, 1984), a large body of evidence suggests that DNA methylation may influence chromatin structure to the effect that DNA is rendered more susceptible to, or protected from, nuclease digestion.

B) *Chromosomal DNA: Differential Accessibility to Carcinogens and Non-Random Distribution of Specific Carcinogen Adducts*

The mode of DNA packaging in the nucleus, as well as differences in the conformation of chromatin fibers consonant with the functional state of DNA, are likely to be important determinants affecting the accessibility of specific DNA constituents to carcinogens (and to cellular enzymes responsible for the elimination and repair of specific carcinogen-DNA adducts; see below).

We have addressed the question to which extent the folding level of DNA in chromatin affects the accessibility of specific molecular sites in DNA to the alkylating N-nitroso carcinogen EtNU (Nehls and Rajewsky, 1985a,b). Using immunoanalytical and radiochromatographic techniques, we have found that — in comparison with native DNA — the degree of

0^6-EtdGuo formation upon reaction with EtNU *in vitro* is reduced by ~ 40% in the DNA of the extended, histone H1-depleted 11 nm chromatin fiber (1st level of DNA folding) and by ~ 60% in the condensed, 30 nm chromatin fiber (2nd level of DNA folding). Folding of chromatin fibers into looped domains does not result in a further reduction of the level of guanine-0^6 ethylation. The frequencies of N ethylation at positions 7 of guanine and 3 of adenine were also investigated. Compared with native DNA, the degree of 7-EtdGuo formation in the extended and condensed chromatin fibers, respectively, is reduced to a similar extent as in the case of 0^6-EtdGuo, while the reduction of 3-EtdAdo formation in the DNA of both types of chromatin fibers is somewhat more pronounced. The core histones thus exert a general protective effect on DNA with respect to ethylation of the 0^6 and N^7 atoms of guanine and the N^3 atom of adenine by the ethyldiazonium ion generated from EtNU. While the N^7 and 0^6 positions of guanine (major groove and base-pairing region) are protected to about the same degree, the N^3 atom of adenine (minor groove) becomes even less accessible. In all cases, however, the presence of histone H1 provides the highest degree of protection.

Several groups have investigated the formation (and repair; see below) of carcinogen adducts in internucleosomal *versus* nucleosomal DNA, and in DNA of chromatin in a transcribable *versus* non-transcribable conformation. In most cases, chromosomal DNA was analyzed after selective digestion with micrococcal nuclease or DNase I (see above). While in earlier studies the overall extent of carcinogen binding to the DNA of different chromatin fractions was determined, more recent work has focused on the quantitation of specific carcinogen-DNA adducts, based on the clarification of their molecular structures and the development of analytical techniques for their specific detection. For the most part, the results of both types of investigations indicate a higher accessibility of nuclease-sensitive DNA sequences (DNA in a transcribable conformation of chromatin; internucleosomal "linker" DNA) to the reactive metabolites of the carcinogens used (Metzger *et al.*, 1976, 1977; Ramanathan *et al.*, 1976; Cox, 1979; Galbraith *et al.*, 1979; Sudhakar *et al.*, 1979; Feldman *et al.*, 1980; Jack and Brookes, 1981; Nose, 1981; see, however, also Metzger *et al.*, 1977; Pegg and Hui, 1978; Marushige and Marushige, 1983).

Like the elegant study by Kaneko and Cerutti (1980) on N-acetoxy-2-acetylaminofluorene (AAAF)-DNA adducts formed in core *versus* linker DNA of human fibroblasts, analyses using nucleosomal monomers and

oligomers with varying ratios of internucleosomal to nucleosomal DNA, have shown a non-random formation of specific carcinogen-DNA adducts due to preferential modification of internucleosomal DNA (Jahn and Litman, 1979; Kootstra *et al.*, 1979; Bailey *et al.*, 1980). Digestion of chromosomal DNA with DNase I has been applied in analyses of rat brain DNA after exposure to N-methyl-N-nitrosourea (MeNU) *in vivo* (Cox, 1979) and of intact fetal rat brain nuclei exposed to EtNU *in vitro* (Nehls and Rajewsky, 1981). In both studies, preferential formation of 0^6-alkyldGuo was found in the DNA of DNase I-sensitive chromatin. Upon digestion with micrococcal nuclease, Ryan *et al.* (1986) observed preferential formation of methyl adducts at the 0^6 and N^7 atoms of guanine and at the N^3 atom of adenine in the DNA of transcriptionally active liver chromatin of rats exposed to N,N-dimethylnitrosamine (DMN) *in vivo.* Using rRNA gene sequences from the liver of aflatoxin B1 (AFB1)-treated rats as an example of actively transcribed genes, Irvin and Wogan (1984) could show that these genes contained a 5-fold excess of the AFB1 adduct to N^7 of guanine as compared to bulk DNA. Similarly, Obi *et al.* (1986) reported preferential formation of benzo(a)pyrene adducts in the DNA of transcriptionally active chromatin regions. Furthermore, it is interesting to note that an elevated degree of adduct formation has also been found in nuclear matrix-associated liver DNA of rats exposed to polycyclic hydrocarbons (Mironov *et al.*, 1983) or DMN (Ryan *et al.*, 1986).

In recent analyses by immuno-electron microscopy (Nehls *et al.*, 1984b), it was found that a small fraction (0.02% of the total genome) of DNA sequences from the brains of fetal rats exposed to EtNU *in vivo*, contained 0^6-EtdGuo residues (as visualized by the corresponding binding sites for anti-0^6-EtdGuo Mab ER-6; see above) at an about 10^2 to nearly 10^4 fold (average value, 750 fold) higher frequency than the calculated average for the total genomic DNA. This clustering highly exceeds the degree of non-randomness in the distribution of 0^6-EtdGuo residues that might be expected on the basis of local effects of DNA sequence, flanking bases, ionic strength, and chromatin structure. A contribution of toposelective DNA repair cannot be invoked here, because the frequency of 0^6-EtdGuo residues in the clusters is far higher than the expected average frequency. A satisfactory explanation for the observed "hot spots" of 0^6-EtdGuo formation must, therefore, await the results of further investigations. However, exposure of intact cells to the carcinogen appears to be required for the effect to occur, since no comparable clustering of 0^6-EtdGuo residues was found after reaction of native DNA

with EtNU *in vitro* (Nehls and Rajewsky, 1984b). Nevertheless, statistical analyses with the aid of a newly developed computer program have also shown some degree of non-randomness in the distributional pattern of 0^6-EtdGuo residues in the latter case (P. Nehls, E. Spiess, E. Weber, J. Berger and M.F. Rajewsky, submitted for publication).

3. *Toposelectivity of Enzymatic DNA Repair*

Although it is evident that repair mechanisms are of primary importance for maintaining the integrity of DNA, the complexity of their genetic control and enzymology has precluded rapid advances regarding their clarification. It is true that some of the repair processes in E. coli are now well understood (see Walker, 1984, 1985); however, the molecular basis of DNA repair in mammalian cells (see recent reviews by Lehmann and Karran, 1981; Lindahl, 1982; Thielmann, 1984; Walker *et al.*, 1985) has not yet been clarified to a similar extent, due to the fact that much less is known about the genetic control of mammalian repair enzymes and their mode of action, and about the presentation (accessibility) of DNA to these enzymes within different conformations of chromatin (see above). Nevertheless, it is clear that mammalian enzymes responsible for the removal and repair of carcinogen-modified DNA components may be differentially expressed in different types of cells and as a function of the cellular state of differentiation; e.g., the 0^6-methylguanine-DNA methyltransferase which removes the methyl group from 0^6-methylguanine in DNA *via* transfer to a reactive cysteine at the active site of the enzyme (see Karran and Lindahl, 1985; Rajewsky, 1985; Cohen and Leung, 1986). Furthermore, some mammalian repair enzymes appear to be regulated in a cell cycle-dependent manner (e.g., hypoxanthine DNA glycosylase; Sirover and Gupta, 1983; Dehazay and Sirover, 1986).

During the past years, a number of studies have focused on the question as to whether the kinetics of repair of carcinogen-DNA adducts differs between nuclease-sensitive *versus* nuclease-resistant chromatin (see above). In general, more rapid removal of adducts (including small alkyl adducts produced by N-nitroso compounds; Ramanathan *et al.*, 1976; Cox, 1979) from nuclease-sensitive chromatin was observed (see Hanawalt *et al.*, 1979; Feldman *et al.*, 1980; Kaneko and Cerutti, 1980; Nose, 1981). Cox (1979) and Ryan *et al.* (1986) found that 0^6-methylguanine was repaired more rapidly in the DNA of nuclease-sensitive rat brain and rat liver chromatin, respectively, while no difference between the two types of (rat

liver) chromatin was observed for the repair of 7-methylguanine and 3-methyladenine (Ryan *et al.*, 1986). Interestingly, the latter authors found that repair of 0^6-methylguanine in nuclear matrix-associated DNA (containing transcriptionally active and/or replicating DNA, besides the attachment sites of DNA loops) was much slower than in the DNA of bulk chromatin.

Like the analysis of 0^6-methylguanine repair in nuclear matrix-associated DNA performed by Ryan *et al.* (1986), other recent studies have aimed at measuring repair kinetics in well-defined genome regions or in individual genes. Thus, Irvin and Wogan (1985) could show that aflatoxin B1-DNA adducts are preferentially removed from rat liver rRNA genes as compared to unfractionated nuclear DNA. For the same type of adducts, and for AAAF-DNA adducts, Leadon and Hanawalt (1986) found that excision repair was deficient in alpha-DNA (a highly repeated 172 bp DNA sequence) of confluent (i.e., non-proliferating) African green monkey cells, but these DNA sequences became accessible to the repair enzyme(s) when the cells were actively proliferating. Studying the repair of UV-induced pyrimidine dimers in excision repair-deficient Chinese hamster ovary (CHO) cells, Bohr *et al.* (1986) could show that within a period of 24 hours, 70% of the dimers were removed from the dihydrofolate reductase (DHFR) gene while only 20% were removed from sequences located $\geqq$ 30 kbp upstream from the 5′ end of the DHFR gene. Repair was most effective in a severely undermethylated region at the 5′ end of the DHFR gene. Rapid removal of dimers from the DHFR gene was also observed in normal human fibroblasts and epidermal keratinocytes, contrary to (excision repair-deficient) human Xeroderma pigmentosum (XP) cells of complementation group C, where almost no removal of dimers from the DHFR gene could be detected (Bohr *et al.*, 1986). In XP cells of complementation group C, Mullenders *et al.* (1984, 1986; see also Karentz and Cleaver, 1986) observed preferential repair of UV damage in nuclear matrix-associated DNA.

Taken together, the data obtained in the studies cited above underline the complexity of DNA repair processes in mammalian cells and their dependency on the accessibility of the damaged DNA in distinct parts of chromatin. Local changes in chromatin structure related to gene function, appear to be closely associated with the efficiency of DNA repair, mostly in the sense that transcriptionally active chromatin is more readily repaired. However, this "rule" may not hold for all repair enzymes, i.e., differences might exist with respect to the enzymatic removal and repair of different types of DNA adducts. Regarding

the process of excision repair, there is now considerable evidence indicating that very soon after completion of repair synthesis, the repaired DNA undergoes a transition from a nuclease-sensitive to a nuclease-resistant state associated with nucleosome cores and histone H1 (see Smerdon and Lieberman, 1980; Lan and Smerdon, 1985; Mathis and Althaus, 1986). It is possible that defined changes in chromatin structure are in fact part of a given type of repair process. Furthermore, if repair processes are initiated by carcinogen-induced, structural damage to specific (perhaps transcriptionally active) gene sequences, then repair might not be triggerable any more in genes that had been silent at the time of carcinogen exposure (and are, therefore, carrying unrepaired adducts) but become transcriptionally active at a later time.

New analytical approaches based on further progress in the study of molecular genetics and chromatin structure will be required to explore the emerging toposelectivity of DNA repair in mammalian cells. Conventional analyses at the level of DNA isolated from tissues or whole cells, cannot provide information on the efficiency of the removal and repair of specific carcinogen-DNA adducts from defined gene sequences. Indeed, misleading results may be obtained in the sense that cells considered repair-proficient are in fact repair-deficient with respect to specific regions of the genome and *vice versa.* It is equally evident that a differential accessibility of damaged DNA to repair enzymes could contribute significantly to the generation of non-random distributions of carcinogen adducts persisting in genomic DNA.

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DRUG AND CARCINOGEN COMPLEXES WITH LEFT-HANDED AND RIGHT-HANDED DNAS

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Abstract

The interactions of the drugs actinomycin D, adriamycin, and ethidium with poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) have been studied under both B- and Z-form conditions. These drugs exhibit cooperative binding to DNA under Z-form conditions. The data from circular dichroism spectroscopy provide evidence that the conformation of the polynucleotide at the intercalation site is right-handed. The adduct of the carcinogen N-acetoxy-2-acetylaminofluorene (AAAF) at the C_8 position of the single guanine in d(CCACGCACC) was prepared and purified. The conformation of the stable duplex of d(CCAC^{AAF}GCACC)·d(GGTGCGTGG) has been investigated by one- and two-dimensional NMR spectroscopy. The conformation of the duplex is highly distorted at the site of the adduct and at the two adjacent base pairs, with one of the adjacent guanines adopting a syn conformation.

Introduction

The carcinogen N-acetoxy-2-acetylaminofluorene (Figure 1) reacts with guanine at the C_8 position to form a covalent adduct (Miller *et al.*, 1966; Kriek *et al.*, 1967), N-(deoxyguanosin-8-yl)-2-acetylaminofluorene. Although minor reactions products are also observed, the present studies focus on the major adduct, the guanine-C_8-AAF adduct. The models which

ETHIDIUM BROMIDE

R=H DAUNOMYCIN

R=OH ADRIAMYCIN

AAAF

ACTINOMYCIN D

ACTINOMINE

FIG. 1. Molecular structures of ethidium bromide, daunomycin, adriamycin, actinomycin D, N-acetoxy-2-acetylaminofluorine (AAAF), and actinomine. Abbreviations: Thr = threonine; Val = valine; Pro = proline; Sar = sarcosine; MeVal = methylvaline. The tertiary nitrogens of actinomine are protonated at ph 7.

have been proposed for the structure of the adduct with DNA include the base displacement model (Grunberger *et al.*, 1970; Nelson *et al.*, 1971; Santella *et al.*, 1976), the insertion denaturation model (Fuchs and Daune, 1971, 1972, 1974; Fuchs, 1975; Fuchs *et al.*, 1976), and the left-handed (Z) DNA binding model (Sage and Leng, 1980; Santella *et al.*, 1981; Wells *et al.*, 1982). In both the insertion-denaturation model and the base-displacement model the guanine base at the site of adduct formation is displaced (denatured) from the helix and a portion of the helix is susceptible to S_1 nuclease, suggesting the presence of single-stranded DNA. The left-handed helical structure was proposed for the interaction of AAF with DNA consisting of alternating pyrimidine-purine sequences. AAF-modified poly(dG-dC)·poly(dG-dC) was not susceptible to S_1 nuclease, suggesting that denaturation of this polymer had not occurred (Santella and Grunberger, 1983). The CD spectrum of highly modified poly(dG-dC)·poly(dG-dC) exhibited a spectrum similar to the high salt form of the polymer, which had been shown to be a left-handed helix. The models just described are based on data from AAAF modified polymeric DNA, which can not yield detailed structural information, and from single-stranded oligonucleotides. The adducts formed with the single-stranded oligonucleotides provide detailed structural information (e.g., the recent work of Box *et al.*, 1984; Sharma and Box, 1986; Evans and Levine, 1986; Evans *et al.*, 1986; Shapiro *et al.*, 1986; and papers cited therein). In this manuscript and in related papers (Sanford, 1986; Sanford, Huang and Krugh, in preparation) we present the first report of one- and two-dimensional NMR studies on an AAF adduct of an oligodeoxynucleotide duplex, and we discuss these data in terms of the structure of the adduct.

The salt-induced conformational transition of poly(dG-dC)·poly(dG-dC) from a right-handed helix to the left-handed (Z) helix (Pohl and Jovin, 1972; Wang *et al.*, 1979) is a striking example of the polymorphism of DNA (for reviews, see Rich *et al.*, 1984; Wells *et al.*, 1980). Pohl *et al.* (1972) showed that ethidium does not bind effectively to poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl (the Z-form) until the free ethidium concentration reaches approximately 20 μM. The intercalation of ethidium is accompanied by a highly cooperative left- to right-handed conformational transition of the polynucleotide, as evidenced by circular dichroism spectroscopy. Several groups have addressed the interesting question of the interaction of various drugs with left-handed DNAs and have made important observations on both the binding preferences of the ligands and the thermodynamics and kinetics of the Z-B equilibrium (e.g., see Rich

et al., 1984; Wells, 1985; Behe and Felsenfeld, 1981; Wu *et al.*, 1981; Chaires, 1983, 1985, 1986a,b,c; Chen *et al.*, 1983; Gupta *et al.*, 1983; Mirau and Kearns, 1983; Zimmer *et al.*, 1983; Shafer *et al.*, 1984; Krugh and Walker, 1985; Walker *et al.*, 1985a,b; Lamos *et al.*, 1986; Neumann *et al.*, 1985; Krugh *et al.*, 1986). In this part of the paper we illustrate the use of optical spectroscopy to investigate the interaction of ligands (Figure 1) with poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) under conditions which favor the left-handed conformation of the polymers. We show that drug binding results in a conformational transition to a right-handed drug-bound polymer with the number of left-handed base pairs converted per bound drug dependent upon the Z-form conditions. It is concluded that this binding scheme is consistent with other studies and may help in understanding enzyme activity under left-handed conditions as observed by van de Sande and Jovin (1982), Durand *et al.*, 1983, and Butzow *et al.* (1984).

Materials and Methods

A high salt buffer consisting of 4.4 M NaCl, 10 mM Na_2PO_4, 10 mM Na_2EDTA, pH 7, will be referred to as 4.4 M NaCl Buffer. A low salt buffer consisting of 50 mM NaCl, 5 mM TRIS, pH 8, will be referred to as 50 mM NaCl Buffer. Other buffers consisted of 50 mM NaCl, 5 mM TRIS, pH 8, and varying amounts of magnesium chloride or cobalt hexamine chloride as indicated; these buffers will be referred to as 2 mM $MgCl_2$ Buffer, 25 mM $MgCl_2$ Buffer, or 40 μM $[Co(NH_3)_6]Cl_3$ Buffer. A buffer consisting of 0.1 M NaCl, 10 mM $NaHPO_4$, 0.1 mM Na_2EDTA, pH 7, was used in the oligonucleotide experiments.

Ethidium bromide (Sigma) was recrystalized from methanol. Actinomycin D (NSC-3053) was a gift of Merck, Sharp and Dohme. Actinomine was a gift of Dr. Sisir Sengupta of the Children's Cancer Foundation. Actinomycin D and the actinomine were checked for purity by TLC and HPLC as previously described. Adriamycin (NSC-123127), which was obtained from the Natural Products Branch of the National Cancer Institute, was used without purification. Drug solutions were prepared immediately before each experiment.

Poly(dG-dC) and poly(dG-m^{5}dC) were purchased from P. L. Biochemicals/Pharmacia and were used without purification. Establishment of the left-handed forms of the polynucleotides and preparation of calf thymus DNA were as described previously (Walker *et al.*, 1985a).

Optical titrations were performed on a Varian CARY 219 spectrophotometer at 25°C using 2, 5 or 10 cm pathlength cells. A detailed description of the experimental procedure and methods of data analysis were previously described (Walker *et al.*, 1985a,b).

Fluorescence spectra were recorded on a Perkin-Elmer MPF44A spectrofluorometer at ambient temperature. Excitation and emission slit widths were 10 nm. Ethidium spectra were recorded with excitation at 510 nm, the isosbestic point between the absorption spectra of free and DNA-bound ethidium.

Circular Dichroism (CD) spectra were recorded on a Jasco J-40 spectropolarimeter at ambient temperature using 1, 2 and 5 cm pathlength cells. The spectra were recorded at 5 nm intervals and were signal averaged and baseline corrected using a Digital PDP 11/34 computer. Sample preparation was as previously described (Walker *et al.*, 1985a,b). Molar ellipticity, [Θ], values are reported in terms of DNA base pairs.

One-dimensional NMR spectra were recorded on a Bruker WH-400 NMR spectrometer (at Rochester); two-dimensional NMR spectra were recorded on a General Electric GN-500 NMR spectrometer located in the NIH NMR facility at Syracuse University. The nuclear Overhauser effect spectra and the two-dimensional NOESY and COSY spectra were recorded by standard techniques as described in Sanford (1986).

Results and Discussion

Ethidium Binding under B and Z Conditions

Parallel binding experiments in 4.4 M NaCl Buffer using both calf thymus DNA and poly(dG-dC)·poly(dG-dC) illustrate the effect of the B-Z transition on ethidium binding. Addition of calf thymus DNA to a 5 μM ethidium solution in 4.4 M NaCl results in bathchromic and hypochromic shifts in the visible absorption spectrum and a large increase in fluorescence intensity (Figure 2). These spectral changes are characteristic of ethidium intercalation and are the basis of absorption and fluorescence titration techniques (LePecq and Paoletti, 1967). Nearly identical spectral changes are observed immediately after addition of B-form poly(dG-dC)·poly(dG-dC) to a solution of 5 μM ethidium in 4.4 M NaCl. Similar spectral properties immediately after mixing are expected for the two samples because ethidium intercalation into B-DNA occurs on a much

faster timescale than the B to Z transition (Mirau and Kearns, 1983). However, differences between the calf thymus DNA and poly(dG-dC)·poly(dG-dC) samples become apparent after B-Z equilibrium is established. After heating the poly(dG-dC)·poly(dG-dC) sample, which hastens B-Z equilibration, the absorption and fluorescence spectra revert back toward the spectrum characteristic of free ethidium, which is consistent with the dissociation of significant amounts of bound drug. A comparison of these samples clearly illustrates that ethidium binding to right-handed DNA is much more favorable than binding to a left-handed poly(dG-dC)·poly(dG-dC) helix in 4.4 M NaCl Buffer. It should be noted that the B to Z conversion and the concomitant dissociation of ethidium occur without heating, but very long equilibration times are required, consistent with the observation by Mirau and Kearns (1983) that the presence of ethidium slows the rate of the B to Z transition.

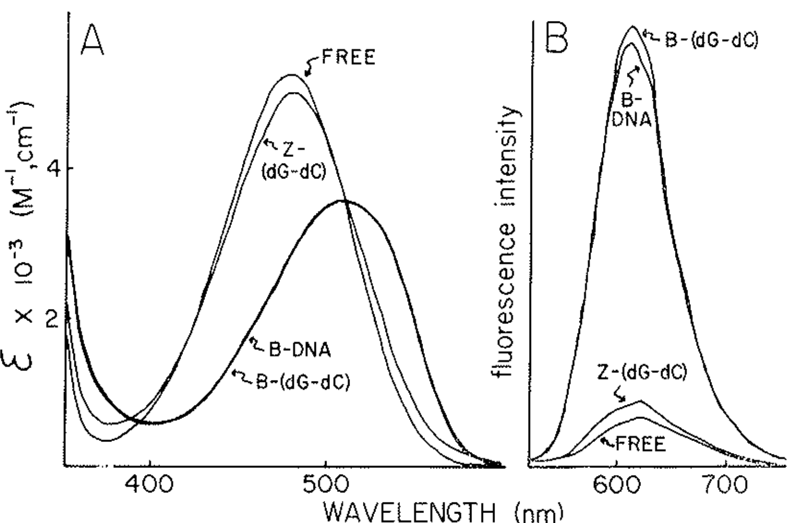

FIG. 2. Absorbance (A) and fluorescence spectra (B) for bound and free ethidium in 4.4 M NaCl Buffer. The spectra correspond to free ethidium [Free], ethidium in the presence of calf thymus DNA [B-DNA], ethidium in the presence of B-form poly(dG-dC) [B-(dG-dC)], and ethidium in the presence of Z-form poly(dG-dC) [Z-(dG-dC)]. B-Z equilibrium in the poly(dG-dC) sample was established by heating for 10 minutes at 60°C. The fluorescence spectra are uncorrected. Further explanation as to experimental conditions are provided in the text.

Cooperative Binding of Ethidium Under Z-Form Conditions

The binding preference of ethidium for right-handed DNA and the highly cooperative B-Z equilibrium results in highly cooperative binding under Z-form conditions. The equilibrium binding isotherm for the interaction of ethidium with poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl is shown in Figure 3. Positive cooperative binding is evident by the positive slope and near zero r/C_f intercept in the Scatchard (1949) plot (Figure 3A) as well as the sigmoidal shape of the r versus C_f plot (Figure 3B) where r is defined as the bound drug to DNA base pair ratio and C_f is the concentration of free drug. The near zero r/C_f intercept results from the free drug concentration reaching approximately 20 μM before significant binding occurs, as illustrated in Figure 3B and as originally reported by Pohl *et al.* (1972).

Shafer *et al.* (1984) have performed ethidium-poly(dG-dC)·poly(dG-dC) experiments in 4.4 M NaCl similar to those described by Figures 2 and 3, but the much greater binding affinity of ethidium for right-handed DNA was not readily apparent due, at least in part, to the high ethidium and poly(dG-dC)·poly(dG-dC) concentrations used in their experiments. At ethidium concentrations much greater than 20 μM and with millimolar poly(dG-dC)·poly(dG-dC) concentrations, a significant fraction of ethidium is expected to bind to poly(dG-dC)·poly(dG-dC) in 4.4 M NaCl under these conditions, thus making it more difficult to observe the marked binding preference of ethidium for the right-handed conformation.

As shown in Figure 3A, the binding isotherm can be satisfactorily represented by the allosteric transition binding model of Bresloff and Crothers (1981) in which the polynucleotide can adopt two conformations, Form 1 (left-handed) and Form 2 (right-handed). In 4.4 M NaCl, poly(dG-dC)·poly(dG-dC) exists as Form 1 DNA at the start of the experiment but changes to Form 2 because of the preferential binding of ethidium to Form 2. The allosteric model describes a sequential conversion of Z-DNA to a right-handed form as ethidium binds (Figure 3C). The model confirms the clustering of ethidium into regions of right-handed DNA, as deduced from the experimental CD data described below. For example, r_2, the r value for ethidium binding to right-handed DNA, rises sharply to a value of $\sim$ 0.35 at low overall values of r, and then remains constant until saturating levels of ethidium are reached (inset of Figure 3C). In contrast, binding of ethidium to left-handed DNA is calculated to occur at very low densities ($r_1 \approx 0.0025$).

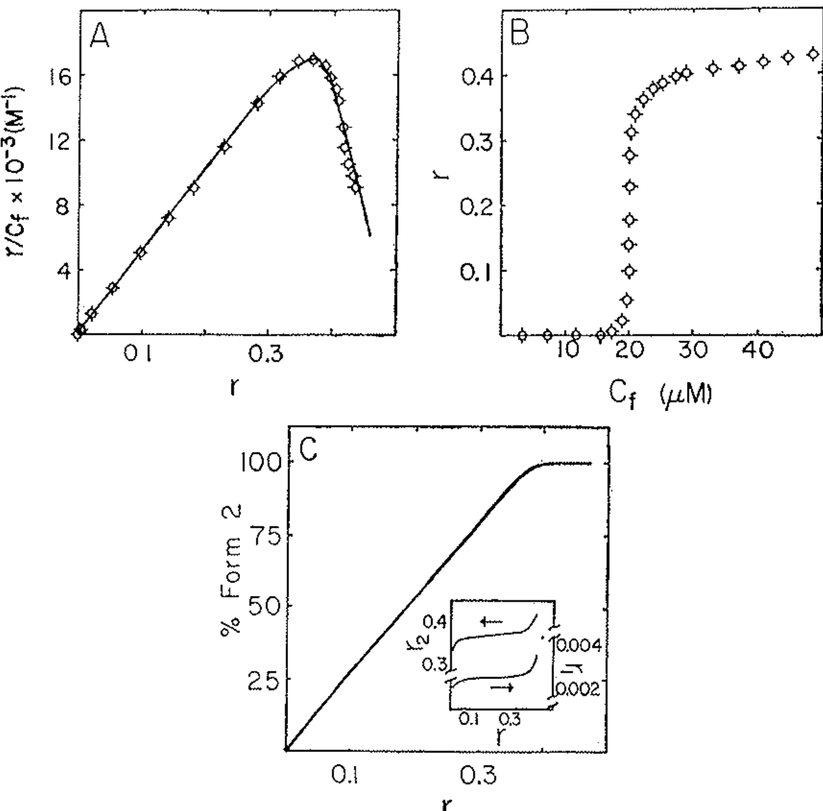

FIG. 3. Equilibrium binding isotherm and binding model fit for the interaction of ethidium and Z-form poly(dG-dC) in 4.4 M NaCl Buffer. The data were obtained by optical titration methods (13). (A) Scatchard plot with the solid line representing a fit to the allosteric transition binding model. (B) Plot of r versus the free drug concentration, C_f, (C) Calculated percent of the DNA in Form 2 as a function of r according to the allosteric transition binding model. The inset contains plots of the average r value for binding to Forms 1 and 2 (r_1 and r_2, respectively) as a function of the overall r value for the polynucleotide. Note the separate scales for r_1 and r_2. The best fit of the model estimates that binding to right-handed DNA occurs with a cooperativity parameter (τ) of 3 and a binding constant which is ~300 times that for binding to left-handed DNA.

Is Cooperative Binding Under Z-Form Conditions Unique to Ethidium?

We have studied the binding of adriamycin, actinomycin D and actinomine under Z-form conditions through a variety of phase partition and optical titration techniques (Krug and Walker, 1984; Walker *et al.*, 1985a,b; Krugh *et al.*, 1986). All four drugs exhibit cooperative binding isotherms under Z-form conditions, although the exact shape of a binding isotherm under a given set of conditions is unique to each drug.

The binding isotherm for adriamycin and Z-form poly(dG-dC) in 4.4 M NaCl is shown in Figure 4. As described for ethidium, the sigmoidal shape of the r versus C_f plot is indicative of positive cooperative binding. Chaires (1986c) has performed similar binding experiments with adriamycin and daunomycin and has obtained similar results to Krugh *et al.* (1986). Note that very little adriamycin binds to poly(dG-dC) in 4.4 M NaCl until the free drug concentration reaches approximately 7 μM (Figure 4). This compares to values of ~ 20 μM for ethidium (Figure 3) and ~ 0.3 μM for actinomycin D (Walker *et al.*, 1985b); these values are influenced by the binding affinity of the ligand to the right-handed form, which in turn is related to the charge of the ligand. In 4.4 M NaCl Buffer

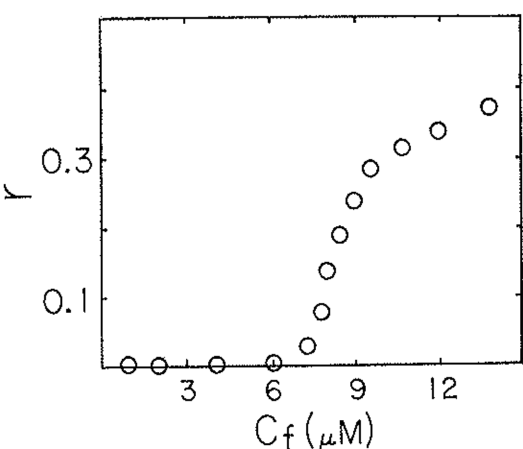

Fig. 4. Equilibrium binding isotherm for the interaction of adriamycin and Z-form poly(dG-dC) in 4.4 M NaCl Buffer. The data are plotted as r versus the free drug concentration, C_f. The data were obtained by optical titration methods using molar extinction coefficients (M^{-1}, cm^{-1}) at 477 nm of 5350 and 9680 for the bound and free forms of adriamycin, respectively.

the binding affinity of actinomine to the right- and left-handed forms is so small that no binding to poly(dG-dC) is observable by absorption spectroscopy even at 100 μM free drug concentrations.

The extent of the left- to right-handed conversion per bound drug has been determined for ethidium, actinomycin D, and actinomine under a variety of Z-form conditions (Walker *et al.*, 1985a,b). The number of base pairs converted per bound drug does not vary significantly among the drugs for a given set of Z-form conditions. The polynucleotide and the particular Z-form conditions employed are the dominant factors controlling the number of left-handed base pairs converted per bound drug.

Effects of Z-DNA Stability and Ionic Strength on Drug Binding

Factors which influence the B-Z equilibrium alter the shape of the binding isotherms, as illustrated by a comparison of ethidium binding to left-handed poly(dG-m^{5}dC) in 2 and 25 mM $MgCl_2$. The stability of the left-handed form of poly(dG-m^{5}dC) increases with higher magnesium concentrations under these buffer conditions. Consequently, the free ethidium concentration (C_f), at which half saturation of the polynucleotide occurs, increases from ~ 0.2 to ~ 3.5 μM in going from 2 to 25 mM $MgCl_2$ Buffer, respectively (Figure 5). Likewise the number of left-handed base pairs

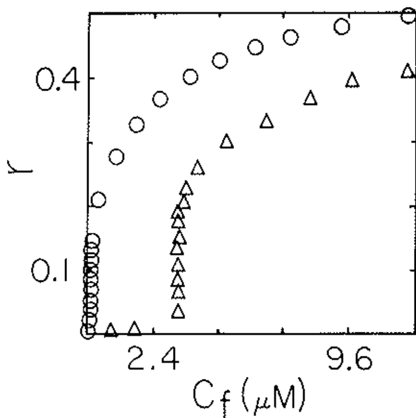

Fig. 5. Equilibrium binding isotherms for the interaction of ethidium and Z-form poly (dG-m^{5}dC) in 2 mM $MgCl_2$ (O) and 25 mM $MgCl_2$ Buffer (Δ). The data are plotted as r versus the free drug concentration, C_f. The data were obtained by optical titration methods (13).

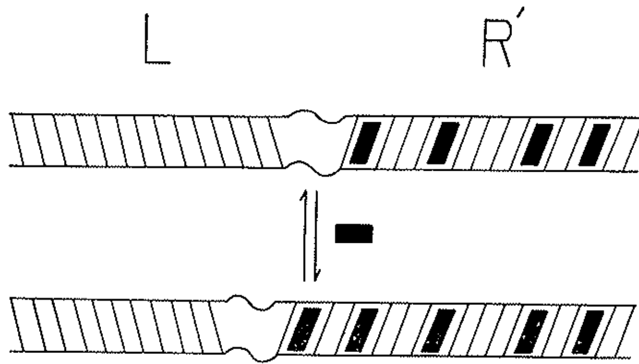

FIG. 6. Illustration of the clustering model for drug binding under Z-form conditions. The left-handed form (L) is converted to a drug-bound right-handed form (R′) with a B′-Z interface separating the two forms of the DNA.

converted per bound ethidium decreases from ~ 7 to ~ 4 base pairs. Analogous behavior is observed with ethidium and poly(dG-dC) as the salt concentration is varied from 2 to 4.4 M NaCl (Walker, 1985; Walker and Krugh, in preparation), as well as with adriamycin and daunomycin (Chaires, 1986b,c).

Under conditions where the Z-form of poly(dG-dC) is only slightly lower in energy than the B-form, more than 20 base pairs of left-handed polynucleotide switch to a right-handed conformation for each bound ethidium. The shape of the binding isotherms also reflects the type of binding. When the Z-form is much more stable than the B-form, the isotherms exhibit a near zero intercept on the r/C_f axis which results from inefficient binding of the drug until a minimum concentration is reached.

Ethidium Clustering Under Z-form Conditions

The CD data above 315 nm, as well as the analysis of the binding isotherms in terms of the allosteric binding model show that ethidium binds in clusters when the polymer is present under conditions strongly favoring the Z-form (e.g., Walker *et al.*, 1985a,b). A representation of the clustering model is shown in Figure 6 in which left-handed and right-handed DNA coexist on the same polynucleotide, separated by a B′-Z interface. While Figure 6 was designed to represent ethidium binding

to poly(dG-dC) in 4.4 M NaCl, it may be taken as a general depiction of drug clustering under Z-form conditions. The tendency of ligands to form nearly saturated regions of right-handed DNA (i.e., clusters) in 4.4 M NaCl Buffer may be understood in terms of the relative stability of B- and Z-DNA, and by the presence of energetically unfavorable B-Z (and B′-Z) interfaces, the number of which are expected to remain at a minimum during the titration. The binding isotherms and CD data obtained with adriamycin and actinomycin D strongly suggest that these drugs bind in clusters also, especially in 4.4 M NaCl, following the same general binding model elaborated above for ethidium. Verification of ethidium clustering has also been obtained through fluorescence detected circular dichroism (FDCD) studies (Lamos *et al.*, 1986).

The Conformation of a Carcinogen-Oligodeoxynucleotide Adduct

The carcinogen N-acetoxy-2-acetylaminofluorene (AAAF) binds covalently to guanine (Miller *et al.*, 1966) and facilitates the transition from B- to Z-form DNA in alternating pyrimidine-purine sequences (Sage and Leng, 1980, 1981; Santella *et al.*, 1981, 1982). The carcinogen was reacted with d(CCACGCACC) to form a covalently modified oligodeoxynucleotide. A modified duplex was formed when the complementary strand, d(GGTGCGTGG), was added (Figure 7). The unmodified duplex (no AAAF) has a CD spectrum characteristic of B-DNA, whereas the CD

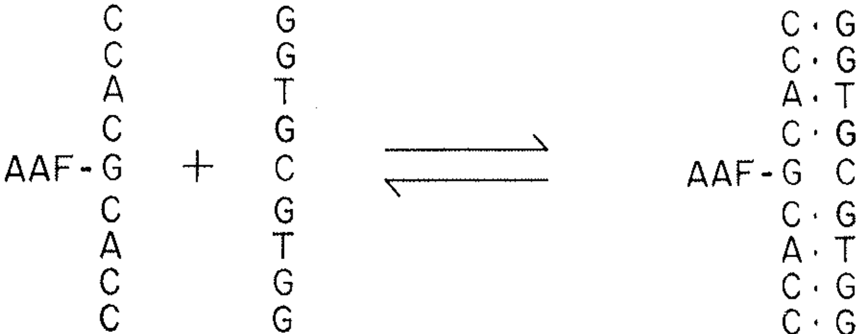

FIG. 7. Schematic representation of the covalent modification of an oligonucleotide and the formation of a modified duplex. AAAF reacts with the single guanine in d(CCACGCACC) to form a covalent adduct. The purified adduct is then mixed 1:1 with the complementary strand d(GGTGCGTGG) to form a modified duplex.

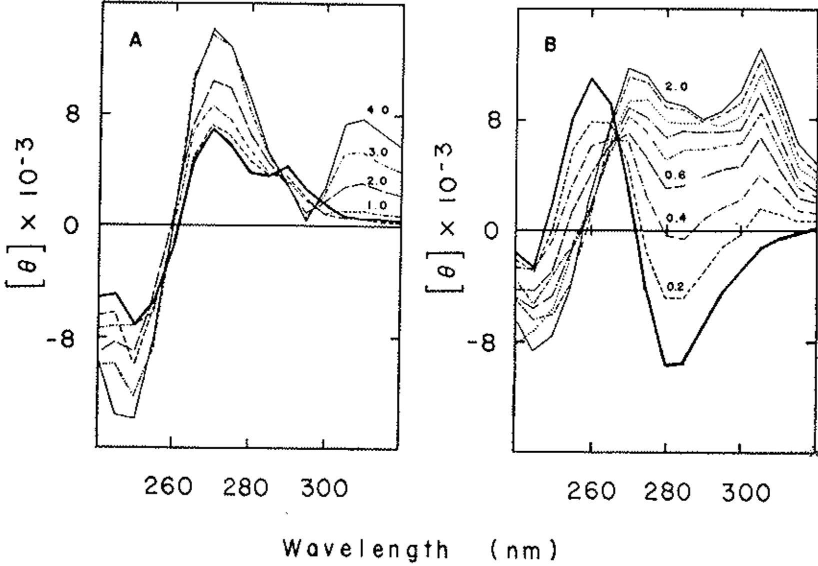

FIG. 8. CD spectra from ethidium titrations of the unmodified (A) and the AAAF modified (B) oligonucleotide duplex described in Figure 10. The bold lines indicate the spectra in the absence of ethidium. The numbers above selected spectra indicate the input ratio of ethidium molecules per duplex. Spectra were recorded at 5°C. The molar ellipticity is given in terms of the nucleotide concentration.

spectrum of the AAAF modified duplex (Figure 8) suggests a conformational change which may involve formation of a left-handed duplex, although CD spectra may be misleading when it comes to structural interpretations. Titration of the modified duplex with ethidium results in conversion of the CD spectrum to one which is quite similar to the CD spectrum of the unmodified duplex with ethidium. The spectra of the unmodified and modified duplexes at saturating levels of ethidium are not identical, consistent with contributions to the CD from the carcinogen, or the effect of the carcinogen on the oligonucleotide duplex conformation. The observed transition in the CD spectrum of the modified duplex upon binding ethidium is reminiscent of the changes that accompany the left- to right-handed conversion of poly(dG-dC) in 4.4 M NaCl and is taken as supportive evidence that the modified duplex has at least a portion of the helix in an altered conformation, presumably a left-handed conformation at low salt and in the absence of ethidium.

Proton NMR Spectra on the d(CCAC^{AAF}GCACC)·d(GGTGCGTGG) Adduct

Extensive one- and two-dimensional NMR experiments have been performed on the d(CCAC^{AAF}GCACC)·d(GGTGCGTGG) adduct, as discussed separately (Sanford, 1986; Sanford *et al.*, in preparation). A major question is whether the bases are hydrogen bonded, as suggested in the schematic of Figure 7. The duplex has a T_m of approximately 40°C at the 2-3 mM concentration used in the NMR experiments and thus, if the conformation of the duplex were similar to the base displacement model (Grunberger, 1970; Nelson *et al.*, 1971; Santella *et al.*, 1976) or insertion denaturation model (Fuchs and Daune, 1971, 1972, 1974; Fuchs, 1975; Fuchs *et al.*, 1976), we would expect to find only a few intact base pairs. The loss of base pairing would be evident in the imino portion of the ^1H spectrum recorded in H_2O solution since these resonances are observed only when the imino protons exchange slowly with the solvent protons, as a result of base pair formation or from other structural constraints such as loop formation (e.g., see Wemmer and Reid, 1985; Patel *et al.*, 1985). On the other hand, if d(CCAC^{AAF}GCACC)·d(GGTGCGTGG) were to adopt a conformation similar to the one proposed in the left-handed (Z) DNA binding model (Sage and Leng, 1980; Santella *et al.*, 1981; Wells *et al.*, 1982) then we would expect to observe all the imino protons because these model building studies show that the AAF moiety may attach to the C_8 position of guanine without requiring major structural distortions in the conformation of the duplex.

The ^1H spectrum of d(CCAC^{AAF}GCACC)·d(GGTGCGTGG) in H_2O solution at both 0°C and at 10°C is shown in Figure 9. Nine imino resonances are observed at both temperatures, indicative of the presence of a stable duplex in which all nine imino protons exchange slowly with the H_2O solvent protons. As the temperature is raised the terminal base pairs (resonances D and G at 0°C) begin to broaden, as expected. The imino proton resonances of all but the terminal guanines are observable up to 30-35°C, which is slightly below the T_m of the duplex. Resonances A and B are from the two thymine imino protons, and exhibit the characteristic NOE to their respective adenine-H_2 protons; at least one of the A·T resonances is downfield of the corresponding resonance in the unmodified duplex (Sanford, 1986). The other seven resonances are from the seven remaining guanines in the 9-mer duplex. The imino proton resonances labelled as H and I in Figure 9 are substantially upfield of

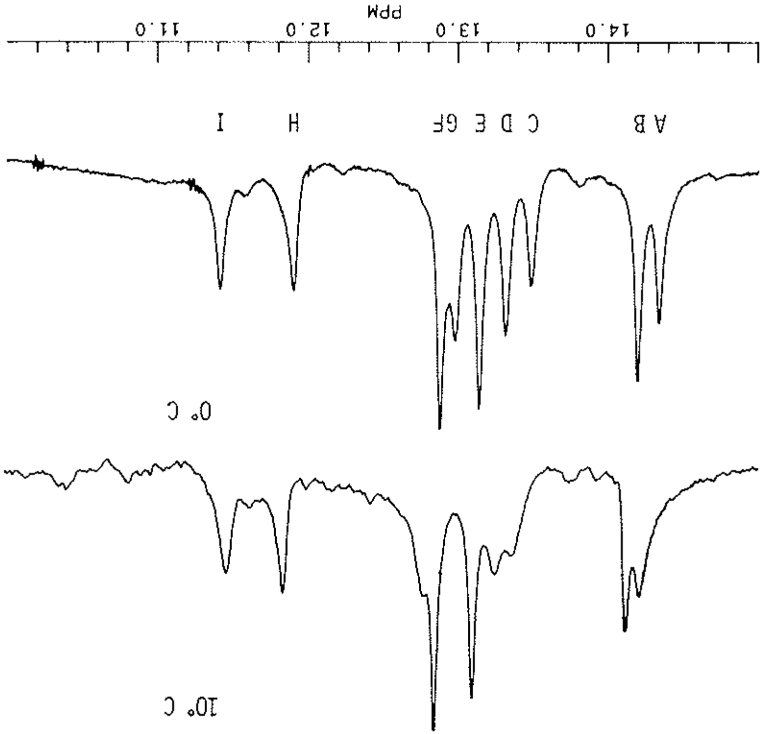

Fig. 9. 400 MHz ^{1}H NMR spectrum of d(CCAC^{AAF}GCACC) · d(GGTGCGTGG) at 0°C (bottom) and 10°C (top) in an H_2O solution containing 0.1 M NaCl. The sample was approximately 3 mM in each strand. The imino resonances are labelled A-I for reference in the text.

normal G·C imino proton resonances, and reflect the structural distortion that accompanies AAF modification.

The changes in the chemical shifts of the imino protons are likely caused by either ring-current effects or changes in the hydrogen bonds. A change in the overlap between adjacent base pairs in going from the unmodified to the modified duplex would result in each imino proton experiencing a different ring current. However, these ring-current effects from base pair overlaps are unlikely to be able to explain the significant upfield shifts observed for resonances H and I, nor the downfield shifts observed for the A·T resonances (A and B in Figure 9). In the insertion-denaturation or base-displacement models the fluorene moiety is stacked within the helix which could lead to substantial changes in the imino proton

resonances due to ring-current effects. However, if G^5 were displaced from the duplex, the G^5 imino resonance would not be expected to be observed, and yet seven resonances from the seven internal guanines are observable at 20°C, even at pH 8 (data not shown) which argues strongly against the base-displacement or insertion-denaturation models as being appropriate for the conformation of the d(CCAC^{AAF}GCACC)·d(GGTGCGTGG) duplex.

```
          1C · G18
          2C · G17
          3A · T16
          4C ? G15
      F - 5G ? C14
     A    6C ? G13
    A     7A · T12
          8C · G11
          9C · G10
```

A series of NOE experiments were used to determine the connectivities of the imino proton resonances. The data in Figure 10 show that resonance I is adjacent to resonance A. Smaller NOEs which are not much larger than the noise in these spectra are observed to resonances E and H. Other spectra show that, neglecting the terminal guanines, the connectivities are E→A→I and F→B→C. This leaves resonance H as the G^5 imino proton from the modified base.

The assignment of the resonances was performed using NOESY, COSY, and 1D spectra. The G^{13}-H_8 resonance was assigned to a peak at 7.19 ppm, which is farther upfield than is usually observed for guanine-H_8 resonances. The G^{13}-H_8 resonance exhibits a strong NOE to an H1′ sugar at 6.48 ppm, as shown in Figure 11. This large H_8-H1′ NOE indicates that G^{13} has adopted a *syn* conformation. The slices through the G^{11}-H_8 and the G^{15}-H_8 resonances show NOEs characteristic of bases with an *anti* glycosidic bond angle. Assuming that the presence of the bulky acetyl group of the adduct forces G^5 into a *syn* conformation, we conclude that two purines are in a *syn* conformation while the majority of the purines are in an *anti* conformation. It is speculative to suggest at the present time that the binding of AAF to the 9-mer results in the formation of a two base pair region of left-handed DNA. However, other duplexes can be made in which two or more carcinogens are bound at adjacent sites of an appropriate sequence which may stabilize a longer region of the altered conformation of the oligomer, as well as generating two clearly defined junctions.

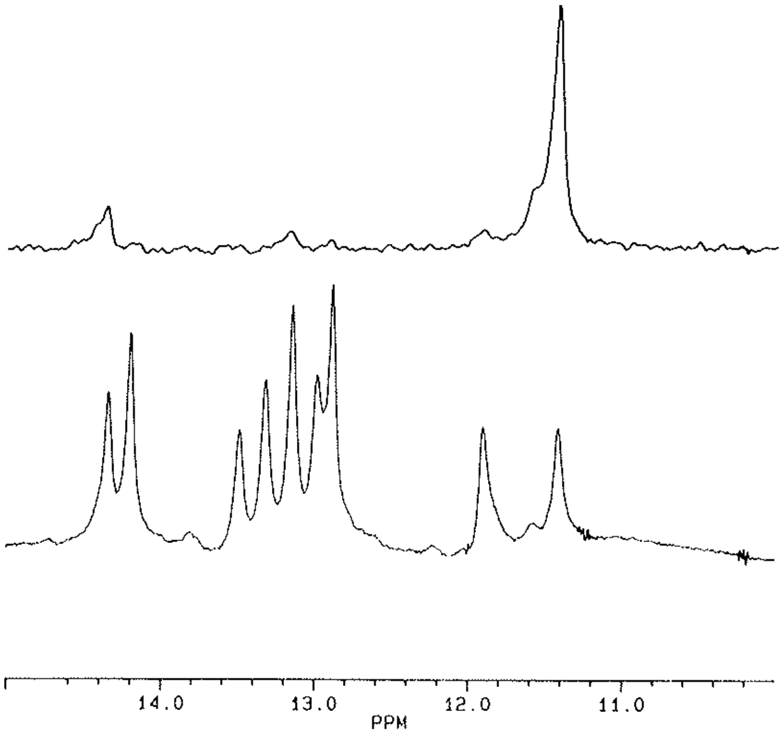

FIG. 10. 400 MHz NOE difference spectrum (top) at 0°C for irradiation of peak I showing a connectivity to resonance A. The control spectrum is shown as the bottom trace.

CONCLUDING REMARKS

The strong preference of ethidium for a right-handed intercalation site is interesting because model building studies do not show stereochemical restrictions for intercalation into a left-handed helix (Gupta *et al.*, 1983). The relative preference for ethidium intercalation into the right- and left-handed forms of poly(dG-dC) in 4.4 M NaCl may be expressed in terms of the ratio of the binding constants as calculated by the allosteric transition model; for ethidium, the K_2/K_1 ratio appears to be as large as 300:1 favoring binding to the right-handed form. With actinomycin D, the K_2/K_1 ratio appears to be substantially larger than 300:1, which may arise from the interaction of the cyclic pentapeptides with the outside of the helix (Walker *et al.*, 1985b). The kinetics of

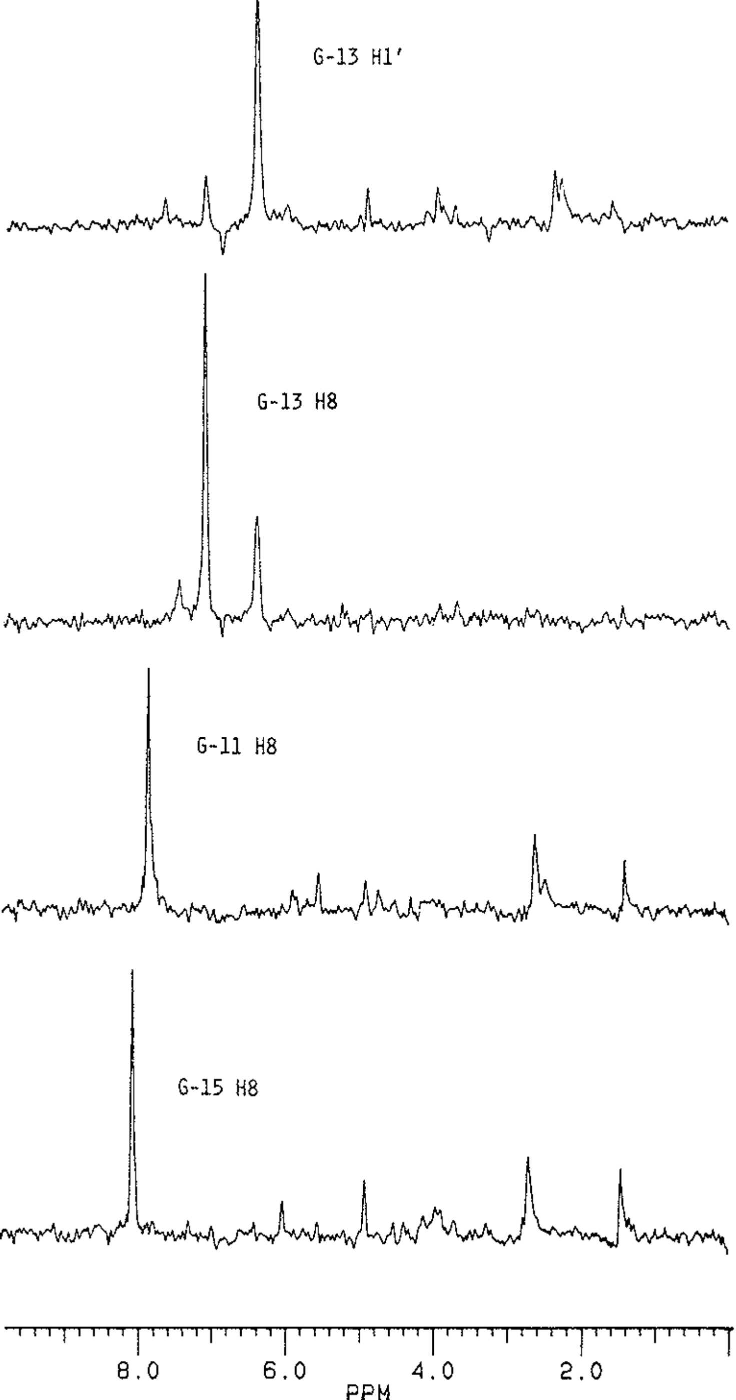

FIG. 11. Two-dimensional NOESY data. The slices through the peaks assigned to the G-15 H8, the G-11 H8, the G-13 H8, and the G-13 H1′ are shown for comparison of the NOEs.

ligand binding in the Z-DNA systems are likely to provide new insights into these systems because the activation free energies will contain both electrostatic and hydration contributions, as evidenced for example, by the dependence of the dissociation rate of actinomycin D from poly(dG-dC) on both the NaCl concentration and the solvent composition (Krugh *et al.*, 1979). For Z-DNA regions stabilized by supercoiling, the activity of the solvent is presumably the same for both left- and right-handed forms, but the electrostatic terms will be quite different for the two structurally different forms, and thus one would expect different rate constants in these systems as well.

The experiments on the interaction of drugs with Z-DNA have direct pharmacological significance if Z-DNA plays an important role in cellular processes. However, even without this justification, this area will continue to be explored because the ligands have proven to be important probes in studying the conformational properties of poly(dG-dC). The NMR studies of carcinogen-oligodeoxynucleotide duplexes promise to provide the detailed structural information needed in this area.

Acknowledgments

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MATAGEN (MASKING TAPE FOR GENE EXPRESSION): A FAMILY OF SEQUENCE SPECIFIC OLIGONUCLEOSIDE METHYLPHOSPHONATES

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INTRODUCTION

It is clear that the properties and behavior of living organisms, including viruses, are governed by their genes and the regulatory mechanisms involved in gene expression. In other words, DNA sequences and DNA sequences expressed in the form of RNA provide basic information about living systems. Thus in DNA replication and transcription, and RNA processing and translation there are specific nucleotide sequences which provide key information about the operation and the specificity of these biochemical processes. During the past decade, an increasing amount of information has been collected concerning these vital sequences. For example, the complete nucleotide sequence of the Human T-cell Leukemia Virus III (HTLV-III) has been elucidated recently (Ratner *et al.*, 1985). More information on gene sequences will surely be collected in the future. This sequence information can potentially be used to understand gene function.

In the past, procedures involving deletion or mutation of gene sequences have been used to study gene expression in viruses and bacteria. Temperature sensitive mutants have also been widely used. In the latter case, the gene of interest can function at certain temperatures, whereas at other, usually higher temperatures, the product of the mutated gene

does not function. Using these mutations, the role or the function of the gene in a living biological system can be ascertained.

However, this approach is not readily applicable to the study of genes of eukaryotic cells. Deletion mutations are not easily obtained at a specific locus and temperature sensitive mutants are even harder to acquire. Therefore, it would be quite useful to develop methods to specifically control gene expression by directly and specifically blocking the nucleic acid sequences of the gene. In practical terms this approach could be used to specifically control or inhibit the growth of pathogens such as viruses.

How can we specifically block gene expression? In order for the gene or its corresponding mRNA to function properly the nucleotide sequences must exist at least transiently in an exposed, single-stranded state. If these exposed sequences were to base pair with a complementary nucleic acid analog, the expression of the gene could be controlled. In order to function within living cells, the nucleic acid analog should have the following properties:

1) The nucleic acid analog must retain the absolute specificity of the Watson-Crick base pairing scheme. Therefore, no change in the structure of the bases should be made. If possible, the mutual interaction between the cellular target nucleic acid and the analogs should be increased without reducing base-pairing specificity.

2) The nucleic acid analog must be able to penetrate cellular membranes and enter the cell.

3) The nucleic acid analog must be resistant to hydrolysis by cellular enzymes. In other words, the chemical structure of the analogs must be such that it is inherently resistant to nucleases.

4) The nucleic acid analog must have a sequence complementary to a specifically targeted, single-stranded cellular nucleic acid so the function of the target nucleic acid will be under the specific control of the analog upon duplex formation.

The above approach should function well for control of foreign nucleic acids such as viral nucleic acids inside mammalian or human cells. Presumably, crucial nucleotide sequences of foreign pathogens will not be the same as those of the host. Therefore, once the crucial sequence of a virus has been determined, a nucleic acid analog complementary to that sequence would then be introduced into mammalian cells in order to control the virus.

A similar approach may be used to study oncogenes. Oncogenes have been recently implicated in the process of carcinogenesis. Over-expression of such genes, or overactivity of the oncogene product, can possibly lead to neoplasia. A variety of molecular events, including gene rearrangement, promoter insertion, gene amplification, point mutation, etc., could result in such over-expression or over-activity. Point mutations can also result in activation of oncogenes in such a way that the activity of the gene product increases. An example of such activation is the mutation in the 12th codon of the H-*ras* gene (Pulciani *et al.*, 1982).

Sequences of oncogenes have been determined from studies on oncogenic viruses. For example, the gene products of the *myc* gene, and therefore the gene sequence, have been elucidated (Ramsay *et al.*, 1984). It is still relatively difficult to study the precise function of these genes in the process of carcinogenesis or metastasis. It would be valuable to have a specific means to suppress the expression of these genes in order to determine their role in neoplasia. It should be kept in mind, however, that many of these genes also exist in normal cells and therefore may have vital functions. The function of oncogenes in normal cells will therefore also require careful evaluation.

It is known from heredity studies that defects in certain genes are related to the development of cancer and that these defective genes function in a recessive manner. This is in contrast to oncogenes, which function in a dominant manner. Such defective genes have been termed "anti-oncogenes", suggesting that these genes are regulatory in nature, their normal function being to regulate or suppress the expression of the oncogenes. Thus a defect in an anti-oncogene results in loss of regulation of oncogenes (Knudson, 1985). In this case, if the nucleotide sequence and the precise mode of action of anti-oncogenes can be understood, then we will have an obvious means to imitate the regulation of the oncogene.

In terms of both basic molecular biology studies and clinical application, the precise role of oncogenes in the early stages of carcinogenesis or at a later stage of tumor progression/metastasis has not been clearly defined. It is possible that such a role would be different for different oncogenes. However, the main goal in clinical applications is to inhibit the growth or metastasis of the tumor. The gene function responsible for such biological properties can be operationally defined as a tumor growth gene(s), which may or may not be equivalent to the viral transforming genes or the oncogenes described above. In the future, genes causally related to tumor growth or metastasis may be identified and sequenced.

Such genes could serve as targets for nucleic acid analogs acting as antitumor agents.

Research done in the Division of Biophysics over the past 15 years has led to the development of a family of oligonucleotide analogs which has the properties described above and which can inhibit specific biological systems, including animal viruses in mammalian and human cells.

We have prepared two types of nonionic oligonucleotide analogs: oligonucleotide alkylphosphotriesters and oligonucleoside methylphosphonates (see Figure 1). The latter analogs are also given the name Matagen (an acronym for "*Ma*sking *Ta*pe for *Gen*e *E*xpression". Figure 2 compares the phosphodiester backbone of the deoxyoligonucleotides and methylphosphonate backbone of Matagen and outlines the differences in the physical, chemical, biochemical and biological properties of the oligomers. The bases, sugar and a portion of the sugar phosphate backbone of these analogs are identical to those of natural nucleic acids. However, the analogs are not charged due to the replacement of the negatively charged phosphodiester linkage with the nonionic methylphosphonate internucleotide bond. This type of modification allows the analogs to form complementary duplexes with single-stranded nucleic acids with a high degree of specificity.

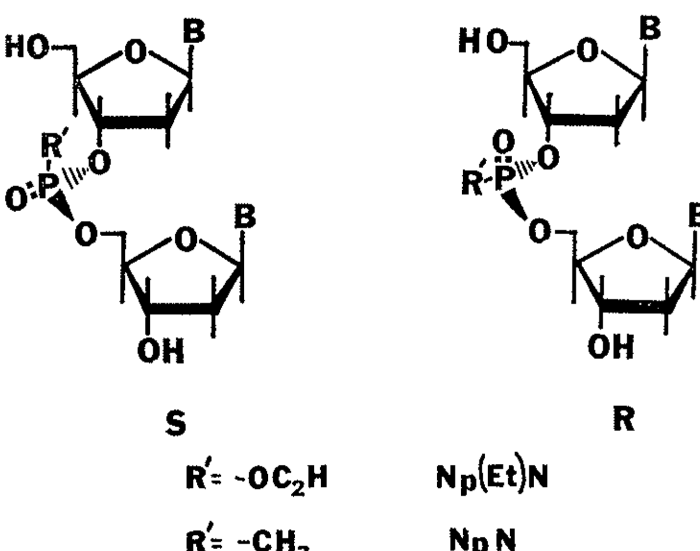


Fig. 1. Diastereoisomers of a dinucleotide ethylphosphotriester [d-Np(Et)N] and a dinucleoside methylphosphonate (d-N*p*N).

$$\left(\begin{array}{ccccccccccccccc} B_1 & & O & & B_2 & & O & & B_3 & & O & & B_4 & & O \\ | & & \| & & | & & \| & & | & & \| & & | & & \| \\ R & - & P & - & R & - & P & - & R & - & P & - & R & - & P- \\ & & | & & & & | & & & & | & & & & | \\ & & O- & & & & O- & & & & O- & & & & O- \end{array}\right)_{2-4} \qquad \left(\begin{array}{ccccccccccccccc} B_1 & & O & & B_2 & & O & & B_3 & & O & & B_4 & & O \\ | & & \| & & | & & \| & & | & & \| & & | & & \| \\ R & - & P & - & R & - & P & - & R & - & P & - & R & - & P- \\ & & | & & & & | & & & & | & & & & | \\ & & CH_3 & & & & CH_3 & & & & CH_3 & & & & CH_3 \end{array}\right)_{2-4}$$

oligodeoxyribonucleotides	oligodeoxyribonucleoside methylphosphonates (Matagen)
Negatively charged phosphodiester backbone	Nonionic methylphosphonate backbone
Forms duplexes with complementary polynucleotides	Forms duplexes with complementary polynucleotides. Stability of duplex higher than that of oligodeoxyribonucleotide duplexes.
Backbone readily hydrolyzed by nucleases	Backbone totally resistant to nuclease hydrolysis
Oligomer usually not taken up intact by cells	Oligomer taken up intact by cells by passive diffusion
	Oligodeoxyribonucleoside methylphosphonates are active in the following cells:
	Transformed Syrian Hamster Fibroblasts (BP-6)
	Transformed Human Cells (HTB 1080)
	Rabbit Reticulocytes
	African Green Monkey Kidney Cells (BSC40)
	Mouse L-Cells

FIG. 2. Comparison of oligodeoxyribonucleotides and oligodeoxyribonucleoside methylphosphonates (Matagen).

These analogs are resistant to nucleases and are taken up intact by mammalian and certain bacterial cells in culture.

The phosphotriester or methylphosphonate modification results in the formation of a pair of diastereoisomers which differ in configuration about the phosphorus atom, as shown in Figure 1. In most cases, the dideoxyribonucleoside methylphosphonate diastereoisomers can be separated by reversed phase high performance liquid chromatography. The configurations of the methylphosphonate linkage of dA*p*T have been determined by x-ray crystallographic studies (Chacko *et al.*, 1983) whereas that of dA*p*A has been assigned by NMR nuclear Overhauser enhancement experiments (Kan *et al.*, 1980). Studies on the interaction of dimers with complementary polynucleotides show that the two diastereoisomers (denoted as S or R, representing pseudo axial and pseudo equatorial configuration) form complexes of different stabilities. For example, the equatorial (R) isomer of d-A*p*A forms a more stable complex with poly U in the d-A*p*A: poly(U) system [(2A: 1U) complex] than does the axial (S) isomer. The base stacking patterns of both isomers of d-A*p*A are very similar to those of the dinucleoside monophosphate, d-ApA, with the stacking of the S isomer being identical to that of the diester (Miller *et al.*, 1979; Kan *et al.*, 1980).

Experiments on the thermal stability of complexes formed between the oligonucleoside methylphosphonates and complementary polynucleotides show that the Tm (melting temperature) of the nonionic oligonucleotide complex is usually higher than that of the complex formed between the naturally occurring oligonucleotides and their complementary polynucleotides. For instance, the poly dT · deoxy A_4 complex (2T: 1A) has a Tm of 35.5°C, while the corresponding poly dT · deoxy A_4 analog has a Tm of 44.5°C under the same salt conditions (Miller *et al.*, 1981). Clearly, the removal of the negative charge from the oligonucleotide analog has increased the stability of the complexes.

Methods have been developed to synthesize and characterize oligodeoxyribonucleoside methylphosphonates and to study their interactions with RNA. Methylphosphonate oligomers of defined sequence are readily prepared in good overall yields on insoluble polymer supports by stepwise additions of protected deoxyribonucleoside methylphosphonic imidazolides to the growing oligomer chain (Miller *et al.*, 1986). The 5′-hydroxyl groups of oligomers which terminate with a nucleoside phosphodiester linkage can be phosphorylated by polynucleotide kinase. The size of the oligomer can be determined by partial hydrolysis of the methylphosphonate linkages

with piperidine followed by polyacrylamide gel electrophoresis which separates the oligomers according to their chainlengths. The sequence of the oligomers can be determined by a modified Maxam-Gilbert procedure. Sequence-specific [^{32}p]-labeled oligomers hybridize with mRNA in agarose gels. Since the oligomers serve as primers for reverse transcriptase, their binding position on mRNA can also be characterized (Murakami *et al.*, 1985). Thus, the methylphosphonate linkages of Matagen are not hydrolyzed by nucleases and this analog can penetrate the membranes of mammalian cells as well as bind to complementary polynucleotides. Evidence is accumulating that methylphosphonate analogs can also form complementary complexes with single-stranded portions of cellular RNA inside the cell.

Two general strategies have been developed to utilize Matagen to block the expression of a gene or to mask the function of the targeted nucleic acid. The first approach is to block the translation of a targeted mRNA and the second approach is to inhibit the mRNA processing, particularly the splicing of pre-mRNA. These approaches are illustrated in Figure 3 and are described in more detail in the following sections. We have also attempted to block the interaction between bacterial 16S rRNA and mRNA by sequence specific oligonucleoside methylphosphonates.

Specific Inhibition of Bacterial Protein Synthesis

We have synthesized a Matagen with the sequence d-A*p*G*p*G*p*A*p*G*p*G*p*T which is complementary to the 16S rRNA of the Shine-Dalgarno sequence, -ACCUCCU-, which in turn is complementary to the initiation region of the bacterial mRNA. The Shine-Dalgarno sequence is absent in 18S ribosomal RNA of mammalian cells. This Matagen blocks the interaction between the 16S rRNA and the bacterial mRNA, and thereby inhibits the translation of bacterial mRNA (Jayaraman *et al.*, 1981). It does not inhibit translation of globin mRNA in a mammalian cell-free translation system. Since the *E. coli* cell wall prevents the uptake of Matagen, experiments were performed with an *E. coli* mutant which has an altered, more permeable cell wall. Experiments in a cell-free *E. coli* system and with the living *E. coli* mutant clearly demonstrated that the d-A*p*G*p*G*p*A*p*G*p*G*p*T Matagen is a powerful, specific inhibitor of protein synthesis and bacterial growth, exerting little inhibitory effect in a mammalian translational system (globin synthesis in rabbit reticulocytes) or on growth of human cells (HT1080, human fibrosarcoma fibroblast).

Activity: Blocks specific sequences of critical single-strended regions of cellular nucleic acids.

I. Inhibition of translation of mRNA - Inhibition of translation by Matagen complementary to the initiation codon region of mRNA

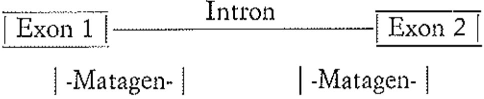
—— A U G ——————

T*p*A*p*C*p*N*p*N*p*N*p*N*p*N

A. Inhibition of rabbit globin synthesis in rabbit reticulocytes and lysates

B. Inhibition of Vesicular stomatitis virus (VSV) protein synthesis in VSV-infected mouse L-cells

II. Inhibition of splicing of pre-mRNA - Inhibition of splicing by Matagen complementary to the splice junctions of pre-mRNA



A. Inhibition of large T antigen synthesis in SV40-infected cells.

B. Inhibition of proteins encoded by immediate early mRNA 4 and 5 in Herpes simplex virus type 1 and 2-infected cells.

FIG. 3. Matagen (*M*asking *T*ape for *G*ene *E*xpression).

MATAGEN DESIGNED TO INHIBIT PROTEIN SYNTHESIS BY MASKING mRNA TRANSLATION

Two systems were used to investigate the ability of sequence specific oligonucleoside methylphosphonates to prevent translation of mRNA. In the first system, Matagens with sequences complementary to various regions of rabbit globin mRNA were tested as inhibitors of globin synthesis in rabbit reticulocyte lysates and cells (Blake *et al.*, 1985). In the second system, Matagens with sequences complementary to the initiation

codon regions of selected Vesicular Stomatitis virus (VSV) mRNAs were studied for their effects on virus protein synthesis both in a cell-free system and in virus-infected cells (Agris *et al.*, 1986). The results of these experiments are briefly described below.

A) *Inhibition of Globin mRNA Translation*

Matagens were synthesized whose sequences are complementary to the 5′ end, the initiation codon region or the coding region of rabbit globin mRNA. The sequences of these Matagens and their binding sites on globin mRNA are shown in Figure 4. These Matagens were shown to interact with their target complementary mRNA binding sites by their ability to serve as primers for the enzyme reverse transcriptase. In several cases the priming efficiency of the Matagen was enhanced when the oligomer was first preannealed with the mRNA. The priming efficiency of the Matagen correlated with the predicted secondary structure of the Matagen binding site on globin mRNA. That is, Matagens whose binding sites are located in the hydrogen bonding stem region of the mRNA are less efficient primers than those whose binding sites are located in single-stranded loop regions.

Matagens inhibit translation of globin mRNA in a rabbit reticulocyte lysate system, as shown in Table I. This inhibition is influenced by the sequence and the chainlength of the Matagen, the location of the Matagen binding site on the mRNA and finally, the presence or absence of secondary structure at the binding site. In general, oligomers which bind to the 5′ end and initiation codon regions of β-globin mRNA inhibit both α- and β-globin synthesis whereas oligomers which bind to the coding region of α-globin mRNA or the coding region of β-globin mRNA inhibit translation of their target mRNA in a specific manner. Of particular interest is the Matagen d-Cp*ATTCTGT* whose nucleotide sequence is complementary to nucleotides 49-56 of β-globin mRNA. Under the usual conditions of the translation experiment, this Matagen has little or no inhibitory effect on either α or β globin synthesis, even at oligomer concentrations up to 200 μ*M*. However, if the oligomer is preannealed to the mRNA prior to translation, inhibition as high as 35-50% is observed.

Matagens also effectively inhibit globin synthesis in rabbit reticulocytes at 25°, as shown in Table II. The effects of the oligomers on cellular globin synthesis are similar to those in the lysate system and suggest that the conformation of globin mRNA is the same in both systems

α Globin mRNA

```
         1           10             30          40                             60           70
G^m pppA^m C^m ACUUCUGG ..........AAGGAACCACCAUGGUGCUG...........................GACCAACAUCA...........(A)N
                                              TACC                                GGTTGTAGT
                                              TACCA
                                              TACCACGT
```

β Globin mRNA

```
         1           10                50             60           70           80
G^m pppA^m C^m ACUUGCUUUU ........AAACAGACAGAAUGGUGCAUCUGUCCAGTGAGGAGAAG......................(A)N
              GAACGAAAA                    TACC
                                           TACCA
                                           TACCACGT
                                     TGTCTTAC
                                TGTCTGTCTTAC                      GTCCTCCT
```

FIG. 4. Partial nucleotide sequence of rabbit α-globin mRNA and rabbit β-globin mRNA. Below each mRNA are shown the sequences of the complementary oligodeoxyribonucleoside methylphosphonates. The underlined portion of each oligomer shows the position of the methylphosphonate internucleotide linkages. [Reprinted with permission from Biochemistry, 1985, *24* (22), 6139-6145. Copyright 1985, American Chemical Society].

TABLE I - *Inhibition of Rabbit Globin mRNA Translation by Oligodeoxyribonucleoside Methylphosphonates in a Rabbit Reticulocyte Lysate at 37°C.*

Oligomer	Binding Site	Conc. μ*M*	% Inhibition α	β
5′-end				
d-Ap*AAAGCAAG*	β 4-12	100	56	51
		100 ([a])	70	70
Initiation codon region				
d-*CCAT*	α 37-40	100	0	0
	β 54-57	200	28	42
d-Ap*CCAT*	α 37-41	100	23	36
	β 54-58	200	67	67
d-Tp*GCACCAT*	α 37-44	100	35	28
	β 54-61	200	73	70
d-Cp*ATTCTGT*	β 49-56	50	2	2
		100	0	7
		200	11	8
		100 ([a])	30	36
		200 ([a])	48	34
d-Cp*ATTCTGTCTGT*	β 45-56	25	17	14
		100	49	50
Coding region				
d-Ap*CAGATGC*	β 59-66	25	5	− 7
		50	5	4
		100	33	24
		100 ([a])	76	66
d-Tp*GATGTTGG*	α 62-70	25	16	8
		50	24	9
		100	38	11
d-Tp*CCTCCTG*	β 72-80	25	8	16
		100	15	36
Other				
d-*ACAGACAT*	none	25	7	− 14
		100	19	8
d-*TTTTTT*	poly A tail	300	0	0

([a]) Oligomer preannealed to mRNA before translation. [Reprinted with permission from Biochemistry, 1985, *24* (22), 6139-6145. Copyright 1985, American Chemical Society].

during translation. For example, d-Cp*ATTCTGT*, which does not inhibit unless preannealed with mRNA, has no effect on reticulocyte globin synthesis. This observation strongly suggests that the secondary structure of globin mRNA in living reticulocytes is similar to that in the lysate and that the conformation of mRNAs in living cells may be probed by Matagen.

B) *Inhibition of VSV Protein Synthesis*

Matagens whose sequences are complementary to the initiation codon regions of N, NS, and G mRNA of VSV were synthesized (see Figure 5). Although these Matagens are completely complementary only to their target mRNA, a computer search revealed that they do have varying degrees of complementarity with other VSV mRNAs (see Table III). The Matagens were tested for their ability to inhibit translation of VSV mRNA in a rabbit reticulocyte lysate translation system and in VSV-infected mouse L cells. In the lysate system, oligomers complementary to N and NS mRNAs inhibited translation of VSV N and NS mRNAs whereas the oligomer complementary to G mRNA had only a slight inhibitory effect on N protein synthesis (Table IV). The N-specific oligomer specifically inhibited N protein synthesis whereas the NS-specific oligomer inhibited both N and NS protein synthesis. This reduced specificity of inhibition may be due to formation of partial duplexes between the NS-specific oligomer and VSV N mRNA. The oligomers had little or no inhibitory

TABLE II - *Effects of Oligodeoxyribonucleoside Methylphosphonates on Globin Synthesis in Rabbit Reticulocytes at 25°C.*

Oligomer	Conc. (μM)	% Inhibition α	% Inhibition β
d-Tp*GCACCAT*	200	42	29
d-Cp*ATTCTGT*	200	0	0
d-Cp*ATTCTGTCTGT*	200	21	17
d-Tp*GATGTTGG*	100	59	59

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effects on the synthesis of globin mRNA in the same lysate system (Table IV). All three oligomers specifically inhibited synthesis of all five viral proteins in VSV infected cells in a concentration dependent manner (Table V). The oligomers had no effect on cellular protein synthesis in uninfected cells or on cell growth. As a control, an oligothymidylate which forms only weak duplexes with poly(rA) was shown to have only a slight effect on VSV protein synthesis or yield of virus. It should be noted all three oligomers have extensive partial complementarity with the coding regions of L mRNA (Table III). The non-specific inhibition of viral protein synthesis in infected cells may reflect the role of N, NS, and/or L proteins in the replication and transcription of viral RNA or result from duplex formation between the oligomers and complementary, plus-strand viral RNA. The oligomers also significantly inhibited VSV production in a manner corresponding to their effects on VSV protein synthesis as shown in Table VI. These results demonstrate that the Matagen can be used to study viral gene expression and to control virus production.

N mRNA

```
       1            10           20
G^m pppA^m ACAGUAAUCAAAAUGUCUCUUACA .................... (A)n
                           TACAGACAA
```

NS mRNA

```
       1            10           20
G^m pppA^m ACAGAUAUCAUGGAUAAUCUCACA .................... (A)n
                        TACCTATTA
```

G mRNA

```
       1            10       30
G^m pppA^m ACAGAGAUC..........CUAUGAAGUGC .............. (A)n
                              TACTTCACG
```

FIG. 5. Matagens complementary to VSV mRNA. [Reprinted with permission from Biochemistry, 1986, 25 (20), 6268-6275. Copyright 1986, American Chemical Society].

TABLE III - *Complementarity Between Oligodeoxyribonucleoside Methylphosphonates and VSV mRNA or Rabbit Globin mRNA.*

Oligonucleoside methylphosphonate	Number of contiguous oligomer bases	Number of complementary sites of VSV mRNA ([a]) VSV mRNA						Globin mRNA		
		L	G	NS	N	M	Total	α	β	Total
d-Ap*ACAGACAT* (I)	9	0	0	0	1	0	1	0	0	0
	8	0	0	0	0	0	0	0	0	0
	7	2	0	0	0	0	0	0	0	0
	6	4	4	0	1	0	9	0	0	0
				1*			1*			
	5	16	1	2	3	1	23	1	3	4
				1*		1*	2*	1*		1*
d-Ap*ATTATCCAT* (II)	9	0	0	1	0	0	1	0	0	0
	8	1	0	0	0	0	1	0	0	0
	7	2	0	0	1	0	3	0	0	0
	6	8	1	0	2	0	11	0	0	0
	5	33	10	5	6	3	57	0	1	1
				1*	1*		2*			
d-Gp*CACTTCAT* (III)	9	0	1	0	0	0	1	0	0	0
	8	1	0	0	0	0	1	0	0	0
	7	1	1	0	0	0	2	0	0	0
	6	9	1	2	0	1	13	0	1*	1*
	5	40	7	8	6	6	67	0	1	1
		2*	1*	2*	1*	1+	8*			

([a]) These values represent the number of nonoverlapping sites on the mRNA which are complementary to 5 or more contiguous bases of the oligomer.

(*) Site is located in the 3′-noncoding region of the mRNA.

(+) Site is located in the 5′-noncoding region of the mRNA.

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MATAGENS TARGETED AGAINST THE SPLICE JUNCTIONS OF PRE-mRNA

As discussed above, Matagens targeted against single-stranded regions of mRNA are more effective inhibitors of mRNA translation than those whose binding sites are involved in secondary structure. Based on our current understanding of mRNA biosynthesis, it would appear that the splice junctions of pre-mRNAs would be ideal targets for Matagens. Recent experiments have demonstrated that the splice junctions interact with the RNAs of small ribonucleoproteins which mediate the splicing process.

TABLE IV - *Effects of oligodeoxyribonucleoside methylphosphonates on VSV and globin protein synthesis in a rabbit reticulocyte lysate at* 30°C ([a]).

Oligomer	Conc. (μM)	% Change ([b]) NS	N	M	α globin	β globin
d-Ap*ACAGACAT* (I)	50	+ 7	−26	+20	− 6	− 9
	100	− 7	−36	+12	−15	− 9
	150	−38	−77	−43		
d-Ap*TTATCCAT* (II)	50	+ 5	− 4	+ 7	+18	+14
	100	−15	−25	+ 2	+63	+50
	150	−24	−35	−18		
d-Gp*CACTTCAT* (III)	50	− 4	−15	0	+26	+20
	100	+ 2	−16	+ 6	+40	+55

([a]) Average of 2 or 3 experiments, with a range of ± 6% (VSV) or ± 4% (globin).

([b]) Minus sign indicates inhibition of translation, while a plus sign indicates stimulation of translation.

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TABLE V - *Effects of oligodeoxyribonucleoside methylphosphonates on VSV protein synthesis in mouse L cells* ([a]).

Oligonucleoside Methylphosphonate	Conc. (μ*M*)	% Inhibition ([b]) L	G	NS	N	M
d-Ap*ACAGACAT* (I)	50	4	+ 3	7	12	13
	100	12	2	12	9	27
	150	69	73	57	67	63
d-Ap*TTATCCAT* (II)	50	20	4	7	4	9
	100	38	31	26	29	38
	150	59	42	43	47	46
d-Gp*CACTTCAT* (III)	50	35	25	45	15	10
	100	94	88	90	80	63
	150	96	94	97	99	92
d-Tp*TTTTTTT* (IV)	50	14	10	9	20	5
	100	15	13	17	22	11
	150	16	30	8	25	15

([a]) Average of 2 or 3 experiments with a range of ± 5%.

([b]) Plus sign indicates stimulation of protein synthesis.

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TABLE VI - *Effects of oligodeoxyribonucleoside methylphosphonates on VSV production* ([a]).

Oligonucleoside	Conc. (μM)	Log reduction (pfu/ml) ([b]) Hours post-infection 6	24
d-Ap*ACAGACAT* (I)	50	0.07	0.07
	100	0.40	0.07
	150	1.08	0.62
d-Ap*TTATCCAT* (II)	50	0.08	0.10
	100	0.36	0.20
	150	0.52	0.39
d-Gp*CACTTCAT* (III)	50	0.40	+0.02
	100	1.18	0.79
	150	1.52	1.02
d-Tp*TTTTTTTT* (IV)	50	0.10	0.07
	100	0.12	0.09
	150	0.15	0.07

([a]) Average of 2 or 3 experiments with a range of ± 0.03 log reduction (pfu/ml).

([b]) Plus sign indicates stimulation of VSV production.

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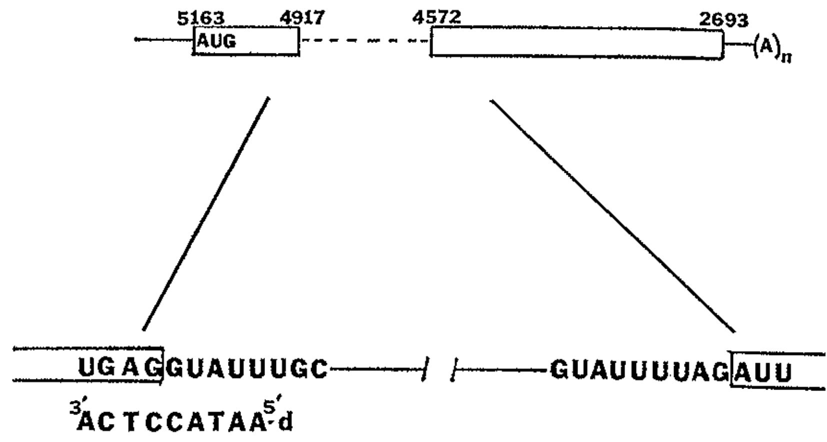
These observations suggest that the splice junctions are in a single-stranded form. We have prepared Matagens to the splice junctions of two viral pre-mRNAs in order to test the possibility of controlling gene expression at the level of mRNA processing.

A) *Inhibition of SV40 Large T-antigen RNA.*

We have synthesized Matagens with sequences complementary to the donor splice junction of SV40 large T-antigen and to the 5′-terminal sequence of U_1 RNA (see Figure 6). U_1 RNA has been shown to be involved in the splicing of mammalian and viral pre-mRNAs. The effects of these compounds on large T-antigen synthesis in SV40-infected African green monkey kidney cells (BSC40) were determined by an immunoprecipitation technique. As shown in the table accompanying Figure 6, the splice junction-complementary oligomer, d-*AATACCTCA*, and the two U_1 RNA-com-



Oligomer	Concentration M	% Reduction of T-antigen positive Nuclei
D-A*p*A*p*T*p*A*p*C*p*C*p*T*p*C*p*A	1	20
	5	30
	25	45
D-G*p*G*p*T*p*A*p*A*p*G*p*	1	10
	5	25
	25	30
D-$(Tp)_5T$	1	6
	5	6
	25	0

FIG. 6. Partial nucleotide sequence of the splice junctions of SV40 large T-antigen pre-mRNA. The sequence of the methylphosphonate oligomer complementary to the donor splice junction is shown below the pre-mRNA sequence. The table shows the effects of this oligomer and oligomers complementary to U_1 RNA on large T-antigen synthesis in SV40-infected BSC40 cells.

plementary oligomers each reduce the level of large T-antigen in the cells, while the non-specific oligomer, d-*TTTTTT*, had no effect. None of these oligomers reduced overall protein synthesis by the BSC40 cells.

B) *Inhibition of Herpes Simplex Virus Type 1.*

We have synthesized the Matagen d-Tp*CCTCCTG* whose sequence is complementary to the acceptor splice junctions of HSV-1 immediate early mRNA 4 and 5 (Smith *et al.*, 1986). The targeted binding site of this Matagen on the HSV-1 pre-mRNA is shown in Figure 7. To test the specificity of the Matagen we have also synthesized a control Matagen,

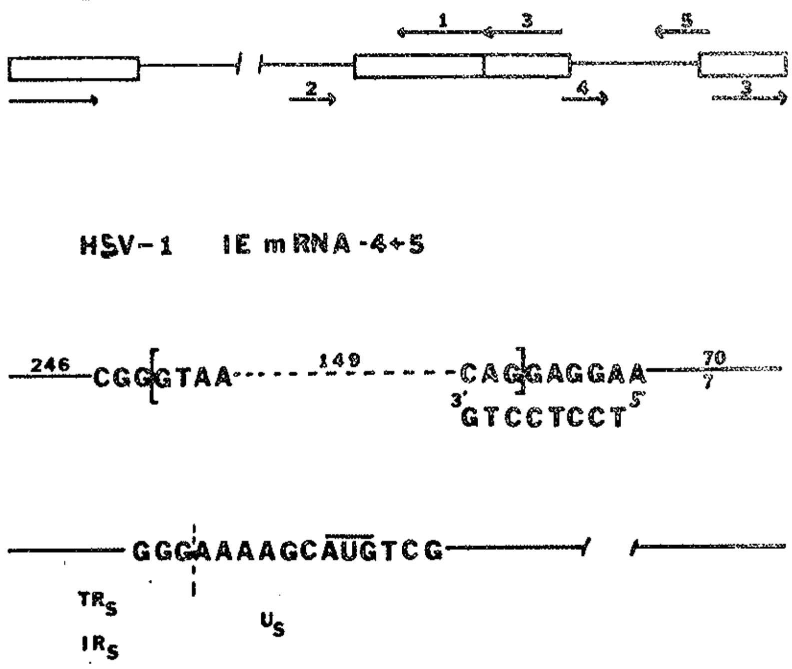


Fig. 7. Location and direction of synthesis (arrows) of IE mRNAs 4 and 5. Open bars represent terminal (TR_L, TR_S) and inverted (IR_L and IR_S) repeats bounding the unique long (U_L) and short (U_S) HSV-1 DNA sequences. Intervening nucleotides are numbers. Intron is bounded by brackets. The sequence of the oligomer is shown below the mRNA sequence.

d-Tp*CCCTCTG* in which positions #4 and #5 are -C T- instead of -T C-. The effects of these Matagens on virus replication are shown in Figure 8. Treatment of HSV-1-infected cells with d-Tp*CCTCCTG* before (1-24 hr) or at the time of infection caused a dose-dependent inhibition of virus replication. Virus titers were decreased 50% and 90% in cells treated with 25 μM and 75 μM oligomer, respectively; at 300 μM, a 99% reduction in virus production was observed. Under the same conditions the control Matagen d-Tp*CCCTCTG* had little inhibitory effect. These data clearly demonstrate the sequence specificity of the Matagen. In addition, the Matagen is more effective against HSV-1 than HSV-2, indicating a strain specificity in the action of Matagen. Finally, the timing of the addition of Matagen is important. As shown in Figure 8a, addition of the Matagen at the time of infection is most effective.

The Matagen specifically reduced viral DNA synthesis 70-75% as shown in Figure 9. A 90% reduction in the synthesis of viral proteins including other immediate early species and viral functional and structural proteins was also observed. The specificity of the Matagen is demonstrated by the observation that Matagen caused minimal reduction in cellular protein synthesis (Figure 10) and minimal effects on the growth rate of uninfected cells (Figure 11).

Synthesis of Matagen on Polymer Supports

A polymer support method was developed to prepare Matagens of defined sequence (Miller *et al.*, 1986). The synthetic scheme is shown in Figure 12. 5′-(Dimethoxytrityl)deoxyribonucleoside 3′-(methylphosphonic imidazolides) are condensed with polymer support-bound oligonucleotide in tetrazole, which appears to act as an acid catalyst. The kinetics of dimer formation linked to different supports in the presence and absence of tetrazole at 22°C are shown in Figure 13. The half-life for dimer formation on the polystyrene support is 5 min and the reaction is 95% complete after 60 min. The reaction cycle for the preparation of Matagen is described in Table VII.

The yields and the HPLC retention times of six Matagens prepared by this procedure are shown in Table VIII. In order to simplify purification and sequence analysis of the oligomer, the 5′-terminal nucleoside unit is linked *via* a phosphodiester bond. The singly charged oligomers are easily purified by affinity chromatography on DEAE cellulose. The chain-lengths of the oligomers were confirmed after 5′-end labeling with poly-

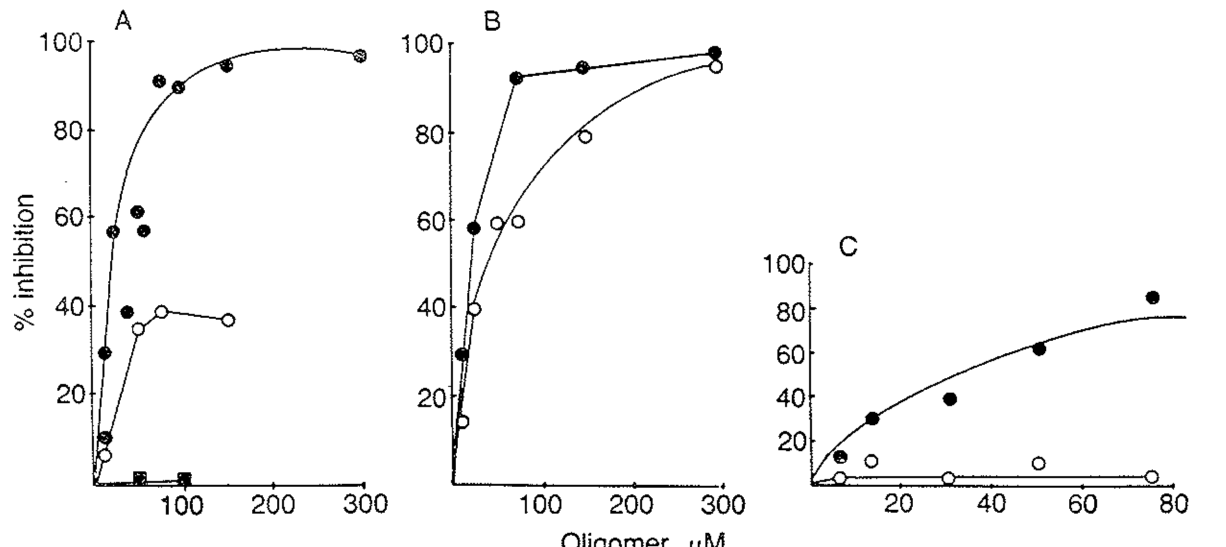


Fig. 8. Inhibition of virus production: (A) HSV-1 (F)-infected Vero cells were exposed to d-Tp*CCTCCTG* at 0 hpi (●) or 1 hpi (○) or to d-Tp*TTTTT* at 0 hpi (■). (B) HSV-1 (F)-infected Vero cells were exposed to d-Tp*CCTCCTG* 24 hr before infection (●). HSV-1 (G)-infected Vero cells were exposed to d-TpCCTCCTG at 0 hpi (○). (C) HSV-1 (F)-infected Vero cells were exposed to d-Tp*CCTCCTG* (●) or d-Tp*CCCTCTG* (○) at 0 hpi.

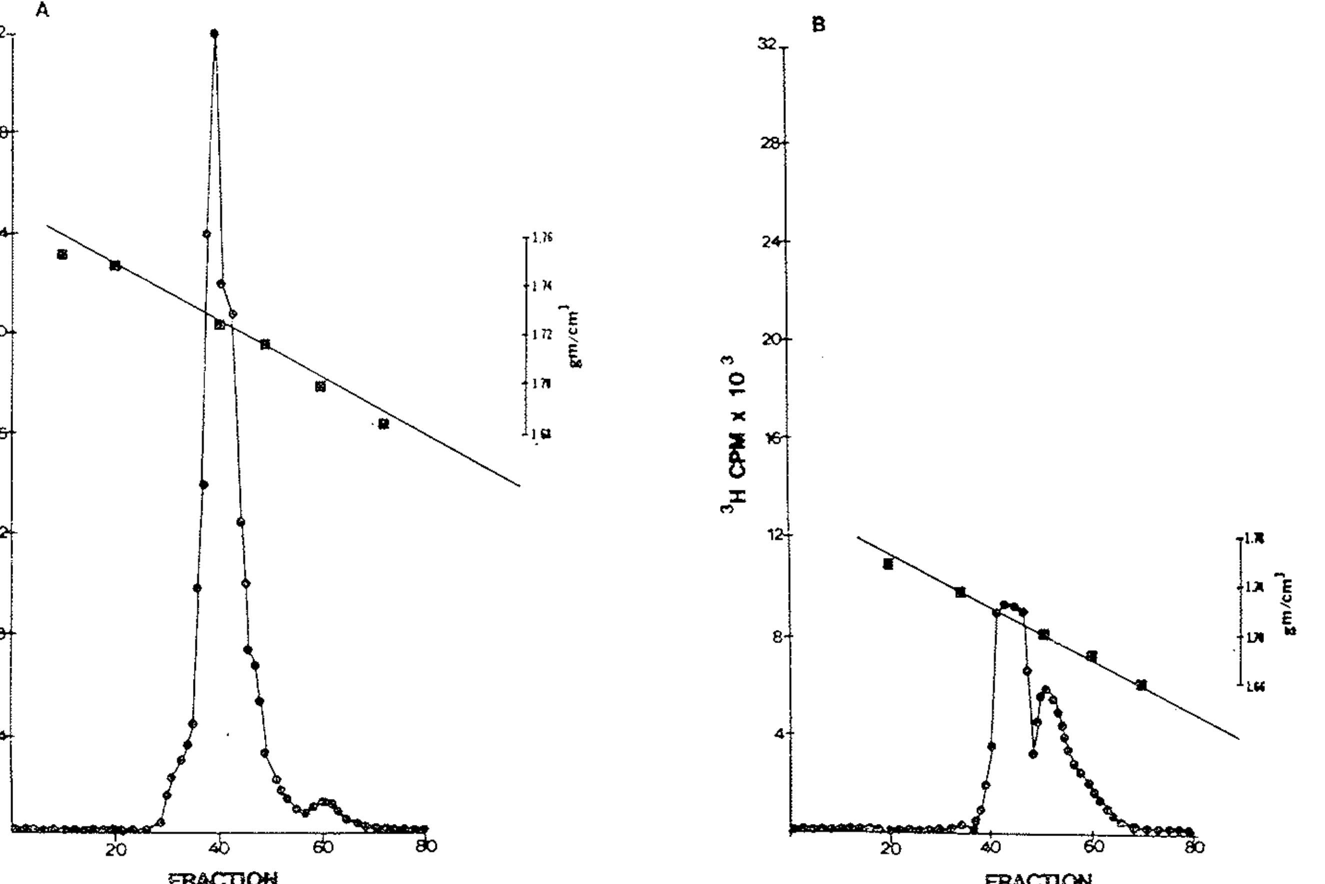


FIG. 9. CsCl density gradients of DNA from HSV-1 (F)-infected Vero cells exposed (A) or not (B) to 250 μM d-Tp*CCTCCTG* added at 0 hpi and labeled with [^{3}H]-thymidine (4-18 hpi).

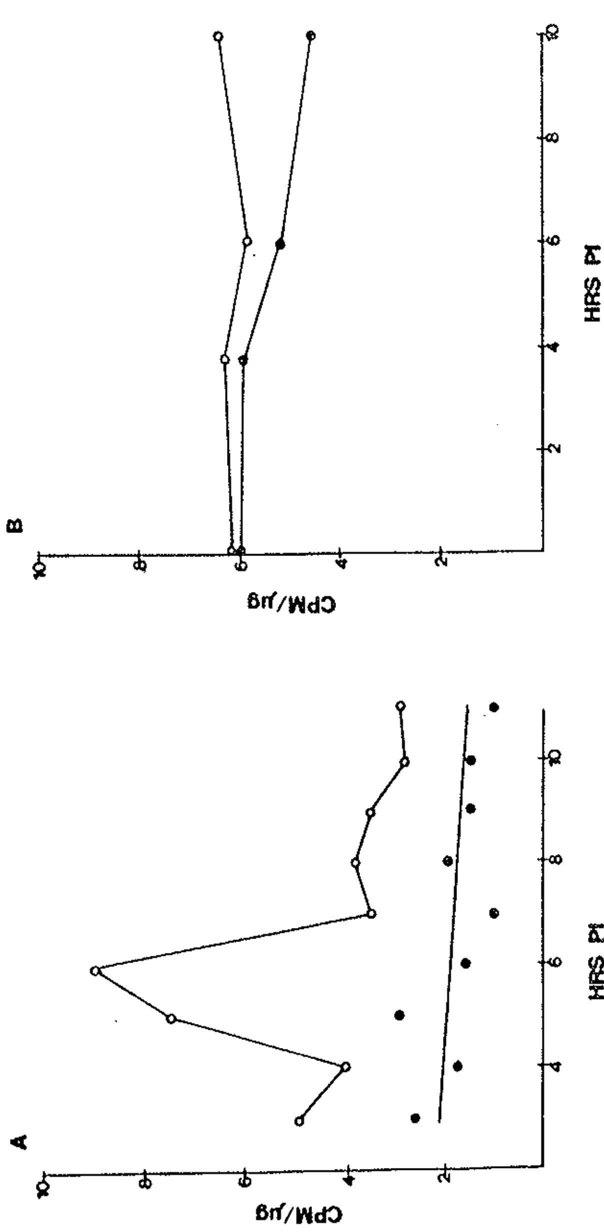


FIG. 10. Protein synthesis in HSV-1(F)-infected (A) and mock-infected (B) Vero cells untreated (○) or treated (●) with 250 μM d-Tp*CCTCCTG* added at 0 hpi. Cultures were labeled for 1 hr with [^{35}S]-methionine.

TABLE VII - *Reaction Cycle for Preparation of Oligodeoxyribonucleoside Methylphosphonates* ([a]).

Step	Reagent		Time (min)
Detritylation	1 *M* $ZnBr_2$ in methylenechloride/ isopranol ([b])	4 × 1 ml 2 × 2 ml	2 each (C, T) 2 each (G, A)
Wash	methylene chloride/isopropanol ([b])	3 × 1 ml	5 last wash
Wash	0.5 *M* TEAA in dimethylformamide	3 × 1 ml	
Wash	acetonitrile	6 × 1 ml	
Dry	vacuum pump		30
Condensation	coupling mixture	400 µl ([c])	60
Wash	tetrahydrofuran	5 × 1 ml	
Acetylation	capping solution ([d])	2 × 1 ml	5 last wash
Wash	methylene chloride/isopropanol ([b])	6 × 1 ml	

([a]) Reactions were carried out on 100 mg or 200 mg of polystyrene support at room temperature.

([b]) 85:15 v/v.

([c]) Use 800 µl for 200 mg of support.

([d]) 1 ml acetic anhydride, 1 ml anhydrous pyridine, 10 mg dimethylaminopyridine.

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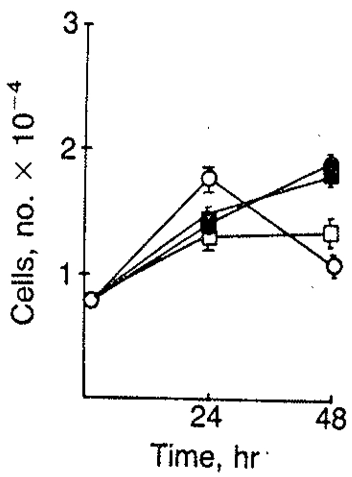

FIG. 11. Growth of uninfected cells. Vero cells (8 × 10^3) were cultured for 24 or 48 hr without (●) or with 10 µM (■), 150 µM (○) or 300 µM (□) d-Tp*CTCCTG*.

FIG. 12. Synthesis of oligodeoxyribonucleoside methylphosphonates on a polystyrene support. [Reprinted with permission from Biochemistry, 1986, *25* (18), 5092-5097. Copyright 1986, American Chemical Society].

nucleotide kinase by partial hydrolysis of the methylphosphonate linkages with 1 *M* aqueous piperidine followed by polyacrylamide gel electrophoresis of the hydrolysate (Murakami *et al.*, 1985). The positions of purine and pyrimidine bases were confirmed by treatment of 5′-end labeled oligomers with acid and hydrazine respectively. The characterization of Cp*ATTTTTGGTTTCCA*, a 15-mer, is shown in Figure 14. These results show that Matagens (up to 15 nucleotides in length) can be synthesized on

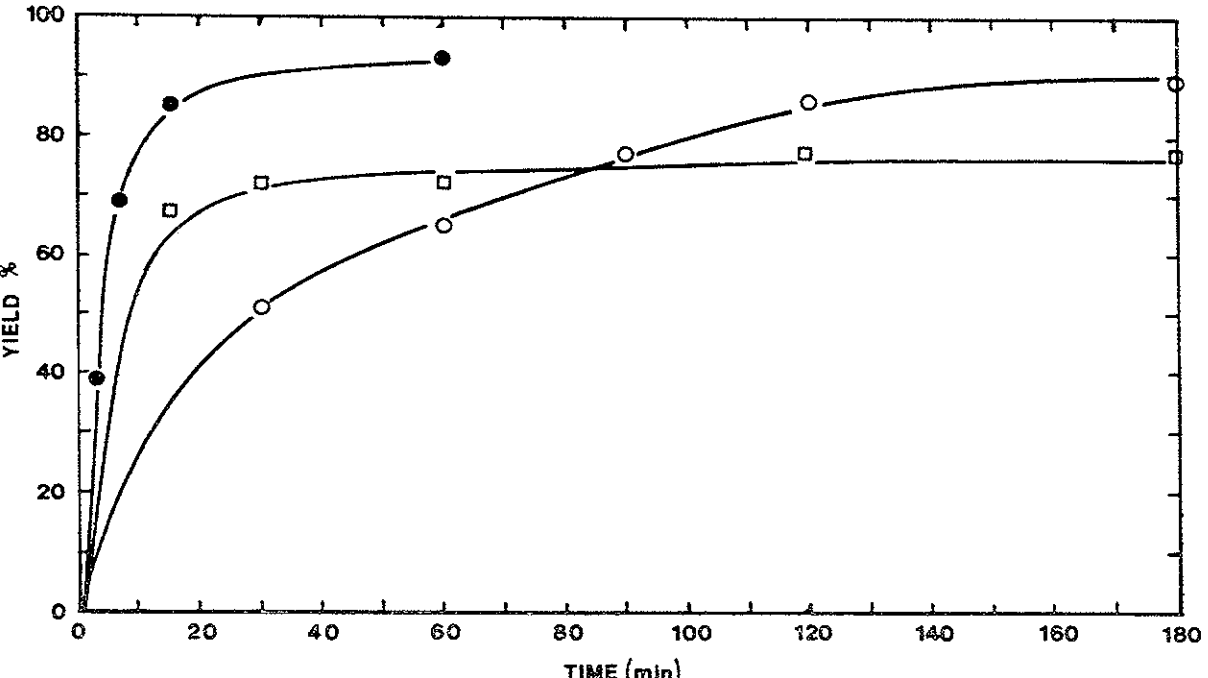

FIG. 13. Kinetics of the coupling reaction between d-[$(MeO)_2$Tr]bzA*p*Im and thymidine-derivatized polystyrene in the absence (○) or presence (●) of tetrazole and thymidine-derivatized controlled pore glass in the presence (□) of tetrazole at 22°C. [Reprinted with permission from Biochemistry, 1986, *25* (18), 5092-5097. Copyright 1986, American Chemical Society].

TABLE VIII - *Syntheses of Oligodeoxyribonucleoside Methylphosphonates on a Polystyrene Support* ([a]).

Oligomer	Average Yield/Phosphonate Condensation Step ([b])	Isolated Yield	HPLC ([c]) Retention Time (min)
d-Tp*CCTCCTG*	89%	15%	13.8
d-Gp*AATCCTG*	90%	21%	14.1
	92% ([d])	24%	14.1
d-Tp*GTTGGTC*	91%	25%	14.2
		18% ([e])	14.2
d-Ap*ACAGACAT*	88%	12%	15.8
d-Tp*AAATAAAAAAAATT*	91%	4% ([e])	16.5
d-Cp*ATTTTTGGTTTCCA*	91%	4%	15.6

([a]) The reactions were carried out on 100 mg of support and the last nucleotide was added using the phosphotriester method unless otherwise noted.

([b]) Determined by analysis of the dimethoxytrityl group after each coupling step.

([c]) ODS-3 reversed phase HPLC using a 50 ml gradient of 0.5% to 30% acetonitrile (8 and 9 mers) or a 0.5% to 35% acetonitrile (15 mers) in 0.1 *M* ammonium acetate (pH 5.8) at a flow rate of 2.5 ml/min.

([d]) Reaction run on 200 mg of polystyrene support.

([e]) 5′ Terminal nucleotide added by phosphoramidite method.

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a polymer support and can be purified, sequenced and characterized in a manner similar to that used for normal oligodeoxyribonucleotides.

FUTURE CHALLENGES

While our experiments with Matagen show a great deal of promise, there are still problems which require further investigation:

1) Thermodynamic considerations indicate that at low binding levels, the association process predominates and binding is efficient, while at high binding levels the dissociation process predominates and binding becomes inefficient. Thus, physical binding above the 50% level is much less efficient and the efficiency approaches 0 as the binding approaches 100%.

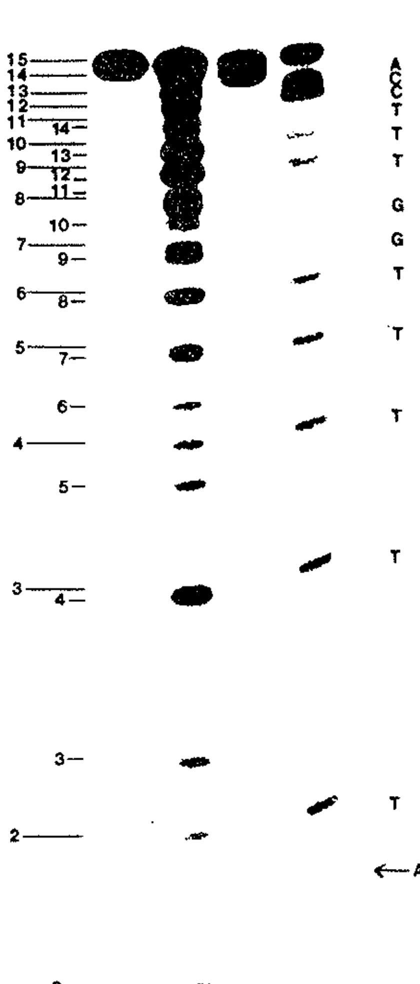

FIG. 14. Characterization of d-^{32}pCp*ATTTTTGGTTTCCA*. The autoradiogram shows: lane 1, 15 mer; lane 2, 15 mer treated with 1 *M* aqueous piperidine at 37°C; lane 3, 15 mer treated with 2 *M* hydrochloric acid at 37°C and lane 4, 15 mer treated with 90% hydrazine at 37°C. The numbers at the far left show the chain length of oligomers terminating with a 3′-hydroxyl group while the next set of numbers shows the chainlength of oligomers terminating with a 3′-methylphosphonate group. The position of purine and pyrimidine residues are shown at the right. The arrow indicates the position of the band which is observed in longer exposures of the gel. This band corresponds to the monomer formed by cleavage of the first A residue in the oligomer. [Reprinted with permission from Biochemistry, 1986, *25* (18), 5092-5097. Copyright 1986, American Chemical Society].

2) Efficient physical binding of Matagen requires high Matagen concentrations and the extent of binding depends on the binding constant. However at high concentrations the specificity of Matagen may be reduced because the Matagen may partially bind to other sequences.

3) The specificity of Matagen binding depends upon base pairing selectivity and the uniqueness of the sequence. It can be demonstrated that a sequence of 15 nucleotides would be unique in a mammalian genome. However, Matagen of that chainlength could participate in partial binding involving 5 to 10 contiguous bases. Under equilibrium conditions, complete binding by the oligomer is most stable and would lead to high specificity. Under transient conditions partial binding to other sites could occur which would lower the specificity.

4) The average size of the mammalian haploid genome is about 3×10^9 base pairs and the average gene expression in any given cell is only 1-3% of the total genome or 3×10^7-1×10^8 base pairs. It is estimated that only 10% of the mRNA sequences or 3×10^6-1×10^7 nucleotides have fully exposed regions of 8-12 nucleotides in length. The number of unique sequences is a combination of 4 bases, equivalent to 4^n, where n is the chain length of the sequence. Thus, 4^{10} equals 1.05×10^6, 4^{12} equals 1.68×10^7, 4^{14} equals 2.68×10^8 and 4^{16} equals 4.16×10^9. According to these considerations, a 12-mer could be a unique sequence in a genome size of approximately 1.5×10^7 base pairs. Thus, on a statistical basis, a 12-mer is sufficiently long to mask a specific mRNA species. In order to mask a specific gene in DNA, a 14-mer, which would give 3×10^8 sequence specificity, may be more appropriate. Since approximately 50% of the DNA is in the form of repetitive or mid-repetitive sequences, a chainlength of 14 may be sufficient to uniquely define the sequence specificity (1.5×10^9 b.p. in a single copy sequence). In any event, a chain length of 16 is sufficiently unique on a statistical basis for the entire mammalian genome, and may be used in solution hybridization experiments.

5) For the use of Matagen as a therapeutic agent instead of as a probe in cellular experiments, two additional problems require consideration.

A) The design of Matagen is based on selectivity at the gene level but it may still *not* be sufficiently selective against several million tumor cells versus billions of normal cells in the body. A targeted delivery system may be required. In addition, this procedure can perhaps be

equivalent to a surgical approach (chemosurgery), which is most effective in reducing the tumor load drastically, say by 95%. However, it may be difficult to eliminate all the tumor cells using such a procedure. Additional treatment to stimulate the host's immunodefense system could be necessary to eradicate the remaining tumor cells.

B) Matagen is expected to persist in the animal for long periods of time. The immunological response to Matagen and its possible side effects in animals are unknown.

Future Directions

In order to overcome some of the problems cited above, two new types of oligonucleoside methylphosphonate derivatives are currently being developed and tested. The schematic concepts of these two new types of Matagen are illustrated schematically in Figure 15.

DERIVATIZED OLIGONUCLEOSIDE METHYLPHOSPHONATES

I. *Psoralen-Modified Matagen* - Able to crosslink with targeted mRNA after irradiation with UV light

Matagen - Psoralen
mRNA → Matagen - Psoralen X
mRNA

II. EDTA(Fe^{2+})-modified Matagen - Able to catalytically cleave phosphodiester backbone of targeted mRNA

Matagen - EDTA(Fe^{2+}) + O_2
mRNA → Matagen - EDTA(Fe^{3+}) -O_{-2}
mRNA ↓ ↓

FIG. 15. Derivatized Matagens.

1) *Crosslinking-Matagen* (*Matagen-C*)

The structure of one type of Matagen-C is shown in Figure 16 and the structure of the cross-link between Matagen-C and a complementary target strand is shown in Figure 17. The formation of a crosslink between the target nucleic acid and the Matagen could not only irreversibly inactivate the target RNA but also provide unambiguous information about the exact binding site of the Matagen. Because the Matagen-C crosslinks only upon photoirradiation, it should be possible to specifically control gene expression at selected times during the cell cycle or virus replication cycle. However, because Matagen-C requires photoactivation in order to elicit inhibition, it may be less useful in experiments involving animals.

2) *Restriction-Matagen* (*Matagen-R*)

This type of Matagen is designed to degrade the sugar-phosphate backbone of targeted complementary nucleic acids. A promising candidate is EDTA-linked Matagen, whose structure is shown in Figure 18. The EDTA portion of Matagen, when complexed with FeII and in the presence of molecular oxygen and a reducing agent, is capable of generating hydroxyl radicals which can cleave the complementary strand of the target nucleic acid.

This class of Matagen does not require specific activation and can presumably act in a catalytic manner. Much work in this area has been done by Dr. Peter Dervan, one of the speakers in this workshop, as well as by Dr. Leslie Orgel's group at the Salk Institute. However, one serious problem with this family of compounds is autodegradation, a problem which appears to be less serious in oligomers without a phosphodiester backbone.

Concluding Remarks

The above discussion indicates Matagen, a family of sequence-specific oligonucleoside monophosphates with chainlengths of 8-15, can be used effectively to inhibit specific gene expression in living cells, when targeted against complementary sequences in mRNA or pre-mRNA. The required concentration of Matagen is moderate, ranging from 50-150 μM, and Matagens targeted against viral mRNAs have been very effective in

reducing virus yield. Sequence-specific Matagen can now be synthesized by machine on a 1 to 3 mg scale within about 5 days, which includes purification, characterization, and sequencing if necessary.

Second generation Matagens, i.e., Matagen-C which can crosslink with target nucleic acid, and Matagen-R which can degrade the complementary targeted nucleic acid, are currently being synthesized and investigated.

FIG. 16. Oligodeoxyribonucleoside methylphosphonate derivatized with aminomethyltrimethylpsoralen (AMT) - Matagen-C.

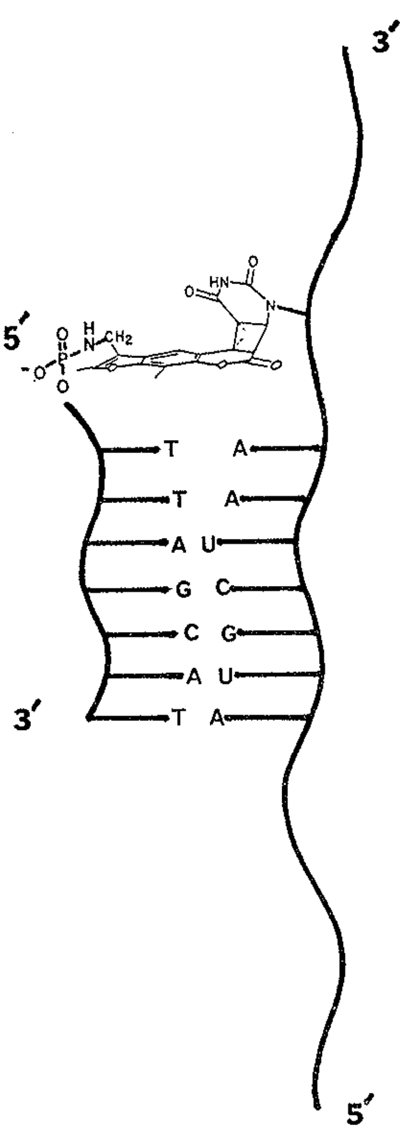

FIG. 17. Psoralen-derivatized oligonucleoside methylphosphonate (left strand) and mRNA (right strand). A cyclobutane bridge is formed between the pyrone ring of the psoralen and the A uracil residue of the mRNA.

FIG. 18. EDTA-Derivatized oligonucleoside methylphosphonate - Matagen-R.

In addition to perfecting the new generations of Matagen-C and Matagen-R, a new procedure for synthesizing substantial quantities for animal experimentation is needed, since these compounds can now clearly be useful in providing valuable information on gene expression in cellular experiments.

Looking to the next challenge, the strategy thus far has been based upon targeting toward single-stranded nucleic acid or nucleic acids that can readily form a complex with a complementary strand. Could Matagen attack a specific gene existing in the form of double-stranded DNA? This may be possible during replication or transcription of the DNA when the target gene exists in a transient single-stranded state. Can Matagen be used as a sequence-specific anti-gene agent? Indeed, a very challenging future lies ahead in answering the above questions. Nevertheless, we have breached the membrane barrier of living cells and are turning living cells into a reaction system in which we might interfere with single-stranded nucleic acid function in a sequence-specific manner. Similar situations might also exist for actively replicating or transcribing genes. Can the Matagen take advantage of the transitory open state of DNA to attack the function of the gene, or even destroy the gene specifically according to base-sequence information?

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OLIGODEOXYNUCLEOTIDES COVALENTLY LINKED TO INTERCALATING AGENTS AND TO NUCLEIC ACID-CLEAVING REAGENTS. NEW FAMILIES OF GENE REGULATORY SUBSTANCES.

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Abstract

Oligodeoxynucleotides were covalently linked to intercalating agents. These composite molecules recognize in a specific way the complementary sequence of the oligonucleotide. The intercalating agent provides an additional binding energy which stabilizes the complex. These substances can be used *in vitro* to block transcription initiation and mRNA translation. *In vivo* they are able to inhibit the cytopathic effect of viruses, including the oncogenic virus SV40.

A reactive group can also be attached to an oligodeoxynucleotide in order to achieve site-directed modifications of the target sequence. Metal complexes of EDTA, phenanthroline or porphyrins induce cleavage reactions of the phosphodiester backbone in both DNA and RNA. Photoactive groups can be used to modify bases in the complementary sequence. These site-directed modifications inhibit biological processes.

These results provide the basis for the rational design of gene-specific

inhibitors which can be used as tools in molecular and cellular biology and as anti-viral, anti-parasitic and anti-tumoral agents.

I. Introduction

Gene expression in all living organisms is controlled at different steps of information processing: transcription of one of the DNA strands; splicing of mRNA precursors; translation of mRNA. In most cases this regulation is achieved by proteins which bind to specific regions of DNA or RNA and either block or stimulate the enzymatic processes (see Hélène and Lancelot, 1982, for a review). Recently it has been shown that small RNAs could play a role similar to that of some regulatory proteins. Upon hybridization with a mRNA these regulatory RNAs may alter the translation process or induce premature termination of transcription (see Green *et al.*, 1986, for a review). Such phenomena have been originally observed in bacteria (Green *et al.*, 1986) but they might also occur in eukaryotes (Heywood, 1986). The discovery of regulatory RNAs has been the starting point for the design of "anti-sense" RNAs. By inserting a gene fragment close to a strong promoter in the reverse direction as compared to that of the gene itself the non-template strand of the gene fragment is now used as a template by RNA polymerase. As a consequence this "anti-sense" transcript is fully complementary to the mRNA. This might block mRNA translation or other post-transcriptional processes such as splicing or mRNA migration from the nucleus to the cytoplasm (Green *et al.*, 1986; Kim and Wold, 1985).

Long before the discovery of regulatory RNAs the idea of using synthetic oligonucleotides complementary to RNA sequences to alter gene expression was put forward in several laboratories (Paterson *et al.*, 1977; Stephenson and Zamecnick, 1978; Jayaraman *et al.*, 1981; Trudel *et al.*, 1981; see also Knorre and Vlassov, 1985; Hélène *et al.*, 1985). Since then it has been shown that oligodeoxynucleotides complementary to mRNAs could block translation either *in vitro* (Blake *et al.*, 1985; Stephenson and Zamecnik, 1978; Paterson *et al.*, 1977) or in microinjected *Xenopus* oocytes (Kawasaki, 1985; Cazenave *et al.*, 1986). The application of these oligodeoxynucleotides to *in vivo* studies faces two main problems: i) their penetration into living cells in culture is very limited; ii) their sensitivity to nucleases makes their lifetime very short. Several attempts have been made to overcome these two difficulties. The phospho-

diester backbone of the oligodeoxynucleotide can be changed to a phosphonate backbone. The loss of negative charges makes these oligophosphonates more efficient in penetrating through the cell membranes and much more resistant to nucleases (Miller *et al.*, 1983). Attachment of oligonucleotides to polymers such as poly-L-lysine increases the efficiency of penetration and makes oligonucleotides active *in vivo* at much lower concentrations (Bayard *et al.*, 1986).

Despite these progresses there is an obvious need of developing new families of gene regulatory substances which could be used *in vivo* to control the expression of undesirable genes, such as oncogenes. This review summarizes here the approach we are following to achieve this goal. The recognition of a nucleic acid base sequence can be easily achieved by using an oligonucleotide of complementary sequence. The stability of the mini-double helix can be increased by covalent attachment of an intercalating agent at one end of the oligonucleotide (Asseline *et al.*, 1983, 1984a,b). In addition the intercalating agent endows the oligonucleotide with a higher penetrability and stability against 3′ or 5′-exonucleases, depending on the attachment site. The other end of the oligonucleotide can be substituted by a reagent which may be induced to modify the target sequence by either chemical or photochemical activation (Boidot-Forget *et al.*, 1986). Specific cleavage of a mRNA target or chemical modification of the bases at the binding site of the oligonucleotide prevents translation of the mRNA. In addition the backbone of the oligonucleotide can be modified in such a way as to make it more resistant to nucleases.

II. Oligonucleotides Covalently Linked to an Intercalating Agent

The minimum size that an oligonucleotide should have in order to recognize a single specific sequence in a genome can be calculated assuming a random distribution of the bases. The frequency of occurrence of a sequence containing n nucleotides (a adenines, g guanines, t thymines and c cytosines with $n = a + g + t + c$) in a genome containing N base pairs is given by equation (1)

$$(f/2)^{a+t} \times ((1 - f)/2)^{g+c} \times 2\,\mathrm{N} \tag{1}$$

where f is the fraction of A.T base pairs in the genome. From equation (1) it can be calculated that a minimum length of 12 nucleotides is re-

quested to find the complementary sequence only once in the *E. coli* genome ($N \approx 4 \times 10^6$; $f = 0.5$). For the human genome ($N \approx 4 \times 10^9$; $f = 0.6$) this minimum length varies from 15 to 19 depending on the oligonucleotide base composition.

However there are additional considerations which lead to a reduction in the minimum length that the synthetic oligonucleotide should have. For example, let us assume that the oligonucleotide is targeted to a messenger RNA. In a given cell type, at a given time, only a small fraction of the genome is expressed as mRNA. If it is assumed that about 0.5% of the DNA is transcribed into mRNA then the complexity of the target sequence is strongly reduced. For a human cell this leads to a reduction of N (equation 1) from 4×10^9 to 2×10^7 and consequently the minimum length n should be between 11 and 15. This can be further reduced if additional constraints are taken into account. For example, the sequence CpG is under-represented in eukaryotic genomes due to its involvement as a signal in gene expression (as a consequence of cytosine methylation in CpG sequences). Also, if the target sequence is located in a regulatory region it should have been selected during evolution in such a way as to appear only once in the genome in order to achieve specific regulation.

On the basis of the above calculations short synthetic oligonucleotides can be designed to recognize selectively a target nucleic acid. This should facilitate both the synthesis (especially if other linkages than phosphodiesters are envisaged) and the penetration across cell membranes. However a short oligonucleotide might not have a high enough affinity towards its target sequence if the number of base pairs involved in complex formation is too small. There are different ways of increasing this affinity. We have chosen to covalently link an intercalating agent to one of the oligonucleotide ends. Intercalating agents are polycyclic aromatic molecules which insert their planar aromatic ring between two consecutive base pairs of double-stranded DNA. They bind much more weakly to single-stranded structures. If the linker between the oligonucleotide and the intercalating agent is appropriately chosen, intercalation can occur in the mini-double helix formed when the oligonucleotide is bound to its complementary sequence. The free energy of binding of the composite molecule (ONBI for *O*ligo*N*ucleotide-*B*ridge-*I*ntercalator) should be the sum of the free energy for binding the oligonucleotide to its complementary sequence (ΔG_{ON}) and that of intercalation (ΔG_I), corrected for an entropy term taking into account the restricted configurational space available to the

intercalating agent when it is covalently linked to the oligonucleotide (entropy of mixing)

$$\Delta G_{ONBI} = \Delta G_{ON} + \Delta G_{I} + T\Delta S_{m} \quad (2)$$

Since ΔS_m in equation (2) is negative, the association constant for the ONBI($K_{ONBI} = \exp - \frac{\Delta G_{ONBI}}{RT}$) should be at least the product of the association constants for the oligonucleotide and the intercalating agent.

$$K_{ONBI} = \alpha K_{ON} \times K_{I} \quad (3)$$

with $\alpha = \exp\left(\frac{-T\Delta S_m}{RT}\right) > 1$

However the presence of the linker might not allow the intercalating agent to form the same intercalation complex as it would if it was free in solution. Also the local double helical structure might be distorted in such a way as to increase ΔG_{ON} and ΔG_{I}. Therefore equation (3) would lead to an overestimation of the association constant for the ONBI. The results presented below show that equation (3) represents nevertheless a good approximation.

III. Binding of ONBI to Complementary Sequences

The intercalating agent we have used in most of our studies is an acridine derivative: 2-methoxy, 6-chloro, 9-amino acridine. It exhibits no specificity of binding with respect to A.T. and G.C. base pairs. Therefore it can be covalently linked to oligonucleotides of different sequences without perturbing the specificity of binding to the complementary sequence. A polymethylene linker was used to tether the acridine ring (via its 9-amino group) to the 3'- or 5'-phosphate group of the oligonucleotide.

Binding of the ONBI to its complementary sequence was followed by several spectroscopic techniques, including absorption, fluorescence, circular dichroism and nuclear magnetic resonance. The results can be summarized as follows:

i) an hypochromism is induced in the visible absorption band of

the acridine derivative upon binding. A shift of the maximum to longer wavelengths is observed which depends on the base sequence of the oligonucleotide (Asseline *et al.*, 1983, 1984a,b; Hélène *et al.*, 1986).

ii) the fluorescence quantum yield of the acridine derivative is increased when the terminal base pairs are all A.Ts.Guanine and G.C. base pairs strongly quench the acridine fluorescence. Fluorescence excitation spectra reveal several environments (at least two) for the acridine ring in the complexes (Asseline *et al.*, 1984a,b; Hélène *et al.*, 1986). The fluorescence of the complexes is highly polarized due to (partial) immobilization of the acridine ring. Fluorescence anisotropy decays reveal that the acridine ring retains a local mobility which is quite similar to that of acridine intercalated in DNA (Hélène *et al.*, 1986).

iii) an induced circular dichroism is observed as a result of the asymmetric environment of the acridine ring in the complexes (Hélène *et al.*, 1986).

iv) proton and phosphorus magnetic resonance studies indicate that the acridine ring is intercalated between the terminal base pairs of the mini-double helix (Lancelot *et al.*, 1985, 1986; Lancelot and Thuong, 1986).

The results of spectroscopic studies led to the conclusion that different complexes could form which are characterized by different locations of the acridine ring, as summarized in figure 1. The equilibrium between these different structures depends on the base sequence of the oligonucleotide, on the site of attachment (3′ or 5′ end) of the acridine ring, on the length of the linker and on the nature of the base which follows the mini-double helix on the target sequence. For example, the stacking interaction between adenine and acridine is quite strong. If an adenine is located on the target nucleic acid next to the last base pair (on the acridine side) the equilibrium will be shifted towards the structure where the acridine stacks on top of the last base pair and interacts with the adenine.

Upon increasing the temperature, complexes dissociate according to a cooperative process. Base pairing and intercalation are disrupted in a single step. From the concentration dependence of the temperature of half-dissociation (T_m) it is possible to derive thermodynamic parameters according to equation (4)

$$\frac{1}{T_m} = \frac{\Delta S}{\Delta H} + \frac{2.3\,R}{\Delta H} \log C_m \quad (4)$$

where C_m is the free ONBI concentration at temperature T_m. Table 1 gives ΔG values obtained for the binding of $(T_p)_8Et$ and $(Tp)_8m_5Acr$ to poly(rA). In $(Tp)_8Et$ an ethyl group has been attached to the 3′-phosphate of an octathymidylate to mimic the pentamethylene linker (m_5) used to tether the acridine ring (Acr) in $(Tp)_8m_5Acr$. The results show that the acridine strongly stabilizes the complex by providing an additional binding energy due to its intercalation between A.T. base pairs.

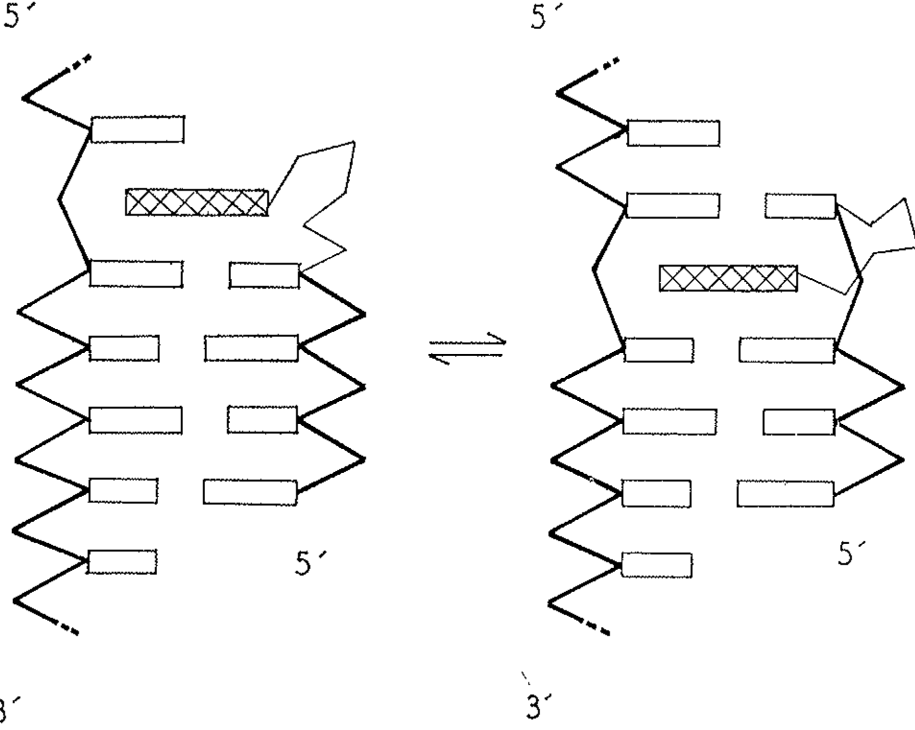

FIG. 1. Models for different types of complexes formed by an oligodeoxynucleotide covalently linked to an intercalating agent with its complementary sequence. The hatched rectangle represents the intercalating agent.

TABLE 1 - *Thermodynamic parameters for the binding of two octathymidylates to* poly(rA) *at* 273 K *in a* pH 7 *buffer containing* 10 mM Na *cacodylate and* 0.1 M NaCl. T_m *refers to the temperature of half dissociation of the complex at* 10^{-5} M *total oligonucleotide concentration.*

	$(Tp)_8$	$(Tp)_8\, m_6\, Acr$
ΔG (kcal. mole^{-1})	-8.8	-14.3
K (M^{-1})	10^7	2.4×10^{11}
T_m (°C)	10.3	38.2

IV. INHIBITION OF TRANSCRIPTION AND TRANSLATION BY OLIGONUCLEOTIDES COVALENTLY LINKED TO INTERCALATING AGENTS

The sequence complementary to the oligonucleotide should be accessible in the target nucleic acid in order for the ONBI to form a complex. This is not the case in double-stranded DNA except if base pairs are locally disrupted either upon binding of proteins or enzymes, e.g., RNA polymerase, or upon introduction of a torsional stress as in supercoiled DNA. If the ONBI target is a messenger RNA, complex formation will be made easier if the target sequence is not engaged in secondary or tertiary structures.

A. *Inhibition of transcription initiation*

When RNA polymerase binds to a promoter it first forms a "closed" complex which then isomerizes to an "open" complex characterized by 10-12 open base pairs. Oligonucleotides covalently linked to an acridine derivative were directed against the transcribed strand in the open complex formed by *E. coli* RNA polymerase with the promoter of the β-lactamase gene. These ONBI of short length (hexa to nonanucleotides) were shown to inhibit transcription initiation provided the target sequence did not extend into the double-stranded region. For example, the oligonucleotide covering region -4 to $+2$ was more efficient than that complementary to the region -3 to $+3$ ($+1$ is the transcription start site; see figure 2).

a

β-LACTAMASE GENE

β-LACTAMASE mRNA

+1 Met Ser

T G A G A C A A T A A C C C T G A T A A A T G C T T C A A T A A T A T T G A A A A A G G A A G A G T A T G A G T
A C T C T G T T A T T G G G A C T A T T T A C G A A G T T A T T A T A A C T T T T T C C T T C T C A G A C T C A

OPEN REGION IN PROMOTER-RNA POLYMERASE COMPLEX

-1 +1

5'——A G A C A A T A A C C C T G A T A A A——3'
3'——T C T G T T A T T G G G A C T A T T T——5'

5'C C C T G A~~Acr
5'C C T G A T~~Acr
5' T A A C C C T G A~~Acr

OLIGONUCLEOTIDES COVALENTLY LINKED TO ACRIDINE AND COMPLEMENTARY TO THE TRANSCRIBED STRAND

b

GENE 32 FROM BACTERIOPHAGE T4

REPEATED SEQUENCE — SHINE-DALGARNO SEQUENCE — Met Phe Lys Arg

5' ...G U U A A U U A A A U U A A A U U A A A A A G G A A A U A A A A A U G U U U A A A C G T

Acr~~A A T T T 5'
Acr~~A A T T T A A T T T 5'
Acr~~A A T T T A A T T T A A T T T 5'

OLIGONUCLEOTIDES COVALENTLY LINKED TO ACRIDINE AND COMPLEMENTARY TO THE REPEATED SEQUENCE OF GENE 32 mRNA

FIG. 2. Target sequences and oligodeoxynucleotides covalently linked to intercalating agents used in the *in vitro* studies reviewed in the text

a β-lactamase promoter region (Hélène *et al.*, 1985; Saison-Behmoaras *et al.*, unpublished results).

b. gene 32 mRNA from bacteriophage T4 (Toulmé *et al.*, 1986).

c

β-GLOBIN mRNA

```
             1             10            8     50                  60
5'G^Mp p p A C A C U U G C U U U . . . A A A C A G A C A G A A U G G U G C A U . . .

                                   Acr~~T T G T C T G T C T T 5'
```

d

INFLUENZA VIRUS

GENOME : 8 DIFFERENT RNAs

CONSERVED SEQUENCE AT THE 3'-END OF THE 8 RNAs

```
3'U C G C/U U U U C G U C C A U...     TYPE A (A/PR/8/34)
          | | | | | | |
       5'A A A G C A G→Acr             SYNTHETIC OLIGONUCLEOTIDE
            | | | |
3'U C G U C U U C G C U/G U C...       TYPE B (B/HK/8/73)
          •       •  •  • •
```

e

TRYPANOSOMES

CONSERVED SEQUENCE AT THE 5' END OF ALL mRNAS
(MINI-EXON)

```
T. VIVAX    5'A A (A) G C T (T) T T A T T A G A A C A G T T T C T G T A C T A T A T T G...
                          10                 20                   30
T. BRUCEI   5'A A C G C T A T T A T T A G A A C A G T T T C T G T A C T A T A T T G...

                  3'T G C G A T A A T A A T 5'
OLIGONUCLEOTIDES  Acr←T G C C A T A A T 5'
                             3'T A A T A A T C T T G T 5'
```

Fig. 2

c. β-globin mRNA (Cazenave *et al.*, 1986).

d. influenza virus mRNA, common sequence at the 3'-end (unpublished results).

e. trypanosome mRNA, 5'-end mini-exon (Cornelissen *et al.*, 1986, and unpublished results).

Control experiments showed that an ONBI inhibiting β-*lactamase* transcription did not have any effect on the *lac* gene promoter.

During the course of studies using a transcription-translation *in vitro* assay (see below) it was discovered that some ONBIs could have a non-specific effect on transcription, most probably as a result of binding to RNA polymerase. These ONBIs were characterized by a high A+T content. The binding site of RNA polymerase on promoters involves an (A+T)-rich sequence (the so-called "*Pribnow*" or "*TATA box*"). The attachment of an intercalating dye to an (A+T)-rich oligonucleotide might enhance binding to RNA polymerase as compared to the unsubstituted oligonucleotide, thereby inhibiting transcription via competition with promoter binding.

B. *Inhibition of messenger RNA translation*

The possibility of blocking gene expression at the level of translation was tested in several systems (Figure 2).

i) gene 32 from bacteriophage T4 was chosen as a prokaryotic example (Toulmé *et al.*, 1986). This gene codes for a protein which binds selectively to single-stranded nucleic acids. The synthesis of this protein was investigated in an *E. coli* acellular system where a gene 32 mutant carried by a plasmid was first transcribed and then translated. An ONBI directed against a repeated sequence upstream of the Shine-Dalgarno ribosome binding site inhibited gene 32 protein synthesis. It was shown that the ONBIs used in this study [d(TTTAA)$_n$ m$_5$Acr with n = 2 and 3] were able to bind to RNA polymerase and blocked transcription in a non-specific way. If transcription was carried out in the absence of the ONBIs and the ONBIs added when translation started, then a specific effect could be observed on gene 32 mRNA translation.

ii) translation of rabbit β-globin mRNA was investigated both in an *in vitro* acellular system (wheat germ extract) and in microinjected *Xenopus* oocytes (Cazenave *et al.*, 1986). An ONBI (11 nucleotides in length) complementary to the β-globin mRNA upstream from the initiation codon was synthesized. Using a mixture of α- and β-globin mRNAs it was demonstrated that this ONBI specifically inhibited β-globin synthesis in the wheat germ extract without any effect on α-globin synthesis (the ONBI target sequence was not present on α-globin mRNA). In micro-injected oocytes the synthesis of β-globin was also inhibited in a con-

centration-dependent process. The ONBI concentration inhibiting 50% of β-globin synthesis was 0.5 μM (internal concentration) as compared to 0.2 μM in the *in vitro* wheat germ extract.

An undecanucleotide with the same base sequence as the ONBI was also tested in both assays. It was unable to block β-globin synthesis in microinjected oocytes even at a 10 times higher concentration than the ONBI concentration required to block 100% protein synthesis. In the wheat germ extract the undecanucleotide had an inhibitory effect on β-globin synthesis. It should be noted that the wheat germ extract contains an RNase H activity which hydrolyzes the RNA strand in RNA-DNA hybrids (Minshull and Hunt, 1986). This might explain the high efficiency of oligodeoxynucleotides in this system because mRNA hydrolysis will obviously block translation. In both the wheat germ extract and microinjected oocytes the effect of the ONBI was sequence specific. An ONBI with no complementary sequence in β-globin mRNA had no inhibitory effect.

V. Oligodeoxynucleotides Covalently Linked to Intercalating Agents as Anti-Viral, Anti-Parasitic and Anti-Tumoral Agents

In order to test the efficiency of ONBIs as potential anti-viral, anti-parasitic and anti-tumoral agents, several systems were investigated.

A. *Inhibition of influenza virus*

The influenza virus genome is segmented and contains eight different RNAs coding for different proteins involved in viral life. The 3′-end sequences of these eight RNAs are identical over 12 bases except for the fourth one in type A viruses (figure 2). An ONBI seven nucleotides in length was able to block the cytopathic effect of the virus using MDCK cells in culture. A type B virus was used as a control. The sequences at the 3′-ends of the eight RNAs are identical over 12 bases except for a degeneracy at the eleventh position. The type A and type B sequences are however different at four positions. The heptanucleotide-containing ONBI which is fully complementary to the type A viral RNAs has three mismatches with the type B viral RNAs. Therefore the complex should be highly destabilized. As a matter of fact this ONBI had no effect on the

cytopathic effect of a type B virus. This experiment demonstrates that the ONBI is acting at the level of the viral RNAs, not on a cellular component essential for viral development.

B. *Inhibition of DNA replication of an oncogenic virus, SV40*

The simian virus SV40 is a DNA-containing virus which replicates in CV1 cells in culture. We have investigated the effect of several ONBIs of sequence $(Tp)_8$, $(Tp)_7Ap$ and $(Tp)_6ApTp$. They are all complementary to an (A+T)-rich region located in the promoter of the T-antigen and in the origin of replication which are overlapping in this region of the viral genome. When CV1 cells infected with SV40 are treated by one of the three ONBIs the cytopathic effect of the virus is strongly inhibited. An oligodeoxynucleotide of sequence $(Tp)_8$ lacking the covalently linked acridine does not have any inhibitory effect nor does a shorter ONBI containing only four thymines. This last experiment demonstrates that the observed effect is not due to the acridine derivative which could have been released upon nuclease digestion of the ONBI. Other ONBIs which did not possess complementary sequences in the SV40 genome were also shown to be devoid of any effect on the SV40 cytopathic effect.

Using an immunofluorescence assay the ONBI $(Tp)_8m_5Acr$ was shown to induce a decrease in the synthesis of T-antigen by about 25%. Concomitantly more than 90% of SV40 DNA synthesis was inhibited. The decrease of T-antigen concentration does not seem sufficient to explain this large effect on replication. The ONBI could block DNA synthesis by hybridizing with the open region at the replication origin. Experiments on SV40 replication using an *in vitro* system should demonstrate whether such an hybridization blocks the initiation of replication.

C. *Inhibition of trypanosome mRNA translation*

Trypanosomes are characterized by an unusual property of their RNA transcripts. All mRNAs possess a common 35-nucleotide sequence at their 5′-end. This sequence originates from a mini-exon (encoded in tandem repeats) which is attached at the end of pre-mRNA either via a transsplicing event or by acting as a primer during gene transcription. Oligodeoxynucleotides complementary to the 35 nucleotides-long sequence (or part of it) inhibit the translation of all mRNAs in an *in vitro* assay. (Cornelissen

et al., 1986; Walder *et al.*, 1986). ONBIs as short as 9 nucleotides act similarly and block translation of all trypanosome mRNAs. They have no effect on RNAs which do not contain the complementary sequence, e.g., Brome Mosaic Virus RNA. An ONBI without target sequence in the 35-nucleotide mini-exon does not inhibit trypanosome mRNA translation.

There are several trypanosome species with different infectivity patterns. Each species has a 35-nucleotide mini-exon sequence in all of its mRNAs but there are several point mutations which differentiate one species from another. An ONBI complementary to the region [2-10] of the mini-exon of *Trypanosoma brucei* has two mismatches when *Trypanosoma vivax* is used instead. These mismatches which occur within a nonanucleotide sequence destabilize the complex. As a matter of fact this ONBI inhibits translation of *Trypanosoma brucei* mRNA but has no effect on *Trypanosoma vivax* mRNA translation in an *in vitro* assay.

D. *Inhibition of oncogene expression*

Cell transformation is associated either with a deregulated expression of cellular genes or expression of mutated genes. The *myc* gene falls into the first category. In the premyelocytic cell line HL60 the myc gene is amplified about 30 times. In *Burkitt lymphoma* it is translocated in the vicinity of highly expressed immunoglobulin genes. The *ras* genes fall into the second category. Mutations usually in the 12th or 61st codons, alter the functions of the ras gene products which leads to cell transformation.

We synthesized ONBIs directed against the *myc* and *ras* genes. *In vitro* experiments showed that translation of *myc* mRNA in a wheat germ extract could be inhibited by short ONBIs directed against a region close to the AUG initiation codon (A. Darveau, unpublished results). No effect was observed on TMV RNA translation. Experiments are presently under way to test the effect of these ONBIs on transformed cells in culture. One might expect that a reduction in oncogene expression level would reverse the transformed to a normal phenotype.

VI. Oligonucleotides Covalently Linked to Intercalating Agents and/or Nucleic Acid Cleaving Reagents

In all the examples described above the oligodeoxynucleotide covalently linked to the intercalating agent binds to a complementary sequence on, e.g., a mRNA, in a specific way. The intercalating agent provides an

additional binding energy which stabilizes the complex but the system retains reversibility. Due to the increased residence time of the ONBI on its target sequence the biological process, e.g., translation, is inhibited. However when the ONBI dissociates, the biological system, e.g., the ribosome, can resume its activity and synthesize the final product. In order to make the system irreversible the target sequence on the mRNA should be chemically modified in such a way that it is no longer recognized by the biological system. This can be achieved by attaching a reactive group at the end of the ONBI or by using the intercalating agent itself as a reactive group.

A. *Site-directed cleavage of nucleic acids*

Several chelating agents, such as EDTA, phenanthroline and porphyrin derivatives, can be attached at the 3′- or 5′-end of an oligodeoxynucleotide. An intercalating agent can be covalently linked at the remaining free end to stabilize the complex formed with the complementary sequence. The metal complexes EDTA-Fe, (Phenanthroline)$_2$-Cu and Porphyrin-Fe (Mn, Co) can generate radical species, especially OH$^{\cdot}$ radicals, in the presence of molecular oxygen and a reducing agent (a thiol derivative, ascorbic acid...). The radicals attack the ribose or deoxyribose and induce cleavage reactions in the phosphodiester backbone (Chu and Orgel, 1985; Dreyer and Dervan, 1985; Le Doan *et al.*, 1986; Boidot-Forget *et al.*, 1986; Chen and Sigman, 1986). These reactions work not only on DNA but also on RNA and can be used to cleave mRNA thereby preventing translation in an irreversible way.

B. *Site-directed chemical modifications of nucleic acids*

Site-directed chemical modification of nucleic acids can be achieved in different ways. An oligonucleotide can be used to bring a reactive group close to the region which is to be modified. Cross-linking of the oligonucleotide to the target sequence has been previously described (Knorre and Vlassov, 1985). We have recently shown that photochemical modifications could also be induced in the target sequence. When acridine derivatives such as 2-methoxy, 6-chloro, 9-amino acridine or proflavine are stacked with a guanine base or a G.C. base pair their fluorescence is quenched, most probably as a result of electron-transfer reactions in the excited state. Upon visible-light excitation, photooxidation reactions are

induced which lead to base modifications in the target sequence near the end of the complementary oligonucleotide where the photosensitizer is attached.

VII. Conclusions

The synthesis of new families of molecules involving oligodeoxynucleotides has been described. An intercalating agent is attached at one (or both) end(s) of the oligonucleotide. It provides an additional binding energy which stabilizes the specific complex formed by the oligonucleotide with its complementary sequence. A nucleic acid-cleaving reagent can be attached at the other end of the oligonucleotide and used to cleave the phosphodiester backbone of the target sequence. A photoactive group can also be attached to the oligonucleotide to induce site-directed irreversible photochemical modifications in the target sequence. The intercalating agent can itself be used as a photoactive group.

These molecules have been shown to inhibit DNA transcription or mRNA translation *in vitro*. They are also active in microinjected *Xenopus* oocytes and block the cytopathic effect of viruses in cells in culture. These results provide the basis for the rational design of gene-specific inhibitors which could be used not only as tools for cellular biology but also as antiviral, anti-parasitic, or anti-tumoral agents. The development of cellular applications requires additional steps to be solved. The penetration of these molecules across the plasma membrane of living cells should be increased. Their resistance to nuclease action should be improved. This raises challenging questions to the chemist and the biochemist. Their resolution could provide new tools for molecular and cellular biologists who cannot use the powerful techniques of bacterial genetics when studying the relationships between gene expression and phenotypic behavior in most eukaryotic systems. The development of therapeutical applications still requires more general problems to be solved such as, e.g., disponibility in biological fluids or cell targeting.

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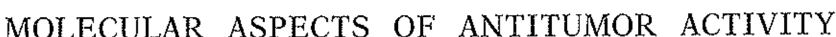

MOLECULAR ASPECTS OF ANTITUMOR ACTIVITY

CHEMICAL DERIVATIVES OF ANTICANCER ANTIBITOTICS WITH DIFFERENT DNA BINDING PROPERTIES

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ABSTRACT

Studies with doxorubicin and distamycin analogs are reported. Although the antitumor anthracyclines show different types of interaction with cell constituents, nuclear DNA is the main target as deduced from biochemical studies and structure-activity relationships. Structural requirements of the DNA intercalation complex seem to be important for the main effects on cell proliferation, whereas redox reactions should not be of relevance in this context. In the distamycin series the addition of a non pyrrole ring at the amino terminal part of the polypeptide system is compatible with DNA binding and antiviral activity. High cytotoxicity is associated with a novel derivative exhibiting both sequence specificity and alkylating properties.

1. INTRODUCTION

Different antibiotics have been shown to have antitumor properties. In our laboratory two main classes of biosynthetic compounds were studied, the anthracyclines and the distamycins. The anthracyclines are a numerous group of aminoglycosidic pigments possessing an anthraquinone chromophore in a tetracyclic aglycone moiety, the best known of which is doxorubicin (Ia) (Arcamone, 1981). Doxorubicin was originally isolated,

together with the related daunorubicin (Ib) from the cultures of *Streptomyces peucetius*, and shown to exhibit superior antitumor properties when compared to the latter in the preclinical studies. The successful clinical development of doxorubicin and its usefulness in the medical treatment of a variety of human cancers has prompted us to proceed through a wide analog program resulting in the second generation antitumor anthracyclines epirubicin (II) and idarubicin (III). A number of new analogs are currently studied in our as well as in other laboratories (Arcamone, 1984). Distamycin (distamycin A, stallimycin, V) was isolated from *Streptomyces distallicus* and also obtained by total synthesis (Arcamone *et al.*, 1964, 1967; Penco *et al.*, 1967). The compound displayed antiviral and antitumor properties (Di Marco *et al.*, 1962), and exhibited a unique oligopeptide structure. Analog development studies and some biochemical properties

Ia : R= OH
Ib : R= H

II

III

IV

V

of the drug were reported from our laboratory (Arcamone *et al.*, 1969a and 1969b; Arcamone, 1973; Arcamone *et al.*, 1975; Verini *et al.*, 1976). Selective binding to AT rich regions of double-helical DNA by distamycin was originally established by Zimmer *et al.* (1971). A recent X-ray diffraction study of Kopka *et al.* (1985) has clarified the structure of the complex between the related netropsin and a B-DNA synthetic dodecamer. The binding of the drug in the minor groove of the B-DNA has been therefore definitely proved.

In this presentation we shall summarize and discuss the DNA binding and other chemical properties of the new derivatives of the anthracyclines and of the distamycins synthetized in our analog development studies, also in relation to the biological activity exhibited by the same.

2. Anthracycline Glycosides

The antitumor anthracyclines show a number of biochemical interactions both of the reversible and of the irreversible type. Some of these are listed in Table 1. Although all interactions shown have been proved in different experimental situations, and likely many of them contribute to the therapeutic and to the toxic effects of the drugs, evidence is available that cell DNA is the main receptor of the antitumor anthracyclines. This is deduced from a variety of cell culture and animal studies (for reviews see Arcamone, 1981 and 1984). Recently, convincing conclusions have been reached according to which the enzyme topoisomerases II of malignant

Table 1 - *Molecular interactions of antitumor anthracyclines.*

REVERSIBLE INTERACTIONS:

- Intercalation into double-helical DNA
- Complex formation with other acidic biopolymers and phospholipids
- Protein binding
- Association with small molecules and metal ions

IRREVERSIBLE INTERACTIONS:

- Redox and radical reactions
- Enzyme catalyzed reactions: reduction at C-13, reductive deglycosidation, glucuronidation at C-4′ (only epirubicin)

cells is intrinsically hypersensitive to poisoning by intercalating drugs. Namely, interference of the drug with the breakage-reunion reaction of the mammalian enzyme causes the formation of a stable DNA-topoisomerase II covalent adduct that ultimately results in a protein-associated DNA cleavage (Tewey *et al.*, 1984; Minford *et al.*, 1986).

In connection with such findings, the molecular features of the binding of doxorubicin and its analogs to DNA have major importance in the interpretation of the pharmacological data and in the process of new drug development. The binding parameters of doxorubicin to calf-thymus native DNA are shown in Table 2. The apparent binding constant is clearly dependent on ionic strength, but different methodologies afford consistent results. As for the structure of the complex, the only available study describing an anthracycline-DNA complex at atom resolution is that of Quigley *et al.* (1980) according to which daunorubicin intercalates between the two C·G base pairs in the self-complementary hexadeoxynucleotide CGTACG. In our laboratory we have determined the binding constant of daunorubicin with $d(CGTACG)_2$ and found it to be only one order of magnitude lower than the binding constants currently reported for the native B-DNAs.

In analyzing the relationship between the DNA complex stability parameter of 26 anthracyclines and the values of the optimal non-toxic dose of the same in P388 leukemia-bearing mice, an acceptable correlation

TABLE 2 - *Values of apparent association constant* (K) *and number of binding sites per nucleotide* (n) *for the doxorubicin-calf thymus DNA complex as determined by different experimental techniques.*[a]

METHOD	K_1 ($10^7 l.mol^{-1}$)	n_1	K_2 ($10^5 l.mol^{-1}$)	n_2
Equilibrium dialysis[b]	1. 0	0.08	2.0	0.11
Fluorescence quenching[c]	1. 1	0.09	2.6	0.13
Fluorescence quenching[d]	4. 9	0.12	1.3	n.d.[e]
Spectrometric titration[c]	0.94	0.10	1.6	0.12

[a] data evaluation according to the model of two independent binding sites
[b] Tris buffer + NaCl, $\mu = 0.19M$
[c] same as (b), $\mu = 0.20M$
[d] same as (b), $\mu = 0.01M$
[e] not determined

was found (Valentini *et al.*, 1985). Similar conclusions were reached in a study concerning a number of analogs modified in the aminosugar moiety (Bargiotti *et al.*, 1983). Also, the compounds that have reached the clinical stage were found to belong to the group with the highest affinity for DNA, suggesting that, although cytotoxicity might be the result of different pharmacological interactions, that exhibited with the DNA receptor seems to be the most favorable for useful activity. Noticeable deviations from a clear correlation are present which can be related, at least in part, to other biological factors like bioavailability, pharmacokinetics and metabolism. However, as it has been pointed out in the case of doxorubicin isomer 4-demethyl-6-0-methyldoxorubicin IV (Zunino *et al.*, 1986), in which results could be rationalized in terms of a steric influence of the bulky methoxy group, also structural features of the DNA complex may be of relevance. In fact, although computations gave a similar intercalation structure for different anthracyclines possessing the daunomycinone-adriamycinone chromophore (Fig. 1), similar data are not yet available for other structural types.

The chromophoric system of the anthracyclines might conceivably be involved in the pharmacological behavior of these drugs also in other ways. Indeed various biochemical mechanisms have been centered on the ability of these molecules to enter into redox chemistry by virtue of their quinone moiety. In the presence of a suitable redox enzyme the quinone may undergo either a one or two-electron reduction. Should this reduction occur under aerobic circumstances, the electrons are rapidly passed from the reduced quinone to molecular oxygen generating $O_2^{\cdot}$, H_2O_2 and the toxic $OH^{\cdot}$ radical (Bachur *et al.*, 1982). The anaerobic reduction yields an unstable hydroquinone which is prone to a rapid intramolecular expulsion of the sugar moiety producing a quinone methide. The quinone methide, in the case of daunorubicin, most frequently undergoes a thermodynamically favourable tantomerization with acquisition of a solvent proton at C-7 to provide 7-deoxydaunomycinone. These 7-deoxyaglycones are mammalian metabolites of daunorubicin and doxorubicin indicating that *in vivo* reductive metabolism under hypoxic conditions is commonplace.

The reductive bioactivation of the anthracyclines has been suggested also in relation to the antitumor efficacy, as the quinone methide is a potentially reactive species which may provide specific alkylation *in vivo* (Moore, 1977). Although there are no direct experimental data in support of this hypothesis, the chemical competence of this quinone methide as

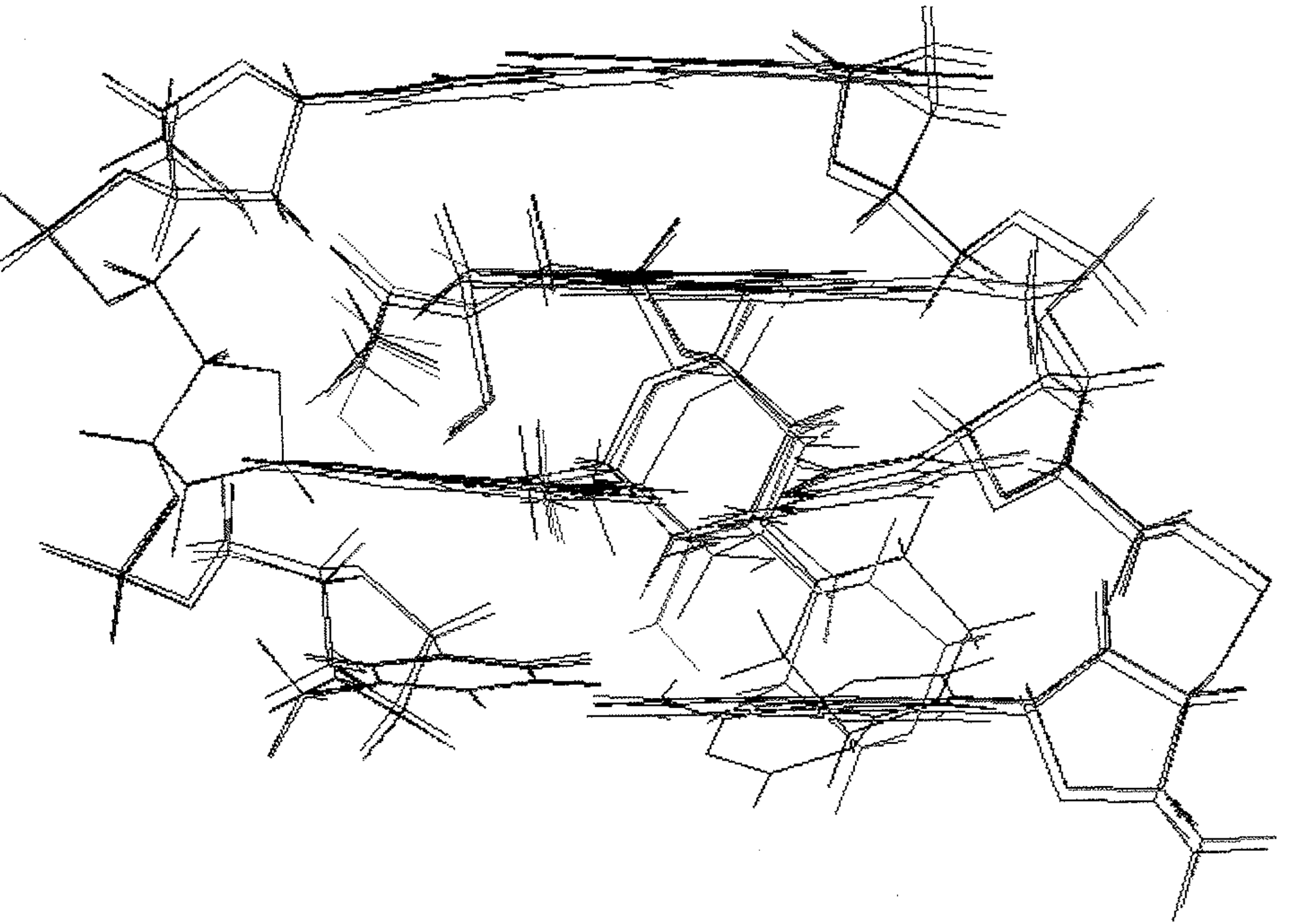

FIG. 1. The DNA intercalation complex as deduced from theoretical computation based on X-ray data (Ughetto, 1986) of daunorubicin

both a nucleophile and an electrophile has recently been proved by Koch (Kleyer and Koch, 1984) and Fisher (Ramakrishan and Fisher, 1986).

As part of our continuing research program aimed at the enlargement of the antitumor spectrum for unresponsive tumors, we have taken into consideration the synthesis of daunorubicin-doxorubicin analogs differing in the substitution pattern of the anthraquinone moiety. In fact a wide range of oxygenated substitution patterns in the chromophore of natural anthracyclines appears to be compatible with the biological activity although the existence of a direct contribution of the said substitution pattern on the antitumor activity has not yet been established. It is of interest to investigate the relationship between phenolic substitution on the aromatic system and the antitumor activity and also to widen our knowledge of how such pattern influences the affinity towards DNA for different series of daunorubicin and doxorubicin analogs. Moreover, the question of interest is whether, in contrast to the poor base pair specificity displayed by daunorubicin and doxorubicin, specificities for (definite) sequences are possible within the newly designed analogs. In fact a different specificity might give rise to a novel subclass of pharmacologically interesting anthracyclines. Further interest is based on the presumption that the redox properties of the chromophore as well as the reactivity of the reduced species is influenced by changing the substitution pattern of the anthraquinone moiety.

The existence of the preferred α-half-chair conformation of ring A (9-OH ax. and 7-OH pseudo ax.) has been provided by NMR as well as by x-ray diffraction experiments. By quantum mechanical calculations, daunorubicin turns out to be more stable than daunorubicin with the β conformation of A ring (9-OH eq. and 7-OH pseudo eq.) by 2.4 Kcal/mol. At least two interactions have a different value in daunorubicin with the α and the β-A ring puckering and tend to favor the α conformation: the interaction 06-07 (the interatomic distance is 3.18A in the α vs 2.43A in the β) and on hydrogen bond 09H-07. Now the result obtained by a quantum mechanical approach refers to the isolated molecule, and may be extrapolated with confidence to the molecule dissolved in a non-polar solvent like chloroform. In a polar solvent, such as DMSO or water, the solvent molecules can compete for hydrogen bonds. The question is whether this competition occurs in daunorubicin and its analogs to an extent such that the alternative conformation of ring A becomes preferred. The PMR results tell us that in addition to daunorubicin, 11-deoxycarminomycin (VIa), 11-deoxy-4-demethoxydaunorubicin (VIb) (as well

as their aglycones), 9-deoxydaunorubicin (VII), either in deuterated chloroform or in deuterated DMSO display the spin coupling values between H7-H8 and $H8_{eq}$-$H10_{eq}$ in good agreement with the α conformation. On the contrary, the 6-deoxy analogs (VIIIa and b) which in deuterated chloroform prefer the α conformation, in deuterated DMSO show spin coupling values corresponding in great extent to the β form. Therefore we may conclude that the conformation flexibility of ring A in anthracycline derivatives depends upon the position of 07 with respect to 06 and H-09. The presence of the 06-07 interaction prevents these molecules from assuming the β-conformation. Only 6-deoxy derivatives are potentially candidates to β-A ring puckering. However, in order for the β form become preferred, the 07-H09 hydrogen bond should be broken. A consequence of such molecular properties of the 6-deoxy analogs is their resistance to reductive deglycosidation (Penco *et al.*, 1986).

We have investigated the influence of the chromophore on the behaviour of selected anthracyclines, in the presence of rat liver microsomes, under anaerobic conditions. In particular we have studied, in comparison to Ib, either a glycoside having the same chromophore, such as VII, or compounds having a different pattern of hydroxylation of the anthraquinone moiety, namely VIa and VIIIa. The enzymic reaction of Ib and of VII gave the corresponding 7-deoxyaglycones, whereas VIa gave the bis (7-deoxyaglycone-7-yl), in agreement with that reported in literature for other 11-deoxyanthracyclines (Oki *et al.*, 1977). The redox enzymic activation of VIa did not result in the splitting of the sugar moiety. The cleavage of the glycosidic bond in Ib, VIa and VII observed in the presence of redox enzymes (microsome NADPH-cytochrome P-450 reductase, xanthine-xanthine oxidase) is also performed electrochemically (Malatesta *et al.*, 1984). The reduction of VIIIa by xanthine-xanthine oxidase produces a large ipsochromic shift from λ_{max} 432 nm to λ_{max} 412 nm, the original spectrum being easily restored by addition of oxygen to the system. The same shift in the UV-visible spectrum was obtained with an excess of dithionite, which reduces the quinones to the corresponding hydroquinones. On the other hand the quinone conversion to the semiquinone radical anion is known to cause a solvent dependent batochromic effect (Anne and Moiroux, 1985). Figure 2 shows the visible spectral changes of 6-deoxycarminomycin, as a function of time, in the presence of the xanthine-xanthine oxidase system. After 2 minutes from the addition of the enzyme, more than 90% of substrate was converted to the hydroquinone. The morphology of the UV-Visible

VIa : R = OH
VIb : R = H

VII

VIIIa : R = OH
VIIIb : R = H

IX

spectrum is time-dependent, and the changes observed at an early stage do not define a unique isobestic point. While the family of curves obtained after 55s and up to 150s have the same absorbance at 412 nm (same behavior was observed when the reaction was carried out in the presence of rat liver microsomes), it is not clear whether the two apparently different families of curves arise because of the intermediate formation of a semiquinone on the way to hydroquinone or if the apparent lack of an isobestic point is due to spectral modifications corresponding to local environment changes during the docking of the anthracycline into the enzymic active site.

We have therefore investigated by ESR the radicals formed from 6-deoxycarminomycin and daunorubicin in the presence of rat liver microsomes. The radical (i.e., semiquinone) concentration formed during the enzymatic reduction is different for the two substrates. Indeed a 55 μM solution of 6-deoxycarminomycin, when incubated under anaerobic conditions with rat liver microsomes and NADPH, gave rise, after a lag

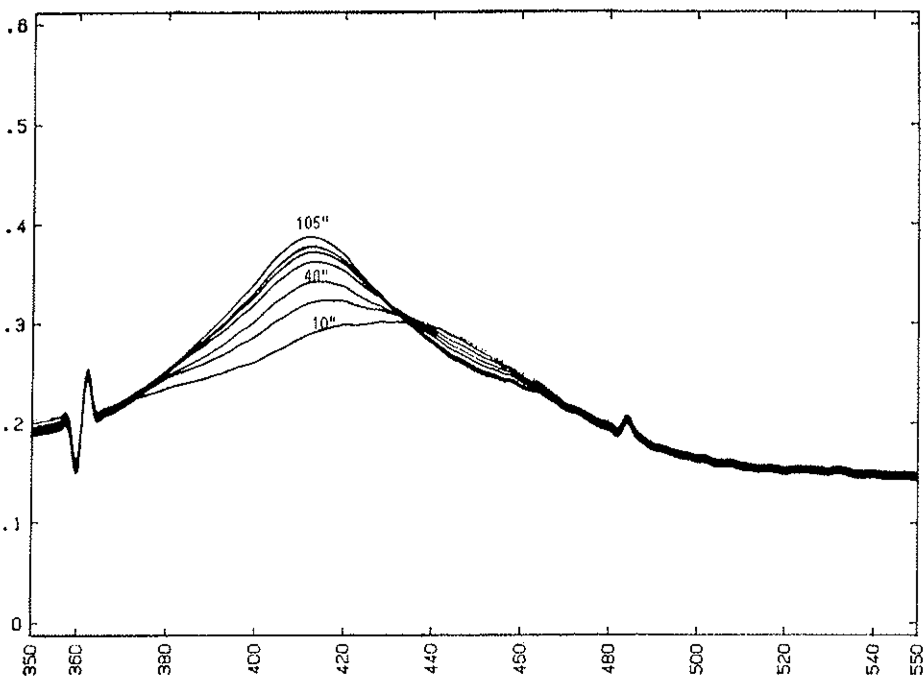

FIG. 2. Visible spectrum of the buffered reaction mixture containing 6-deoxycarminomycin (0.2 μM), xanthine (240 μM) and the enzyme, xanthine oxidase at different times (in seconds) of incubation at 37°C.

period of about 10 minutes, to an ESR signal (g = 2.0035) which lasted for at least two hours. The amount of free radicals formed (probably arising by a disproportionation process) was calculated to be about 2-2.5% of the original 6-deoxycarminomycin molecules present in solution. The daunorubicin solution did not give any signal under the same experimental conditions. However, when the substrate concentration was increased to 10^{-3}M, a signal was detected after 4 minutes. Double integration of this ESR signal and comparison with a concentration standard allowed us to calculate a yield of the free radicals equal to or lower than 0.04% which was not affected by the ratio drug/enzyme/NADPH. The result is in keeping with a previous report (Bachur *et al.*, 1979) in that the authors observe the radicals at relatively high values of the drug concentration and the efficiency of formation is low (not emphasized previously).

As for the correlation between redox potentials, DNA association constant and cytotoxicity, we can note what follows (Table 3). The

substitution of a hydroxyl group with a hydrogen atom on the B ring of the anthraquinone system results in a decrease of value of the DNA association constant, without much change of the cytotoxicity. On the contrary, the introduction of a hydroxyl group at C-1 in the D-ring as in IX, determines a decrease of both the DNA affinity and cytotoxicity. The data related to VII are noteworthy: absence of C9-OH determines the loss of the biological activity, although the value of the DNA association constant is similar to that of the parent drug. On the contrary, VIb is very active against Gross leukemia and against solid tumors such as advanced mammary carcinoma in the mouse. The compound resulted also more cardiotoxic than doxorubicin on the basis of the hystological evaluation of the atrial and ventricular lesions (unpublished data). These findings suggest that the antitumor activity as well as toxic effects cannot be correlated with the redox behavior of the anthracyclines. The partial relationship of the DNA binding properties with the bioactivity confirms that the type of intercalation complex formed is of relevance. In this regard it is of interest to recall the high biological potency of the cyano-morpholino compound X that has been found to give rise to an irreversible binding mode with native calf-thymus DNA *in vitro* (Menozzi *et al.*, 1984)

TABLE 3 - *Polarographic half-wave potential, apparent binding constant for the complex with calf-thymus DNA and cytotoxicity on cultured HeLa cells of different anthracycline aminoglycosides.*[a]

COMPOUND	El/2 (mV)	($10^6 M^{-1}$) K_{app}[b]	ID_{50}[c] (ng/ml)
DAUNORUBICIN (Ib)	−572[d]	1.9	15
IDARUBICIN (III)	−588[d]	3.0	4.2
11-DEOXYCARMINOMYCIN (VIa)	−492[d]	0.71	14
11-DEOXYIDARUBICIN (VIb)	−468[d]	0.31	25
6-DEOXYCARMINOMYCIN (VIIIa)	−530	0.79	3.7
6-DEOXYIDARUBICIN (VIIIb)	−524	0.19	17
11-DEOXY-1-HYDROXY-IDARUBICIN (IX)	−520[d]	0.71	250
9-DEOXYDAUNORUBICIN (VII)	−592[d]	2.2	1200

[a] Partly umpublished data
[b] According to a model of one class of independent binding sites
[c] After 24 h exposure
[d] Deglycosidation is observed

and with rat hepatocytes (Westendorf *et al.*, 1985). On the other hand, we have recently found that L-xylo-4′-aminoglycoside XIb, showing a low affinity for native DNA ($K_{app} = 0.32 \times 10^6 M^{-1}$), is practically devoid of cytotoxicity ($ID_{50} = 1600$ ng/ml in HeLa cell culture), whereas "iso-daunorubicin" XIa shows $K_{app} = 1.63 \times 10^6 M^{-1}$ and $ID_{50} = 7$ ng/ml. Needless to say, both compounds are reductively deglycosidated in anaerobic conditions with the microsomal preparation.

3. The Distamycin Analogs

The first group of synthetic analogs of distamycin prepared in our laboratory were the compounds corresponding to the structures XII-XIV. The study of biological activities of these analogs indicated that whereas activity in the bacterial systems was almost restricted to distamycin (V), antiviral activity on vaccinia virus in cell culture was present in all compounds of type XII and XIII, the most effective ones being the derivatives of structure XIII with n = 4 or n = 5. The amidino on the C-terminal side and the formyl group at the N-terminal side were essential for the exhibition of activity in both the bacterial and the mammalian systems (Arcamone, 1972).

The derivatives with 2 to 5 pyrrole rings at 4×10^{-5}M concentration inhibited the reaction catalyzed by the DNA-dependent RNA polymerase of *E. coli.* Compounds XIII, n = 4 and XIII, n = 5 exhibited an 80% reduction of AMP-^{3}H incorporation, calf-thymus DNA being the template (Chandra *et al.*, 1972). Circular dichroism measurements showed a highly favored interaction of the same compounds with A·T base pairs of DNA

X

XIa : R^1 = H, R^2 = OH
XIb : R^1 = OH, R^2 = H

A = CH_2, $(CH_2)_3$,

XII

n = 1, 2, 4, 5, 6

XIII

R = CH_3, CH_2CH_2, CHONHCH$_2$, CHONHCH(CH_3), CH$_2$CH NHCHO

XIV

(Luck *et al.*, 1977), although XIII, n = 4 and XIII, n = 5 show a significant affinity also for A·T and C·G alternating sequences (Zimmer *et al.*, 1983).

Following up this information we planned studies aimed to (1) establish the effect of substituting the pyrrole ring with other aromatic or heterocyclic moieties on affinity and selectivity for DNA (and also on bioactivity) and (2) synthetize new alkylating agents possessing high affinity for the DNA molecule and able to bind covalently to it, possibly with some kind of sequence specificity.

As for the first point we have carried out a study concerning the synthesis of new analogues of distamycin (Arcamone *et al.*, 1986b). The compounds were tested in cell cultures infected with Herpes virus (HV) or with Moloney Sarcoma virus (MSV) and the results are shown in Table 4. Three compounds, namely XVI, XVIII and XIX, showed significant activity against HV, of the same order of magnitude as the parent compound. The higher effect against HV of XVI as compared to XV is in agreement with the higher potency of XIII (n = 4) in respect to V (Verini *et al.*, 1972) in this biological system. As regards the activity

against the RNA virus system, the activity of XVIII is noteworthy and is approximately 15 times higher than that of the parent compound. Therefore the answer to the first question might be that, at least when present at the formylated amino terminus, a different ring is not incompatible with antiviral activity. It has also been shown that DNA affinity and preference for A·T base pairs are still present in this series. However, compound XXI, in which the added benzene ring is in the interior of the pyrrole-peptide sequence, shows less affinity for DNA and shows no significant antiviral activity in the HV system.

CHONH–(N-methylpyrrole)–CONH–C_6H_4–CO[NH–(N-methylpyrrole)–CO]$_3$NHCH$_2$CH$_2$C(=NH)NH$_2$

XXI

TABLE 4 - *Inhibition of viral multiplication by the new distamycin analogues in murine cell culture.* (Arcamone *et al.*, 1986a).

R[NH–(N-methylpyrrole, CH_3)–CO]$_n$–NHCH$_2$CH$_2$C(=NH)NH$_2$

COMPD.	R	n	ID_{50} (10^{-6} M) HV	ID_{50} (10^{-6} M) MSV
V	CHO	3	2.7	58
XV	CHONH–C_6H_4–CO (benzene)	3	19.5	20
XVI	″	4	1.7	>11
XVII	CHONH–C_6H_4–SO_2 (benzene)	3	>1.2 (a)	30
XVIII	CHONH–(thiophene)–CO	3	5.0	3.9
XIX	″	4	4.1	(a)
XX	CHONH–(pyridine)–CO	3	9.8	56

(a) Not measurable because of cytotoxicity.

SCHEME 1 - *Synthesis of bis (N-chloroethyl)-N-deformyldistamycin.*

XXII

Along the line of investigation aimed at the development of cytotoxic distamycin derivatives, we have synthetized compounds bearing electrophilic reactive groups. An example is the bis (N-chloroethyl) derivative XXII (Scheme 1), a compound that exhibited both the preference for A.T rich sequences and the ability to form stable adducts with DNA. This suggests the possibility that the stability of the adducts is due to formation of a covalent bond between the alkylating derivative and the DNA (Arcamone *et al.*, 1986a).

Compound XXII displays inhibition of viability in cultured P388 cells. A comparison with distamycin indicates that XXII is 20 times more effective than the parent compound in this system. This compound and similar derivatives recently synthetized in our laboratory are DNA specific alkylating agents, as the high selectivity of the pyrrole-amidine polypeptide system for B-DNA would insure a special reactivity toward this target. The pharmacological consequence of the recognition of determined base sequences possibly comprised in regulating sites of DNA cannot be established at the present time, although the potential remains for the discovery of more selective drugs with lower incidence of toxic side effects and useful anticancer activity.

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MOLECULAR PHARMACOLOGY OF THE ANTICANCER AGENT MITOXANTRONE AND RELATED STRUCTURES

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ABSTRACT

Several chromophore modified anticancer agents have been developed in an attempt to obviate the severe clinical limitation of risk of cardiotoxicity in the treatment of human malignancies by doxorubicin. These agents include the anthracene derivatives 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9, 10-anthracenedione (mitoxantrone) and the related ametantrone as well as 5-[(aminoalkyl)amino]-substituted anthra-[1,9-cd]pyrazol-6(2H)-ones (anthrapyrazoles) and related structures. Biochemical evidence indicates that their anticancer action is due, at least in part, to their interaction with cellular DNA. The characteristics of the intercalative interaction of these agents with DNA is examined by electron microscopy, determination of binding constants and their DNA base dependence, by the kinetics of the SDS-driven dissociation and by high field ^{1}H- and ^{31}P-NMR studies. Binding constants for mitoxantrone and related structures are $\sim 1 \times 10^{6}\ M^{-1}$ while those of the anthrapyrazoles are somewhat lower $\sim 2 \times 10^{5}\ M^{-1}$ under comparable conditions. Theoretical analysis of the preferred binding sites of mitoxantrone reveal a sequence dependence which is verified by ^{1}H and ^{31}P-NMR studies. The chromophores of mitoxantrone, ametantrone and the anthrapyrazoles are resistant to reductive enzymatic activation and therefore, as expected,

exhibit low augmentation of microsomal oxygen consumption which correlates with their low cardiotoxicity. They are however subject to extensive peroxidative enzymatic metabolism. The primary metabolite of mitoxantrone results from oxidative cyclization to position 2 and is itself subject to subsequent redox reactions. The metabolite in its oxidized form is a strong electrophile and can be reduced by biologically and physiologically relevant electron donors including ascorbic acid, *L*-cysteine and glutathione. In addition certain of these anthracene derivatives act as photosensitizers with visible light illumination, a process which, in the presence of oxygen, can give rise to reactive oxygen species including singlet oxygen, superoxide ion, hydrogen peroxide and the hydroxyl radical. These latter species can, for example, cause facile oxidation of ascorbic acid and 3,4-dihydroxyphenylalanine (Dopa). Whilst this photodynamic effect is not marked in the cases of mitoxantrone and ametantrone, (evidently because of the relative position of the amine functions), it is however prevalent for certain anthrapyrazoles, particularly those lacking intramolecular hydrogen-bonded structures. This latter property extends the potential clinical application of these new agents to include the possibility of photodynamic therapy.

I. Introduction

The anthracycline antitumor antibiotics daunorubicin (daunomycin) and doxorubicin (adriamycin) (Fig. 1) are considered to have the broadest range of clinical utility of all anticancer agents in current use (Arcamone, 1981; Wiernik, 1980). However their clinical utility is limited by associated toxicities, primarily cardiotoxicity which ranges from a delayed, dose-related and insidious cardiomyopathy to irreversible congestive heart failure (Young *et al.*, 1981; Smith, 1969). Consequently intensive efforts have been undertaken to elucidate the mechanism of action of these agents (Arcamone, 1981; Lown, 1983) and to understand the underlying molecular origin of the cardiotoxicity (Doroshow *et al.*, 1980; Goodman and Hochstein, 1977; Lown, 1983), so that it may be obviated or minimized by structural modification (Tong *et al.*, 1979) or by the design and *de novo* synthesis of less toxic agents (Lown *et al.*, 1982; Showalter *et al.*, 1986).

These latter synthetic efforts have been based on the premise that the mode of action of anthracyclines depends, at least partly, on intercalative binding to DNA. The new synthetic anthracenedione DNA-

Anthracyclines and Anthracenediones

R=H Daunomycin

R=OH Adriamycin

Compound	R_1	R_2
(Mitoxantrone)	$(CH_2)_2NH(CH_2)_2OH$	OH
(Ametantrone)	$(CH_2)_2NH(CH_2)_2OH$	H

1, 4-Diaminoanthraquinones

Compound	Z	R	Y
1	H	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$
2	7,10 $(OH)_2$	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$
3	7(OH)	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$
4	10(OH)	$(CH_2)_2NH(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$
5	7,10$(OH)_2$	$(CH_2)_2OH$	$NH(CH_2)_2NH(CH_2)_2OH$
6	7,10$(OCH_2Ph)_2$	$(CH_2)_2NCH_2Ph(CH_2)_2OH$	$NH(CH_2)_2CH_3$

Anthrapyrazoles

Fig. 1. Structural formulae of anthracyclines and anthracenediones.

intercalating agents, mitoxantrone and ametantrone Fig. 1 (Murdock *et al.*, 1979; Wallace *et al.*, 1979) have shown to date a relatively low incidence of cardiac failure in the clinic. Mitoxantrone is more effective than doxorubicin against P388 and L1210 leukemias, B-16 melanoma and colon tumor 26. Mitoxantrone is used primarily in the treatment of breast cancer and the acute leukemias (Smith, 1983; Zee-Cheng and Cheng, 1983; McDonald *et al.*, 1984). The relatively narrow spectrum of activity of mitoxantrone and ametantrone, the problem of tumor resistance and their color liability (deep blue) has in turn led to the development of additional types of chromophore modified agents, including the anthrapyrazoles (Showalter *et al.*, 1986).

The significant promise of these agents has prompted several studies on their molecular pharmacology. Biochemical evidence suggests that, in common with the anthracyclines, nucleic acids are among the principal cell targets of these drugs (Traganos *et al.*, 1980; Waldes and Center, 1982; Bowden *et al.*, 1981; Citarella *et al.*, 1982). For example, mitoxantrone binds to both DNA and RNA in the nuclear chromatin and produces profound changes in its structure, prevents progression of cells at the G_2 phase of the cycle, and inhibts CHO cell colony forming ability.

Whilst there is compelling evidence that mitoxantrone, ametantrone and the anthrapyrazoles do indeed bind intercalatively to DNA (*vide infra*), the experience with other clinically useful anticancer agents, particularly of the quinonoid type, would suggest parallel modes of action involving other cellular macromolecules (Lown, 1983). Consequently in this report we will consider not only the characteristics of the DNA binding but also biologically relevant properties of these new agents including possible redox activity, peroxidative enzymatic metabolism and the concomitant generation of free radical species, as well as, in certain cases, photosensitizing properties and the possible application to photodynamic therapy.

II. Characteristics of the Binding of Mitoxantrone and Related Structures to DNA

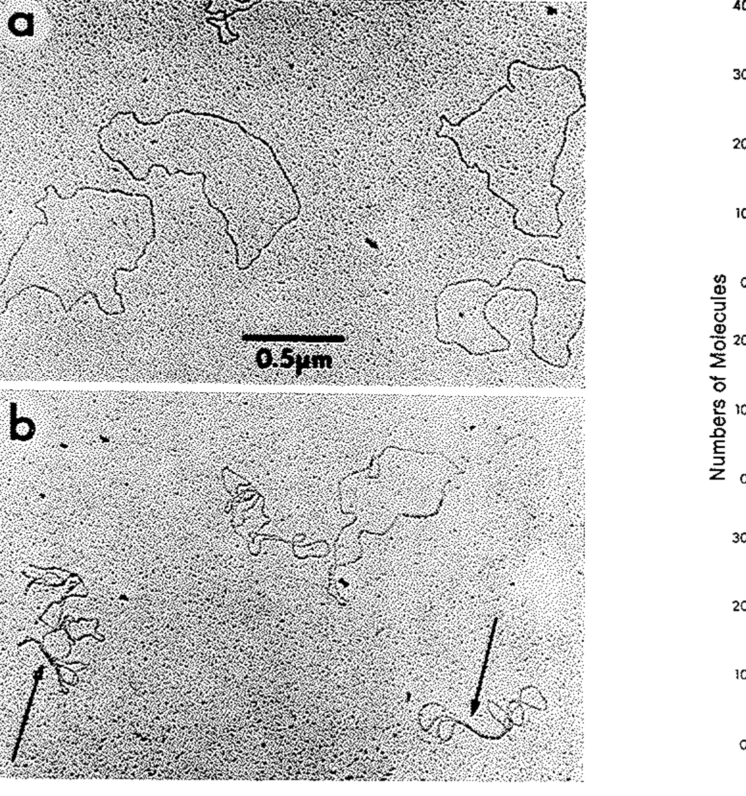
1. *Electron Microscopy Evidence for Intercalation*

A full description of the modes of binding of mitoxantrone, ametantrone and the anthrapyrazoles has yet to be given. Although these drugs bear the requisite planar aromatic chromophores recognized as an es-

sential requirement for intercalation (Lerman, L.S., 1961) the presence of the two extended side-chains on positions 1 and 4 preclude smooth incorporation of all parts of the molecule, which is supported by the greater extent of binding of the less hindered ethidium. This has led to various suggestions including partial intercalation and external binding for mitoxantrone (Kapuscinski *et al.*, 1981; Foye *et al.*, 1982). Electron microscopy employing pBR322 in the form of nicked circles and linears shows pronounced increase in length characteristic of intercalative binding by mitoxantrone (Lown *et al.*, 1984). This causes a 13% average length increase in pBR322 corresponding to ca. 580 drug molecules bound per circle at saturation. Consideration of the slight G.C. preference for non-nearest binding of mitoxantrone (*vide infra*) and inspection of the sequence of pBR322 suggest that, under these conditions, the available intercalation sites are occupied and that additional external electrostatic binding of the cationic drugs also occurs. Ethidium causes a $21.3 \pm 3.6\%$ length increase in nicked, open circular PM2 DNA or 2100 molecules bound per circle, compared with mitoxantrone which causes a 16.6% length increase in the nicked PM2 equivalent to $\sim$ 1700 drug molecules per circle (Fig. 2). E.M. measurements on relaxed PM2 with progressively increasing proportions of mitoxantrone from 1.4:1 to 14:1 drug molecules per base pair revealed the unique property of this drug to cause extensive inter-DNA cross-links leading to compaction into networks of linked molecules Fig. 3 (Lown *et al.*, 1984). These observations provide experimental verification of previously suggested compaction and distortion of chromatin by mitoxantrone (Waldes and Center, 1982; Kapuscinski *et al.*, 1981). Examination of the behavior of several mitoxantrone analogues revealed that the charged side arms are responsible for DNA network formation. A necessary but not sufficient requirement is to have two OH groups in ring C of the chromophore. An additional requirement is to have two basic groups in the side arms separated by two carbon atoms (Lown *et al.*, 1984).

2. *DNA Binding Affinity of Mitoxantrone and Congenors and Base Pair Selectivity*

Independent evidence for binding of mitoxantrone and congenors to DNA was obtained from absorption spectral determination of binding constants (K) (Lown *et al.*, 1985; Krishnamoorthy *et al.*, 1986). Scatchard



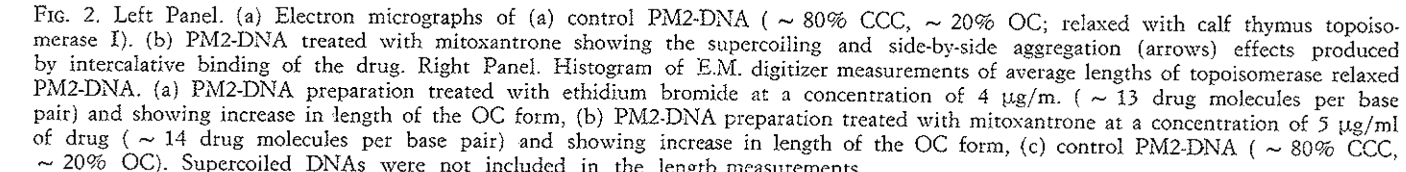


FIG. 2. Left Panel. (a) Electron micrographs of (a) control PM2-DNA (~ 80% CCC, ~ 20% OC; relaxed with calf thymus topoisomerase I). (b) PM2-DNA treated with mitoxantrone showing the supercoiling and side-by-side aggregation (arrows) effects produced by intercalative binding of the drug. Right Panel. Histogram of E.M. digitizer measurements of average lengths of topoisomerase relaxed PM2-DNA. (a) PM2-DNA preparation treated with ethidium bromide at a concentration of 4 μg/m. (~ 13 drug molecules per base pair) and showing increase in length of the OC form, (b) PM2-DNA preparation treated with mitoxantrone at a concentration of 5 μg/ml of drug (~ 14 drug molecules per base pair) and showing increase in length of the OC form, (c) control PM2-DNA (~ 80% CCC, ~ 20% OC). Supercoiled DNAs were not included in the length measurements.

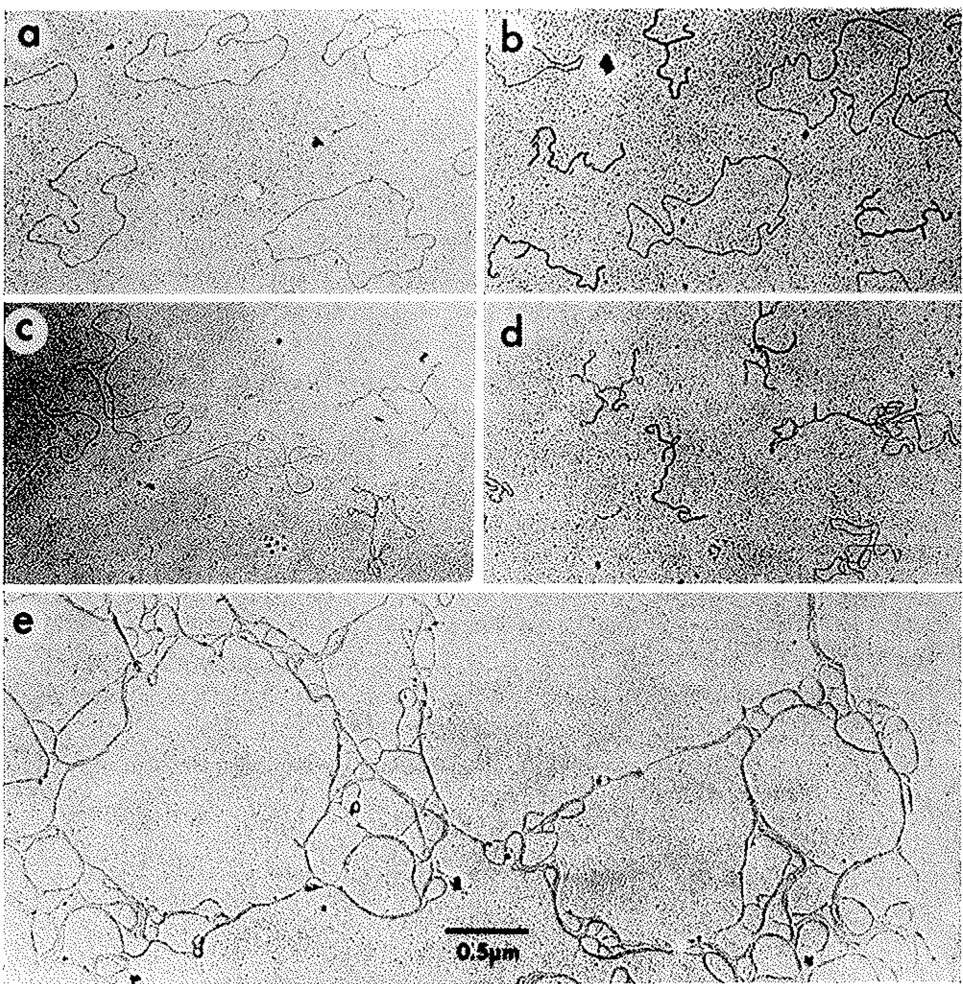


FIG. 3. Electron micrographs of (a) control PM2 (~ 80% CCC) relaxed by treatment with calf thymus topoisomerase I; (b) PM2-DNA as in (a) treated with ethidium bromide, showing length extension of OC forms and supercoiling of CCC forms; (c) PM2-DNA as in (a) treated with mitoxantrone at 0.14 eg. drug per base pair showing progressive supercoiling of the DNA; (d) PM2-DNA treated with mitoxantrone at 1.4 eg. drug per base pair showing more extensive supercoiling; (e) PM2-DNA treated with mitoxantrone at 14 eg. drug per base pair demonstrating extensive formation of networks of linked DNA molecules.

plots afforded values for K and n (the average number of base pairs occupied) from the equation (McGhee and von Hippel, 1974)

$$\nu/c = K[1-n\nu]\ \ [(1-n\nu)/(1-(n-1)\nu)]^{n-1}$$

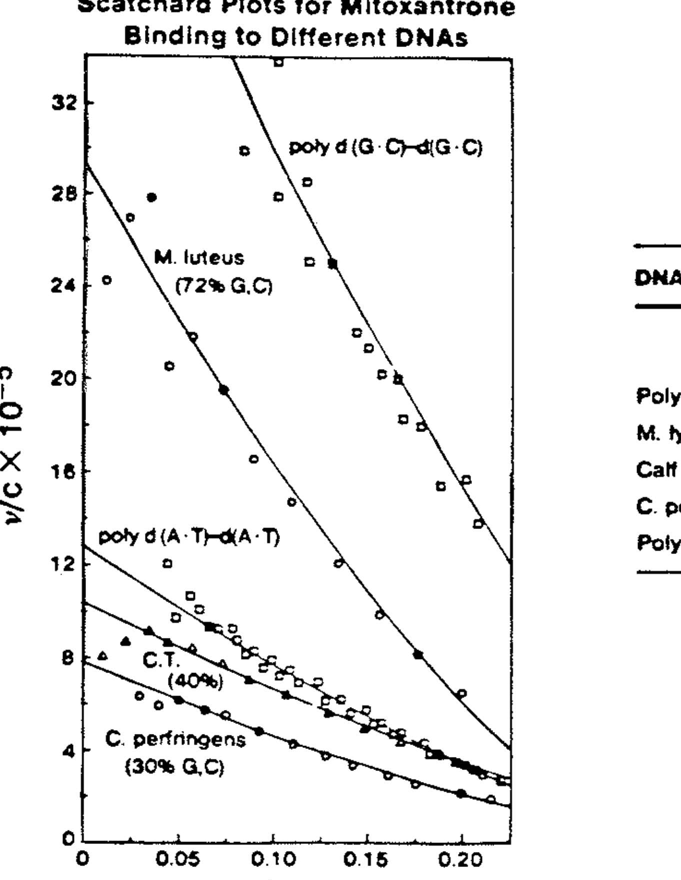
(where c is the free intercalator molarity).

The compounds bind to double helical DNA and fall into three groups based on the equilibrium constants: mitoxantrone and compounds which bear the same OH substitution pattern on the aromatic ring and which have side chain OH groups, have K values near 2×10^5; a second group has the same ring OH substituents but no side chain OH have K values close to 1×10^5; and those compounds lacking OH substituents or possessing a different ring substitution pattern have K values near 0.2×10^5 (Krishnamoorthy *et al.*, 1986). A general value of n is 22.5 ± 0.5 which is in the range typically observed for intercalators.

Mitoxantrone and its congenors exhibit a slight preference for alternating sequences, particularly those of G.C. base pairs, and the extent of overall base preference depends on the nature of the substituents on the anthraquinone chromophore (Krishnamoorthy *et al.*, 1986; Foye *et al.*, 1982). However there also appears to be some DNA sequence preference to the interaction since the binding to poly d(A-T)·d(A-T) is greater than for calf thymus or *C. perfringens* DNA samples which have higher G.C content than the polymer (Fig. 4) (see also discussion below). Removal of the OH substituents from the side chains results in a significant decrease in the G.C base pair binding preference.

3. *Kinetics of Binding of Mitoxantrone and Ametantrone and Sequential versus Competitive Binding*

The SDS driven rate constant for dissociation of mitoxantrone from its calf thymus DNA complex is 1.3 sec^{-1}. This rate constant is in the same range as the dissociation rate constants observed for anthracycline-DNA dissociation (Wilson *et al.*, 1976) but is much lower than those observed for other intercalators such as ethidium (Ryan and Crothers, 1984; Chandrasekaran *et al.*, 1984). Further investigation employing stopped flow kinetics revealed the biphasic nature of the dissociation processes from calf thymus DNA. (Fig. 5) Analysis of the effects of ionic strength changes on the two rate constants for daunorubicin and mitoxantrone suggests that both



Base Pair Specificity in Binding

DNA Type	K ($\times 10^{-5}$)[a]	n
Mitoxantrone		
Poly d (G-C)·d(G-C)	47.5	2.4
M. lysodeikticus	23.9	2.8
Calf thymus	10.4	2.4
C. perfringens	7.7	2.6
Poly d(A-T)·d(A-T)	12.9	2.6

FIG. 4. Scatchard plots for the binding of mitoxantrone to different DNA samples in PIPES 20 buffer. (□) poly [d(G.C)-d(G.C)]; (○) *M luteus* DNA; (□) poly [d(A.T)-d(A.T)]; (△) calf thymus DNA: (○) *C. perfringens* DNA; from top to bottom in the figure. The points are from the spectrophotometric titration, and the curves are the best-fit K and values by using eq. 1. K and n values are collected in the Table.

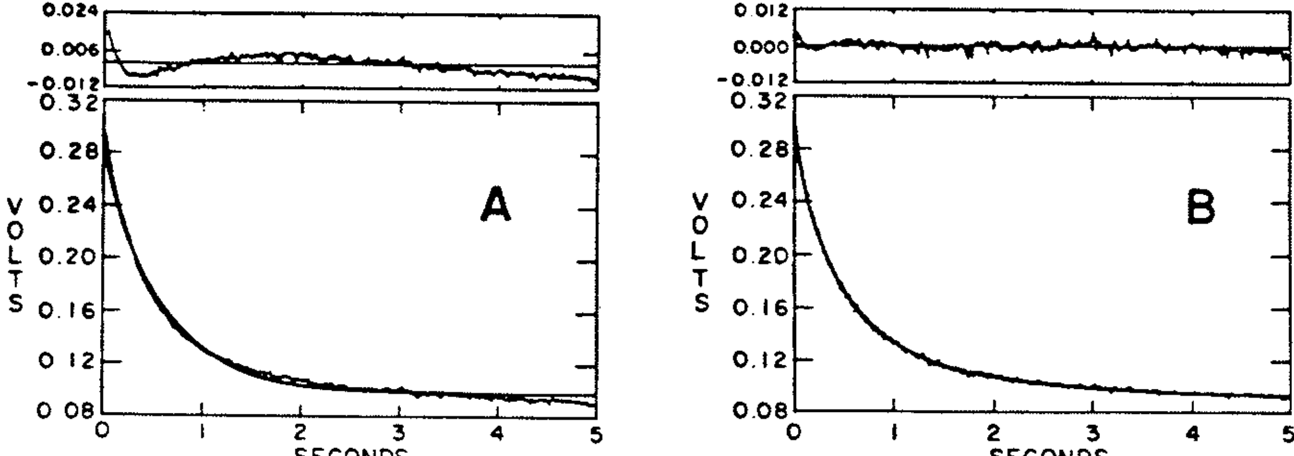


FIG. 5. SDS driven stopped-flow dissociation reactions at 20°C in PIPES 10 buffer for DNA-mitoxantrone complex. The DNA concentration in base pairs is 5×10^{-5} M which is ten times the concentration of mitoxantrone. 200 data points were collected and plotted. The smooth line in panel A is a single exponential non-linear least squares best fit values to the experimental data and panel B is a two exponential fit for the same data. Residual plots for both data sets are shown above the experimental plots.

of these compounds bind to DNA via mechanisms which involve relatively fast formation of an initial exterior complex followed by a relatively slow intercalation. Daunorubicin and mitoxantrone have similar dissociation rate constants which are lower than those for ametantrone. The effects of temperature and ionic strength on both rate constants for each compound are similar, and activation energies for these processes are in the range of 16-24 kcal/mole. The daunorubicin dissociation results from poly $d(G\text{-}C)_2$ and poly $d(A\text{-}T)_2$ can be fitted with a single exponential function and the rate constants are quite similar. The polymer dissociation results for ametantrone can also be fitted with a single exponential function but in this case the dissociation rate constants for the poly $d(G\text{-}C)_2$ complexes are approximately 10 times lower than for the poly $(A\text{-}T)_2$ complexes (Krishnamoorthy *et al.*, 1986). The simplification of the dissociation reactions for these compounds in moving from natural DNA to the synthetic polymers suggests that the complexity in their dissociation kinetics from DNA is due to a combination of base pair and sequence dependent interactions which are broken with different rates on dissociation from different sequences. With the uniform base pair composition and sequence of the polymers, both the interactions and the dissociation reactions are thus simplified.

Mitoxantrone also exhibits a much slower dissociation rate from poly $d(G\text{-}C)_2$ than from poly $d(A\text{-}T)_2$ but its dissociation from both polymers exhibits biphasic kinetics. In all cases to date multiphasic SDS driven dissociation rate processes from complexes with natural DNA samples have simplified to single exponential reactions with the polymers poly $d(A\text{-}T)_2$ and poly $d(G\text{-}C)_2$. This has been the case even with complex molecules such as the antibiotics nogalamycin and echinomycin which require at least the sum of three exponential curves to fit their dissociation kinetic data with natural DNA samples (Fox *et al.*, 1981, 1985). There are two possible explanations for the unusual behavior of mitoxantrone: (i) the drug has two significantly different DNA binding modes, both with side chains in the same groove or with one side chain in each groove and/or (ii) mitoxantrone binds and dissociates differently with sites that are purine (5′ - 3′) pyrimidine versus pyrimidine (5′ - 3′) purine. Recent theoretical calculations serve to discriminate between these alternatives (*vide infra*).

Chaires *et al.* (1985) have pointed out that the two observed SDS driven dissociation rate constants for the daunorubicin. DNA complex could arise from two limiting mechanisms of the following types:

$$D + S \rightleftharpoons C_1 \rightleftharpoons C_2 \rightleftharpoons C_3 \quad (1)$$

$$D + S \rightleftharpoons C_1 \begin{matrix} \nearrow\!\!\swarrow C_2 \\ \searrow\!\!\nwarrow C_3 \end{matrix} \quad (2)$$

C_1 is formed in a rapid bimolecular reaction and is identified as a weakly bound exterior complex. Alternative intercalation complexes C_2 and C_3 are formed either sequentially as in Scheme 1 or independently/competitively from C_1 as in Scheme 2. The rate differences from C_1 to C_2 or C_1 to C_3 could arise from specific base pair, sequence and/or conformational effects. An analysis of the ionic strength dependence on the rate constants suggests that Scheme 2 represents the correct mechanism for the biphasic dissociation of both daunorubicin and mitoxantrone from DNA.

Mitoxantrone and daunorubicin, both of which have shown excellent anticancer activity, bind strongly to DNA under physiological conditions and have slow dissociation kinetics from their DNA complexes. It has been suggested that slow dissociation kinetics may be essential for activity of drugs whose cytotoxicity is due, in whole or part, to DNA binding (Muller and Crothers, 1968).

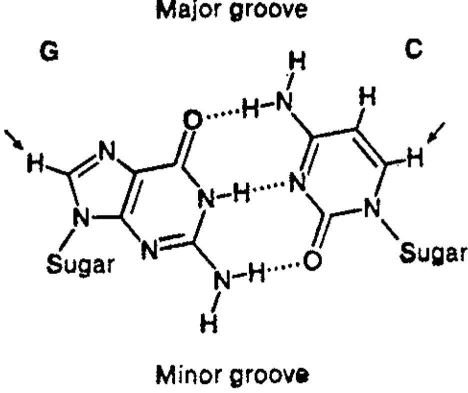
4. *Anthrapyrazole-DNA Interactions*

The anthrapyrazoles shown in Fig. 1 are close structural analogues of mitoxantrone. Mitoxantrone extensively self-associates in solution and is more difficult to study quantitatively than daunomycin, ethidium, etc. The anthrapyrazoles are even more difficult to handle. They extensively self-associate under physiological conditions and exhibit significant dimerization in the 10 micromolar concentration range (dimerization equilibrium constants $> 10^4$). The compounds also show some tendency to adsorb to glass and must be studied in special cells. Binding isotherms for these compounds must be corrected for any residual adsorption and self association and, therefore, have somewhat higher error than with intercalators which do not exhibit these adverse properties. From the biological activity standpoint, it is of interest to determine how these association properties of anthrapyrazoles and anthraquinones affect their ability to pass through membranes and cause reactions such as DNA condensation. Molecules which extensively self-associate may be particularly suited for DNA condensation and aggregation reactions.

Addition of the anthrapyrazole 1 (Fig. 1) to calf thymus DNA causes large increases in viscosity which closely parallel those seen with mitoxantrone (Lown *et al.*, 1985) indicating that the anthrapyrazole also binds to DNA by intercalation. The visible spectrum of the anthrapyrazole has a peak at 493 nm ($E = 1.6 \times 10^4$) $M^{-1} cm^{-1}$, a slightly smaller overlapping peak with an adsorption maximum centered at 468 nm, and another small peak at 386 nm. All of these bands, as with spectra for other intercalators, exhibit hypochromicity and spectral shifts to longer wavelengths on addition of DNA. There is a pseudo-isosbestic point at approximately 504 nm. Corrected spectrophotometric binding isotherms for this compound in neutral pH buffer with 0.2M NaCl give an approximate binding constant for the interaction with calf thymus DNA of 2×10^5. This is slightly lower than the value for mitoxantrone in the same buffer ($\sim 1 \times 10^6$) but both of these values are quite large relative to other intercalators. Preliminary studies with the alternating AT and GC polymers have not demonstrated any significant base pair binding specificity for the anthrapyrazoles (Wilson *et al.*, 1986).

5. *Characterization of Mitoxantrone: Oligomer Intercalation Complexes*

In view of the slight G.C preference for mitoxantrone binding, high field two-dimensional ^{1}H-NMR studies were undertaken with $d[CpGpCpGp]_2$ (Lown and Hanstock, 1985). The spectral data are in accord with 1:1 stoichiometry and intercalation symmetrically in the tetramer in which the chromophore is aligned approximately perpendicular to the axis of the central base pair (Fig. 6). In addition, NOE and 2D-NOESY measurements confirm projection of both of the side chains into the major groove in accord with conclusions drawn from computer graphics modelling (Islam *et al.*, 1985) Fig. 6. In addition 2D-^{1}H and ^{31}P-^{1}H correlation NMR studies of the interaction of mitoxantrone with $d[CpGpApTpCpG]_2$ in H_2O indicate by examination of the imino protons (rendered visible by selective H_2O signal suppression) selective shifts at the CpG sites (Kotovych *et al.*, 1986). Moreover, maximal and selective shifts were observed with stoichiometries of drug:DNA of 1:1 and 2:1, corresponding to selective intercalation of the drug at the two CpG termini. This result is in accord with the slight C.G base preference suggested by the spectroscopic binding studies with native DNAs and synthetic oligonucleotides and by the electron microscopy studies (Foye *et al.*, 1982; Lown *et al.*, 1984, 1985).



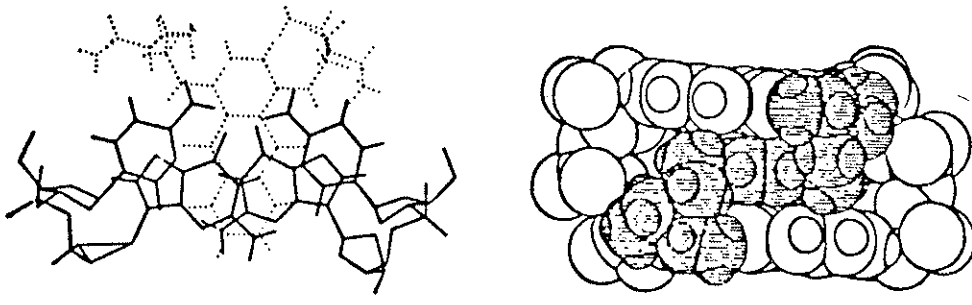


FIG. 6. (a) Points of contact of the mitoxantrone side-chains in the 1:1 complex with [d(CpGpCpG)]$_2$ deduced from NOE differences in the major groove. (b) Elevation computer projection of intercalation of 1,4-bis[[(diethylamino)ethyl]amino]-anthraquinone showing perpendicular orientation of the chromophore and having side chains in the major groove. (c) Space-filling computer graphics depiction of major groove intercation of the anthracenedione.

A characteristic property of intercalators is to produce not only length extension of the DNA helix (16.6% for mitoxantrone versus 21.3% for ethidium), but unwinding of the helix. The unwinding angle of mitoxantrone was determined independently by viscosity measurements and by a novel assay employing calf thymus topoisomerase and shows excellent agreement for a value of 17.5° (Lown *et al.*, 1985). This latter value was utilized in the interpretation of the ^{1}H-NMR of the mitoxantrone complexes and in theoretical calculations on the sequence selectivity of the binding.

6. *Theoretical Analysis of the Sequence Selective Binding of Mitoxantrone to Double Stranded Oligodeoxyribonucleotides and Experimental Verification*

Recently Pullman and coworkers have applied refined theoretical techniques to the question of the mode of binding and particularly on the sequence preference of mitoxantrone for double-stranded nucleotides (Chen *et al.*, 1986). The following sequences were considered — i: $d(GCGC)_2$; ii: $d(CGCG)_2$; iii: $d(ATAT)_2$; iv: $d(TATA)_2$; v: d(GTGT)·d(ACAC) and vi: $d(CCGG)_2$. For all sequences the intercalation of the chromophore was assumed to take place in the center of the oligomer, namely, between base pairs 2-3′ and 3-2′. Sequences i-iv are prototypes of the regularly alternating polymers poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT). Computations were performed with SIBFA (Sum of Interactions Between Fragments computed Ab initio) method (Gresh *et al.*, 1984, 1985) which uses empirical formulae based on *ab initio* SCF computations. The most favorable binding configuration of mitoxantrone locates the chromophore orthogonal to the intercalation site base pair axis and with the side chains both in the major groove (Fig. 7). This result is in accord with that found experimentally from 2D-NMR studies on a $d(CGCG)_2$-mitoxantrone complex (Lown and Hanstock, 1985) and is also consistent with the computer graphics study of a $d(CG)_2$-mitoxantrone complex (Islam *et al.*, 1985). This finding of major groove orientation of the side chains is in striking contrast to the results, both experimental (Quigley *et al.*, 1980) and theoretical (Chen *et al.*, 1985; Nakata and Hopfinger, 1980; Newlin *et al.*, 1984), obtained for the anthracyclines daunorubicin and doxorubicin.

A marked preference is predicted for intercalation of the chromophore of mitoxantrone in a pyrimidine (3′-5′) purine sequence. This type of general preference of intercalative compounds is well documented (Sobell

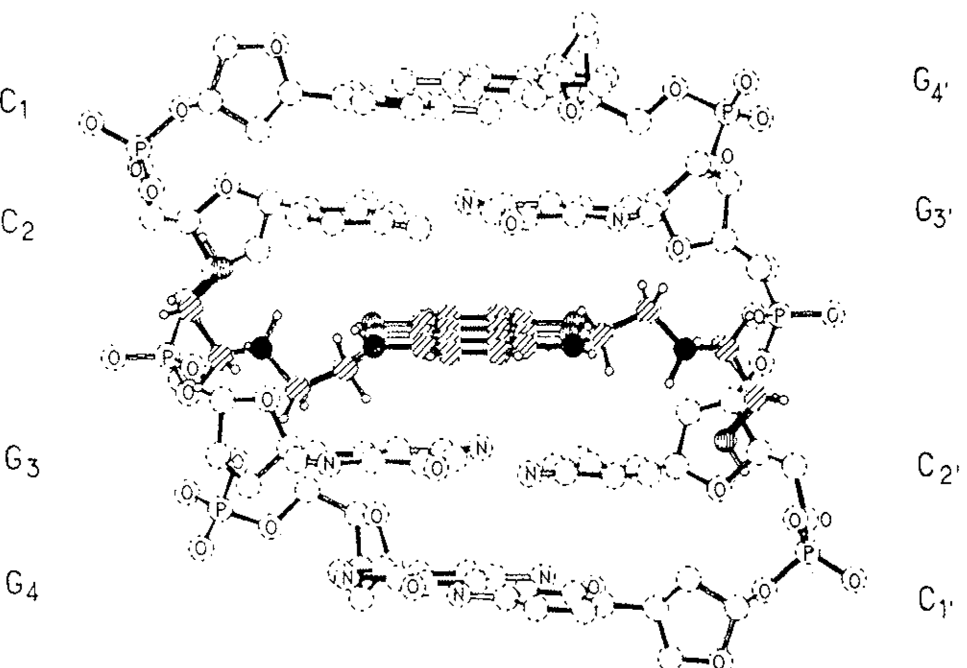


FIG. 7. Representation of the intercalative binding configuration calculated for mitoxantrone bound in the major groove of $[d(CpCpGpG)]_2$.

et al., 1982), the only notable exception being provided by the chromophore of actinomycin D which prefers the purine (3'-5') pyrimidine sequence, a preference ascribed to interactions of the peptide side chains.

In addition, for mitoxantrone the following order of preferential major groove binding is predicted: $d(CCGG)_2 > d(GCGC)_2 > d(GTGT) \cdot (ACAC) > d(ATAT)_2 > d(CGCG)_2 > (TATA)_2$ These findings on the preferred site of binding provide a rationale, all other factors being equal, for the prediction of intercalation sites of mitoxantrone on longer oligomers. An experimental test case is provided by $d[CGATCG]_2$. The two termini are the only prospective intercalation sites which can provide two 5'-purines and should therefore be preferred. This inference is borne out experimentally by 2D-NMR techniques which, as mentioned above, show maximal and selective shifts of the terminal using protons with drug: DNA stoichiometries of 1:1 and 2:1 (Kotovych *et al.*, 1986).

III. Enzymatic Activation of Anthraquinone-Derived Anticancer Agents

1. *Anthraquinone Redox Potentials and Augmentation of Microsomal Oxygen Consumption*

Although the molecular origin of the anthracycline cardiac toxicity remains incompletely understood, recent biochemical studies have suggested that this side effect may be related to the redox reactions of the quinone moiety giving rise to damaging free radicals *in vivo* (Bachur *et al.*, 1978; Myers, 1982; Lown, 1983). Both cardiac sarcosomes and mitochondria can reduce daunorubicin and doxorubicin to their respective semiquinones, thereby initiating, in the presence of oxygen, a free radical cascade (Doroshow, 1983). Reactive oxygen species generated in this cascade are thought to be associated with peroxidative injury to membrane lipids in cardiac tissue (Mimnaugh *et al.*, 1981, 1983, 1985; Thayer 1984; Myers *et al.*, 1977) which is more susceptible to such damage because of suppressed levels of detoxifying enzymes superoxide dismutase, catalase and glutathione peroxidase in this organ (Doroshow *et al.*, 1980). This rationale gave rise to the concept of chromophore modified anthracyclines. The prototype structure 5-iminodaunorubicin has a very negative quinonoid redox potential which prevents *in vivo* reduction and does, in fact, display diminished cardiotoxicity (Lown *et al.*, 1979). Additional examples which include 6,9,11-trihydroxyxantho[2,3-g]tetralin glycosides in which redox activity of the chromophore is suppressed but in which anticancer activity is retained (Lown and Sondhi, 1984), serve to support this rationale for the structural separation of anticancer cytotoxicity from cardiac toxicity.

Mitoxantrone exhibits a redox potential of -0.897^{v} (Sinha *et al.*, 1983) i.e., substantially more negative than 5-iminodaunorubicin at -0.67^{v} (Lown *et al.*, 1979) and which is known not to be subject to *in vivo* reductive metabolism (Peters *et al.*, 1984). Ametantrone and similar anthracenediones exhibit redox potentials similar to that of mitoxantrone and therefore, as expected, attempts to establish if mitoxantrone, ametantrone and related structures are subject to reductive metabolism similar to that of doxorubicin, mainly employing the NADPH-microsomal system, have indicated a low level of activity (Doroshow, 1983; Sinha *et al.*, 1983; Basra *et al.*, 1985).

2. *Peroxidase-induced Oxidative Metabolism of Mitoxantrone and Related Structures*

The redox potential of the quinone moiety of mitoxantrone evidently precludes significant reductive enzymatic activation of this and structurally related drugs. However the possibility of oxidative metabolism must be considered since mitoxantrone contains within its structure functional groups known to be susceptible to enzymatic oxidation. For example enzymatic oxidation of hydroquinones (Yamazaki, 1977) and phenylene-diamines (Person and Fine, 1961; Vinogradov *et al.*, 1979) is well established. When mitoxantrone is treated with horseradish peroxidase and hydrogen peroxide the characteristic absorption of mitoxantrone at 608 and 662 nm decreases. Further reaction results in the appearance of a new absorption band with a maximum at 586 nm (Reszka *et al.*, 1986a) (Fig. 8). In this phase of the reaction isosbestic points are observed at 526 nm and 696 nm (Fig. 8). The appearance of the new absorption at 586 nm is due to the primary metabolite in which a new ring has been formed and whose structure was confirmed by independent synthesis. It is likely that the primary site of the enzymatic oxidation is at one of the amino groups adjacent to the ring and not at other oxidizable groups, such as OH at the 1,4-positions. This interpretation finds support in the observation that ametantrone (a mitoxantrone analogue lacking the chromophoric OH groups) undergoes similar enzymatic oxidation (Kolodziejczyk *et al.*, 1987). Earlier studies reviewed by Mason (1982) have shown that amines are good substrates for enzymatic oxidation. The horseradish peroxidase and ceruloplasmin catalyzed oxidation of some amines to free radical cations has been reported (Peisach and Levine, 1963). The formation of the mitoxantrone metabolite is accompanied by the concomitant generation of a free radical species detected by EPR spectroscopy (Fig. 9). The intensity of the latter signal is dependent on the ratio of mitoxantrone to oxidant as well as on the pH of the medium (Reszka *et al.*, 1986a). The increased stability of the free radical species at lower pH conditions is in accord with its tentative designation as a cation radical. The enzymatic conversion of mitoxantrone to the primary metabolite is irreversible. However the latter is itself subject to further peroxidase action in the presence of H_2O_2 and the resulting oxidized iminoquinone form can then be reduced by biologically and physiologically relevant electron donors including ascorbic acid, *L*-cysteine and reduced glutathione. These latter redox processes (in contrast to the initial enzymatic conversion of mitoxantrone and subsequent reduction, Fig. 10) are fully reversible (Fig. 11). Mitoxantrone itself is capable of

FIG. 8. Oxidation of mitoxantrone (1) by HRP/H_2O_2 system. The new absorption spectrum with maximum at 586 nm is ascribed to the cyclic metabolite (3). Arrows indicate direction of changes.

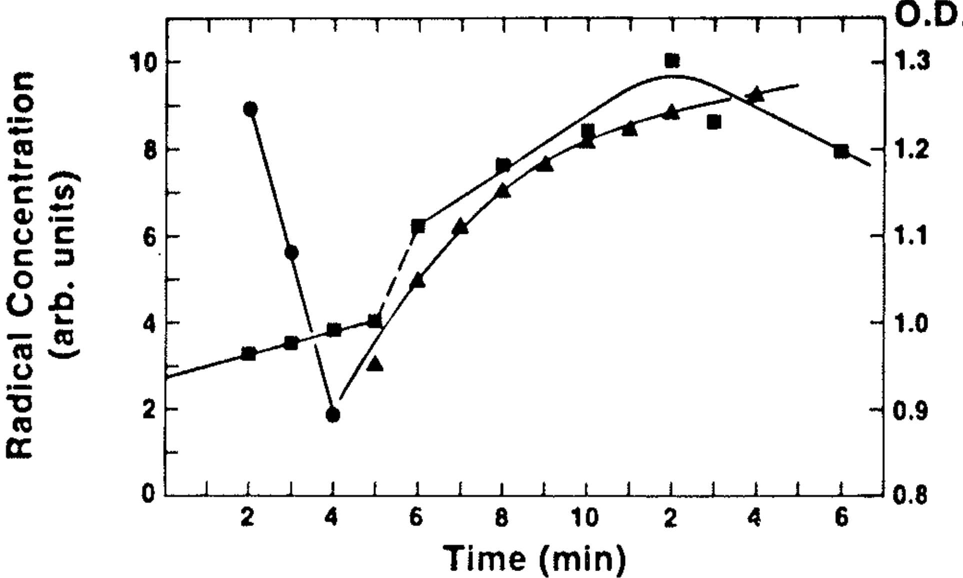


FIG. 9. Horseradish peroxidase-catalyzed oxidation of mitoxantrone. Time course of reaction showing disappearance of drug monitored at 608 nm (●), appearance of the primary metabolite (▲) monitored at 586 nm and appearance of free radical species (■).

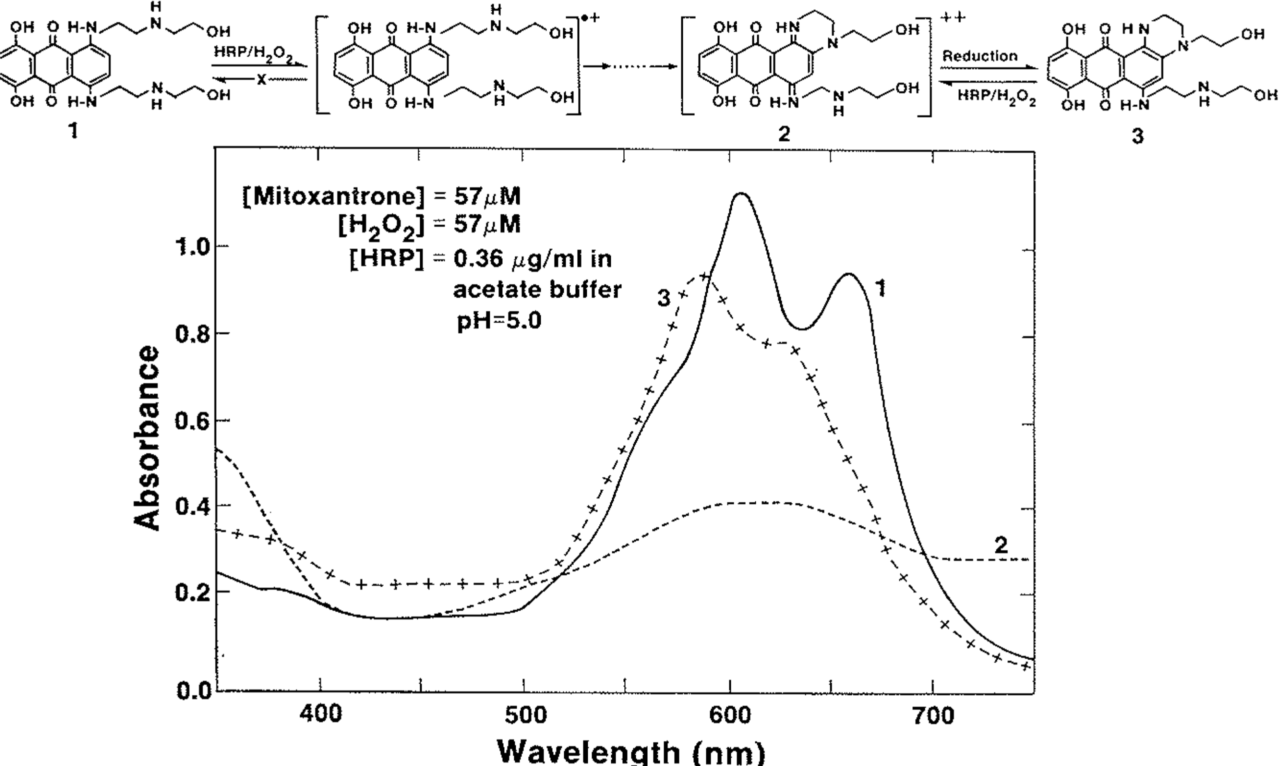


FIG. 10. Horseradish peroxidase-catalyzed oxidation of mitoxantrone (1) monitored spectroscopically and showing schematically formation of the principal metabolite (3) via free radical intermediates.

reducing the diimino (oxidized) form of the primary metabolite. It appears likely that the reaction of the peroxidase with the cyclic metabolite also proceeds via free radical intermediates in accord with the known mechanism of action of the peroxidase (Chance, 1952). In fact a strong EPR signal was detected when the cyclic metabolite was incubated with HRP/H_2O_2 (Kolodziejczyk *et al.*, 1987).

These novel reactions of mitoxantrone summarized in Fig. 12 may be relevant to its biological activity. In particular, detection of the relatively long-lived free radical species and a strong electrophile may shed light on possible alternative modes of action of this clinically useful drug.

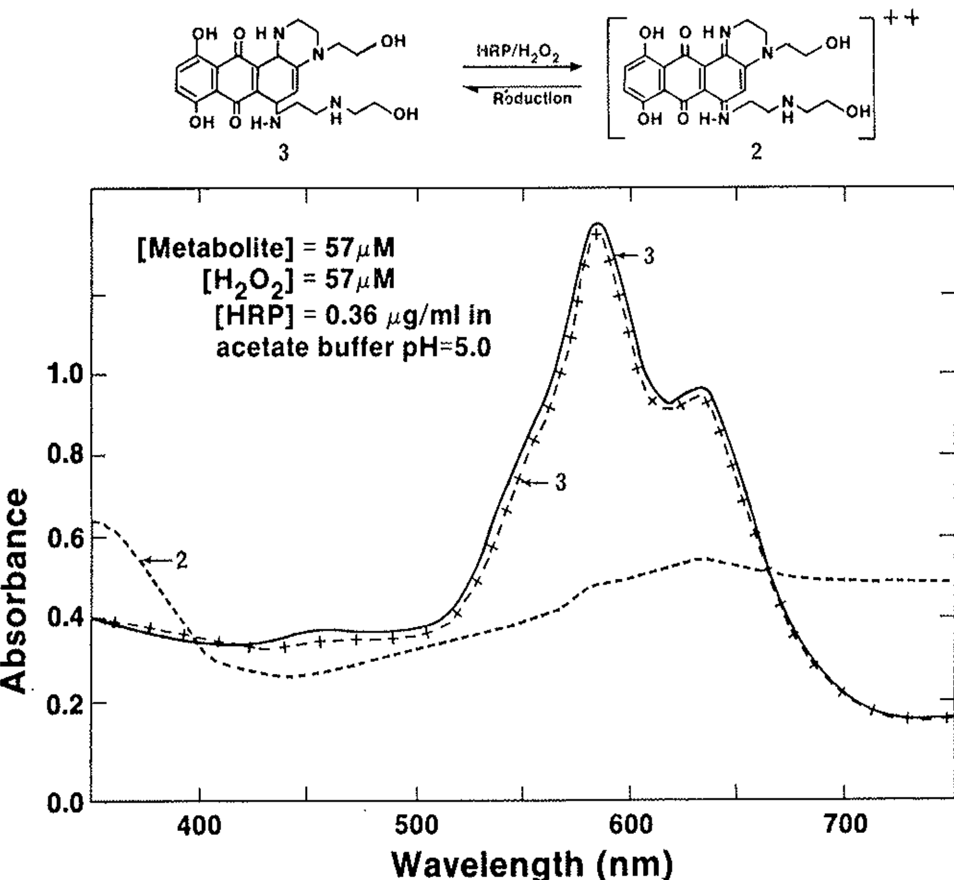


FIG. 11. Peroxidase-catalyzed oxidation of metoxantrone primary metabolite (3 → 2) and reversible reduction of 2 with ascorbic acid.

HRP - Catalyzed Oxidation of Mitoxantrone:
Formation of Principal Metabolites via Free Radical Intermediates.

X=OH Mitoxantrone

HRP/H_2O_2

FIG. 12. Schematic representation of horseradish peroxidase-catalyzed irreversible oxidation of mitoxantrone via a radical cation intermediate followed by cyclization to the primary metabolite and subsequent reversible oxidation and reduction of the latter by biological reductants (e.g., ascorbate, glutathione) and equilibrium disproportionation-comproportionation reaction of the fully oxidized metabolite. The N,N-diacetyl derivatives of the parent drugs, while subject to peroxidase action via free radical intermediates, do not cyclize.

Comparable reactions have been observed for mitoxantrone with other enzyme systems such as cytochrome C and ceruloplasmin (Kolodziejczyk *et al.*, 1987).

IV. Photodynamic Action of Anthraquinone-Derived Agents

1. *Photosensitized Generation of Singlet Oxygen from Anthraquinone Derived Anticancer Agents*

The phenomenon of photosensitization by certain chemotherapeutic agents, including anticancer drugs, is now well recognized. For example, the photodynamic action of daunorubicin has been demonstrated (Sanfilippo *et al.*, 1968). There is increasing evidence that the photosensitization caused by antibiotics and antitumor agents is mediated by the same species produced during the irradiation of well known sensitizers such as methylene blue and rose bengal. These intermediates include oxygen derived radicals ($O_2^{\cdot-}$, $OH^{\cdot}$) and singlet oxygen $^1O_2$. The generation of oxyradicals has been observed during photochemical reactions sensitized by doxorubicin and daunorubicin (Carmichael *et al.*, 1983; Gray *et al.*, 1982).

It has been pointed out above that the rationale in synthesizing chromophore modified anthracycline analogues such as 5-iminodaunorubicin (Lown *et al.*, 1979), xantho[2,3-g]tetralin glycosides (Lown and Sondhi, 1984), 9,10-anthracenedione derivatives including mitoxantrone and ametantrone (Murdock *et al.*, 1979) as well as the anthrapyrazoles (Showalter *et al.*, 1986) is to render the C-ring electron deficient quinone chromophore more resistant to enzymatic reduction to one or two electron reduced species while retaining characteristics of the chromophore necessary for DNA binding. In addition however mitoxantrone suffers from a color liability in clinical applications in being bright blue. Therefore an additional consideration in developing the anthrapyrazoles was to address this color problem. The prototype anthrapyrazoles (Fig. 1) exhibit absorption maxima in the region 470 to 516 nm with extinction coefficients in the range 1.46 to 2.00×10^4 $M^{-1} \cdot cm^{-1}$ i.e., they are yellow, orange or red like the anthracyclines. An unexpected consequence of this structural modification, however, is to render some of the anthrapyrazoles effective photosensitizers (Reszka *et al.*, 1986b,d). Thus oxygen consumption, photoinduced by visible light, and sensitized by the anthrapyrazoles to a greater or lesser extent depending on the structure, has been observed in solution. The generation of singlet oxygen under these conditions was demonstrated

for mitoxantrone, ametantrone and anthrapyrazoles using a $^1O_2$ acceptor, 2,5-dimethylfuran [DMFu] (Fig. 13). The procedure for measuring the concentration of oxygen in solution relies on the dependence of the line-width ΔH of the EPR line of a stable free nitroxide radical marker on the oxygen concentration (Reszka and Chignell, 1983)

$$\Delta H = \Delta Ho + b\,[O_2]$$

(where ΔHo is the linewidth in deoxygenated solution in G (gauss) and b is a calibration parameter).

Thus the rate of oxygen consumption can be measured using equation

$$\frac{-d[O_2]}{dt} = \frac{1}{b}\,\frac{d\Delta H}{dt}$$

(where $d\Delta H/dt$ is the rate of narrowing of the EPR signal). The effects of specific singlet oxygen quenchers, such as sodium azide, on this process confirm the intermediacy of singlet oxygen.

There is a marked difference in the photosensitizing properties of different anthrapyrazoles (Fig. 13). The essential structural modification which influences this property is the presence of one or two hydroxyl groups. The positions of these groups are such that they can form intra-molecular hydrogen bonds. It is known that intramolecular hydrogen bonding facilitates radiationless deactivation of the singlet and triplet excited states (Beckett and Porter, 1963; Mordzinski and Grabowska, 1982; Werner, 1979). As a result, lifetimes of the excited states are shortened and accordingly their deactivation via energy transfer to $^3O_2$ becomes insignificant. Alternatively structural modifications can affect the yield of the triplet state formation, ϕ_T. Since singlet oxygen is most likely produced via the triplet quenching (Fig. 14), a decreased ϕ_T can also lead to a lower yield of $^1O_2$.

Mitoxantrone and ametantrone can generate singlet oxygen upon irradiation with visible light (Fig. 13). The efficiency of sensitization is considerably lower than in the case of anthrapyrazole 1, for example. Thus the ostensibly similar second generation alternatives to the anthra-cyclines, mitoxantrone and anthrapyrazoles, differ in at least two important respects, namely, anthrapyrazoles are much more difficult to reduce and, in general, are more reactive as photosensitizers in generating $^1O_2$.

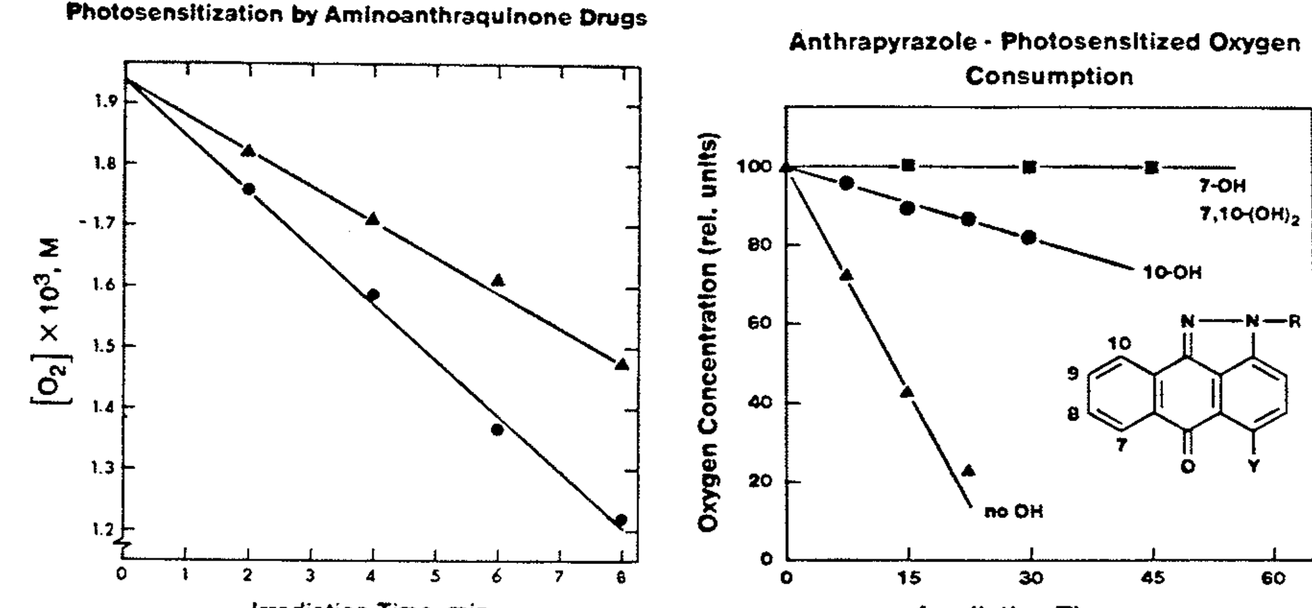


FIG. 13. Visible light photosensitization by aminoanthraquinone drugs: left panel; mitoxantrone (●) and ametantrone (▲) photosensitized oxygen consumption in methanolic solutions. Concentrations of the drugs were such as to achieve the cptical densities of ~ 0.4 and ~ 0.3, respectively. [DMFu = 2.63 mM]; right panel; visible light photosensitized oxygen consumption by anthrapyrazoles in ethanolic solutions and showing the marked effects of hydroxyl substituents. The concentration of oxygen does not change when either drug or DMFu was absent. Such DMFu dependent oxygen uptake suggests involvement of single oxygen in this reaction.

Photochemical Generation of Singlet Oxygen

FIG. 14. Schematic representation of photochemical generation of singlet oxygen.

2. *Anthrapyrazole Sensitized Photooxidation of Biological Reductants*

Recent studies of the photosensitizing properties of anthrapyrazoles and related structures have revealed contributions from mechanisms other than $^1O_2$ generation under certain conditions (Reszka *et al.*, 1986c). These processes are most readily apparent during anthrapyrazole sensitized photo-oxidation of biological reductants in aqueous media (Fig. 15). Illumination of the anthrapyrazole and ascorbic acid with blue light in aerated aqueous solutions causes superoxide dismutase and catalase sensitive oxygen consumption indicating involvement of both superoxide radical and hydrogen peroxide in this process. Electron paramagnetic resonance showed that the ascorbyl radical is also produced during the photooxidation. When 3,4-dihydroxyphenylalanine (Dopa) is used as a substrate, production of hydrogen peroxide is evidenced by catalase-sensitive oxygen consumption. In addition the generation of hydroxyl radicals during illumination of the drug and ascorbic acid (or Dopa) in the presence of catalytic amounts of Fe(III)/EDTA complex is demonstrated using EPR and spin trapping techniques. For example, Fig. 15 illustrates the production of the DMPO-hydroxylethyl spin adduct in the presence of ethanol.

On the basis of these results the anthrapyrazole can be regarded as an active photosensitizer capable of inducing oxidation of sensitive biomolecules by both free radical and singlet oxygen mechanisms. Such photosensitizing properties may be considered in the application of this, or related agents, in photodynamic therapy. One aspect, currently under

investigation, is whether the photosensitizing properties are manifested when the drugs are bound to DNA.

As has been pointed out above, hydroxylated anthrapyrazoles [7-10$(OH)_2$ on ring A Fig. 1] do not possess photosensitizing properties of the nonhydroxylated structure (Reszka *et al.*, 1986d). Therefore should the photosensitization result in deleterious side effects in the clinical application of anthrapyrazoles then the desired structural modification is available to obviate this property. Alternatively this property could possibly be developed for photodynamic therapy.

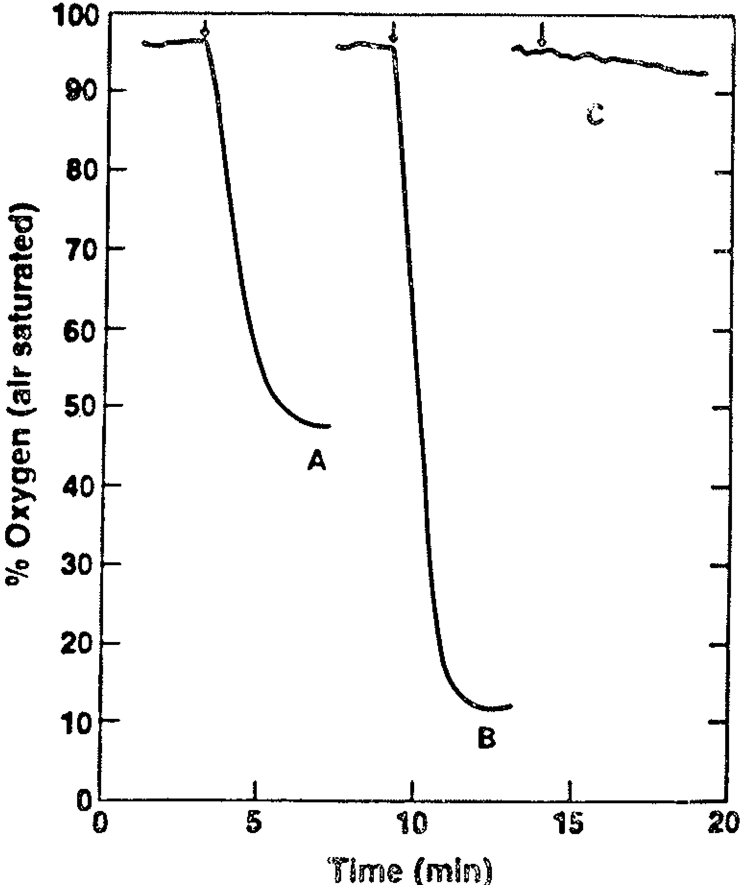


FIG. 15. Oxygen consumption during blue light illumination of a solution containing anthrapyrazole [1] = 16.5 μM and [ascorbate] = 150 μM (A), 290 μM (B); 1 omitted, [ascorbate] = 290 μM (C). [diethylenetriamine-pentaacetic acid] 0.1 mM was present in all samples as metal ion scavenger. Arrows indicate initiation of illumination.

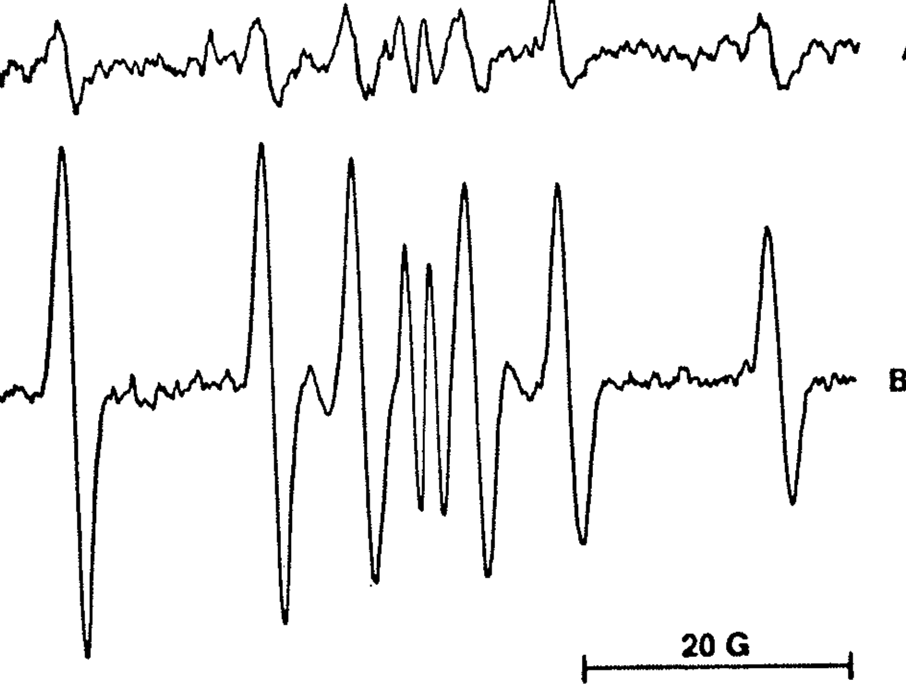


Fig. 16. Effect of ethanol on the EPR spectra from anthrapyrazole photosensitized oxidation of ascorbic acid in aerated phosphate buffer, pH 7.4 A: [1] = 0.33 mM, [ascorbate] = 1 mM, [Diethylenetriaminepentaacetic acid] = 0.1 mM; [5,5-dimethyl-l-pyroline-N-oxide] = 88 mM, [(Fe(III)/EDTA] = 1.2 μM, [EtOH] = 1 M in the dark; B - as in A but upon illumination. EPR instrument-settings were: modulation amplitude 1 G, receiver gain 6.3×10^5, time constant 0.5 sec, microwave power 20 mW.

V. Conclusions and Prospects

Mitoxantrone, ametantrone, the anthrapyrazoles and related structures were synthesized in response to the need for less cardiotoxic clinical alternatives to the anthracyclines. The limited clinical experience obtained to date with these agents has been encouraging, in some cases, particularly with regard to suppressed cardiotoxicity. This has in turn stimulated interest in their biochemical pharmacology. It is evident that intercalative binding to cellular DNA accounts, in part, for the anticancer properties of these agents. Significant progress has been made in understanding the nature of this interaction with regard to the base, sequence, and major groove selectivity, orientation of the chromophore and the kinetics of the

dissociation process, at least for mitoxantrone and ametantrone. More precise information in this area is needed for the newer anthrapyrazole agents.

Experience with the more thoroughly investigated anthracyclines should however alert us to the possibility of alternative modes of action. The rationale for the synthesis of the anthracenedione agents was to suppress enzymatic reductive activation which, in the case of the anthracyclines, appears to lead indirectly to the undesired cardiotoxicity. While this premise appears justified in that reductive metabolism is minimal for these agents, however oxidative enzymatic action is clearly prevalent for all these agents. The biochemical and possible clinical implications of these latter processes are, at the moment, not evident but clearly require extensive investigation. Another unforeseen consequence of chromophore modification to remove the blue color liability of mitoxantrone (to produce the anthrapyrazoles) has been to render certain of the latter active photosensitizers. Progress has been made in understanding the structural parameters controlling the latter phenomenon and of the biochemical processes that ensue and which are mediated by reactive oxygen species. This latter property may have clinical potential in certain cases, in the photodynamic therapy of cancer. In conclusion, the anthracenedione agents represent an active area for research and afford the opportunity for testing predictions on the design of new anticancer agents and to increase our understanding of the biochemical origin of anticancer activity as well as undesired toxicities.

ACKNOWLEDGEMENTS

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MONOMERS, DIMERS AND TRIMERS OF ACRIDINES AND 7H-PYRIDOCARBAZOLES AS ANTITUMOR DRUGS: NMR-DERIVED STRUCTURES OF DNA-COMPLEXES AND STRUCTURE-ACTIVITY RELATIONSHIPS

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ABSTRACT

Due to its higher DNA binding affinity and its reduced mobility in the intercalation site, the 6-chloro-2-methoxy-9-aminoacridine was shown to be preferable to the unsubstituted 9-aminoacridine to prepare DNA bis and tris-intercalators. The DNA affinities of dimers and trimers with carboxamidoalkyl chains are only 1,000 times higher than that of the parent monomer whereas the use of the corresponding reduced aminoalkyl chains leads to the first reported trimer with a DNA affinity (Kapp $\sim 10^{14} M^{-1}$) in the range of DNA regulatory proteins. These differences are likely to be due to the presence of cationic charges and the increased flexibility of the linker. None of these dimers and trimers exhibited strong antitumor properties. ^{1}H NMR studies demonstrate bis-intercalation of an amidoalkyl linked acridine dimer into a d[CpGpCpG]$_2$ minihelix. The complex is in fast equilibrium with the linker located in the major groove. The same situation occurs with ditercalinium, a highly potent antitumor dimer of 7H-pyridocarbazole, although in this case the dimer is in slow equilibrium, a result in agreement with the higher DNA affinity of the two 7H-pyrido-

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carbazole rings. A geometry of the complex between ditercalinium and the tetranucleotide is proposed from ring current shifts and NOE experiments. Discrete changes in the flexibility of the linking chain lead to large differences in antitumor properties but the loss of potency of an acridine-7H-pyridocarbazole heterodimer shows that the presence of the two isoellipticine rings is absolutely required for activity. All these results seem to indicate that only a particular deformation of the DNA is associated with antitumor activity.

Introduction

The selective expression of oncogenes in tumor cells designates DNA as the most appropriate target for the design of potential antitumor drugs. Ideally such compounds would inhibit genetic expression by specific binding to regulatory sequences of the oncogenes. Given the repetitive structure of nucleic acids only molecules made up of subunits complementary to genomic sequences would be susceptible to give the required affinity and specificity. These oligomeric compounds could belong to the group of oligonucleotides bearing an intercalating agent (C. Hélène *et al.*, this volume) or to polyfunctional DNA intercalators endowed with sequence specificity, for instance through hydrogen bonding (this paper and P. Dervan *et al.*, this volume). Nevertheless the efficiency of these promising approaches has yet to be demonstrated.

Another non-specific approach based on the development of cytotoxic agents which behave as DNA monointercalators has led to clinically used antitumor agents such as daunomycin, actinomycin, ellipticines, amsacrine. The cytotoxicity of these compounds could be related to: inhibition of polymerase progression, blocking of DNA regulatory proteins like topoisomerases or interference with DNA repair processes (review in Mechanisms of DNA Damage and Repair, 1986). Theoretically these properties should be dependent on the residence time of the intercalating agent into DNA binding sites.

Accordingly, over the last 15 years, much effort has been devoted to the design of DNA intercalating agents exhibiting a very high DNA affinity and possibly a sequence specificity. The substituted or unsubstituted 9-aminoacridine ring has often been chosen as an intercalating moiety because: i) several acridine derivatives have been shown to exhibit antimalarial, antibacterial (Albert, 1966), and antitumor properties (Cain and Atwell, 1974) in clinical medicine; ii) intercalation of acridines into DNA

induces large changes in their fluorescence properties; iii) the chemistry of the commercially available 9-aminoacridines is relatively simple. The extensive use of monomeric and oligomeric acridines as DNA intercalators and their antitumor properties have been recently comprehensively reviewed (Denny *et al.*, 1983; Wakelin, 1986).

This paper will focus on: i) the molecular requirements necessary to design oligomeric intercalators with DNA affinities in the range of DNA regulatory proteins — this includes a comparison of monomeric and oligomeric acridines containing aminoalkyl or carboxamidoalkyl chains; ii) NMR studies on the mode of binding of bis-intercalating acridines investigating the differences between the DNA binding properties of substituted and unsubstituted bis and tris acridines. Finally this article will discuss the application of the results obtained with acridines, in the design of bis-pyridocarbazoles, especially the clinically investigated ditercalinium (Roques *et al.*, 1979). The use of NMR allowed the first demonstration of bis-intercalation of ditercalinium in a minihelix of $d[CpGpCpG]_2$ (Delbarre *et al.*, 1986). The modulation of the antitumor potency in the series of bis-pyridocarbazoles is discussed in terms of geometry of the DNA complex and change in the flexibility of the linking chains (Garbay-Jaureguiberry *et al.*, 1986).

I - DNA POLYINTERCALATION: REQUIREMENTS FOR BIS AND TRIS INTERCALATION IN THE SERIES OF UNSUBSTITUTED AND SUBSTITUTED 6-CHLORO-2-METHOXY-9-AMINOACRIDINES.

The theoretical free energy of interaction of a n-mer with DNA, $\Delta G_{th}(n)$ can be estimated (Laugâa *et al.*, 1985) by:

$$\Delta G_{th}(n) = n\Delta G(1) - (n-1)RTLn(55.6)$$

where $\Delta G(1)$ is the free energy of interaction of the monomer and RTLn(55.6) corresponds to the entropy of mixing. This expression is only valid in the absence of interaction between the various subunits. Thus the derived affinity constant is given by:

$$K_{th}(n) = (55.6)^{n-1}K_1^n$$

For $K_{mon} = 10^5 M^{-1}$, the theoretical affinities range $K_{dim} \simeq 10^{10} M^{-1}$ and $K_{tri} \simeq 10^{15} M^{-1}$ (Fig. 1).

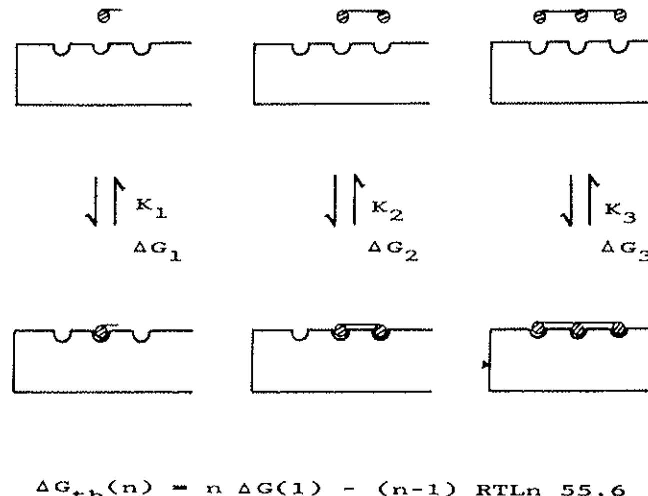

FIG. 1. Schematic interactions of n-mers (n = 1,2,3...) with a macromolecule.

I.1 - *Differences in DNA intercalating ability of dimers derived from either unsubstituted 9-aminoacridine or 6-chloro-2 methoxy-9-amino-acridine.*

Given the structure of DNA, bis-intercalation can involve binding of the two aromatic rings on either side of a base pair or spanning two base pairs according to the "nearest neighbour exclusion principle".

These two binding modes require respective distances of at least 6.8 Å and 10.2 Å between the intercalating moieties. Both DNA binding processes were investigated using dimers belonging to two series of 9-aminoacridines and bearing linkers of different lengths and chemical structures (Wakelin, 1986).

As shown in table 1, the dimer *1* seems to bis-intercalate in DNA by spanning one base-pair, while introduction of substituents on the acridine ring in *2* leads to a monointercalating dimer. This result was previously explained by the steric hindrance brought about by the Cl and OCH_3 groups (Delbarre *et al.*, 1981). This hypothesis seems to be confirmed by the bis-intercalating properties of compound *3* in which the larger substituted ring probably intercalates first into DNA, allowing the unsubstituted acridine to search freely for the energy minimized configuration in the adjacent intercalation site. Such a required conformational freedom is limited when the two acridine rings are substituted.

TABLE 1 - *Differences in DNA intercalation properties of substituted and unsubstituted acridine dimers.*

Nº	R_1	H_2	R_1'	R_2'	d, Å	Linker	unwinding angle ϕ^o	Helix extension slope
1	H	H	H	H	8.8	$-(CH_2)_6-$	34	3.56
2	OCH_3	Cl	OCH_3	Cl	8.8	$-(CH_2)_6-$	26[a]	1.14[a]
3	H	H	OCH_3	Cl	8.8	$-(CH_2)_6-$	32	3.46
4	H	H	H	H	16.1	$-(CH_2)_3-NH$ $(CH_2)_4$ $-(CH_2)_3-NH$	14	3.00
5	OCH_3	Cl	OCH_3	Cl	16.1	$-(CH_2)_3-NH$ $(CH_2)_4$ $-(CH_2)_3-NH$	38	4.80

d Interchromophore distance estimated ± 1 Å.

ϕ^o Helix unwinding angles are deduced from viscosity variations of covalently closed circular DNA. The value of 17° is obtained for the monointercalating 9-aminoacridines.

Helix extension slopes are obtained from viscosimetric variations (η) of sonicated rodlike fragments of DNA by addition of the drugs. Data are fitted from the equation $\log \eta/\eta_o = s.\log(1 + 2r)$ where r is the ligand binding ratio defined in nucleotide units (Le Pecq *et al.*, 1975). The value of the slope is expected to vary between 2 and 3 for monofunctional ligands, to lie between 4 and 6 for bisintercalators and to be greater than 6 for tris-intercalating drugs.

([a]) Results from Wakelin (1986). The representation used is based on the equation $L/Lo = 1 + mr$, where L/Lo is the relative increase in contour length in the presence of the drug, approximated to the cube root of the ratio of measured reduced viscosities (η) of rodlike fragments of DNA, r is the ligand binding ratio, per base pairs. The theoretical slopes m expected in this representation are 1 for monointercalator and 2 for bisintercalators.

In agreement with the length of the linker, the first reported synthetic bifunctional acridine *5* (Barbet *et al.*, 1975), bis-intercalates into DNA as shown by the helix unwinding angle of closed circular DNA (38°) and the increase in the contour length of sonicated rodlike DNA fragments, which were twice those found for the corresponding monomer (Table 1). Bis-intercalation of *5* was shown to occur following the excluded site model and, as expected, its DNA binding affinity Ka $\simeq 10^{11}M^{-1}$ was close to the square of the monomer affinity (Le Pecq *et al.*, 1975). Strikingly, the dimer with unsubstituted acridine rings *4* led to unwinding angle and contour length enhancement reminiscent of those of the monofunctional analog, although NMR experiments with $d(AT)_5 \cdot d(AT)_5$ argued in favour of bis-intercalation (Assa-Munt *et al.*, 1985). More generally, dimers with unsubstituted acridines gave anomalous behaviour regarding DNA bis-intercalation (Denny *et al.*, 1985a), a result which could be related to differences in the DNA binding properties of both types of acridines (Gaugain *et al.*, 1981). Accordingly, the 6-chloro-2-methoxy-9-amino-acridine was shown by NMR to exhibit a strong pyrimidine-3′,5′ purine preference, with an orientation of the 9-amino group in the minor groove similar to that found by X-ray analysis in minihelical IodoCpG-drug complex (Reddy *et al.*, 1979). In contrast, at the level of autocomplementary oligonucleotides, the unsubstituted 9-aminoacridine displays no sequence preference in both the solvated (Gaugain *et al.*, 1981) and solid states (Sakore *et al.*, 1979). Moreover the presence of the chloro and methoxy substituents increases the size of the ring with a concomitant reduction in the degree of freedom in the intercalation site and enhances the ring polarisability, leading to compounds with DNA affinities at least 10 times higher than that of the unsubstituted 9-aminoacridine. Accordingly, in the series of 9-aminoacridine related dimers, the apparent anomalies between the results of NMR experiments and those derived from helix extension parameters and unwinding angles could be due to the occurrence of different types of DNA complexes for the same molecule including monointercalation, bis-intercalation in both grooves, interduplex cross-linking etc.

I.2 - *DNA polyintercalation of acridine dimers and trimers with carboxamidoalkyl linkers.*

In order to obtain molecules able to bind to DNA with binding constants even higher than that of the spermine diacridine *5* (Table 2),

TABLE 2 - *DNA intercalating properties of acridine monomers, dimers and trimers with carboxamidoalkyl linkers.*

$NH-(CH_2)_n-CONH_2$ Mo B

$-(CH_2)_n-NHCO-(CH_2)_m-CONH-(CH_2)_n-$ Di A

$-(CH_2)_n-CONH-(CH_2)_m-NHCO-(CH_2)_n-$ Di B

$-(CH_2)_n-NHCO-CH_2-CH-CH_2-CONH-(CH_2)_n-$ Tri A

$-(CH_2)_n-CONH-(CH_2)_m-NHCO-CH_2-CH-CH_2-CONH-(CH_2)_m-NHCO-(CH_2)_n-$ Tri B

N°	series			n	m	d, Å	φ°	s[a]	m[b]
6	Mo	B	X	2	—	—	17	2.6	0.90
7	Mo	B	Y	2	—	—	17	2.3	0.90
8	Di	A[b]	X	3	3	17	28.5	—	1.49
9	Di	B[b]	X	2	3	14	30	—	1.05
10	Di	B	X	2	5	17	32	7.5	—
11	Di	B	Y	2	5	17	51	7.3	—
12	Tri	A[b]	X	7	—	13	22	—	1.32
13	Tri	B	X	2	5	17	55	7.0	—
14	Tri	B	Y	2	5	17	ND	10.6	—

d is the estimated interchromophores distance (± 1 Å).

Helix unwinding angle φ° are obtained from viscosity variations of circular closed DNA.

([a]) Slopes of helix extension *s* or *m* obtained from viscosimetric measurements with sonicated rodlike DNA: *s* values are fitted following Le Pecq *et al.* (1975) and *m* values are fitted following (Wakelin, 1986) (see legend table 1).

([b]) Results from Wakelin (1986). Series: X ($R_1 = R_2 = H$), Y ($R_1 = OCH_3, R_2 = Cl$).

acridine trimers have been synthesized by several groups. Due to the difficulty of preparing such compounds, the first described trimers were made up of 9-aminoacridine moieties linked by carboxamidoalkyl chains of various lengths (Atwell *et al.*, 1983; Gaugain *et al.*, 1984).

As shown in Table 2, the DNA unwinding angle and the extension of rodlike DNA are in agreement with a bis-intercalation for the dimers whose separation between the aromatic rings ranges 17 Å while a shorter distance (14 Å) led to more ambiguous results. It is interesting to observe that the inversion of the amide bond, between compounds *8* and *10*, does not change the bis-intercalating ability.

The importance of the inter-chromophore distance for poly intercalation is clearly illustrated in the case of the trimers reported in Table 2. Even though a distance of 13 Å was shown to be sufficiently long to span one and even two base-pairs in the drug-DNA complex, the trimer *12* behaved once more as a bifunctional intercalator. In contrast, the DNA length increase induced by compounds *13* and *14* was at least 3 times higher than those of the corresponding monomers *6* and *7*. Moreover for the trimer *13* the unwinding angle also favoured a tris-intercalation. No definite conclusion on the tris-intercalation of *14* into DNA could be drawn as the complex between *14* and circular closed DNA was insoluble.

The dimerization of 9-aminoacridines by a carboxamidoalkyl chain led to dimers *10* and *11* with a threefold order of magnitude increase in DNA affinity (Kapp $\sim 10^7 M^{-1}$) but addition of a third ring in trimers *13* and *14* did not significantly enhance their DNA affinity. This seems to indicate that introduction of several rigid carboxamido groups into the linker produces unfavourable entropic factors. Nevertheless it is interesting to note that in contrast to the trimer *12*, the introduction of two additional amide bonds in the linking chain of *13* and *14* led to a less stringent steric parameter for the linker, allowing a favourable orientation of the three acridine rings to tris-intercalate in DNA.

The position of the carboxamido group in *13* and *14* has been chosen to allow a preferential recognition of G-C base-pairs, a feature already encountered with the parent monomer *7* and related to the formation of a hydrogen bond between the CO of the chain and the amino group of a guanine (Gaugain *et al.*, 1981; Markovits *et al.*, 1981). Using DNAs of various C-G content, the dimers *10* and *11*, as well as the trimers *13* and *14*, have been shown to be devoid of sequence specificity. Nevertheless, due to the low probability of finding 3′-5′ C-G-C-G-C-G sequences in DNA,

this result requires confirmation, e.g., by use of footprinting with DNA fragments of known sequences.

On the other hand, the dimers *8* and *9* exhibited cytotoxicity higher than their parent monomer (Atwell *et al.*, 1986) suggesting that the antitumor properties of *10* and *11* should be tested. Nevertheless neither *13* nor *14* showed important antitumor properties, since for *13* doses higher than 10^{-6}M were required for inhibiting growth of L1210 cells and blocking their cloning, while *14* was almost completely inactive.

I.3 - *Intercalation of the carboxamidoalkyl chain dimer,* 11 *by NMR.*

The 6-chloro 2-methoxy 9-aminoacridine dimer bearing a carboxamidoalkyl chain *11* exists in solution (acetate buffer, 25 mM Na^+, pH 5.5, 27°C) in a folded conformation since, even at low concentrations (8×10^{-5} M) all aromatic protons of its NMR spectrum are still shielded (0.2 to 0.5 ppm) with respect to the monomer. All folded dimers, at high concentration ($> 10^{-4}$ M) are able to form larger stacked aggregates with an oligomerization constant Ks $\simeq$ 1150 M^{-1} (non-cooperative process) very similar to that of the corresponding monomer.

Upon interaction with a self complementary deoxytetranucleotide $d(CpGpCpG)_2$ lines broaden and all species remain in fast exchange on the NMR time scale (400 MHz) (Fig. 2). All aromatic protons of the dimer are strongly upfield shifted (0.8 to 1 ppm) when compared to the free monomer at low concentration. These shifts are unchanged when the dye to helix ratio is increased (up to 0.67) suggesting that even at high ratio almost all the dimer is bound to the helix. The magnitude of the upfield shifts is indicative of bis-intercalation of the dimer.

To obtain more information on the interaction of the dimer with $d(CpGpCpG)_2$, NOE experiments (Fig. 3) were performed on a solution containing 2 dimer molecules for 3 helices (ratio 0.67). The following points emerged:

i) The absence of NOE between $^2G_8$ and $^1C_{2'}$ protons, $^4G_8$ and $^3C_{2'}$ protons concomitantly with the persistence of NOE between $^3C_6$ and $^2G_{2'2''}$ protons indicate that the dimer bisintercalates in the ^1C-^2G and ^3C-^4G sites in agreement with the excluded site model.

ii) The NOE's observed between the dimer methoxy group and both $^1C_6$, and $^2G_8$, as well as between the dimer H_7 and the $^3C_6$ and $^4G_8$ protons allow the following conclusions to be drawn: — the complex retains the twofold symmetry of the helix (dimer rings are inverted with

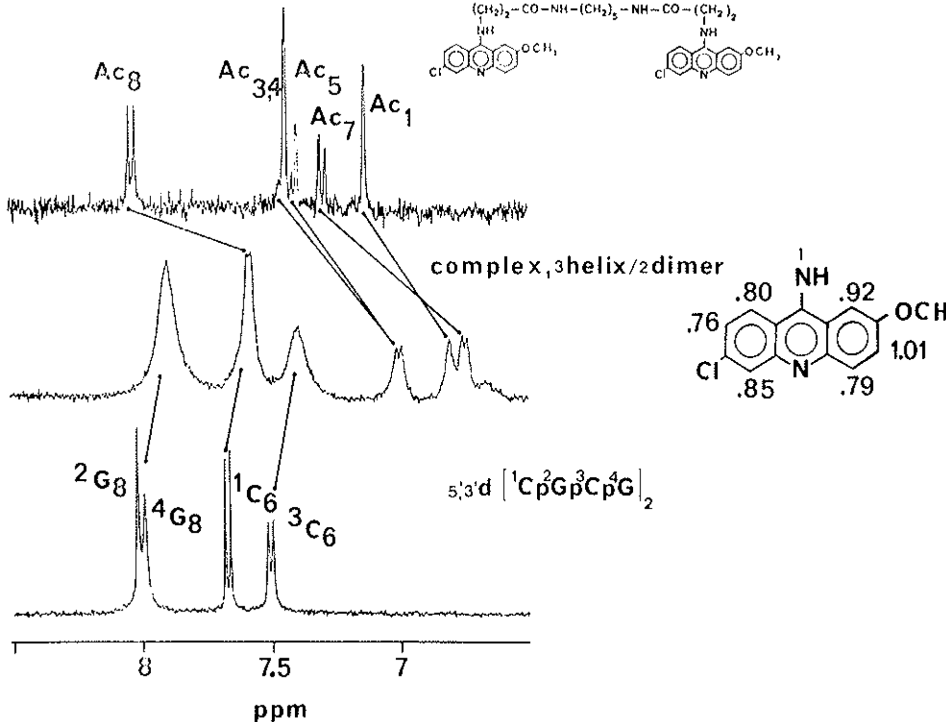

FIG. 2. Carboxamidoalkyl chain linked dimer *11*, -d[CpGpCpG]$_2$ interaction by NMR in 25 mM NaCl, CD_3CO_2D buffer pH 5.5, 27°C. Top: dimer *11*; bottom: oligonucleotide; middle: complex (2 drug/3 helices).

respect to each other); — dimer H_7 and methoxy groups point towards the major groove of the helix. Since these protons are on the same side of the acridine ring as the linking chain, the latter also lies in the major groove.

Major groove binding, though unusual for intercalators, is commonly shared by DNA binding proteins since selective sequence interactions can be obtained through hydrogen bonding (Anderson *et al.*, 1981). It is noteworthy that the methylene protons of the linking chain close to the NH group are nonequivalent, indicating a strong hindered motion at this level. Furthermore examination of a Dreiding model for the d(CpGpCpG)$_2$-acridine dimer complex indicates that hydrogen bonding could occur between the O_6 of the two central guanines on the opposite strands and the

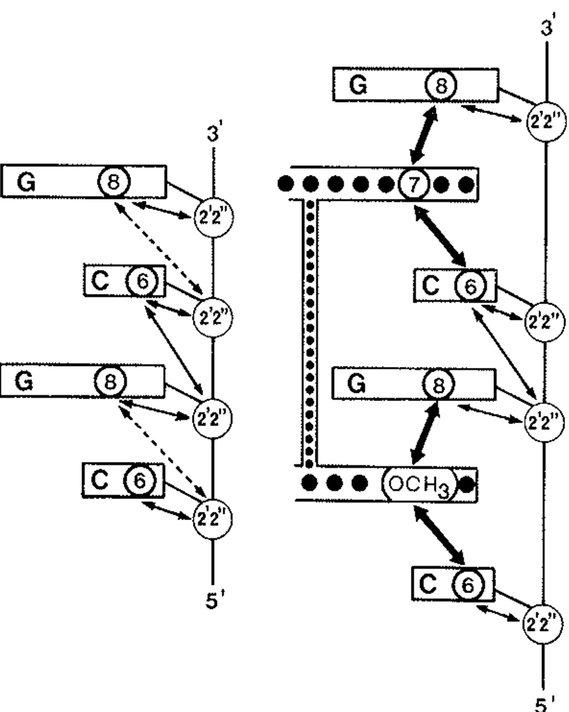

FIG. 3. Proposed mode of intercalation of dimer 11 into d[CpGpCpG]$_2$ from NOE data. Arrows correspond to NOE effects observed either in d[CpGpCpG]$_2$ (left) or in the complex (right). *Dashed arrows*: NOEs disappearing upon complexation. *Solid arrows*: NOEs persisting upon complexation. *Solid large arrows*: Intermolecular NOEs between dimer 11 and d[CpGpCpG]$_2$.

two NHs of the linking chain. Experiments are now in progress to shed some light on the latter point.

I.4 - *A trimeric acridine with DNA affinity in the range of DNA regulatory proteins.*

The preceding results emphasize the crucial role of the nature of linking chain, since acridine dimers with positively charged polyamine chains such as *5* showed DNA binding affinities several orders of magnitude higher than that of the corresponding acridine with a carboxamidoalkyl linker. Therefore a trimer *15*, bearing an aminoalkyl chain

of enhanced flexibility, was synthesized through reduction of the amide groups of *14* (Laugâa *et al.*, 1985). It was not possible to obtain the trimer corresponding to *13* because the 9-aminoacridine moieties of the desired product were very rapidly transformed in acridone.

The length of the linking chain was selected to allow intercalation of each chromophore according to the excluded site model. ^{1}H NMR studies have shown that, at 5 mM NaCl, pH 5, the acridine trimer occurred under a folded conformation stabilized by stacking interactions between the three aromatic rings. DNA tris-intercalation of the dye at a low dye-base pair ratio was shown by measurements of both the unwinding of PM2 DNA and the lengthening of sonicated rodlike DNA. The main DNA binding properties of the trimer *15* and the corresponding dimer *5* and monomer are reported in Table 3. The trimer exhibits a high DNA affinity for poly[d(AT)] (Kap = 8×10^8 M^{-1}, 1M NaCl) as shown by competition experiments with ethidium dimer. Kinetic studies of both the association with poly[d(AT)] and the exchange between poly[d(AT)] and sonicated calf thymus DNA have been performed as a function of the ionic strength. In 0.3 M Na^+ the on-rate constant ($k_1 = 2.6 \times 10^7$ M^{-1} s^{-1}) is similar to that reported for other mono or diacridines, whereas the off-rate constant is very small (k_{-1} = 1.2 10^{-4} s^{-1}), leading to an equilibrium binding constant as large as $K_{ap} = 2.2 \times 10^{11}$ M^{-1}. A plot of log (k_1/k_{-1}) as a function of log[Na^+] yielded a straight line whose slope shows that 5.7 ion pairs (out of a potential 7) are formed upon the interaction with DNA. From this linear relationship a K_{ap} value of 10^{14} M^{-1} in 0.1 M Na^+ can be estimated. Such a value reaches and even goes beyond that of some DNA regulatory proteins. This acridine trimer appears to be the first synthetic ligand with such a high DNA affinity. Only one other trifunctional acridine with aminoalkyl chains has been described (Hansen *et al.*, 1984). The distance between each unsubstituted 9-aminoacridine ring lies around 6 Å suggesting that a "one base pair sandwich" tris-intercalated complex can occur only through strong deformation (bending or kinking) of the DNA.

II - Comparative antitumor properties of mono and poly-functional acridines.

Amsacrine (mAMSA), a representative of the series of monomeric acridine, is one of the more recently introduced drugs in cancer chemotherapy (Clinical Trial, 1978). Its antineoplasic action has been studied

TABLE 3 - *DNA binding characteristics of 6-chloro-2-methoxy 9-amino-acridine derived monomers, dimers and trimers with aminoalkyl linking chains.*

$(CH_2)_3-NH-(CH_3)_2$

AcMo

$(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3$

AcDi

$(CH_2)_3-NH-(CH_2)_5-NH-(CH_2)_2-CH-(CH_2)_2-NH-(CH_2)_5-NH-(CH_2)_3$

AcTri

	d, Å	Kap (M^{-1})	unwinding angle ϕ^o	helix extension s
AcMo		3.10^6	20	2.1
AcDi	17	2.10^{11}	41	4.8
AcTri	17	10^{14}	66	9.

Apparent binding constants Kap are very high and were deduced from kinetic experiments (Kap = k_1/k_{-1}). The on-rate constants for binding to poly[d(A-T)] were measured by stopped flow using fluorescence detection. Off-rate constants were measured by displacing the dyes bound to poly [d(A-T)] by an excess of GC rich DNA.

Helix extension slopes were obtained from viscosimetric measurements on sonicated rodlike fragments of DNA and the data fitted as described in the legend of Table 1.

Helix unwinding angles are obtained from viscosimetric variations of covalently closed circular DNA.

in detail and seems to be related to its ability to generate protein associated DNA strand breaks in mammalian cells (Nelson *et al.*, 1984). In this mechanism topoisomerase II appears to be the primary target. The biological activity of several acridine dimers with alkyl linking chains of increasing length was extensively studied by Canellakis and colleagues (1976a,b). As a general feature bis-intercalating dimers are more potent in inhibiting the growth of P 388 leukemia cells in culture than their monofunctional homologues. However, significant *in vivo* activity was only observed in the series of 9-aminoacridines with C_6, C_7 and C_8 linkers (Wakelin *et al.*, 1986). One of these (a C_6 diacridine) was evaluated in clinical trials (Golding, 1981), but was soon discarded as the soluble form displayed undesired secondary effects and only marginal antitumor activity (Schepartz *et al.*, 1981).

A recent review of Wakelin (1986) summarizes all the data reported in the series of diacridines. Briefly, among all the dimers linked by aminoalkyl or amidoalkyl chains so far synthesized, some have been claimed to be potential antitumor drugs, based on their cytotoxic effect on cell cultures; however, they did not reveal significantly active *in vivo* (Wakelin, 1986; Denny *et al.*, 1983) except those with rigid linker recently described by Denny (1985b).

Analysis of DNA binding kinetics of various diacridines formed at the 9-position by different linkers led Denny (1985b) to separate them into several classes: i) the diacridines linked by flexible chains which have slower dissociation rates as compared to the monomers but show relatively fast chromophore exchange kinetics among DNA binding sites and are not active *in vivo*; ii) the diacridines linked by aminoalkyl chains, whose exchange kinetics are slowed by the positively charged linker but remain inactive; iii) the diacridines linked by polar relatively rigid carboxamide chains which are disappointingly inactive; iv) the diacridines linked by rigid dicarbamoylpyrazole chain with slow exchange kinetics and greatly reduced chromophore exchange between the sites which seem to exhibit significant *in vivo* antileukemic activity.

III - Bis-7H-pyrido[4,3-c]carbazoles as new antitumor agents.

The results obtained using the acridines as intercalating agents have shown that bis-intercalators with high affinity for DNA can be obtained. Nevertheless the antitumor properties of the acridine dimers or trimers appeared somewhat deceptive. Therefore in order to obtain new com-

pounds with high antineoplasic activity we replaced the acridine subunits by larger pyridocarbazole rings and used a rigid chain to prevent the unfavourable intermolecular self-association of the dimer. Among the synthesized molecules (Pélaprat *et al.*, 1980) one of them, ditercalinium, showed strong antitumor activity and is now under preclinical investigation.

A number of closely related analogs of ditercalinium, in which the 7H-pyrido[4,3-c]carbazole subunits were replaced with various pyridocarbazole rings, displayed a strongly reduced cytotoxicity. These features are indicative that the geometry of the complexes is another important parameter to be considered. Intercalation might provoke modifications in the DNA conformation which, in turn, could possibly alter the binding properties of the polymer with regard to its natural ligands (e.g., regulatory proteins, polymerases, topoisomerases, methylases, ...). The structure of the complex formed, in aqueous solution at pH 5.5, between ditercalinium and the self complementary tetranucleotide $d[CpGpCpG]_2$ was investigated by 400 MHz ^1H NMR. For a 1:2.5 drug to helix ratio the dimer occurred only under bound form, whereas free and complexed tetranucleotide were in slow exchange, allowing unambiguous assignment of the protons in the complex through exchange polarisation transfer measurements. The tetranucleotide existed as a right handed double helix in the complex. The strong upfield shifts measured on most aromatic protons on both drug and nucleobases, as well as on DNA imino protons, were consistent with bisintercalation of the dimer. According to the negative NOE's generated on protons on the convex edge of the bound drug rings by saturation of sugar protons, it was concluded that ditercalinium was intercalated with its rigid bis-ethylbispiperidine spacer fitting the major groove of the helix (Fig. 4).

IV - Modulation of the antitumor activity by the linking chain rigidity in the series of ditercalinium.

As previously discussed, the most active dimers were found in the series of 7H-pyrido[4,3-c]carbazole, in which the nitrogen in position 2 was quaternarized by a rigid bis-ethylbipiperidine chain, indicating that the nature of the linker was essential for activity (Pélaprat *et al.*, 1980). The reduced flexibility of the spacer prevents the intramolecular stacking of the two aromatic rings, a feature unfavourable for the DNA bisintercalation process (Delbarre *et al.*, 1981). All these results underline the crucial role that a restricted flexibility of the DNA bisintercalating compounds seem

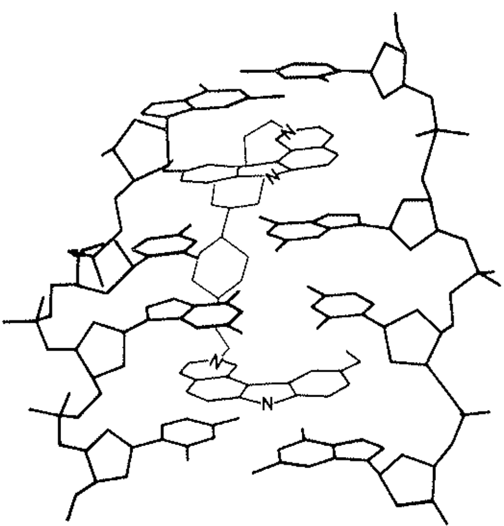

FIG. 4. Structure of the ditercalinium d[CpGpCpG]$_2$ complex proposed from NMR analyses.

to play in the occurrence of antitumor activity. Since our dual purpose was to obtain more active dimers and to elucidate the precise structural features yielding antitumor activity, we synthesized analogs of ditercalinium only modified at the center of the dimerization chain by an extension of one and two methylenes. The pharmacological activity of these compounds was then compared in relation to their conformational and DNA binding properties. The results reported in Table 4 show that the biological activity is associated with a short spacer ($m = 0$ or $m = 1$ as *16* and *17*) and decreases progressively with its lengthening. The delayed toxicity observed for all compounds, as shown by a large difference between ED_{50} and CE_{37} values and the appearance of small abortive clones in semisolid medium (Esnault *et al.*, 1984), argues for a similar mode of action. With regard to conformation both types of dimer, the more active *16* and the less active *19*, behave similarly whereas compound *17* can adopt a "kinked" structure, similar to that recently found in the case of triostin (Scheldrick *et al.*, 1984) which decreases the strong tendency to form intermolecular stacking observed with *16* and *18*. The lack of stacking, favoring DNA intercalation might explain the higher DNA-affinities observed for compounds of the *17* series. All these compounds are DNA bisintercalators but the two most active series display the largest and weakest DNA binding affinity (10^8 and $10^7\,M^{-1}$). This emphasizes again

TABLE 4 - *Antitumor activities on L 1210 murine leukemia of 7H pyridocarbazole dimers.*

2 $CH_3SO_3^-$,

2 CH_3SO_3H

Compounds		Cellular Toxicity on L1210		Antitumor Activity in vivo on L1210		
	m	ED_{50}[a]	CE_{37}[b]	M.T.D.	O.D.	T/C%
16	0	0.2	0.026	10	10	182
17	1	1.2	0.07	30	7	175
18	2	1.5	0.25	20	5	143
19	3	0.5	1.	10	5	n.s.

Cellular toxicity: ([a]) Dose (µg/ml) which inhibits 50% of the cell growth after 24 hr exposure to the drug. ([b]) Dose (µg/ml) required to inhibit the cloning efficiency to a factor of 37%.

Antitumor activity in vivo on L1210 infected mice. M.T.D.: Maximal tolerated dose (mg/kg); O.D.: Optimal dose (mg/kg); T/C %: Treated mean survival time per control mean survival time; n.s.: Non-significative values.

that a high DNA affinity is required for antitumor activity but that this characteristic alone is not sufficient. Likewise circular dichroism analyses indicate that the biological activity does not correlate with the ability of dimers to bind differentially to B and Z DNA in the present 7H-pyridocarbazole series.

The critical relationship between the antitumor potency and the degree of freedom of the linking chain, led us to investigate the importance of having two identical intercalating moieties for activity. A rigid heterodimer made up of a 7H-pyrido[4,3-c]carbazole and a 6-chloro 2 methoxy 9-aminoacridine linked by a bis-ethyl bipiperidine chain (Fig. 5) was therefore synthesized. As compared to ditercalinium, this new compound was found to be inactive on the various biological tests.

FIG. 5. Formula of the acridine 7H-pyridocarbazole heterodimer.

Biologically active dimers are cytotoxic on E. coli *pol* A mutant and not on *pol* A *uvr* A double mutant suggesting that some dimers are able to induce *in vivo* DNA conformational changes which are recognized by the *uvr* ABC repair system in E. coli (Le Pecq and Roques, 1986). Thus the activity of these dimers would require both a particular distortion in DNA structure and a residence time long enough for the distortion to be recognized by DNA enzymes. Studies are now in progress, especially by means of NMR to analyse what kind of distortion, able to induce the cytotoxicity, is involved in the DNA drug-complex.

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MECHANISM OF THE ANTITUMOR ACTION OF ELLIPTICINES, ISOELLIPTICINES AND ANALOGUES: COMPARISON OF MONO- AND BIFUNCTIONAL INTERCALATORS

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ABSTRACT

Many recent studies suggest strongly that the cytotoxic effect of DNA monointercalating agents is mediated through the action of DNA topoisomerase II. After the cells are treated with these drugs, the enzyme remains inserted at specific sequences in the DNA double helix. DNA breaks are revealed by detergent or proteinase K treatment. Bifunctional intercalators derived from 7H-pyridocarbazole appear to elicit their action by a completely different mechanism, possibly involving the DNA repair system.

Among the various drugs which are used in the treatment of human cancer, the DNA binding molecules represent some of the most active and useful agents. Many of these drugs are from natural origin (daunorubicin, adriamycin, actinomycin D, bleomycin, echinomycin, ellipticine...). A few have been obtained through chemical synthesis (m-AMSA,

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mitoxantrone, ditercalinium...). The interaction of these compounds with DNA has been studied by many scientists and these studies were recently reviewed (Wilson and Jones, 1981; Waring, 1981; Zimmer and Wähnert, 1986; Wakelin, 1986). These studies have culminated with the structure determination of drug-oligonucleotide mixed crystals at atomic resolution by X-ray diffraction. These studies confirmed that most of these agents bind to DNA by intercalating an aromatic ring between two adjacent base pairs according to the model first proposed by Lerman (1961). In addition the study of the echinomycin-oligonucleotide complex revealed that the binding of such a drug could be associated with unusual DNA conformational changes such as the formation of Hogsteen base pairs (Quigley *et al.*, 1986). One could have thought that such detailed characterization and understanding of the DNA-drug complexes at the molecular level would lead to parallel understanding of the mechanism of the pharmacological action of these agents. Unfortunately, this has not yet been the case, although important progresses have recently been achieved. One of the main difficulties is to identify what are the features of the drug DNA complex which are of pharmacological significance, if DNA is really the pharmacological target of these drugs. One must also understand what are the physiological consequences of the DNA structural alteration by the drug binding and how the drug-DNA complex is recognized by the various proteins and enzymes using DNA as a template or a substrate. Such a problem can be approached by studying in parallel the physico-chemical characteristics of the DNA complexes and the biological and pharmacological properties of a large series of related compounds.

One important physico-chemical parameter which characterizes the DNA drug complexes is the value of the binding constant. To determine the influence of this parameter on the pharmacological activity of various compounds, this binding constant has to be varied by several orders of magnitude.

In this context, ellipticine derivatives constitute a very interesting series. Ellipticine is an antitumor alkaloid found in plants growing in the Pacific islands and which derives from 6H-pyridocarbazole. Many analogues have been prepared. One of them (N-methyl-9-hydroxy ellipticinium, NMHE) has recently been introduced in clinic and elicits therapeutic activity against bone metastasis of breast cancer (Paoletti *et al.*, 1980). Molecules with intercalating rings differing slightly in shape and size have been obtained by preparing derivatives of 7H-pyrido-

carbazole (Pelaprat *et al.*, 1980a), 11H-pyridocarbazole (Lescot *et al.*, 1986) and 5H-pyridopyrroloisoquinoline (Ducrocq *et al.*, 1979), the structure of which is shown in figure 1.

In addition, bifunctional intercalating molecules which are characterized by extremely high DNA binding constants (Le Pecq *et al.*, 1975; Capelle *et al.*, 1979; Laugaa *et al.*, 1985; Le Pecq and Roques, 1986) could be prepared from different pyridocarbazole rings (Pelaprat *et al.*, 1980b). Therefore, the numerous molecules of this series are of particular interest to study the structure activity relation of DNA intercalating drugs.

Before discussing the mechanism of action of any DNA binding drug, a basic question must be answered. It must be decided whether the biological action of these derivatives results directly or indirectly from their binding to DNA *in vivo.* Until very recently, it was generally accepted that, indeed, this was the case. It had been observed that the binding of these drugs to DNA inhibited *in vitro* DNA polymerases and DNA dependent RNA polymerases (rev. Waring, 1981). Extrapolating these results to *in vivo* situation, it was proposed that the cell toxicity of these compounds resulted from unscheduled DNA synthesis arrest. Tumor cells were thought to be more sensitive because of their higher rate of cell division. Such a simple model has recently been challenged. Tritton and Yee (1982) even suggested that DNA intercalating molecules could kill the cells from without. This proposition resulted from the following experiment. Adriamycin was attached to large particles which cannot enter cells. These adriamycin-loaded particles were found able to kill efficiently the cells without reaching of course the cell DNA. This experiment is quite interesting and provocative. But it can be argued that adriamycin covalently linked to a particle is no longer adriamycin and that a large particle with its surface covered with adriamycin might have properties quite unrelated to adriamycin itself.

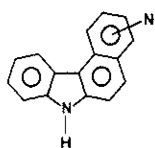
It is worth mentioning two sets of data which demonstrate more directly that DNA replication arrest is not the primary cause of the cell death induced by DNA intercalating agents.

Cells are killed at doses which do not block DNA-RNA or protein synthesis.

Charcosset *et al.* (1985) studied the effects of ellipticine derivatives on two cell lines derived from lung Chinese hamster. A parental cell line (DC-3F) was found very sensitive to these drugs and a variant cell line

6H-pyridocarbazole

5H-pyridopyrroloisoquinoline



7H-pyridocarbazole

11H-pyridocarbazole

2-NCH_3 10-OCH_3 7H-pyridocarbazolium

2-NCH_3 8-OCH_3 5-CH_3 11H-pyridocarbazolium

Ellipticine (E)

$R=(CH_2)_3-N(C_2H_5)_2$

BD 40

FIG. 1. Comparative structure of pyridocarbazole ring and derivatives

(DC-3F/9-OH-E) was rendered resistant by adaptation to increasing concentrations of 9-hydroxy ellipticine (Salles *et al.*, 1982. Interestingly, the membrane permeability of the variant cell line to ellipticine derivatives was found unmodified (Charcosset *et al.*, 1983). The sensitive cell line was killed at 9-OH-ellipticine doses, which did not induce any inhibition of DNA, RNA or protein synthesis. Contrastingly, in the case of the resistant cell line, the drug doses which induced the cell death markedly inhibited both DNA and RNA synthesis.

This experiment clearly demonstrates that tumor cells sensitive to 9-hydroxy-ellipticine are not killed by this drug because of DNA synthesis arrest. DNA inhibition is observed at doses which are much higher than those inducing cytotoxicity. This experiment also underlines that results obtained in one cell line cannot be extrapolated to others without caution.

Most of the DNA intercalating drugs block cells in the G2 part of the cell cycle.

The effect of drugs on cell cycle can be very easily studied by flow-cytofluorometry. It is observed for most of the DNA monointercalating drugs that exposed cells go through S phase where DNA synthesis occurs without being slowed down. Block occurs only in the G2 phase after DNA synthesis is completed (rev. Rao, 1979). These experiments show clearly that DNA synthesis is not prevented *in vivo* in the presence of the drugs at pharmacological doses. Experiments where DNA synthesis inhibition was measured in non-synchronized cells, using labelled thymine incorporation after long incubation times, have been very misleading. The apparent thymine incorporation inhibition observed in these experiments resulted certainly from the arrest of the cell in G2 and therefore from the impossibility for them to enter the S phase where DNA synthesis takes place.

It can therefore be concluded that DNA synthesis arrest does not appear as the primary cause of the cell toxicity of DNA intercalating drugs. However this does not permit to conclude as it has been done sometimes that DNA is not involved directly or indirectly in the mechanism of action of these drugs. In absence of detailed studies on the mechanism of action of these compounds at the cellular level, the only way to approach this question was to study the relation between the DNA binding characteristics and the cytotoxicity of a large series of chemically related derivatives.

Studies performed on acridine and pyridocarbazole derivatives have

led to the same conclusion. DNA binding ability is required to get biological activity, but this property alone is not sufficient (Le Pecq *et al.*, 1974). In these two series, no molecule with no DNA binding ability has ever been found. Among the DNA binding derivatives, there is only a slight correlation between DNA affinity and cytotoxicity. In the acridine series, one of the most striking illustrations of this phenomenon is given by the comparison of the properties of m-AMSA and o-AMSA (Structure figure 2) (Ferguson and Denny, 1980; Denny *et al.*, 1983). These two compounds differ only by the position of a methoxy group on a phenyl ring which is probably not involved in the DNA interaction. The DNA binding characteristics of the two compounds are very similar, but only m-AMSA is cytotoxic and has antitumor action.

In the pyridocarbazole series as seen in tables 1 and 2 minor modifications on the molecules lead to small variations of their DNA affinities and dramatic changes of their biological properties. The comparisons of 6H (ellipticine), 7H (Pelaprat *et al.*, 1980a), 11H-pyridocarbazole (Lescot *et al.*, 1986) and desmethyl ellipticine (Gouyette *et al.*, 1980) are of interest from this point of view. Abolishing DNA binding by partial reduction of the aromatic ring suppressed in all cases the biological activity.

These data suggest therefore that DNA might be involved in the biological activity of these compounds, but that some other factors are required, such as for instance proteins recognizing some specific features of the DNA-drug complexes.

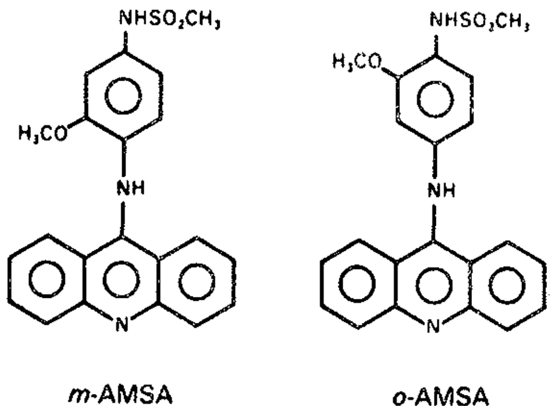


FIG. 2. Structure of o-AMSA (inactive) and m-AMSA (active).

TABLE 1 - *Structure of ellipticine derivatives.*

	R_2	R_5	R_6	R_7	R_9	R_{11}
9-OH-E	—	CH_3	H	H	OH	CH_3
6-CH_3 9-OH-E	—	CH_3	CH_3	H	OH	CH_3
NMHE	CH_3	CH_3	H	H	OH	CH_3
11-desmethyl NMHE	CH_3	CH_3	H	H	OH	H
9-NH_2E	—	CH_3	H	H	NH_2	CH_3
9-OCH_3-E	—	CH_3	H	H	OCH_3	CH_3
11-desmethyl 9-OH-E	—	CH_3	H	H	OH	H
E	—	CH_3	H	H	H	CH_3
2-CH_3E	CH_3	CH_3	H	H	H	CH_3
11-desmethyl 9-OCH_3-E	—	CH_3	H	H	OCH_3	H
9-F-E	—	CH_3	H	H	F	CH_3
7-OH-E	—	CH_3	H	OH	H	CH_3
9-Br-E	—	CH_3	H	H	Br	CH_3

Recent studies performed on DNA monointercalating compounds on one hand and on DNA bisintercalating compounds on the other hand have confirmed this proposition.

I. DNA TOPOISOMERASE II-DNA COMPLEX: A TARGET FOR MONO-INTERCALATING AGENTS INCLUDING ELLIPTICINE DERIVATIVES

Ellipticine derivatives action on DNA topoisomerase II resembles very much that of other monointercalating agents. Therefore the action of these agents on DNA topoisomerase II in relation with their antitumor activity must be discussed. The effect of ellipticine derivatives on this enzyme, which differs from that of other intercalating agents, will be underlined.

TABLE 2 - *Comparison of cytotoxicity on* L1210 *cells in tissue culture of a series of ellipticine derivatives with DNA binding constant* (0.1 M NaCl, Tris-HCl 0.05 M pH 7.4). *Compounds have been ordered according to their cytotoxicity.*

Compound	ID^{e}_{50} (μM)	DNA binding M^{-1} constant	Reference
9-OH-E	0.015	2×10^6	a
$6-CH_3$-9-OH-E	0.022	1.2×10^7	a
$2-CH_3$-9-OH-E (NMHE)	0.05	1.1×10^6	a
$2-CH_3$-11-desmethyl-9-OH-E	0.25	9×10^4	b
$2N-CH_3-10-OCH_3$-7H-Pyr	0.3	3×10^5	c
$9-NH_2$-E	0.53	1.2×10^6	a
$9-OCH_3$-E	0.6	1×10^5	a
11-desmethyl-9-OH-E	0.85	4×10^5	b
E	1	1.5×10^5	a
2 CH_3-E	1.7	2.3×10^5	a
11-desmethyl-$9-OCH_3$-E	3.8	1.1×10^5	b
9-F-E	3.9	6.4×10^5	a
7-OH-E	5.4	1×10^5	a
9-Br-E	13	4×10^5	a
$2N-CH_3-8-OCH_3-5-CH_3$-11H-Pyr	20	1×10^5	c
Actinomycin D	0.004	2.3×10^6	d
Adriamycin	0.025	3×10^6	d

Results for actinomycin D and adriamycin are given for reference. a) Paoletti *et al.* (1980); b) Gouyette *et al.* (1980); c) Lescot *et al.* (1986); d) Esnault *et al.* (1984); e) concentration of agent causing a 50% inhibition of growth after 48 h incubation of L1210 cells.

It was first observed that DNA intercalating agents such as adriamycin, ellipticine derivatives, m-AMSA induced DNA strand breaks in mammalian cells (Paoletti *et al.*, 1979; Ross *et al.*, 1978, 1979; Ross and Bradley, 1981; Zwelling *et al.*, 1981, 1982). Proteins were found associated at the breaks. These breaks occurred well at doses which corresponded to the pharmacological action. Studies with purified DNA topoisomerase II showed that, indeed, the DNA strand cleavage reaction could be reproduced *in vitro* (Nelson *et al.*, 1984; Tewey *et al.*, 1984a,b). Interestingly, m-AMSA, an active compound, could induce DNA breaks *in vitro* as o-AMSA, an inactive analog, could not. Later, it was confirmed

that *in vivo* DNA topoisomerase II was indeed associated with the DNA breaks in treated cells. DNA extracted from these cells could be precipitated by antibodies directed against DNA topoisomerase II (Mindford *et al.*, 1986; Yang *et al.*, 1985). Recently, a strong correlation between topoisomerase II linked DNA breaks, both *in vitro* and *in vivo*, and drug cytotoxicity for a variety of m-AMSA analogues was reported (Rowe *et al.*, 1986).

DNA topoisomerase II (rev. Wang, 1985) catalyses the passage of a double stranded DNA chain across another one. In covalently closed circular DNA, this changes the topological linking number by step of two. The supposed mechanism of action of the enzyme is illustrated in figure 3. The enzyme gets first inserted in the double helix forming covalent links between tyrosine of the protein and 5′ phosphate termini of DNA. The enzyme allows the concerted passage of a double stranded DNA through the protein like a boat passing through a lock. DNA intercalating agents can block the process at various steps as illustrated in figure 3. Single strand or double strand breaks with topoisomerase subunit linked to the 5′ end are obtained. No swiveling can occur at those breaks in constrained structures (Pommier *et al.*, 1984a). The breaks are hidden and are only revealed after sodium dodecyl sulfate or proteinase K treatments. Depending on the intercalating agent, one observes mainly single strand breaks (m-AMSA), double strand breaks (N-methyl 9-OH-ellipticinium) or a mixture of the two types of breaks (anthracyclines). Ethidium bromide inhibits the enzymatic reaction but does not promote the DNA cleavage (Tewey *et al.*, 1984b). Inhibition occurs very likely at an early step, before strand cleavage takes place. Interestingly, ethidium inhibits the the formation of breaks caused by other intercalating agents *in vitro* and reduces the cytotoxicity of these drugs *in vivo* (Rowe *et al.*, 1985; Tewey *et al.*, 1984b; Riou *et al.*, 1986b). Therefore, it seems likely that active compounds are those which are able to interact with both DNA and the protein within the enzyme substrate complex to cause an abortive termination of the enzymatic process at different intermediate steps. Such a model is in agreement with the results of the structure activity relationship discussed above.

Another series of antitumor drugs, the epipodophyllotoxin derivatives seem to have a very similar mechanism of action (Chen *et al.*, 1984; Ross *et al.*, 1984). Those compounds do not interact with DNA. However, ethidium inhibits both *in vitro* and *in vivo* the action of epipodophyllotoxin (Rowe *et al.*, 1985).

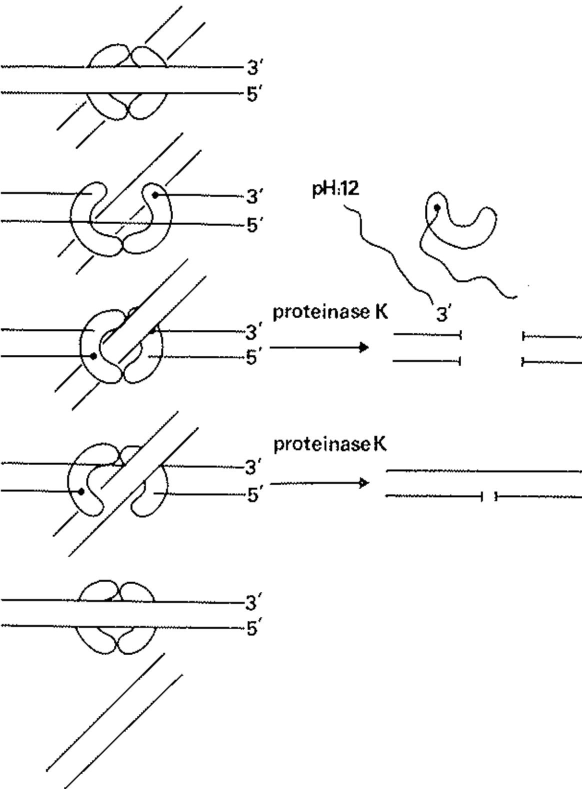


FIG. 3. Schematic representation of DNA topoisomerase II mechanism of action. Interruption of the enzymatic process at various steps by DNA intercalating agents can lead to hidden protein DNA associated breaks which are revealed by sodium dodecyl sulfate or proteinase K treatment.

This suggests that epipodophyllotoxin derivatives interact preferentially with the topoisomerase in the DNA-DNA topoisomerase complex.

Recent studies (Yang *et al.*, 1984; Pommier *et al.*, 1986b; Riou *et al.*, 1986a,b; Rowe *et al.*, 1986) show that *in vivo* the DNA breaks do not occur at random in the genome after the cells have been treated with intercalating agents or epipodophyllotoxin. SV40 chromosome was cleaved inside infected cells at specific sites. One of the major sites was mapped in the DNase I hypersensitive region (Yang *et al.*, 1985).

The action of ellipticine derivatives and analogues resembles that of other intercalating agents but differs significantly in several aspects.

Ellipticine analogues promote mainly double strand breaks. In addition, a biphasic phenomenon is observed in cells. At low concentrations, the double stranded breaks appear whereas at high concentration they are suppressed (Pommier *et al.*, 1985a, Riou, 1986; Vilarem *et al.*, 1986).

As mentioned above, the DNA breaks which are induced do not occur at random. DNA topoisomerase II itself induces a low level of breaks which occur preferentially at given sequences. Sanders and Hsieh (1985) proposed the following consensus sequence for the *Drosophila melanogaster* topoisomerase II:

GTN(T)A(T) ATTNATNN(G)
A C Δ A

Intercalating drugs increase the probability of hidden breaks at these sequences but the effect is far from being uniform. Ellipticine analogues promote breakage at positions different from other intercalating drugs. This is illustrated on figure 4 reproduced from the work of Riou (1986). This figure shows the electrophoretic migration of a DNA fragment (1) treated with DNA topoisomerase II from calf thymus (2) with DNA topoisomerase II in presence of m-AMSA (3) or 9-hydroxy-ellipticine (4). Clearly the last two patterns are different. Compare band number 5 in the two channels. An interesting aspect of this experiment is that the DNA fragment contains the sequence for the protooncogene *c-myc* and that *in vitro*, the breaks can be mapped in the gene. The DNA breaks in this gene could be mapped also *in vivo* in tumor cells where this gene is amplified. *In vivo* cleavage was observed at locations close to that observed *in vitro*. Furthermore, the major sites were registered in the DNase I hypersensitive regions (Riou *et al.*, 1986a,b; Riou, 1986). Similar results were also obtained in the case of the protooncogene *c-fos* with epipodophyllotoxin derivatives (Darby *et al.*, 1986).

These results open quite new perspectives for the understanding of the action of intercalating agents, which appears to be modulated by the accessibility and structure of DNA in specific genes at specific sequences. These factors could well play a crucial role in determining the specificity of cytotoxic action in malignant cells. Definite proof that DNA topoisomerase is indeed involved in the antitumor action of intercalating agents might well soon be obtained using variant cells made resistant to these agents. Already, it has been observed that in the Chinese hamster lung cell line (DC-3F/9-OH-E) resistant to ellipticine derivatives, intercalating agents do not induce protein associated DNA strand breaks. In addition,

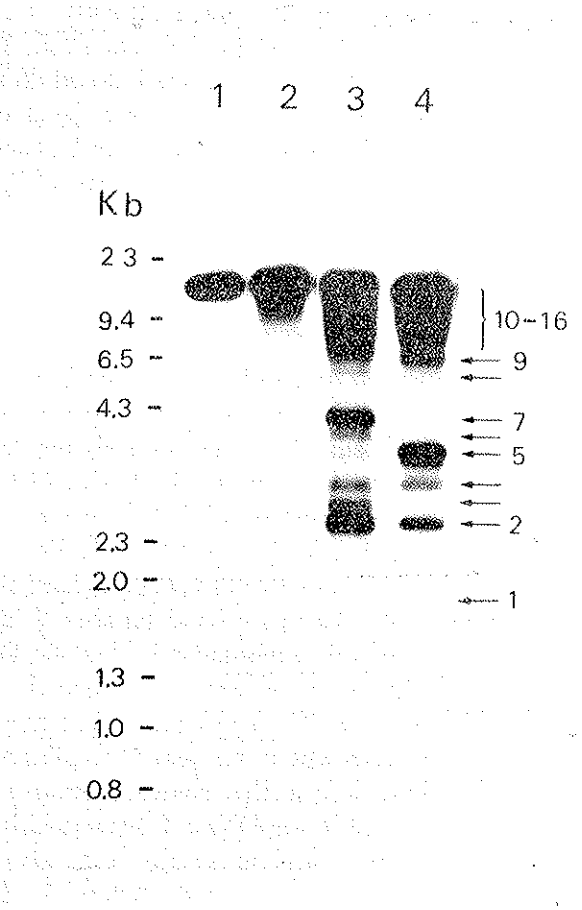


FIG. 4. *In vitro* cleavage of *c-myc* gene induced by calf thymus DNA topoisomerase II: Comparison of the action of m-AMSA and 9-OH-ellipticine. DNA (λ K 76) containing *c-myc* gene was digested by EcoR1 restriction nuclease. DNA alone (1), DNA treated with topoisomerase (2), DNA treated with topoisomerase +m-AMSA (1 μM) (3), or with 9-OH-E (1 μM) (4) were separated through agarose gel (1.2%) electrophoresis and revealed by the Southern (1975) procedure with a *c-myc probe*. Migration positions of standard have been used to determine the size of the fragments (scale in kilobases, Kb on left). Reproduced from Riou (1986).

these cells are resistant to epipodophyllotoxin derivatives (Pommier *et al.*, 1986a). On-going studies suggest that a protein associated with DNA topoisomerase might be altered in the variant resistance to epipodophyllotoxin. Furthermore, a cell line selected for resistance to epipodophyllotoxin has recently been obtained. A qualitative change of DNA topoisomerase II in these cells seems able to confer at the same time resistance to epipodophyllotoxin and to intercalating agents (Glisson *et al.*, 1986a,b).

Finally, an important problem remains without solution. It is not understood at all how the protein associated DNA breaks induce cell death. Surprisingly, it is observed that, after drug removal, the DNA breaks disappear rapidly (Zwelling *et al.*, 1981; Ross and Smith, 1982; Pommier *et al.*, 1984). It has been suggested that the DNA breaks could induce sister chromatide exchange or other DNA rearrangements which could be the true cause of cell death (Pommier *et al.*, 1985), but this hypothesis remains for the present conjectural.

DNA topoisomerase II from trypanosome and plasmodium have recently been purified (Douc-Rasy, 1986; Riou *et al.*, 1986c). The enzyme from trypanosome appears extremely interesting. It differs markedly from other known DNA topoisomerases II because it does not need ATP. This suggests that its structure is sufficiently different from that of the enzyme of mammalian cells to constitute a specific target for chemotherapy of parasitic diseases. DNA topoisomerase II is an important target for antibiotic action. It could well be that, in the future, this enzyme would represent also a useful target for antiparasitic and anticancer drugs. This would then open the way for a rational design of these drugs.

This possibility has recently been discussed in detail by Ross (1985).

II. Mechanism of Action of the Antitumor Bifunctional Intercalator Ditercalinium

Recently, a new class of synthetic intercalating agents, the polyfunctional intercalators have been prepared (rev. Le Pecq and Roques, 1986; Wakelin, 1986).

Molecules with two or three intercalating rings linked by chains of appropriate length and structure to allow the DNA intercalation of each subunit have been obtained. Those molecules bind to DNA with an extremely high binding constant (up to $10^{14}\,M^{-1}$ for trisintercalating molecules) (Laugaa *et al.*, 1985).

Among these molecules, several dimeric molecules deriving from 7H-

pyridocarbazole such as ditercalinium (figure 5) elicited strong antitumor activities (Roques *et al.*, 1979; Pelaprat *et al.*, 1980b). As for the other DNA binding drugs, high DNA affinity was required to obtain biological activity, but this property alone was not sufficient (Pelaprat *et al.*, 1980b).

Studies on the mechanism of action of these molecules (Bendirdjian *et al.*, 1984; Esnault *et al.*, 1984; Markovits *et al.*, 1986), indicated that the mechanism of action of ditercalinium was completely different from that of other antitumor drugs.

One of the most intriguing observations was that ditercalinium and its analogues induced a delayed cytotoxicity. Mammalian cells treated with lethal doses of ditercalinium were still able to grow for five to six generations after drug treatment before getting arrested. Treated cells gave rise to small abortive clones when plated on semi-solid medium (Esnault *et al.*, 1984; Bendirdjian *et al.*, 1984). This effect is illustrated in figure 6 with the L1210 mouse leukemic cell line. It can be seen that after a drug exposure of 24 hours, cells can still grow for 5 to 6 generations before getting arrested.

As discussed above, monointercalating agents block the mammalian cells in the G2 phase of the cell cycle. No such block was observed when cells treated with ditercalinium were analysed by flow-cytofluorometry (Esnault *et al.*, 1984).

Because, as discussed above, monointercalating antitumor drugs have a specific action on DNA topoisomerase II and because this action is well correlated with the antitumor property, it was of interest to measure the potential action of ditercalinium on this enzyme.

The effect of ditercalinium upon the DNA of a L1210 mouse leukemia cell line and one of its variants made resistant to ditercalinium L1210/PyrDi 1 was studied (Markovits *et al.*, 1986). Alkaline elution assays

Fig. 5. Structure of ditercalinium.

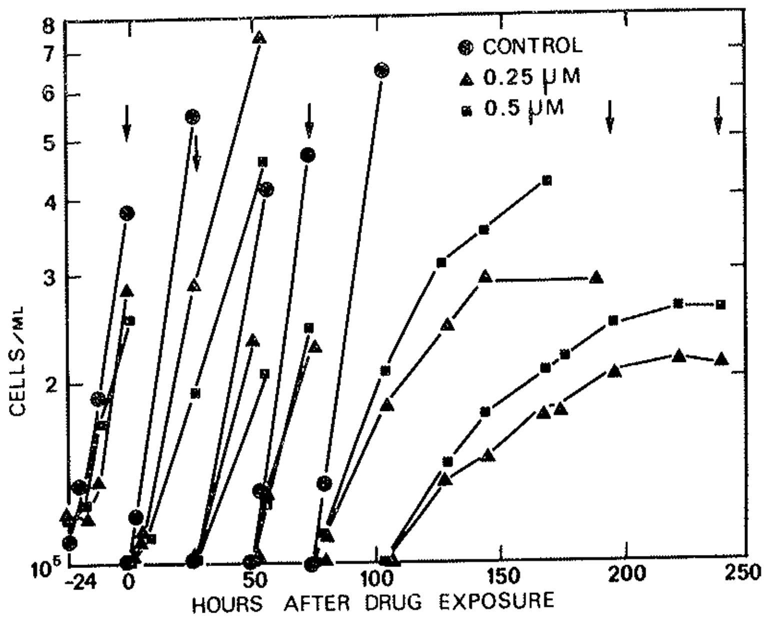


FIG. 6. Delayed toxicity of ditercalinium on L1210 cells. Cells were exposed for 24 hours (t = −24 to t = O) to 0.25 μM or 0.5 μM of ditercalinium. They were then washed and resuspended in fresh medium. The cells were regularly diluted to 10^5 cells/ml in fresh medium. The growth of untreated cells was registered in the same conditions (●—●). Reproduced from Esnault *et al.* (1984).

demonstrated that ditercalinium did not produce any-DNA-protein associated breaks such as those observed with monointercalating agents. None of the other DNA damages which can be registered with alkaline elution were observed. In addition, the structure of chromatin was studied by measuring the rate of sedimentation of nucleoids after ditercalinium treatment. A marked nucleoid compaction, which was dose dependent, was observed in sensitive cells, whereas no chromatin alteration was detected with this technique in the drug resistant variant cell line.

The effect of ditercalinium upon purified L1210 topoisomerase II *in vitro* in presence of ATP using supercoiled SV40 DNA was also studied. The effects of ditercalinium on this enzyme can be seen in figure 7.

a) Ditercalinium at any concentration assayed (10^{-7}M-10^{-5}M) failed to stimulate DNA cleavage by DNA topoisomerase II (lanes 3-6) as does 5 μM m-AMSA (lane 7);

b) In contrast, ditercalinium at concentration ⩾ 0.5 μM inhibited

DNA cleavage and consequently the formation of the cleavable covalent complexes between the enzyme and DNA (lanes 4-6);

c) The presence of 1 or 10 μM ditercalinium in the reaction mixture (lanes 8-9) containing 5 μM m-AMSA inhibited completely the formation of linear DNAs (of full size (form III) and short segments).

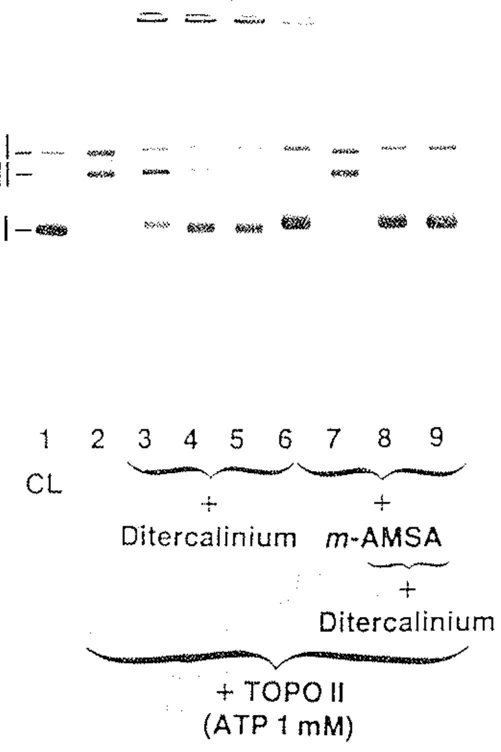


FIG. 7. Effect of ditercalinium on SV40 DNA in the presence of L1210 topoisomerase II. Inhibition of m-AMSA-induced cleavable complexes by ditercalinium. Reactions were carried out with 0.4 μg SV40 DNA and 1 mM ATP. Lane 1 is DNA alone and lane 2, DNA reacted with topoisomerase II. Ditercalinium (10^{-7} M in lane 3, 5×10^{-7} M in lane 4; 10^{-6} M in lanes 5 and 8; 10^{-5} M in lanes 6 and 9) was reacted for 5 min with SV40 DNA. m-AMSA (5 μM) was then added to reaction mixtures of lanes 7, 8 and 9, and L1210 DNA topoisomerase II (130 ng) to all reaction mixtures except to 1. After 30 min at 37°C, reactions were stopped by the addition of 0.5% SDS and 0.5 mg/ml proteinase K (final concentrations). Electrophoresis was in 1% agarose in tris-acetate buffer containing 0.1% SDS (2 v/cm). Reproduced with permission from Markovits *et al.* (1986).

d) Ditercalinium at concentration greater than 1 μM prevented DNA relaxation by topoisomerase II (see the presence of supercoiled SV40 DNA in lane 5 and 6);

e) At concentration close to 1 μM, ditercalinium provoked the formation of high molecular weight DNA (DNA remaining at the top of the gel electrophoresis). This DNA is constituted by DNA catenanes or aggregates. These *in vitro* results are consistent with those obtained in the whole cells. This suggests that ditercalinium might act as a DNA-condensing agent for DNA topoisomerase II, favoring the formation of DNA catenanes.

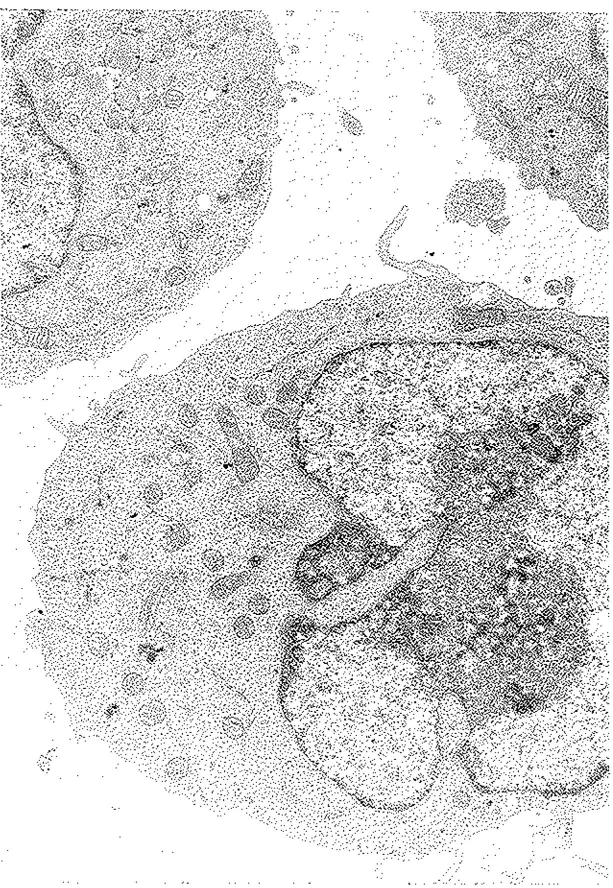
In parallel with these experiments performed on mammalian cells, the action of ditercalinium was studied on *E. coli*.

Wild-type *E. coli* cells are totally resistant to this compound. An *E. coli* strain extremely sensitive to ditercalinium was selected after mutagenesis (Lambert and Le Pecq, 1982). For this strain, cells were killed at concentration close to 10 ng/ml.

This mutation was later identified and located in the *polA gene* (Lambert *et al.*, unpublished results). The *polA* cells reverted with high frequency to a resistant phenotype when selected in presence of drug. All the cells which acquired the resistant phenotype after reversion had still the *polA* mutation. These cells had therefore acquired an additional mutation which suppressed the sensitive phenotype conferred by *polA*. 50% of these revertants were extremely UV sensitive. It was observed that a strain having the two mutations *polA* and *uvrA* was completely drug resistant. In *polA* strain, the SOS system was efficiently induced as seen by β galactosidase synthesis induction in the *polA* (λ(*sfiA::lacZ*) (Huisman and d'Ari, 1981) and by important filamentation of the cells.

These results indicated that the non-covalent DNA ditercalinium complex was recognized by the uvrABC repair system in *E. coli*. In the case of monointercalating agents, the non-covalent DNA complexes are not recognized by DNA repair enzymes. Formation of a covalent bond between the drug and DNA is required to activate the repair system (Lambert *et al.*, 1986). The fact that the "DNA lesion" which is recognized by the uvrABC system is induced by a drug which binds reversibly to DNA has unexpected consequences and could lead to a futile DNA repair process (Lambert *et al.*, unpublished results).

These results prompted us to study whether such a phenomenon could take place in mammalian cells. Preliminary cytologic studies show (figure 8) that ditercalinium treated cells do not show noticeable modi-



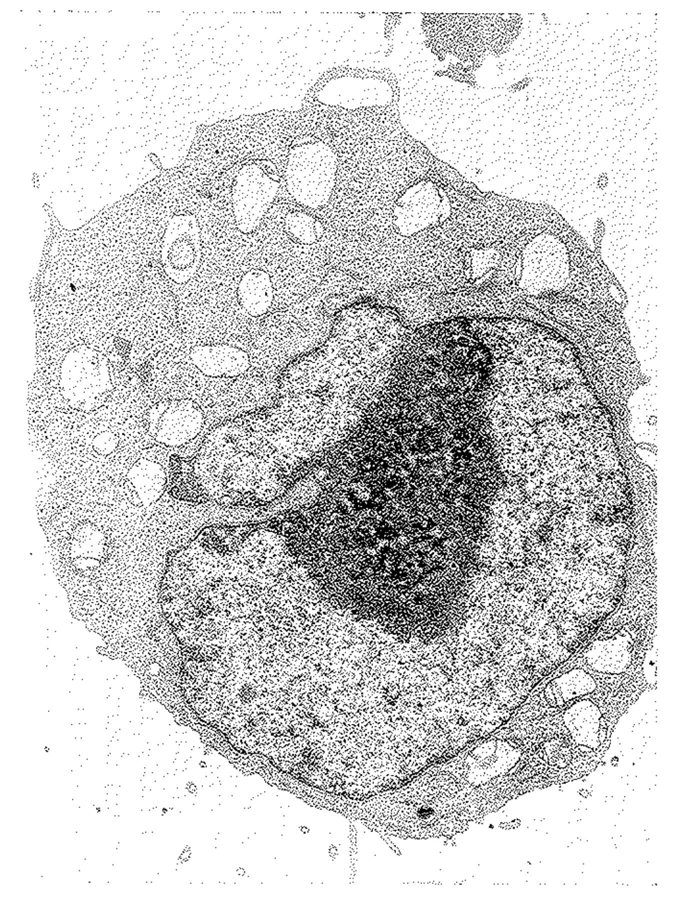


FIG. 8. Electronic microscopy of untreated L1210 (A) and treated L1210 cells (B) (0.2 μM of ditercalinium for 24 hrs). Compare the structure of mitochondria in the two cases.

fications of nucleus structure; in contrast with what is observed for cells treated with an ellipticine monointercalating derivative (Paoletti *et al.*, 1980). However a clear alteration of the mitochondria was observed. This cytological alteration was associated with a decrease of ATP synthesis (Esnault *et al.*, unpublished results) and a stimulation of fermentation (Segal-Bendirdjian *et al.*, unpublished results). These preliminary results suggest that in addition to nuclear DNA, mitochondrial DNA might be a potential target for these drugs. Recent report underlines frequent alteration of mitochondria in carcinomal cells (Chen *et al.*, 1985) and suggests that mitochondria could well represent a specific target for antitumor drugs.

Conclusion

The identification of the DNA topoisomerase II complex as a potential target for DNA monointercalating antitumor agents appears of great importance for the rational design of such drugs. Ellipticine derivatives and azaellipticine analogues (BD40) behave as other intercalating agents. However each compound induces protein associated DNA breaks at different sequences. The physiological consequences of the preferential cleavage in specific regions of the genome are presently unknown, but can now be investigated. It is tempting to speculate that an increased sensitivity to cleavage of genes like oncogenes expressed in malignant cells could contribute to the specificity of action of these antitumor agents.

The mechanism of the antitumor action of ditercalinium, a bifunctional intercalator derived from 7H-pyridocarbazole, appears different from that of DNA monointercalating agents. It is proposed that the cytotoxicity of this molecule is related to the DNA conformational change induced by the binding of this molecule. DNA repair enzymes might be involved in the recognition of the induced DNA alteration.

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The authors are very grateful to Dr J.F. Riou for fruitful discussions and for permission to reproduce some of his results. Support of Association pour la Recherche sur le Cancer, Fondation pour la Recherche Médicale and Université Pierre et Marie Curie (Paris VI) is gratefully aknowledged.

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RECOGNITION OF DNA BY QUINOXALINE ANTIBIOTICS

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Abstract

Quinoxaline antibiotics are powerful anti-cancer drugs which bind selectively to DNA at sites containing the CpG nucleotide sequence. Often the binding reaction seems to cause the propagation of structural disturbances into helical regions flanking the antibiotic binding sites. Molecular features which are critical for recognising the preferred base-pair sequences have been identified; they include peptide N-methylation as well as the CO and NH functionalities of the L-alanine residues. Analogues of the natural antibiotics having modified chromophores have been prepared by directed biosynthesis, and footprinting experiments have revealed that their sequence-recognition properties are altered. Binding of echinomycin to reconstituted nucleosome core particles causes a shift in the positioning of *tyr*T DNA about the histone octamer, equivalent to rotation of the entire DNA molecule by about half a turn.

1. Introduction

Among the drugs in current use for treating cancer patients are numerous substances which bind to DNA (Waring, 1981; see Figure 1). No-one these days needs to be reminded that such drugs generally lack the selectivity needed to kill cancer cells without harming normal host tissues. The argument runs that whatever we can learn about the mode

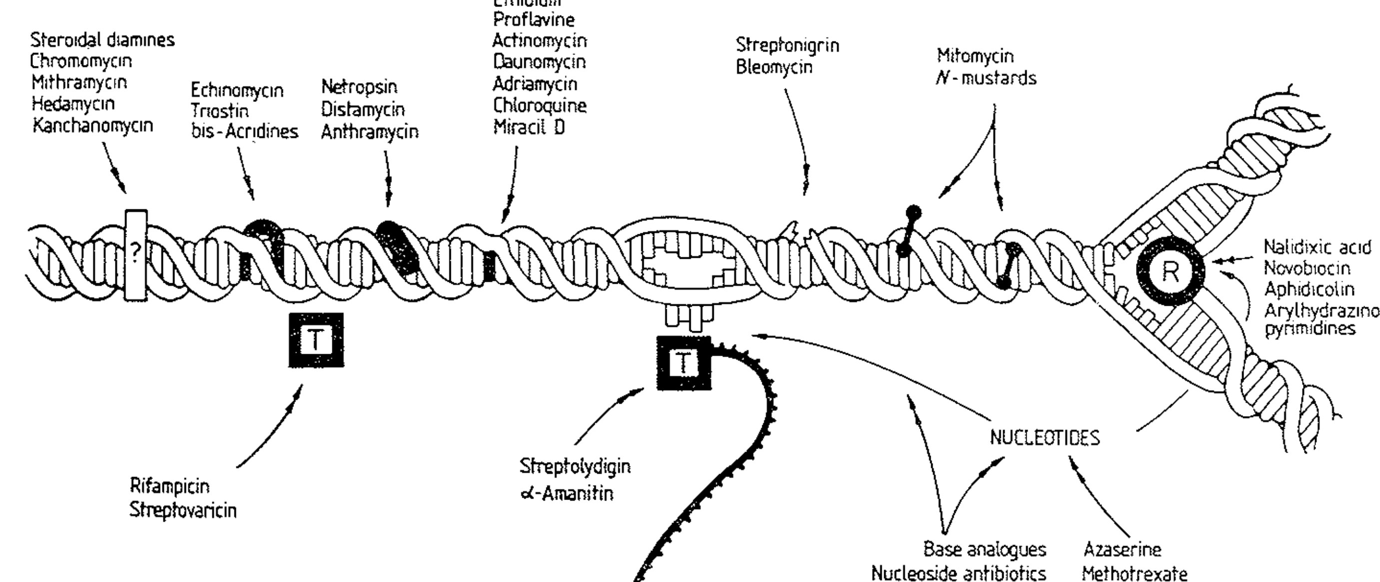

Fig. 1. Diagrammatic representation of a molecule of DNA engaged in transcription by RNA polymerase (T) and undergoing duplication by the replicating enzyme complex (R), subject to interference by various antibiotics and drugs whose principal sites of action are indicated by double-headed arrows. From Gale *et al.* (1981).

of action of compounds presently employed in cancer therapy, particularly at the molecular level, is likely to be of value for our much-needed efforts to improve selectivity. Although the benefits of such studies might not be immediately apparent there is hope that sooner or later they can be interfaced with new discoveries about the fundamental nature of the disease (the role of oncogenes, for example) to yield results in the clinic. This paper is concerned with one particular approach which is focussed on a group of cyclic depsipeptide antibiotics called the quinoxalines, of which echinomycin (Figure 2) is the best-known member (Waring, 1979; Waring and Fox, 1983).

The quinoxalines were discovered more than thirty years ago and seem to be of fairly wide occurrence in nature (reviewed by Katagiri *et al.*, 1975). They are very powerful antitumor agents, and the results of biochemical investigations soon led to the conclusion that their biological activity could be attributed to interaction with DNA so as to distort its structure and function — most probably via selective inhibition of DNA-directed RNA synthesis (Ward *et al.*, 1965; Sato *et al.*, 1967; Waring and Makoff, 1974). These findings led to a detailed investigation of the binding of echinomycin to DNA, mainly using a novel technique of solvent (phase) partition (Waring *et al.*, 1975), which resulted in the discovery that echinomycin binds to DNA by the hitherto unknown mechanism of bifunctional (*bis*-) intercalation (Waring and Wakelin, 1974).

FIG. 2. Structural formula of echinomycin (quinomycin A).

This prompted a survey of related antibiotics and synthetic quinoxaline compounds, which established that bifunctional intercalation seems to be a group-specific property of these substances (Lee and Waring, 1978). Moreover, with echinomycin a distinct preference for binding to GC-rich sequences in DNA became apparent (Wakelin and Waring, 1976). The true nature of that selectivity (actually better called preference) only became fully apparent with the advent of "footprinting" methods for detecting antibiotic binding sites on natural DNA fragments of known nucleotide sequence, employing either DNAase I or the synthetic reagent methidium-propyl-EDTA-Fe(II) as a probe for accessibility of inter-nucleotide bonds to cleavage (Low *et al.*, 1984a; Van Dyke and Dervan, 1984).

In this paper we will first outline the evidence for sequence-selectivity in the binding of quinoxaline antibiotics to DNA, then we shall consider briefly what has been learned about the molecular basis of sequence recognition by these antibiotics. That done, we shall examine the proposition that sequence-selectivity can be experimentally manipulated by making biosynthetic modifications of the basic antibiotic structure, and finally we shall address the question as to what influence(s) the antibiotic might exert on the structure of chromatin.

2. Sequence-Selective Binding to DNA

The results of a classical footprinting experiment with echinomycin are illustrated in Figure 3. Six or seven antibiotic binding sites can be clearly identified on the 160 base-pair DNA molecule employed here (visualized as gaps in the ladder of fragments produced by DNAase I digestion) and each is centred around one or more CpG steps in the nucleotide sequence (see the differential cleavage plot represented in Figure 4). With the possible exception of the region around position 35, all CpG sequences are strongly protected from nuclease attack by the binding of the antibiotic. Also in evidence is the curious phenomenon of enhanced cutting at some sites in the presence of echinomycin; these regions of enhancement always occur at sequences flanking strong antibiotic binding sites, but not all flanking sequences are affected. Enhanced cutting is not an artifact of the footprinting methodology because it can also be seen in experiments using other cutting probes: DNAase II (Low *et al.*, 1984a) and even MPE-Fe(II) (Van Dyke and Dervan, 1984). More-

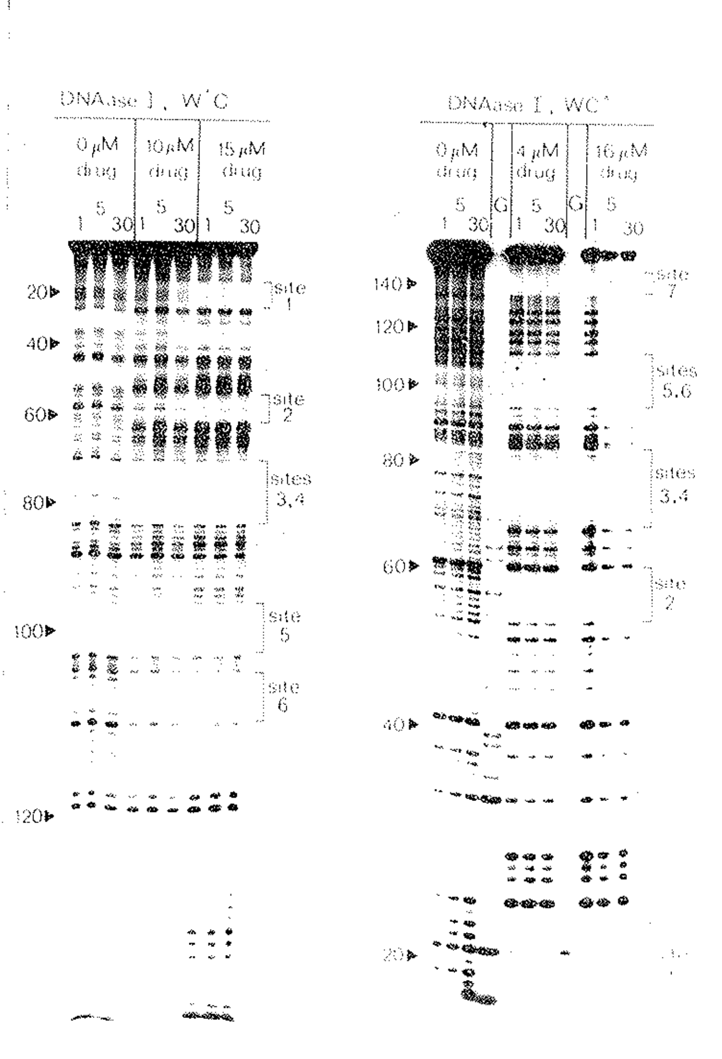

Fig. 3. DNAase I footprinting of echinomycin bound to a 160 base-pair DNA fragment from *E. coli* containing the *tyrT* promoter sequence. Symbols W*C and WC* indicate which of the two strands (Watson or Crick) bears a radioactive 3'-end label. Time in minutes (1, 5, 30) after the addition of enzyme is shown at the top of each gel lane. Tracks labelled « G » are dimethyl sulphate-piperidine markers specific for guanine. Numbers on the left refer to the numbering scheme shown in Figure 4, while sites of protection from DNAase I digestion are identified on the right. For details see Low *et al.*, (1984a).

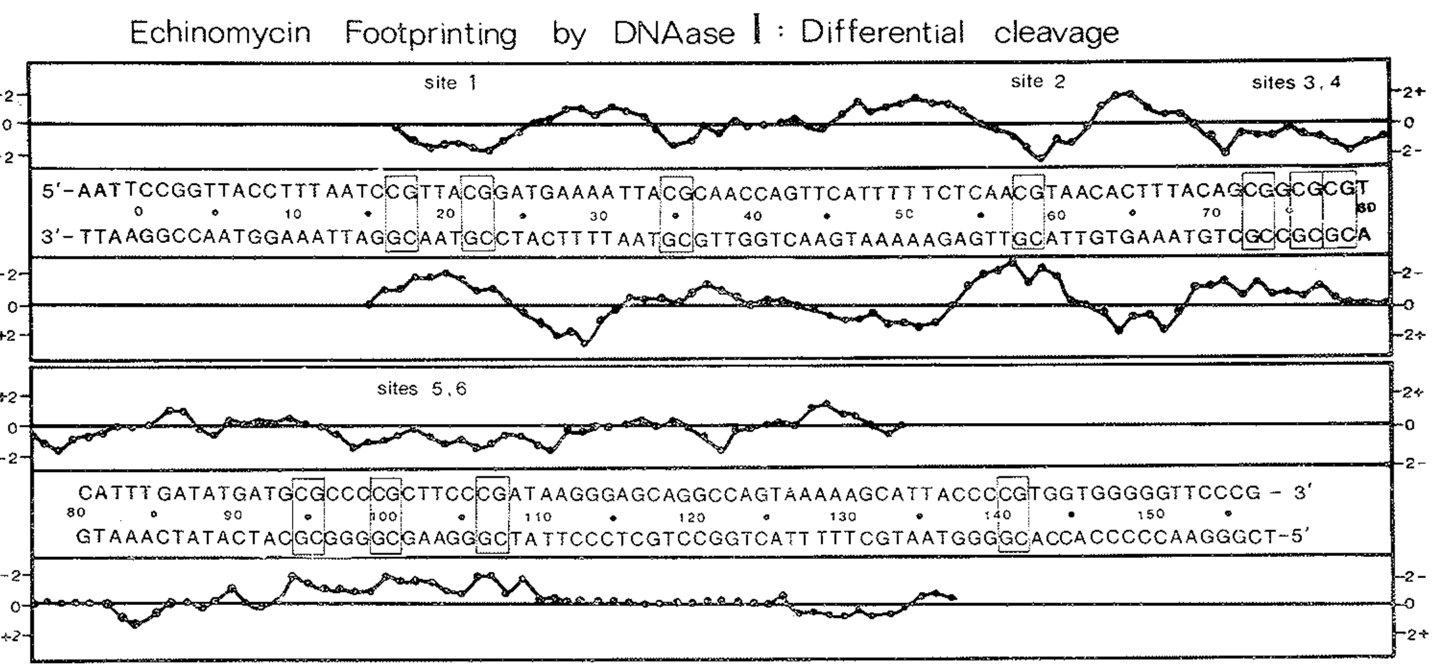

FIG. 4. Echinomycin-induced differences in the susceptibility of *tyr*T DNA to DNAase I digestion. The upper Watson strand reads 5′ to 3′ left-to-right, while the lower Crick strand reads 5′ to 3′ right-to-left. Vertical scales on both sides are in units of ln (f_a) - ln (f_c), where f_a is the fractional cleavage at any bond in the presence of 15 μM antibiotic and f_c is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion (approx. 30% of the starting material in both cases). Positive values indicate enhancement, negative values blockage. Sites of protection are labelled as in Figure 3. From Low *et al.*, (1984a).

over, it occurs at sequences flanking the binding sites of other antibiotics such as actinomycin and distamycin, whose nucleotide sequence selectivity is quite different from that of quinoxaline antibiotics (Fox and Waring, 1984a). We conclude that when sequence-selective antibiotics bind to DNA it is a common phenomenon for local changes in the structure of the helix to be propagated into regions flanking the binding site(s). Those changes can result in enhanced susceptibility to nuclease attack, prompting the notion that they might also influence the interaction between DNA and other types of protein molecules, as will be considered in the last section.

It is important to realise, however, that although footprinting analysis has identified CpG steps as constituting the critical determinant of preferred binding sites for echinomycin in DNA, they are not the *only* possible sequences. The mere fact that echinomycin (in common with other quinoxaline antibiotics) will bind to poly dG·poly dC as well as to poly(dA-dT) (Wakelin and Waring, 1976) is sufficient to establish that the CpG step cannot be mandatory and that other sites, presumably of lower affinity, must exist. Kinetic studies have indeed produced evidence that natural DNAs contain multiple binding sites characterised by widely differing affinity for quinoxaline antibiotics (Fox *et al.*, 1981; Fox and Waring, 1981, 1984b). Moreover, the evidence points strongly to the existence of a "shuffling" phenomenon whereby antibiotic molecules initially associate relatively nonspecifically with many types of binding sites but subsequently migrate along the DNA lattice to locate their preferred (most tightly binding) sites from which they dissociate most slowly (Fox and Waring, 1985, 1986).

3. The Molecular Basis of Sequence Recognition

Until quite recently there was little information as to the precise conformation of any quinoxaline antibiotic (made worse by the existence of an error in the structural formula of echinomycin as originally published, reviewed in Waring, 1979). Consequently any ideas about how echinomycin might recognise the CpG step in a *bis*-intercalated complex were of necessity speculative (see the reviews of Waring, 1977, 1979; and Waring and Fox, 1983). That situation changed radically in 1984 when the first crystal structure for a quinoxaline antibiotic was published (Sheldrick *et al.*, 1984), sharing many features in common with a structure previously

reported for the closely related synthetic depsipeptide TANDEM (Viswamitra *et al.*, 1981; Hossain *et al.*, 1982). It was soon followed by crystal structures for intermolecular complexes formed between quinoxaline antibiotics and self-complementary oligonucleotides (Wang *et al.*, 1984; Ughetto *et al.*, 1985; Quigley *et al.*, 1986). In all these structures the earlier predictions of indirect work based on solution studies were nicely borne out, i.e., that the antibiotic molecules should adopt a staple-like conformation having the intercalative quinoxaline rings attached to a fairly rigid peptide moiety in such a fashion that they could neatly sandwich two DNA base-pairs between them (Figure 5). It was a reasonable guess that the sandwiched base-pairs would take the form of a CpG step, as turned out to be the case in the antibiotic-oligonucleotide complexes, and a fairly detailed picture of intermolecular contacts which govern the sequence-selectivity of binding can now be drawn up. Steric complementarity in the form of van der Waals contacts is important, reinforced by specific hydrogen-bonding interactions between the carbonyl oxygen and NH groups of alanine residues in the antibiotic structure (see Figure 5) and the 2-NH_2 and N(3) functionalities of guanine bases in the DNA. A curious feature of the antibiotic-oligonucleotide complexes is the occurrence of Hoogsteen pairing between the purine and pyrimidine bases on either side of the sandwiched CpG step (Quigley *et al.*, 1986). Whether this unorthodox pairing arises as a necessary consequence of the formation of a *bis*-intercalated complex, and indeed whether it could occur in macromolecular DNA on binding echinomycin, is an open question — as is its possible relation to the propagated structural changes in the double helix mentioned above.

The availability of a crystal structure for triostin A (Sheldrick *et al.*, 1984) as well as for TANDEM, its des-N-tetramethyl analogue (Viswamitra *et al.*, 1981; Hossain *et al.*, 1982), provides a unique opportunity to assess the role of peptide N-methylation in the natural antibiotics. Triostin A, like echinomycin, recognises the GpG dinucleotide step in DNA whereas TANDEM is specific for alternating AT sequences, most probably centred around the dinucleotide step TpA (Figure 6; Low *et al.*, 1984b 1986a). Comparing the crystal structures (Figure 7) we see that the critical difference lies in the formation of two symmetry-related hydrogen bonds in the TANDEM molecule, involving the NH groups of the L-valine residues as donors and the CO groups of the L-alanine residues as acceptors. These interactions are, of course, not possible in the natural antibiotics because the L-valine residues are N-methylated.

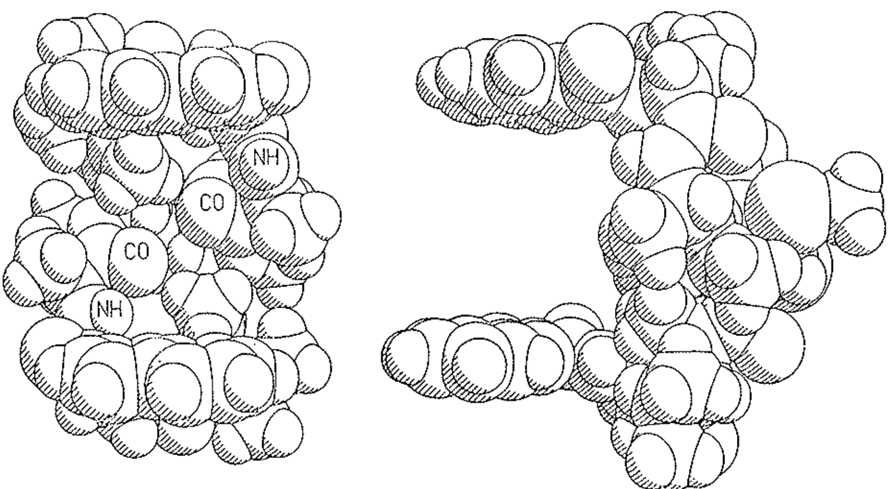

FIG. 5. Crystal structure of a *bis*-quinoline analogue of echinomycin prepared by directed biosynthesis (Gauvreau and Waring, 1984). In the illustration on the left the molecule is viewed down its quasi-dyad axis with the aromatic chromophores projecting out towards the viewer; this reveals the face of the peptide ring presented towards the DNA base-pairs, with the NH and CO groups of the two L-alanine residues lying in a diagonal array from lower left to upper right. In the illustration on the right the molecule is viewed from the « side », i.e., having been turned through 90° about a vertical axis, so as to show its staple-like arrangement which is ideally suited for *bis*-intercalation. Small rotations have been applied to the points of attachment of the aromatic rings so as to bring their planes exactly parallel. Unpublished work of G.M. Sheldrick, P.G. Jones, E.F. Paulus and M.J. Waring.

The effect of the internal hydrogen bonding, apart from satisfying the acceptor properties of the alanine CO groups, is to bring about minor but significant changes in numerous torsional angles which have the effect of drawing the bulky *iso*-propyl side-chains of the valine residues forward into a position where they partially occlude the space between the intercalative quinoxaline rings. Thus positioned they impede interaction between substituents on the DNA base pairs and the Ala CO groups present in the depsipeptide ring, most particularly the hydrogen bonding to the 2-NH_2 groups of the guanines which is supposed to confer selectivity for CpG sequences in the natural antibiotics. The "residual" selectivity of TANDEM for alternating AT base pairs is presumed to derive from the retained capacity to form H-bonds from the alanine NH groups to acceptors on the base-pairs (Viswamitra *et al.*, 1981).

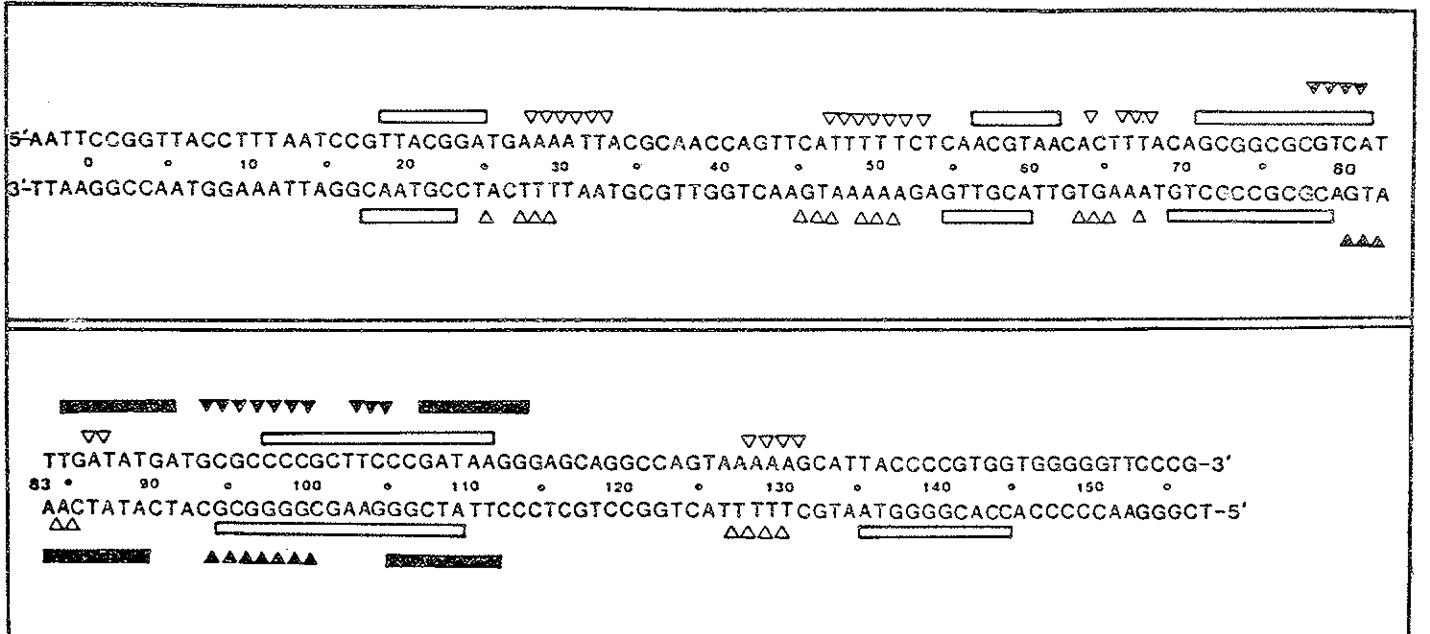

FIG. 6. Summary of DNAase I footprinting results for triostin A (unfilled bars, open triangles) and TANDEM (filled bars, filled triangles). Bars indicate protected regions while triangles show positions of enhanced cutting. The upper sequence represents the 'Watson' (antisense) strand and the lower sequence the 'Crick' (sense, coding) strand. These maps were compiled from visual inspection of numerous gels as well as from densitometric tracings, and may be considered as a set of averaged values. Note that the increased sensitivity to DNAase I in the presence of TANDEM is much less pronounced than that seen with triostin A. From Low *et al.*, (1984b).

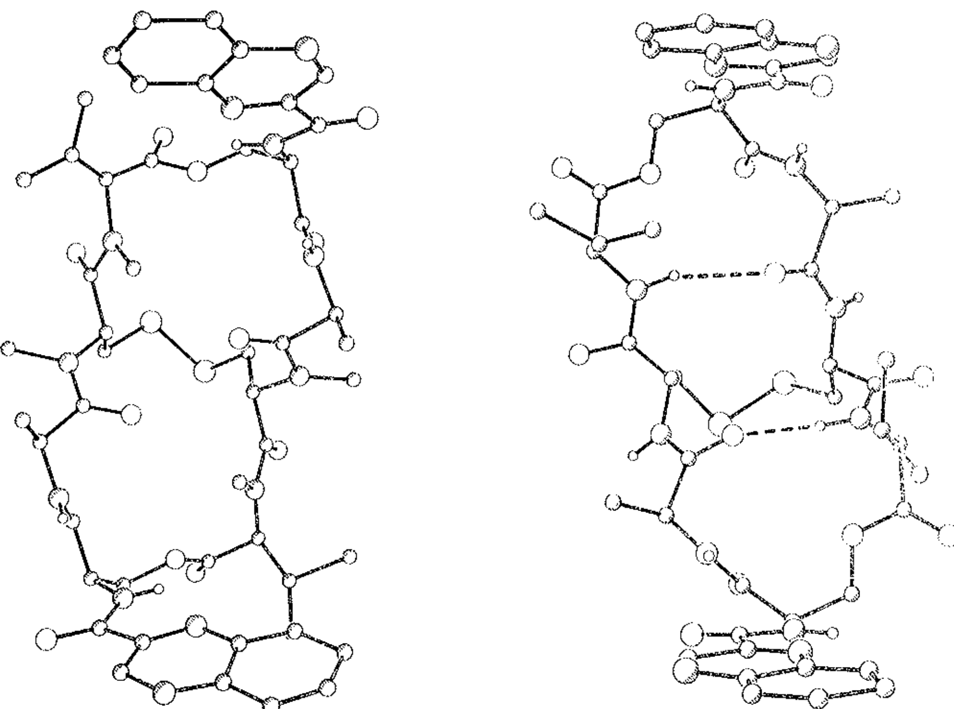

FIG. 7. Comparison of crystal structures for triostin A (left, Sheldrick *et al.*, 1984) and its des-N-tetramethyl derivative TANDEM (right, Viswamitra *et al.*, 1981; Hossain *et al.*, 1982), to show the effect of internal hydrogen bonding in the latter molecule (broken lines).

Support for these ideas about the molecular basis for recognition of specific nucleotide sequences, and its relation to structural properties, has come from an examination of the DNA-binding behaviour of TANDEM analogues (Figure 8; Low *et al.*, 1986a; Olsen *et al.*, 1986). A compound having one L-valine residue methylated, but not the other (and thus capable of forming only one of the internal H-bonds) fails to footprint on DNA and presumably does not bind to the polynucleotide at all. The same is true of a *bis*-lactyl analogue of TANDEM in which the L-alanine residues are replaced by L-lactic acid residues, in effect substituting oxygen atoms for the critical NH moieties of the alanines. But an analogue having the L-cysteine residues methylated binds well to DNA and yields a footprinting pattern on *tyr*T DNA identical to that of TANDEM; it also footprints well on other DNAs containing the TpA dinucleotide step and selectively inhibits the action of a restriction enzyme whose recognition

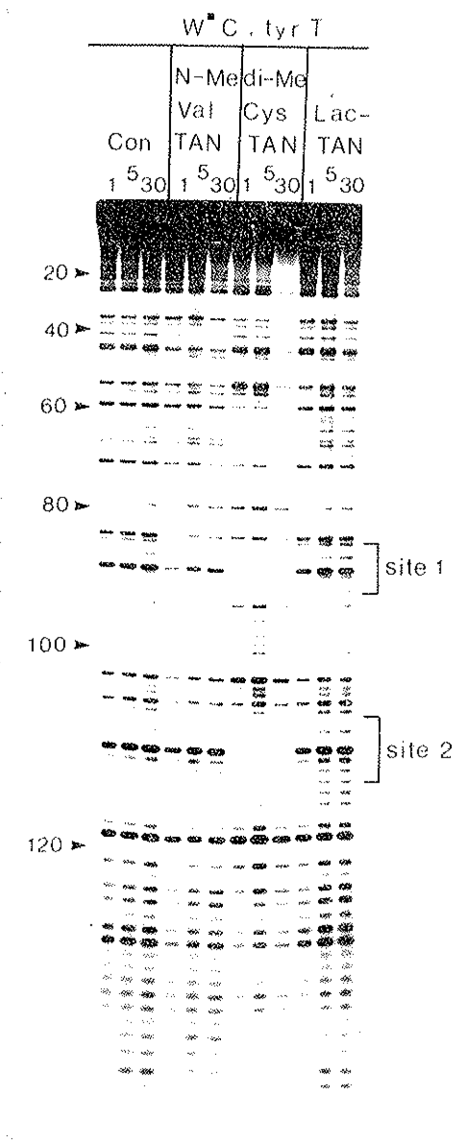

FIG. 8. DNAase I digestion patterns for *tyr*T DNA in the absence (CON) and presence of [N-MeVal[4]]TANDEM (N-MeValTAN) or [N-MeCys[3], N-MeCys[7]]TANDEM (diMeCysTAN) or [Lac[2],Lac[6]]TANDEM (Lac-TAN, an analogue having L-lactic acid residues in place of each L-alanine residue). The symbol W*C indicates that the Watson (upper) strand bears the radioactive 3′-end label. Time in minutes (1,5,30) after the addition of enzyme is shown at the top of each gel lane. The extent of digestion was limited to 20-40% of the starting material so as to minimise the incidence of multiple cuts in any one strand. Numbers on the left refer to the numbering scheme shown in Figure 4, while sites of protection from DNAase I digestion by [N-MeCys[3], N-MeCys[7]]TANDEM are identified on the right. Unpublished experiment of C.M.L. Low and M.J. Waring; for details see Low *et al.*, (1986a).

site contains the sequence GTAC but not one which cuts at GATC sequences (Low *et al.*, 1986a). These observations underline the critical importance of both valine N-methyl groups (but not the cysteine N-methyls), together with the L-alanine NH functionalities, as determinants of which DNA sequences are recognised by the ligands.

4. Biosynthetic Analogues of Quinoxaline Antibiotics

It is obvious from the foregoing paragraphs that the peptide ring system in quinoxaline antibiotics plays a direct role in recognising DNA nucleotide sequences, but the aromatic chromophores are not merely inert plugs which serve to position the peptide moiety within one of the grooves of the helix (the minor groove, by common consent [Wakelin and Waring, 1976; Waring, 1979; Ughetto *et al.*, 1985]). They are capable of significantly modifying the sequence binding preferences of the antibiotics, and thus the production of chromophore-modified analogues by a process of directed biosynthesis is a viable route to the preparation of new drugs of potential clinical importance (Williamson *et al.*, 1982; Cornish *et al.*, 1983; Santikarn *et al.*, 1983; Gauvreau and Waring, 1984). By the simple expedient of adding aromatic acids to cultures of antibiotic-producing streptomycetes such as *S. echinatus* a range of novel, semi-biosynthetic analogues has been produced having chromophores modified as indicated in Figure 9. Many of these new materials are unworkable for one reason or another, but several have proved important as experimental probes (such as the *bis*-quinoline analogue of echinomycin [Fox *et al.*, 1980], code-named 2QN, which provides the only crystal structure of a quinomycin antibiotic in the free state available to date). Figure 10 illustrates how the footprinting pattern characteristic of echinomycin and triostin is modified by substitution of the quinoxaline chromophores with one (1QN) or two (2QN) quinoline moieties, or by substitution with 3-aminoquinoxaline ring systems. The pattern for the quinoline analogues is similar to that seen with echinomycin, but the poorly-protected CpG sequence at position 35 now appears as a strong footprint site. Evidently replacement of the natural chromophores with quinoline rings has led to some broadening of the acceptable range of sequences. By contrast, the addition of a 3-amino group to the quinoxaline ring system leads to more stringent discrimination between sequences: now only two or three distinct sites in *tyr*T DNA are strongly protected,

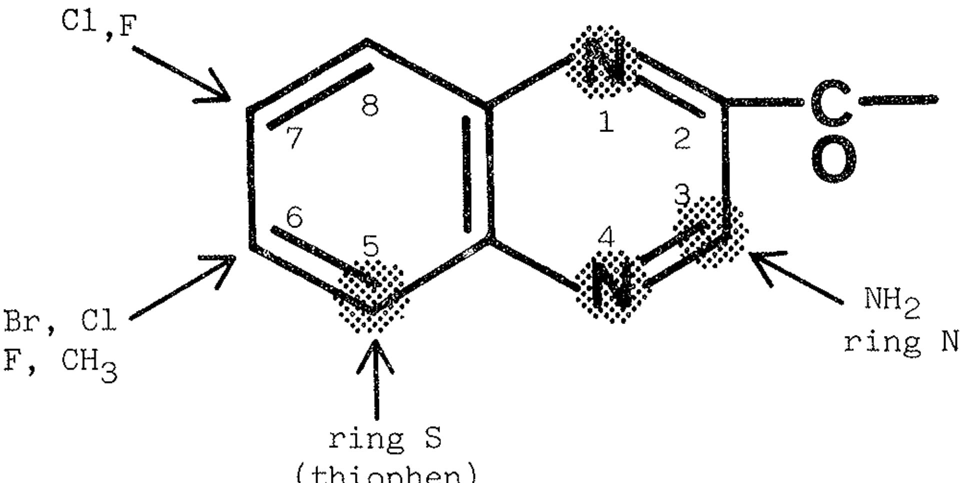

FIG. 9. Substituted chromophores which have been incorporated into quinoxaline antibiotics by directed biosynthesis. Stippled circles indicate positions where the nitrogen heteroatom present in the natural chromophore has been replaced by CH, or vice versa.

as opposed to six or seven with echinomycin or triostin, so that an additional preference for one or more AT base pairs flanking the "sandwiched" CpG sequence can be discerned (Low *et al.*, 1986b). There is reason to believe that further efforts directed towards biosynthetic replacement of the quinoxaline chromophores will yield fruit in respect of biological activity: hopefully selectivity.

5. Effects of Antibiotic Binding to Nucleosome Core Particles

No-one would deny that crystallographic and solution studies have convincingly established the nature of the interaction between antibiotics and purified DNA, but in living cells the DNA is bound to a collection of histones and other chromosomal proteins. Can antibiotics bind equally well to DNA *in situ*, as it were, and what effects might they have on nucleosome structure? To address this question we have investigated the interaction between echinomycin and reconstituted nucleosome core particles containing the same 160 bp *tyr*T DNA molecule employed for the experiment depicted in Figure 3. Happily the same method of analysis is applicable — limited DNAase I digestion followed by electrophoretic

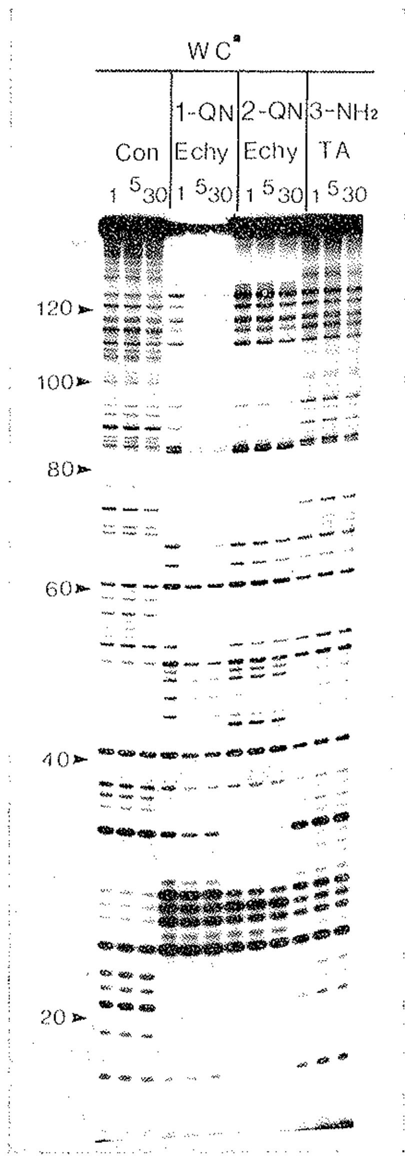

Fig. 10. DNAase I footprinting patterns for mono- and bis-quinoline analogues of echinomycin (1-QN and 2-QN Echy) and bis-3-amino triostin A (3-NH_2TA) on the *tyr*T DNA fragment (Con = control). Symbol WC* indicates that the Crick (lower) strand bears the radioactive 3′-end label. Time in minutes after the addition of enzyme is shown at the top of each gel lane. Numbers at the left refer to the nucleotide sequence numbering scheme as represented in Figure 4. From Low *et al.*, (1986b).

separation of labelled fragments — enabling direct comparison with the effects of the antibiotic on naked DNA. Typical results are shown in Figure 11. Comparing the control core track with the free DNA control track, it is evident that there is an approximately ten-fold periodicity in intensity of the bands, reflecting the accessibility of particular inter-nucleotide bonds to nuclease cleavage. This periodic variation in cutting results from the specificity of nucleosome positioning, i.e., the "phasing" of the DNA sequence with respect to its winding around the protein core, as described by Drew and Travers (1985). In the presence of echinomycin the products of digestion are distinctly different. Many new bands appear (marked with asterisks in Figure 11) at positions approximately mid-way between those cut well in the control. At the same time there is a 50-70% reduction in intensity of the old bands. The observed changes cannot be explained in terms of displacement of a certain fraction of the DNA from attachment to the histone octamer because many of the new bands (those at positions 61, 71 and 82, for example) are totally new in the core digest and virtually missing in free DNA complexed with echinomycin; while others (such as 51, 103 and 114) are strongly enhanced in the nucleosome core but suppressed by the antibiotic in free DNA. We conclude that echinomycin must have caused a substantial fraction of the DNA to change its positioning on the surface of the protein by a translational shift of about five base-pairs, which corresponds to a change in rotational orientation of about half a helical turn (180°). To confirm this interpretation we measured corresponding data for the complementary ("Crick") strand and calculated a differential cleavage plot representing the antibiotic-induced changes in susceptibility of each bond to nuclease attack (Figure 12). Visual inspection of this plot suggests a regular fluctuation in relative accessibility of internucleotide bonds, modulated with a periodicity of about ten nucleotides, and staggered across the helix by about 2-3 bonds towards the 3′ end of each strand, as occurs with free DNA (cf. Figures 3 and 4). The interpretation of these antibiotic-induced perturbations of the nuclease cleavage pattern is materially aided by subjecting the data to Fourier analysis. The computer confirmed the existence of a strong, regular variation with maximum amplitude at a period of 10.64 bonds. No other periodic variations modulated within the range 5.0 to 19.0 bonds were detectable even with amplitudes half the maximum value.

Why should exposure to echinomycin cause DNA to rotate by about half a turn on the surface of the nucleosome, and how much antibiotic

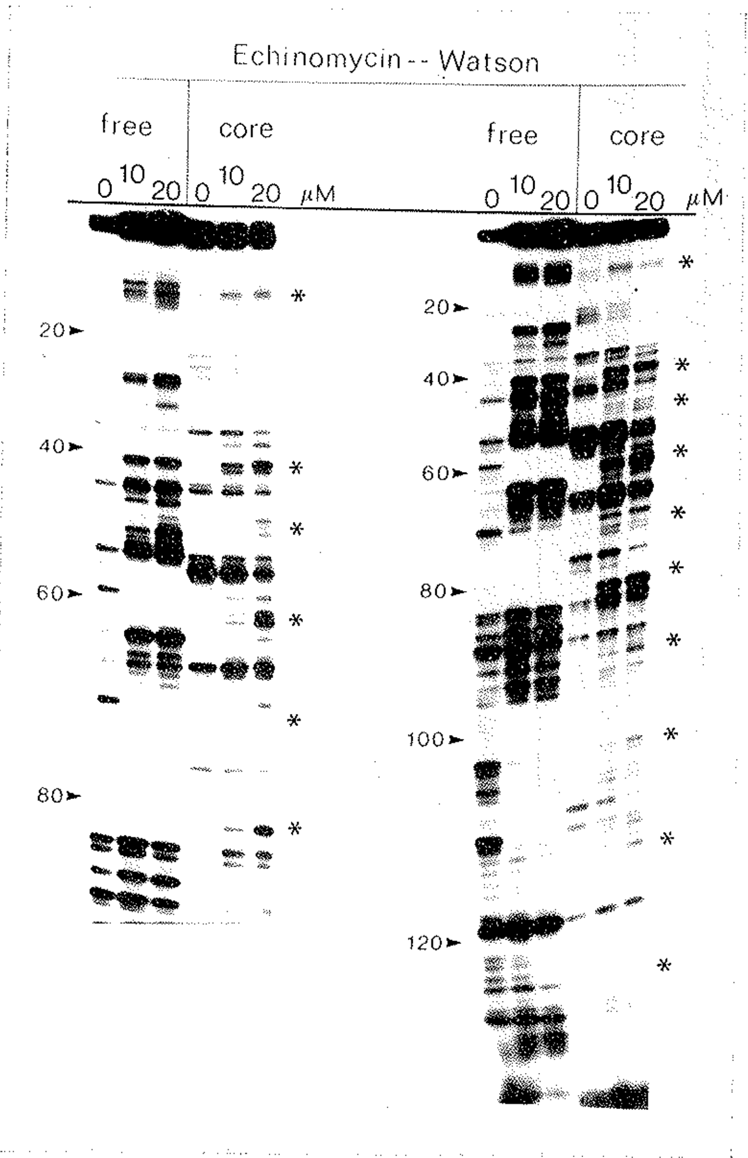

FIG. 11. Effects of echinomycin on the DNAase I digestion pattern of free *tyr*T DNA or reconstituted nucleosome core particles. Two gels derived from the same set of digestion mixtures are shown: that on the left was run for a long time so as to improve resolution of the longer fragments (bands 10-90). In this experiment the « Watson » strand (upper sequence, 5′ to 3′ left-to-right in Figure 4) was labelled at its 3′ end. Each set of three tracks represents a control (no antibiotic) together with samples containing 10 or 20 μM echinomycin as shown at the top of each lane. Numbers at left refer to the numbering scheme shown in Figure 4. Asterisks indicate the new bands which appear in digests of core particles treated with echinomycin. From Low *et al.* (1986c).

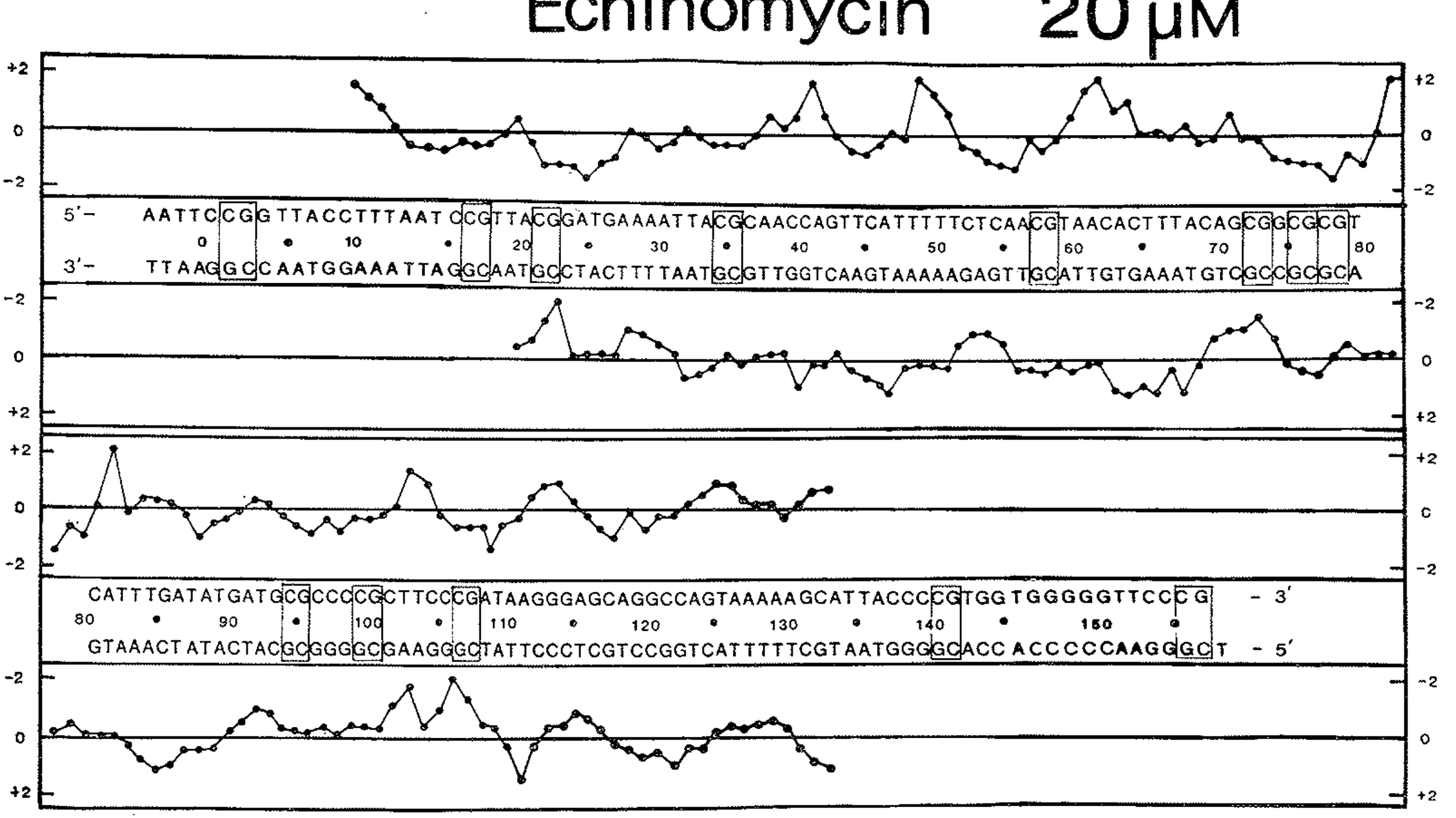

FIG. 12. A plot of differential cleavage representing the extent to which echinomycin (20 μM) affects the susceptibility of nucleosome core particles to attack by DNAase I. The method of plotting is as described in the legend to Figure 4. From Low *et al.*, (1986c).

binding does it take? Our best estimate for the latter is about 2-2.5 antibiotic molecules on average per nucleosome core particle, based on the change in Fourier periodicity from unperturbed DNA (Low *et al.*, 1986c). Granted that each echinomycin molecule which bis-intercalates into DNA unwinds the helix by 48° (Gale *et al.*, 1981) it is natural to suspect that the induced rotation of nucleosomal DNA in some way reflects the unwinding process. That does not seem to be the case. Very similar changes in rotational orientation of the *tyr*T DNA fragment have been observed with distamycin (Low *et al.*, 1986c), netropsin and berenil (Portugal and Waring, 1986) yet none of these substances affects the helical winding of DNA to any great extent. Conversely, actinomycin and nogalamycin, antibiotics which are well-known intercalators causing 26° and 18° helical unwinding respectively (Gale *et al.*, 1981), fail to produce the characteristic pattern of changes in digestion products from nucleosomal DNA (Portugal and Waring, 1986). So far as echinomycin is concerned, the most likely explanation available at present is that it binds to certain sites containing the recognition sequence CpG that are exposed on the outside of the DNA superhelix in the antibiotic-free core particle, and induces them to turn inwards to face the histone octamer. Presumably this serves to optimise non-bonded contacts between the octapeptide ring of the antibiotic and the polynucleotide backbones. Further work with a variety of ligands and different preparations of nucleosome core particles, containing quite different DNA sequences, will be required to probe the generality (or otherwise) of this phenomenon and the nature of the forces which drive it.

Acknowledgements

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THE MOLECULAR MECHANISM OF INTERACTION OF NON-INTERCALATIVE GROOVE BINDING ANTITUMOUR DRUGS WITH DNA

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ABSTRACT

The binding properties of several types of nonintercalative *DNA* binding drugs with respect to possible relationships to anticancer activity and to other biological effects have attracted numerous studies in the past. Members of two recently investigated classes of nonintercalators, related to the *dA·dT* specific antibiotics *netropsin* and *distamycin A*, are investigated in solution for their *DNA* binding properties in relation to their structural modification. The novel oligopeptides, named *lexitropsins*, exhibit a significant reduction of the dA·dT specificity of *netropsin* due to substitution of *pyrrole* by *imidazole*. Although the results indicate that presence of *imidazole* groups in the oligopeptide permits binding to *dG·dC* base pairs, a clear-cut *dG·dC* preference in the binding of *lexitropsins* is not observed.

For a second class of nonintercalators consisting of six-membered aromatic ring systems (*bisquaternary ammonium salts*) it is shown that the *dA·dT* preference in the minor groove binding is influenced by structural factors of the ligand. The *DNA* binding ability of *bisquaternary*

ammonium salts is affected by modification of the aromatic skeleton and the spacer region of the drug molecule. The results suggest that the hydrogen bonding facility of the ligand strongly contributes to base sequence specificity, but other factors also play an important role in the specific recognition of the base sequence. Current *DNA* binding data of nonintercalators in relation to ligand structure and antitumor activity as well as other biological effects are reviewed.

INTRODUCTION

The interaction of small organic ligands to *DNA* has high interest not only in biophysical and biochemical studies [1-5] but also with respect to several areas in biological applications such as molecular pharmacology [3, 6, 7] or carcinogenesis [8]. A large number of *DNA* binding ligands are known as antibacterial, antiviral, antiprotozoal and antitumour active compounds [3-7]. There is evidence for a concept that the biological activity of drugs may be a consequence of their ability to bind specifically and reversibly to *DNA* [3-6, 9]. It has been shown that intercalating as well as nonintercalating *DNA* binding agents exhibit a significant inhibitory effect on nucleic acid synthesis *in vitro* and *in vivo* as well as on protein synthesis [for reviews 1, 5, 6] and these effects are correlated to some extent to antibiotic and antitumour activity or to other different pharmacological activities. The widely investigated antibiotics *netropsin* (Nt) and *distamycin A* (Dst-3) representing nonintercalative DNA groove binders [2, 3, 5, 10] also exhibit antitumour activity but are relatively cytotoxic agents [1, 5]. Another class of nonintercalators comprising to large extent *bisquaternary ammonium heterocyclic* compounds show a significant antitumour activity as measured by a life extension assay with L 1210 leukemia of mice [Table 1, ref. 4, 7]. For those drugs a pronounced correlation between antitumour activity and *DNA* binding ability, estimated by an *ethidium* displacement assay, has been reported [4, 11]. Cain *et al.* [12] proposed structural requirements for the design of active anticancer compounds related to the group of *bisquaternary heterocycles* [4, 7]. The suggested rules include features of a lipophilic-hydrophilic balance, the presence of two charged cationic ends separated by a more or less rigid aromatic skeleton, but the formation of an overall curved conformation for creating an antitumour selective property. These requirements are, however, limited to the antileukemic *bisquaternary ammonium heterocycles* while several natural occurring nonintercalative antibiotics such as

olivomycin, *chromomycin* A_3, *sibiromycin* or *anthramycin* are related to different structural types [for review 3, 5]. The aspects of *DNA* antibiotic binding which are important for the biological effects involve the binding mode, selectivity for *dA·dT* or *dG·dC* base pairs and the preference for *DNAB* conformation. These factors are related to a great extent to the structure and flexibility of the interacting ligand itself. Sequence specificity of the drug binding is of particular interest since this may affect the activity of distinct genes. New information combined with structure-activity relationships of drugs may increase the prospects for rational anticancer drug design.

On the other hand the binding mechanism of nonintercalative small organic molecules with double-stranded or single-stranded *DNA* is fundamental to important molecular recognition processes. The reading of base sequence information, stored in the local *DNA* conformation, by nonintercalative drugs increase our understanding of specific processes in gene expression and control.

The present article deals with *DNA* groove binding properties of two classes of nonintercalative ligands bearing five-membered or six membered aromatic rings. Features of the binding of those ligands are compared with other types of potential nonintercalative anticancer drugs (Table 1).

1) *General features of nonintercalating antitumour antibiotics.*

Among the large number of minor groove binding antibiotics and anticancer drugs two main classes with base pair specificity may be distinguished:

— *dG·dC* preference including the *anthramycin*- and *mithramycin* group.

— *dA·dT* preference including *pyrrole-amidine* antibiotics, *bisquaternary ammonium* compounds, *bisquanylhydrazones* and other structure types such as the antitumour active compound *CC-1065*.

As natural occurring antitumour antibiotics which have been developed to a stage permitting pharmacological studies, *anthramycin*, *sibiromycin* as examples of one group and *mithramycin*, *chromomycin* A_3 as well as *olivomycin* of another one are known to bind preferably to *dG·dC* pairs. This preference is reflected by their inhibitory effect on *DNA*-dependent *RNA* polymerase reactions [Table 1, for rev. see refs. 1, 5]. The source of the *dG·dC* specificity of *chromomycin* A_3, *anthra-*

TABLE 1 - *Comparison of* DNA *binding ability and antitumour activity of nonintercalating* DNA *binding agents.*

Ligand	Base Pair AT	Binding Ability GC	Antitumour Activity	Other Biological Activities	References
Netropsin (Nt)	++	–	(+)	antiviral, antibacterial, cytotoxic	[reviews 1-5]
Distamycin A (Dst-3)	++	–	+	+	[reviews 1-5]
Distamycin Analogues:				cytotoxic, antiviral active	[5, 37]
Distamycin-2	++	–	n.k.	.	
Distamycin-4	++	+	n.k.	weak cytotoxic, weak antiviral	[5, 37]
Distamycin-5	++	+	n.k.	weak cytotoxic, weak antiviral	[5, 37]
Lex.: ImPy	+	–	–	moderately antiviral weak cytotoxic	[24]
Lex.: PyIm	+	–	–	+	[24]
Lex.: Im_2	+	+	–	weak antiviral weak cytotoxic	[24]
Lex.: Im_3	+	+	+	weak antiviral cytotoxic	[24]
Chromomycin A_3	–	++	++	antiviral, antibacterial	[3, 5]
Mithramycin	+	++	++	+	[3, 5]
Olivomycin	–	+	++	+	[3, 5]
Anthramycin	–	++	++	n.k.	[3, 5, 42]
Sibiromycin	–	++	++	antiviral	[3, 5]
Tomaymycin	–	++	++	n.k.	[5, 42]
Bisquaternary Salts:					
SN-6999	++	–	++	antibacteriophage active (cytotoxic)	[4, 33, 34]
SN-6136	++	–	+	+	[4, 33, 34]
NSC-101327	+	+	++	+	[4, 34]
SN-13232	+	–	–	+	[4, 5, 33, 34]
SN-16814	+	+	++	+	[4, 5, 33, 34]
SN-18071	+	+	++	+	[4, 5, 33, 34]
Cc-1065	++	–	++	cytotoxic	[review 5, 35]

Preferred base pair binding ability is based on CD, UV absorption measurements (this work, ref. 30, 36) and on an *ethidium* displacement

mycin and analogs has been attributed to the *2-amino* groups of *guanines* in the minor groove [5, 13, 14].

The best examples among the group of *pyrrole-amidine* antibiotics are *Nt* and *distamycin A* which as natural compounds representing a kind of prototype of nonintercalators with high preference to dA·dT sequences [5, 10, 15, 16]. The sequence specificity is influenced by modification of their chemical structure. It has been demonstrated that chain elongation in the *distamycin* series binds to longer dA·dT sequences but the *pentamethylpyrrolecarboxamide* compound which covers 6 to 7 base pairs [17] may also tolerate binding to dG·dC pairs [5]. Synthesis of bifunctional *Nt*-like compounds leads to increased binding affinity for longer dA·dT sequences [18]. A new concept for structural modifications of *netropsin*-type *oligopeptides* was based on data of x-ray crystal structure analysis of Dickerson and coworkers [19, 20]. It was suggested that replacement of *pyrrole* by *imidazole* in the Nt-molecule should alter the strict dA·dT base pair preference. Lown and Krowicki [21] developed a new strategy of a total synthesis of the lead *oligopeptides Nt* and *Dst-3* and have successfully applied it to *lexitropsins* [22].

2) *Novel oligopeptides containing five-membered aromatic rings.*

A series of *oligopeptide* structures of *lexitropsins* were synthesized [22] containing the base-contact elements *pyrrole-amide* of Nt (Fig. 1A) substituted by *imidazole-amide* groups (Fig. 1B,C). An analog consisting of three *imidazole-amide* units was also available (Fig. 1D).

For binding studies in solution circular dichroism (CD) as a powerful technique for optically inactive nonintercalators have been applied with advantage, since most of these drugs exhibit induced Cotton effects outside the CD spectral range of DNA, which directly reflect bound ligand molecules [15, 16, for rev. ref. 5]. As an example of *lexitropsin* binding to DNA the CD spectra of complexes of DNA and poly(dA-dT)·poly(dA-dT) with *Py-Im* are shown in Fig. 2. The CD binding signal is located around 315 nm to 320 nm which is similar for other *lexitropsins*. Essential binding data of various *lexitropsins* to synthetic DNA duplex polymers are displayed in plots of their binding signal, $\Delta\varepsilon$, as a function of the total ratio, r', of ligand per nucleotide (Fig. 3). It is obvious that binding to poly(dA)·poly(dT) of *lexitropsins* is less efficient relative to that of *Nt(Py-Py)*. The ligands *Im₂* and *Im₃* show the weakest binding tendency for the dA·dT-homopolymeric duplex. Different from this behaviour *Nt* and *Im₂*

FIG. 1. Chemical structures of nonintercalative ligands containing five-membered rings: *netropsin* (A), *distamycin* A (B) and *lexitropsins* (C, D).

C: Im-Py (Imidazole-Pyrrole), Z = N, Y = CH
Py-Im (Pyrrole-Imidazole), Z = CH, Y = N
Im-Im, abbreviated by Im_2, Z = N, Y = N
D: Im-Im-Im, abbreviated by Im_3.

exhibit the lowest binding effect to poly(dA-dC)·poly(dG-dT) whereas *Im-Py, Py-Im* and Im_3 bind to a higher extent to this polymer at moderate ionic strength (Fig. 3). We also observed a greater binding efficiency of the latter ligands to natural GC-rich DNA than to AT-rich DNA [23]. This change in the base preference upon replacing *pyrrole* by *imidazole* seen in

the present CD binding behaviour was also demonstrated by a gradual melting temperature decrease of *lexitropsin* complexes formed with dA·dT-rich and dG·dC-rich DNA's [22, 24]. As a further binding parameter the binding site size (n) of *lexitropsins* was estimated from CD titration data. Table 2 shows values of n from 4 to 5.5 bp which is valid for different DNA polymers. As expected, the binding site sizes correspond to that of Nt, which has been previously determined to be n = 4 bp [5]. The variation of the binding selectivity to poly(dA-dT)·poly(dA-dT) and to poly(dA-dC)·poly(dG-dT) is also reflected in their inhibitory effect on the DNAase I cleavage kinetics of the two DNA's (Table 3).

Most striking alterations in the binding affinity to DNA sequences *of imidazole* containing *lexitropsins* were observed at very high salt con-

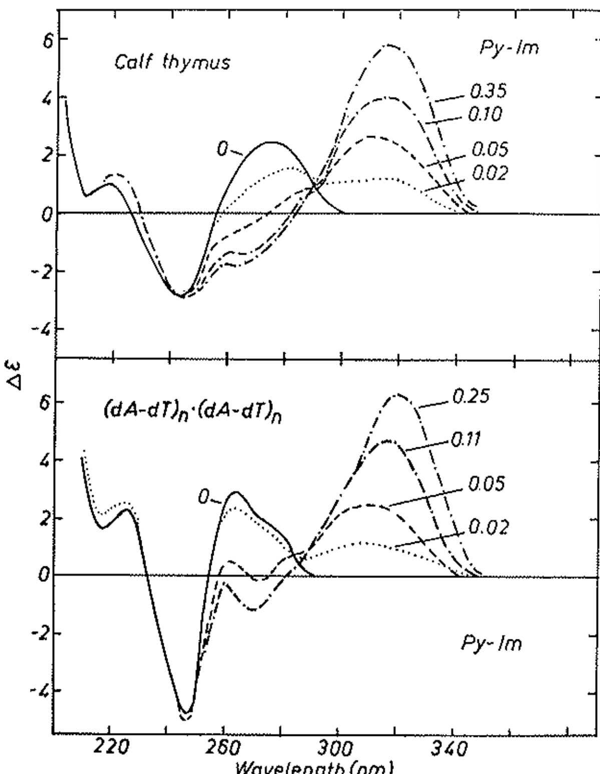


FIG. 2. CD spectra of the *lexitropsin* complexes of *Py-Im* with calf thymus *DNA* and *poly(dA-dT) · poly(dA-dT)* in 0.1 M NaCl, pH 6.8; attached numbers indicate molar ratio (r′) of ligand per nucleotide added.

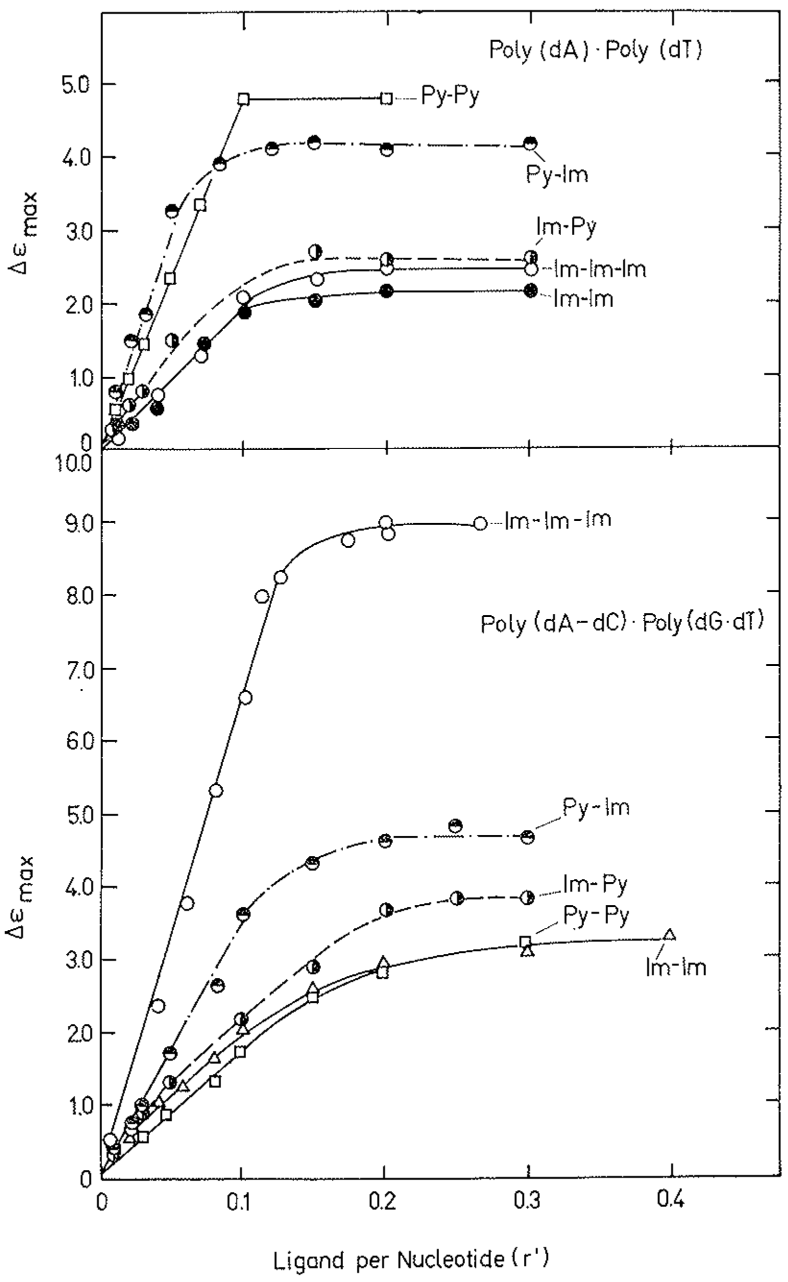


FIG. 3. CD titration at 315 nm to 320 nm of *DNA* duplex polymers with *netropsin (Py-Py)* and the *lexitropsins Py-Im, Im-Py, Im_2* and *Im_3* at 0.1 M NaCl (according to Burckhardt *et al.*, 1986 [23]).

TABLE 2 - *Apparent binding site size of* lexitropsins, *in base pairs* (n).

DNA	Py-Py (netropsin)	Py-Im	Im-Py	Im-Im
Poly(dA) · Poly(dT)	5	5.5	5.5	4.2
Poly(dA-dT) · Poly(dA · dT)	5	5	(3)	(3)
Poly(dA-dC) · Poly(dG-dT)	—	3.9	(3.2)	(2.6)

(n) was estimated from CD titration curves (see Fig. 4) at saturation of the binding, data evaluation is reliable in first approximation only for curves showing a sharp initial rise; a, values are not realistic due to low binding affinity.

centration [23]. In high salt media ionic interactions are suppressed and mainly specific contacts are maintained. Arguments for the dA·dT base sequence specificity of *Nt* [2, 5, 15, 16] and for a binding affinity to *dG·dC* pairs of the *distamycin* analog Dst-5 [25] were based on such studies. A summary of the lexitropsin binding behaviour at high salt is presented in Table 4, in which $\Delta\varepsilon_{rel}$ values close to 1.0 indicate high salt stability of the *DNA*-ligand complex suggesting high specificity. The most stable complexes are formed for pure *dA·dT* duplex *DNA*'s with *Nt* (*PyPy*) while those of Im_2 andIm_3 are not stable. However, replacement of only one *pyrrole* by *imidazole* has not a large influence relative to that of *Nt*. In contrast to that poly(dA-dC)·poly(dG-dT) exhibits the lowest salt stability for *Nt* (*Py-Py*) whereas the presence of one *imidazole* leads to slightly

TABLE 3 - *Protection of duplex* DNA'*s against* DNAase I *cleavage by interaction with* lexitropsins *in Tris buffer solution.*

Ligand	Degree of Protection from Enzymatic cleavage (%) Poly(dA-dT) · Poly(dA-dT)	Poly(dA-dC) · Poly(dG-dT)
Py-Py (Netropsin)	100	0
Im-Py	100	23
Py-Im	89	13
Im-Im	2	8
Im-Im-Im	6	40

measured at r′ = 0.3 (ratio of ligand per nucleotide)

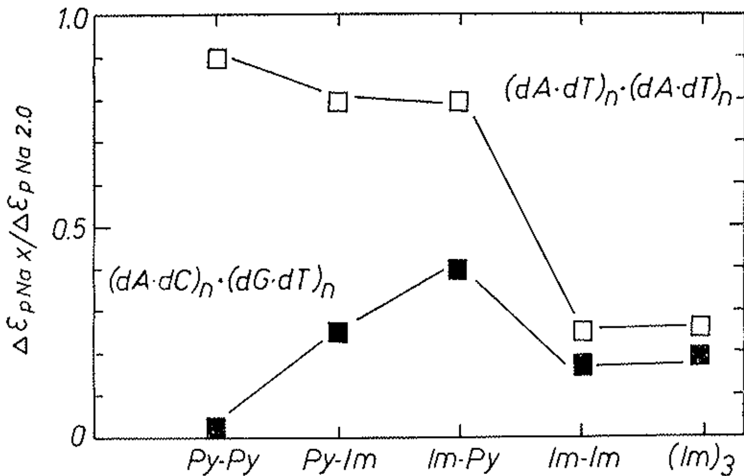


FIG. 4. Schematic representation of the salt stability of lexitropsin complexes of two *DNA* duplex polymers by replacement of *pyrrole* (Py) in the *netropsin* molecule (Py-Py) with *imidazole* (*Im*), measured by CD. Ordinate: normalized $\Delta\varepsilon$ at 315 nm, X = 1 M NaCl.

higher stability of this duplex *DNA* upon binding of *Im-Py* and *Py-Im* (Table 4).

Fig. 4 compares the essential binding effects of *lexitropsins* for *poly(dA-dT)·poly(dA-dT)* and *poly(dA-dC)·poly(dG-dT)* at 1 M NaCl. The *dA·dT* specificity of *Nt (Py-Py)* is drastically decreased by substitution of both *pyrroles* by *imidazole* as shown for Im_2 and Im_3. A significant binding ability to *poly(dA-dC)·poly(dG-dT)* occurs for *Py-Im* and *Im-Py* while *Nt* with two *pyrroles* has no affinity and Im_2 as well as Im_3 are only weakly bound. Obviously there are no remarkable differences in the binding ability between Im_2 and Im_3 to both sequences of the polymers at 1 M NaCl (Table 4, Fig. 4). Comparison with the alterations of recognition elements of *lexitropsins* for base sites (Table 4) suggests that the elimination of base contact sites of *pyrroles* is responsible for the dramatic reduction of the dA·dT specificity measured by the CD data in Figs. 3 and 4 and in Table 4. Important base contacts of *lexitropsins* are based on suggestions of Dickerson and coworkers derived from x-ray crystal structure analysis [19, 20] and are involved in the minor groove binding as schematically displayed in Fig. 5. Thus our solution studies support the concept on the importance of van der Waals contacts in the recognition of *dA·dT* pairs by *Nt* as proposed by Dickerson and coworkers [19, 20]. On the other

TABLE 4 - *Binding Specificity of Netropsin* (Py-Py) *and Lexitropsins in Relation to AT- and GC-Recognition Elements.*

Ligands	$\Delta\varepsilon_{rel}$ at high salt			Number of Recognition Elements		
	Poly(dA-dT) · Poly(dA-dT)	Poly(dA) · Poly(dT)	Poly(dA-dC) · Poly(dG-dT)	AT(NH)	AT(CH)	GC(N)
Py-Py	0.90	0.94	0	5	4	0
Py-Im	0.80	n.d.	0.23	5	3	1
Im-Py	0.75	n.d.	0.38	5	3	1
Im-Im	0.15	0	0.10	5	2	2
Im-Im-Im	0.05	0	0.08	6	2	3

$\Delta\varepsilon_{rel} = \Delta\varepsilon$ at 2M NaCl/$\Delta\varepsilon$ at 0.01M NaCl; $\Delta\varepsilon_{rel}$ is taken as a measure of the binding specificity monitored at 10^{-2}M NaCl and 2M NaCl; at high salt concentration unspecific contacts are mainly suppressed while base contacts dominate; $\Delta\varepsilon_{rel} = 1.0$ at 10^{-2} M NaCl. (NH), (CH) and (N) are contact sites of the ligands.

hand the presence of *imidazole* allows hydrogen bonding with NH_2 of *guanine* in the minor groove [Fig. 5, refs. 19, 20] and therefore should contribute to enhancement of a dG·dC specific binding of the *oligopeptide*. Although *imidazole* in the *lexitropsin* structure obviously tolerates binding to *dG·dC* pairs in *poly(dA-dC)·poly(dG-dT)* (Fig. 3) and to *dG·dC* pairs in DNA [22] an increase in the stability of *lexitropsin* complexes with dG·dC containing duplex *DNA*'s is not observed [23]. Binding of Im_2 and Im_3 to *poly(dG-dC)·poly(dG-dC)* has been found at moderate ionic strength but it is completely abolished at 0.5 M NaCl [23]. In agreement with recent results on melting temperature changes and footprinting experiments [22] *imidazole* containing *oligopeptides* recognize *dG·dC* pairs adjacent to *dA·dT* pairs. Binding data at high salt suggest, however, that the acceptance of *dG·dC* pairs by these *lexitropsins* is favoured by electrostatic interactions and recognition of neighboured *dA·dT* pairs. The molecular electrostatic potential in the minor groove of *dA·dT* and *dG·dC* sequences [26, 27] is another important factor which very likely influences the specificity of *lexitropsin* binding in a certain sequence.

3. *Studies on antitumour drugs containing six-membered aromatic rings: bisquaternary ammonium compounds.*

This group of anticancer compounds has been synthesized by Denny *et al.* [7] and concerns ligands shown in Fig. 6. The essential structural features that distinguish these ligands from the *netropsin*-type are the presence of six-membered aromatic rings linked in para position by distinct spacer segments (Fig. 6). This limits the flexibility of the ligand molecules with respect to formation of a highly curved structure. On the other hand ligand molecules of the *distamycin*-type, bearing *benzamide* units linked together in meta position, are strongly curved [28]. Previous *DNA* binding measurements demonstrated a *dA·dT* preference and minor groove binding of *bisquaternary ammonium heterocycles* [4, 29, 30]. The binding behaviour may be, however, very different depending on the chemical structure of the ligand [30, 31]. Two compounds, *SN-18071* and *pentamidine* (Fig. 6) are particularly interesting since they are devoid of NH groups but show also a binding ability to dA·dT pairs. Detailed studies were previously devoted to *NSC-101327* [29] and reviewing comparative measurements included several synthetic ligands of this group [30, 31]. Recent investigations include *SN-6999*, *SN-13232* and *SN-16814*. The two latter compounds are interesting since it is believed that the flexible aliphatic

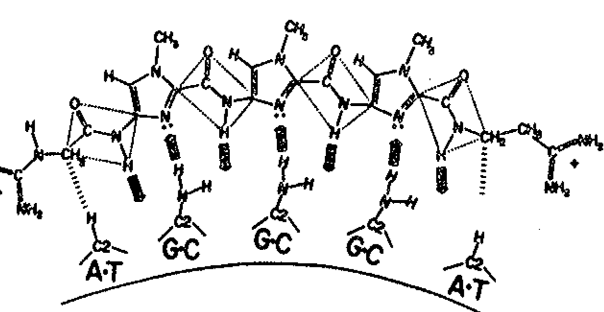


Fig. 5. Features of the binding of *netropsin* (*Py-Py*) and *lexitropsins* (*Im-Py, Py-Im*, Im_2, Im_3) to base pairs in the minor groove of *B-DNA* demonstrating van der Waals' non-bonded contacts (barred lines) and hydrogen bonds for *NH* donor groups to acceptor direction. The latter involves O(2) of *thymine* or (and) *N(3)* of *adenine* for *dA·dT* pairs (based on suggestion of Dickerson and co-

SN 6999 (NH_2- : NSC 176319)

NSC 101327

SN 16814

SN 13232

SN 18071

Pentamidine

FIG. 6. Chemical structures of nonintercalative ligands containing six-membered aromatic rings spaced by linkers in para position: *bisquaternary ammonium heterocycles* and *pentamidine*.

link in the aromatic skeleton of *SN-13232* is responsible for the absence of an antitumour activity due to a low binding affinity for *DNA* found in an *ethidium* displacement assay [4]. On the other hand the insertion of a rigid *bicyclo-octyl* group resulted in a good *in vivo* antitumour activity [Table 1, ref. 4].

As an example differences in the binding behaviour of *SN-13232* and *SN-6999* to *poly(dA-dT)·poly(dA-dT)* are more clearly monitored by CD spectral studies of their complexes. As shown in Fig. 7A the induced CD signal located outside the spectral range of *DNA* is a good measure for bound ligand molecules. The corresponding binding curves displayed in Fig. 7B indicate a broad curve in case of *SN-13232* but a clear-cut sharp rise of $\Delta\varepsilon$ up to saturation for *SN-6999*. This suggests a relatively strong binding effect of the latter and a weaker binding of *SN-13232*. Significant differences in the binding behaviour to various *DNA*'s have been found for *SN-6999* and *SN-18071* (Fig. 8). Whereas the CD titration curves in Fig. 8 exhibit a pronounced preference for *dA·dT*-rich *DNA*'s and a poor affinity to *dG·dC*-rich *DNA*, *SN-18071* shows broad binding curves suggesting a much lower base pair affinity. Although it has some preference for *dA·dT* pairs binding to *dG·dC* containing duplex *DNA* is observed. This indicates that distinct structural alterations including lacking of reactive *NH* residues in *SN-18071* and partially in *pentamidine* affect the stability of the *dA·dT* selective binding.

Binding data have been obtained for various *DNA*'s with different antitumour drugs which are listed in Table 5. Like N[t], *SN-6999* shows the highest selectivity for *dA·dT* pairs while other drugs may bind to some extent to *dG·dC* sequences and to RNA.

Further evidence for structure-related differences in the binding to *dA·dT* sequences emerges from melting temperature changes of *dA·dT* containing *DNA* duplex polymers upon interaction with these antitumour compounds. As seen in Table 6, *SN-6999* causes the most pronounced melting temperature increase indicating a strong binding effect very similar as observed for *Nt*. In accordance with the CD binding results the ΔT^{m} values of the related ligands are decreased showing the weakest stabilization of *DNA* in complexes with the ligands devoid of *NH* groups (*SN-18071* and *pentamidine* in the middle).

Competitive binding studies with *Nt* as a potential minor groove binder also suggest a clear-cut relationship between structure and binding ability. Fig. 9 shows that addition of increasing *Nt* amounts to preformed complexes of *SN-18071* and *pentamidine* with *poly(dA-dT) · poly(dA-dT)*

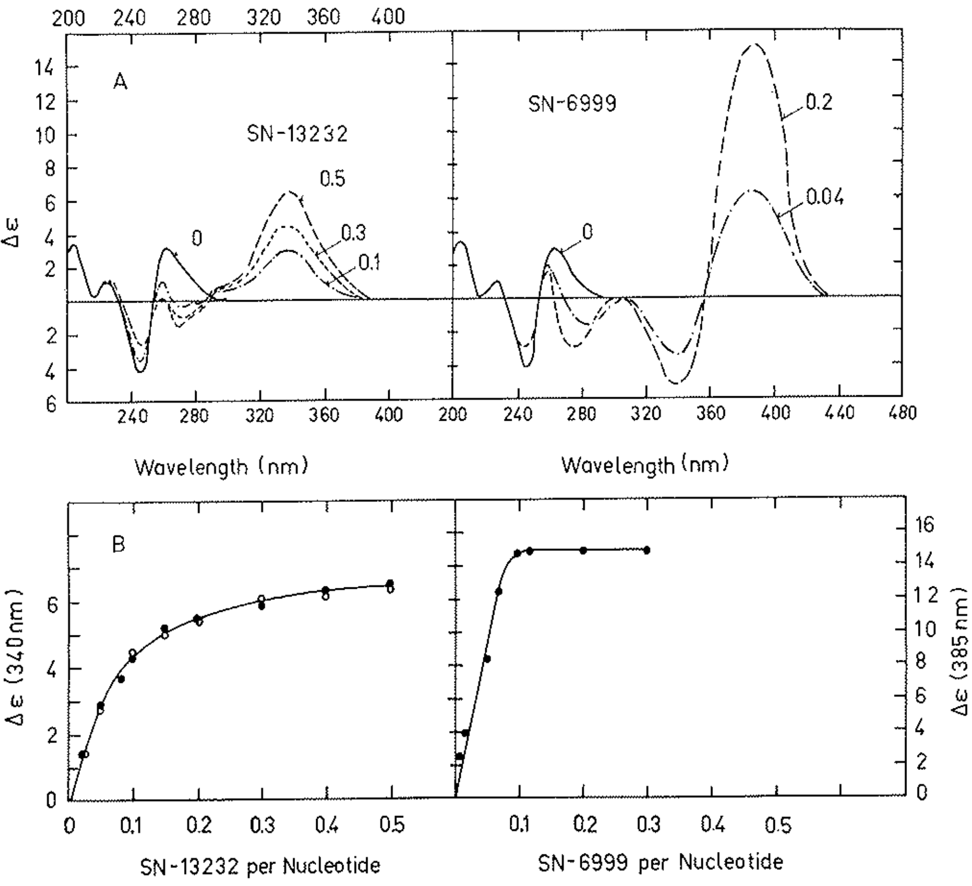


FIG. 7. A: CD spectra of *Poly(dA-dT)·poly(dA-dT)* complexes with *SN-13232* and *SN-6999* in 0.1 M NaCl, attached numbers designate r′ (molar ligand ratio per nucleotide added). B: CD titration curves showing different binding strength.

lowers the binding signal to zero indicating a complete release of these ligands from their complexes. On the other hand *SN-6999* and its analogue are only partially displaced from their complexes by *Nt*. Conversely *SN-6999* is able to remove *Nt* from its complex to some extent (Fig. 9B). These results permit two main conclusions. Firstly, all ligands under consideration are minor groove binders, and secondly, compounds devoid of NH groups do not significantly compete with the interaction of *Nt*. Taken together all experimental binding as well as stability measurements, it is clear that *SN-6999* possesses the highest affinity to $dA \cdot dT$ sequences

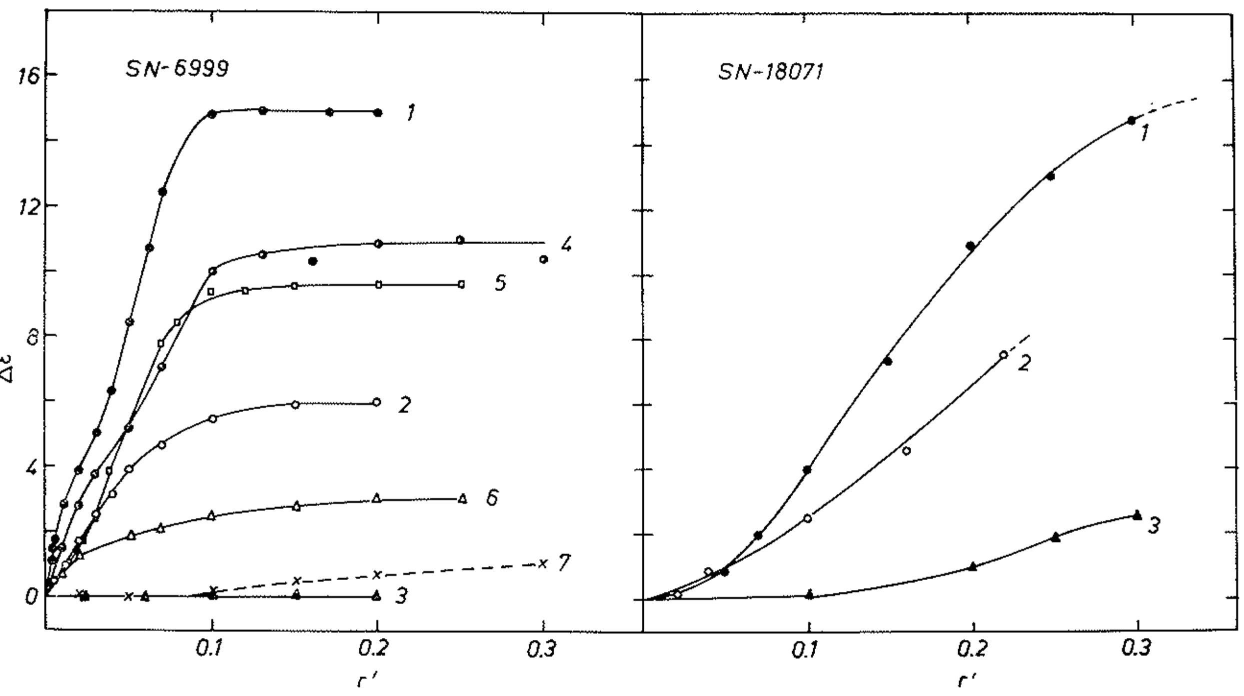


FIG. 8. CD titration curves of various *DNA*'s of *SN-6999* (A) and *SN-18071* (B) in 0.1 M NaCl, presented by plots of Δε as a function of r′; ratio of drug per nucleotide. 1, *poly(dA-dT) · poly(dA-dT)*, 2, calf thymus *DNA* (58 mole-% A+T); 3, *poly(dG-dC) · poly(dG-dC)*; 4, *poly(dI-dC) · poly(dI-dC)*; 5, *poly(dA) · poly(dT)*; 6, Str. chrysomallus *DNA* (28 mole-% A+T); 7, *poly Br(dG-dC) · poly Br(dG-dC)* existing as *DNA* in Z-form.

TABLE 5 - *Binding effects of nonintercalators with nucleic acids measured by circular dichroism.* $\Delta\varepsilon$ ($M^{-1} \cdot cm^{-1}$).

DNA	Netropsin (315nm)	SN-6999 (385nm)	NSC-101327 (325nm)	SN-16814 (308nm)	SN-13232 (338nm)	SN-18071 (405nm)	Pentamidine (280nm)
$(dA)_n \cdot (dT)_n$	4.8	10.0	10.2	1.3	5.0	—	—
$(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$	4.0	14.8	9.3	2.5	4.5	4.0	2.5
DNA (58 mole-% AT)	3.2	5.5	1.0	1.0	0.6	0.5	1.0
$(dG\text{-}dC)_n \cdot (dG\text{-}dC)_n$	no CD	no CD	−1.5	no CD	—	1.0[a]	—
$(rA)_n \cdot (rU)_n$	no CD	no CD	6.3	0.7	no CD	0.9	—

$\Delta\varepsilon$ values were measured at $r' = 0.1$ (ligand per nucleotide) at 0.1 M NaCl.

TABLE 6 - *Melting temperature changes* (ΔTm) *of DNA duplexes upon binding with bisquaternary ammonium heterocyclic ligands and netropsin* (Nt) *in* 0.1 M NaCl, *at* r′ (*molar ratio of ligand per nucleotide*) = 0.1.

Ligand	ΔTm (°C)	
	$(dA\text{-}dT)_n \cdot (dA\text{-}dT)_n$	$(dA)_n \cdot (dT)_n$
Nt	22.5	31.8
SN-6999	22.3	21.9
NSC-101327	15.4	16.5
SN-13232	12.8	9.9
SN-16814	7.0	5.4
SN-18071	3.8	5.3
Pentamidine	4.8	8.1

among this group of antitumour compounds, and it shares common binding regions with *Nt* of about 4 to 5 base pairs. The order of the relative binding ability for *dA·dT* sequences of these minor groove binders appears as follows:

Netropsin > SN-6999 > SN-6136 > SN-13232 > SN-16814 > SN-18071, pentamidine. It is important to note that a very similar variation of the binding behaviour of some of these ligands has been concluded from computation of the complexation energies for *poly(dA-dT)·poly(dA-dT)* [32]. On the basis of CPK space filling models features of the minor groove binding have been proposed for *SN-6999*, *SN-13232* and *SN-6136* [30] which are displayed in Fig. 10. Possible contacts to base edges and to sugar moieties are schematically drawn in the binding model (Fig. 10). Because of its relatively short aromatic skeleton *SN-6999* fits snugly in the minor groove and may probably replace the spine of hydration on the *dA·dT* base pair region in analogy to that proposed for *Nt* [19, 20]. The drastic differences seen in the binding behaviour between nonintercalating drugs consisting of *NH* groups and those devoid of *NH* groups suggest that hydrogen bonding facility of the ligand strongly enhances the specificity for the interaction with the target sequence.

As pointed out by Dickerson and coworkers [19, 20] van der Waals contacts are essential for base sequence reading of the ligand, that means

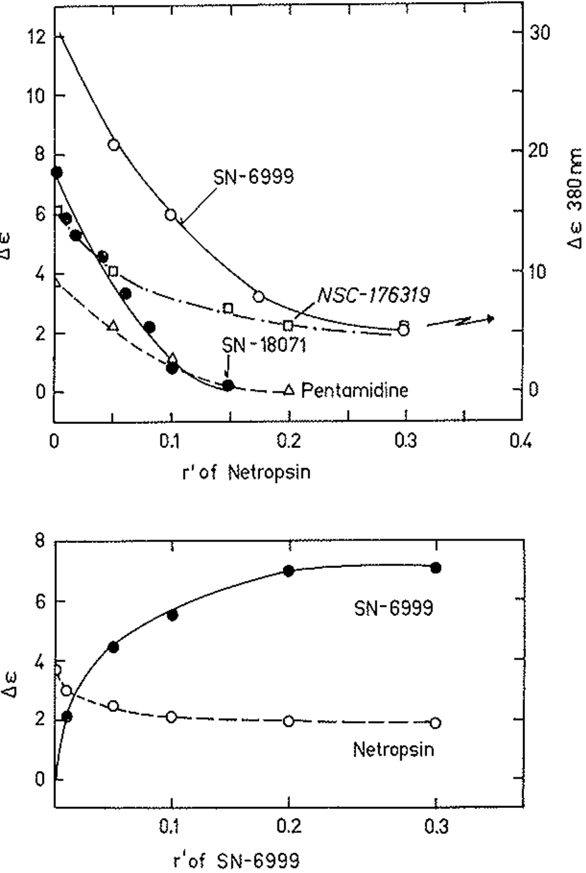
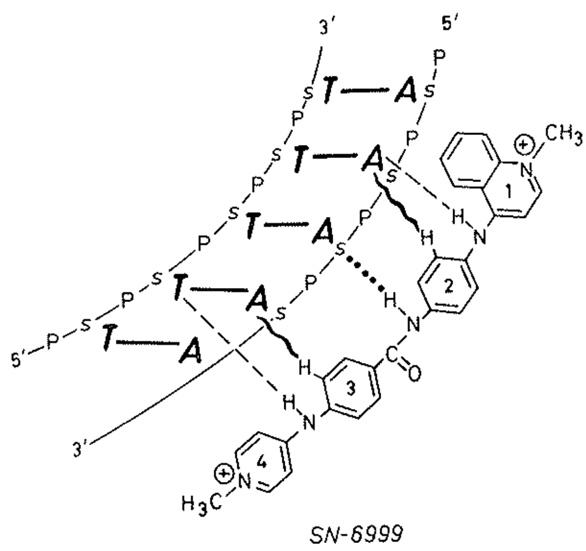


FIG. 9. Competitive binding data. *Upper part*: Changes of the binding of antitumor drugs (*SN-6999, NSC-176319, SN-18071*) and *pentamidine* to *poly(dA-dT)* · *poly(dA-dT)* caused by titration with increasing r′ of *netropsin*. Binding signals, Δε, of the ligands are located outside of that of *netropsin*. *Lower part*: CD titration of the preformed *netropsin* complex of *poly(dA-dT)* · *poly(dA-dT)* (r′ = 0.3 Nt per nucleotide) with *SN-6999*. Binding signals do not overlap each other to significant extent, Nt at 305 nm, *SN-6999* at 380 nm (according to Luck *et al.*, 1986 [36]).

to distinguish between *dAdT* and *dGdC* pairs, the process of which does not necessarily require a tight binding. However, in our opinion high sequence specificity of the recognizing ligand implies tight binding and a selective location, factors which are clearly governed by hydrogen bonding capacity as evidenced by the investigated non-intercalative ligands.



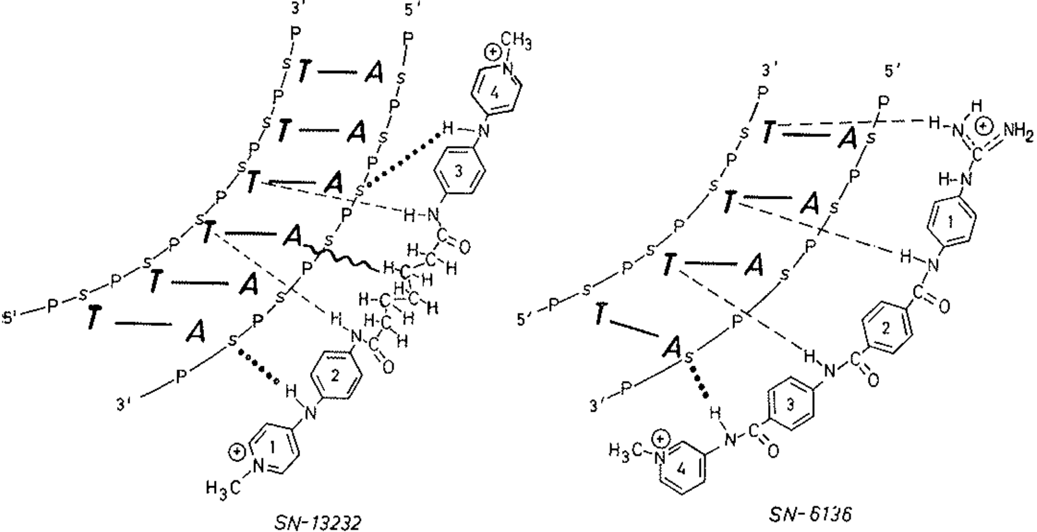


Fig. 10. Schematic representation of binding models of three antitumour active compounds (*SN-6999, SN-13232, SN-6136*) with a homopolymeric duplex segment of *dA·dT* pairs in the minor groove of *B-DNA*. Dashed lines: hydrogen bonds to *O(2)* of *thymine* and *N(3)* of *adenine*, dotted lines to *O1'* of sugar, wave-lines possible van der Waals' contacts (based on examination of space filling models, suggested van der Waals contacts are proposed in analogy to the *netropsin-dodecamer* model of Kopka *et al.*, [19, 20]).

Thus, van der Waals contacts together with hydrogen bonding and ionic contacts as well as other factors, such as conformational adaptability of the ligand structure are important elements that contribute to the base-sequence specificity of the ligand.

4) *Correlation between DNA binding ability and biological activity.*

The relationship between ligand structure, DNA binding ability and biological activity including the cytostatic activity contain a number of complicated and unsolved problems. Accumulated data favours the concept that base sequence selectivity of DNA-drug binding in some way may be the source of the inhibitory effects of drugs in living cells. Structure-relationships for nonintercalators were considered in several reviews [3-7, 9] and have been recently extended to the novel *oligopeptides* related to *netropsin*, the so-called *lexitropsins* [24].

Examples of some main classes of nonintercalators summarized in Table 1, indicate correlations of a base pair preference of these ligands to their antitumour activity, which is not *a priori* similar for all other biological effects. It may be seen that members of the *mithramycin-* and *anthramycin*-group showing a preference for *dG·dC* base pairs [37] are antitumour active throughout [for review see ref. 5]. Antitumour activity or cytotoxicity effects are also well known for *bisquaternary ammonium* salts which exhibit a selectivity for *dA·dT* pairs. In these cases a general concept for the structural requirements has been developed which was applied for the design of synthesis of antitumour active compounds [4, 7]. However, since some of these compounds may bind to *dG·dC* sequences a strict relation of a *dA·dT* base pair preference to their biological activity cannot be generalized. As further seen in Table 1 the cytotoxicity and weak antitumour activity of *Nt* and *distamycin A* are decreased for related *pyrrole-imidazole dipeptides* (*ImPy*, *PyIm*) and the *imidazole dipeptide* (*ImIm*)

There is also a gradual change in the antiviral activity [24]. Previous studies [38] have shown that chain elongation in the *distamycin* series synthesized by Arcamone and coworkers, [see ref. 5] is associated with an increase in antiviral activity and a lowering of the cytotoxicity [38]. It seems also that the *formyl* side chain is critical for the biological activity of *distamycins* [24, 38].

These considerations demonstrate that the *in vitro* binding behaviour must be treated with care in drawing conclusions for *in vivo* effects of

drugs. It is clear from our current knowledge of biophysical data on intercalating and nonintercalating *DNA*-binding drugs that binding effects *in vitro* correlated to biological activities are not sufficient for the understanding of the effects *in vivo* and for general conclusions. Structural factors and properties related to other targets of the cell have to be included. This is underlined by a few examples. Although *distamycin A* is known to bind strongly to *DNA* and it exhibits a drastic inhibitory effect on *DNA*-dependent *nucleic acid* syntheses its effect on transforming cells of B. subtilis originates from an influence on the bacterial cell wall by blocking the penetration of donor *DNA* [39]. Inhibition of phage multiplication of *Dst A* is also unrelated to its *DNA* binding ability; more likely the drug interacts with the membrane or cell wall preventing phage adsorption [40]. Likewise the inhibition by *Nt* or *Dst A* of stable protoplasts type L-forms, e.g., of *E. coli*, is most probably related to a drug interaction with the membrane rather than with *DNA* [41].

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DESIGN OF SYNTHETIC SEQUENCE SPECIFIC DNA BINDING MOLECULES

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ABSTRACT

The design of sequence specific DNA binding molecules has advanced in recent years due, in part, to analytical techniques such as footprinting and affinity cleaving which allow rapid and precise analysis of hundreds of potential DNA binding sites on sequencing gels (Dervan, 1986). *Nona*-N-methylpyrrolecarboxamide, a synthetic analog of the natural product distamycin, binds 11 contiguous base pairs of A,T rich DNA in the minor groove. A synthetic polypeptide, 52 amino acid residues in length and derived from a recombinase protein, binds uniquely 12 base pairs of DNA, most likely in the major and minor groove. The construction of synthetic molecules that bind in the minor and major groove of DNA with incrementally increasing sequence specificity is the first step toward defining a set of rules for the three-dimesional readout of double helical DNA. This may lead to new research tools for use in cancer research, diagnosis of disease states at the level of DNA (oncogenes), and novel chemotherapeutic strategies such as artificial repressors for inactivation of these genes.

1. INTRODUCTION

The local structure of right-handed double-helical DNA depends on base sequence (Dickerson *et al.*, 1982; Kennard, 1984; A. H.-J. Wang *et*

al., 1979; Rich *et al.*, 1984; Calladine, 1982; Calladine and Drew, 1984). Many (but not all) low molecular weight natural products isolated as antiviral, antibiotic or anticancer compounds appear to bind in the minor groove of DNA (Gale *et al.*, 1981). Sequence-specific DNA-binding proteins appear to bind, at least in part, in the major groove of DNA (Takeda *et al.*, 1983). Therefore, the design aspects for the construction of sequence-specific DNA-binding molecules focus on understanding the molecular basis for the binding of low molecular weight drugs in the minor groove and of polypeptides derived from proteins in the major groove. The tools of synthetic organic chemistry are used in combination with nucleic acid techniques such as high resolution gel electrophoresis to define, in part, the scope and limitations of this problem (Dervan, 1986).

Examination of natural products that bind to specific sequences in the minor groove of B-DNA reveal that some of these DNA-binding molecules are flat, and this shape allows them to intercalate between the base pairs (Gale *et al.*, 1981). Other natural products with less obvious structural features fit snugly in the minor groove of the DNA helix. We would like to understand the relative contributions of nonbonded stabilizing and destabilizing interactions that allow structurally diverse natural products such as netropsin and distamycin to bind A,T-rich sequences of DNA; and echinomycin, triostin A, bleomycin, actinomycin D, and chromomycin to bind G,C-rich sequences of DNA. A small but significant number of crystal structures of small molecule-oligonucleotide complexes such as actinomycin D (Jain and Sobell, 1972), daunomycin (Quigley *et al.*, 1980), triostin A (Wang *et al.*, 1984), echinomycin (Ughetto *et al.*, 1985), and netropsin (Kopka *et al.*, 1985) are now available. In the absence of crystal structures for other natural products, such as chromomycin bound to duplex DNA, we rely on model building with consensus nucleotide sequences gained from footprinting experiments on several DNA restriction fragments. Plausible models are tested by synthesizing simpler molecules that are believed to contain key recognition features of the more complex natural product. CPK space-filling models of DNA-small molecule complexes are useful for indicating what is unrealistic. As computer graphics model building and adequate molecular mechanics programs become available, there is hope for improvement in this area (Kollman, 1985).

2. The Analytical Problem

There are four bases possible for each nucleotide position on each strand of the DNA helix, and, within the constraints of the A,T and G,C complementary nature of double-helical DNA, for a binding-site size of n base pairs there are $(4^n)/2$ distinguishable sequences for odd n and $(4^n)/2 + (4^{n/2})/2$ for even n. Natural products that are in the molecular weight range of 500 to 2000 are sufficiently large to cover two to six contiguous base pairs. For binding-sites that are two to six base pairs in size, there are 10 to 2080 unique combinations of base pairs or specific binding sites on double helical DNA, respectively (Table 1). During the past few years, a key issue has been the development of the analytical methods to analyze the sequence specificities of either natural or synthetic DNA-binding small molecules. These methods are footprinting (Galas and Schmitz, 1978; Van Dyke *et al.*, 1982; Van Dyke and Dervan, 1982;

Table 1 - *Binding Site Frequency for DNA Binding Molecules.*

Size Site (n)	Unique Sites (N)	Unique Specificity[a]	Unique A·T Sites	A·T Specificity[b]
1	2	5.0×10^{-1}	1	5.0×10^{-1}
2	10	1.0×10^{-1}	3	3.0×10^{-1}
3	32	3.1×10^{-2}	4	1.3×10^{-1}
4	136	7.4×10^{-3}	10	7.4×10^{-2}
5	512	2.0×10^{-3}	16	3.1×10^{-2}
6	2,080	4.8×10^{-4}	36	1.7×10^{-2}
7	8,192	1.2×10^{-4}	64	7.8×10^{-3}
8	32,896	3.0×10^{-5}	136	4.1×10^{-3}
9	131,072	7.6×10^{-6}	256	2.0×10^{-3}
10	524,800	1.9×10^{-6}	528	1.0×10^{-3}
11	2,097,152	4.8×10^{-7}	1,024	4.9×10^{-4}
12	8,390,656	1.2×10^{-7}	2,080	2.5×10^{-4}
13	33,554,432	3.0×10^{-8}	4,096	1.2×10^{-4}
14	134,225,920	7.5×10^{-9}	8,256	6.2×10^{-5}
15	536,870,912	1.9×10^{-9}	16,384	3.1×10^{-5}
16	2,147,516,416	4.7×10^{-10}	32,896	1.5×10^{-5}

([a]) = 1/N; ([b]) = Unique A·T Sites/N.

Scamrov and Beabealashvilli, 1983; Lane *et al.*, 1983; Van Dyke and Dervan, 1983, 1984; Low *et al.*, 1984; Fox and Waring, 1984; Harshman and Dervan, 1985) and affinity cleaving (Schultz *et al.*, 1982; Taylor *et al.*, 1984; Schultz and Dervan, 1983, 1984; Youngquist and Dervan, 1985; Dervan, 1986).

Footprinting and affinity cleaving techniques, which exploit the analytical power of high resolution sequencing gels, allow a rapid and precise solution to the "product analysis" problem and are an underpinning to recent advances in the molecular recognition of DNA area.

A) *Footprinting*

With the availability of restriction endonucleases and techniques that allow the isolation of discrete DNA fragments, uniform DNA substrates are available that have a sufficiently large number of base pairs or combinations of base pairs to be representative of all possible small molecule-binding sites on DNA. With routine enzymatic procedures, DNA fragments (typically 100 base pairs in size or larger) are tagged on one end of one strand (5′ or 3′) with ^{32}P. Footprinting can be carried out with DNA-cleaving agents such as the enzyme DNase I or the synthetic reagent methidiumpropyl-EDTA (MPE), which cleave double-helical DNA at every base position. After cleavage of a ^{32}P-labeled restriction fragment, the set of labeled DNA cleavage fragments differing in length by 1 nucleotide is resolved on a high-resolution denaturing polyacrylamide gel. A bound ligand protects the DNA-binding site from cleavage by covering the base pairs it binds. This is visualized on the autoradiogram of the high-resolution gel as a gap in the ladder of DNA fragments. A chemical sequencing lane run alongside as a marker permits precise identification of these protected regions.

MPE contains the DNA intercalator, methidium, covalently bound by a short hydrocarbon tether to the metal chelator EDTA (Hertzberg and Dervan, 1982, 1984). In the presence of ferrous ion, reducing agents such as dithiothreitol and dioxygen, MPE at micromolar concentrations produces single-strand breaks at 25°C (pH 7.0) in double-helical DNA (Hertzberg and Dervan, 1982, 1984). The synthetic molecule, MPE·Fe(II), is often employed in footprinting experiments because it mimics DNase I as a DNA-cleaving reagent but affords more accurate resolution of binding-site sizes.

The DNA helix accommodates some of these small molecules by

changing its shape. How far from the bound site altered DNA structure extends undoubtedly depends on both the DNA binding molecule and the neighboring DNA sequences. DNase I is sensitive to local DNA structure and enzymatic cleavage can be inhibited or enhanced by altered DNA structure. Using DNase I footprinting, several groups have observed enhanced rates of cleavage flanking the binding sites of the antibiotics distamycin, actinomycin, and echinomycin (Scamrov and Beabealashvilli, 1983; Lane *et al.*, 1983; Van Dyke and Dervan, 1983; Low *et al.*, 1984; Fox and Waring, 1984). This enhanced susceptibility to cleavage by DNase I has been interpreted as alteration of the width of the minor groove (Fox and Waring, 1984). DNase I footprinting is proving to be a sensitive technique for determining the extent and sequence dependence of altered DNA structure in solution induced by small molecules proximal and distal to specific binding sites on DNA. Understanding the extent of conformational changes produced by ligand binding, whether they are restricted to the actual binding site or distributed over neighboring regions of DNA, will influence the choice of coupling strategies for synthetic hybrids of different DNA-binding natural products.

B) *Affinity Cleaving*

Attachment of EDTA·Fe(II) to a DNA binding molecule creates an efficient DNA-cleaving molecule (at 25°C and pH 7.0) (Hertzberg and Dervan, 1982, 1984). The EDTA attachment converts a sequence-specific DNA-binding molecule to a sequence-specific DNA-cleaving molecule (Dervan, 1986). Analysis of the cleavage products from a ^{32}P-end-labeled restriction fragment on the autoradiogram of a high-resolution denaturing polyacylamide gel allows the binding locations, site size, and orientation of synthetic molecules on double-helical DNA to be visualized (Dervan, 1986). The resulting cleavage patterns are the positive image visualized on an autoradiogram with respect to the negative image produced by footprinting.

The antibiotic distamycin is a crescent-shaped tripeptide containing three N-methylpyrrolecarboxamides that binds in the minor groove of B-DNA with a strong preference for A,T-rich sequences (Zimmer, 1975; Krey, 1980; Gursky *et al.*, 1982; Zimmer and Walnert, 1986). The EDTA moiety was tethered to the amino or carboxyl terminus of *tris*-N-methylpyrrolecarboxamide to give distamycin-EDTA (DE) and EDTA-distamycin (ED), respectively (Taylor *et al.*, 1984). DE·Fe(II) and ED·Fe(II) at micromolar concentrations cleave pBR322 plasmid DNA at discrete locations

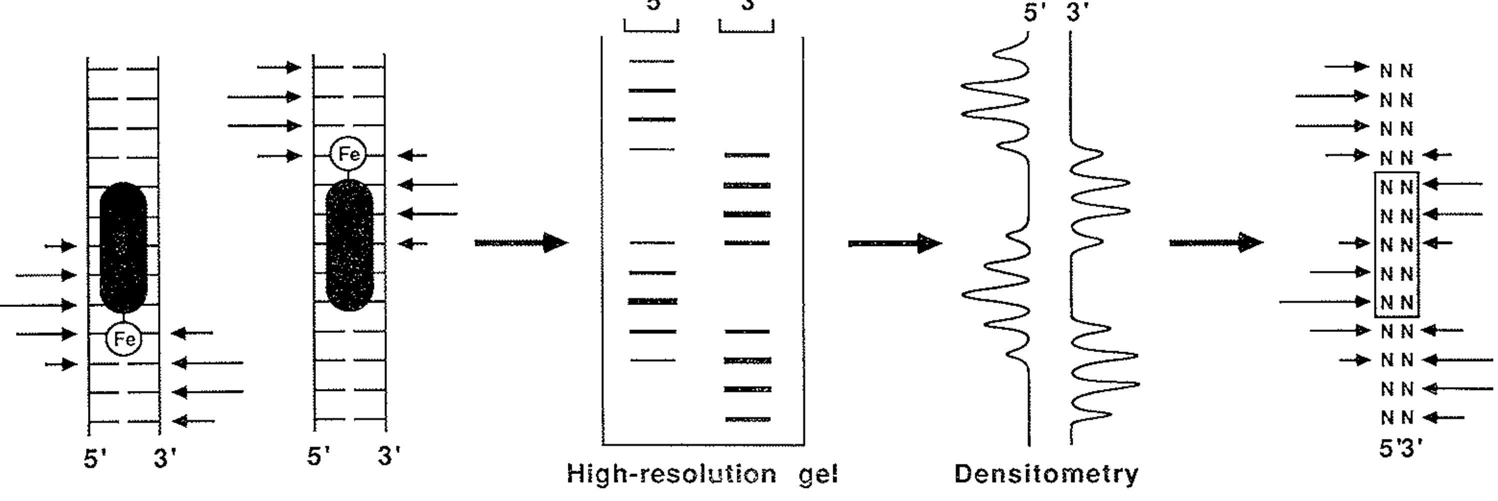

Fig. 1. Scheme for the affinity cleaving method (Dervan, 1986).

in the presence of oxygen and reducing agents such as dithiothreitol. From sequencing gel analyses, it has been found that DE·Fe(II) and ED·Fe(II) afford DNA-cleavage patterns covering four contiguous base pairs adjacent to 5-bp sites consisting of A,T-rich DNA. The multiple contiguous cleavages at each site are taken as evidence for a diffusible oxidizing species, most likely hydroxyl radical (Taylor *et al.*, 1984; Hertzberg and Dervan, 1984). The relative intensity of cleavages on each side of the 5-bp site permits assignment of major and minor orientations of the tripeptide binding unit. The cleavage patterns on opposite DNA strands are asymmetric, shifted to the 3′ side, which can be understood by examination of a model of B-DNA. In the minor groove of right-handed DNA, the proximal deoxyriboses on opposite strands are 2 bp apart to the 3′ side. Assuming that the multiple cleavage events result from a diffusible reactive species, the average position of the EDTA·Fe(II) group is given by the approximate twofold symmetry of the cleavage pattern. From this position, the site of the attached DNA-binding unit can be estimated.

3. Recognition of Large Sequences of A,T-Rich DNA in the Minor Groove

From recent X-ray analysis of the complex of netropsin with the B-DNA dodecamer 5′-CGCGAATTCGCG-3′, Dickerson and co-workers have provided a molecular basis for the sequence-specific recognition of DNA by a *bis*-N-methylpyrrolecarboxamide and, by extension, distamycin (Kopka *et al.*, 1985). Netropsin sits symmetrically in the center of the minor groove of right-handed DNA and displaces the water molecules at the spine of hydration. Each of its three amide groups forms a bridge between adajacent adenine N-3 or thymine O-2 atoms on opposite helix strands. Dickerson and co-workers suggest that the base specificity of netropsin for contiguous A,T-rich sequences in B-DNA is provided not by hydrogen bonding but by close van der Waals contacts between adenine C-2 hydrogens and CH groups on the pyrrole rings of the oligopeptide molecules. Because increased binding-site size would afford increased sequence specificity for DNA-binding molecules, the question arises as to whether higher numbers of N-methylpyrrolecarboxamides in synthetic oligopeptides would fit the natural twist of the B-DNA helix (Arcamone *et al.*, 1969; Zimmer *et al.*, 1983).

Tetra-, penta-, and hexa, hepta-, octa-, and nona-(N-methylpyrrolecarboxamide)s equipped with EDTA (P4E, P5E, P6E, P7E, P8E, and P9E) have been synthesized in our group. The sequence specificities, binding site sizes and orientation preferences have been compared using the affinity cleaving method (Youngquist and Dervan, 1985, 1986). The homologous oligopeptide-EDTA·Fe(II) molecules cleave restriction fragments at common locations rich in A,T that differ incrementally in the size of the binding site (Youngquist and Dervan, 1985, 1986). From analysis of the cleavage patterns visualized by high-resolution denaturing gel electrophoresis, the oligopeptides with four-nine N-methylpyrrolecarboxamide units and containing five-ten amide groups bind sites of A,T-rich DNA consisting of six-eleven contiguous base pairs, respectively. The general rule of n amides affording binding-site sizes of $n+1$ base pairs is explained by the solid state structure of the netropsin: DNA duplex.

From the relative intensities of the cleavage patterns flanking the

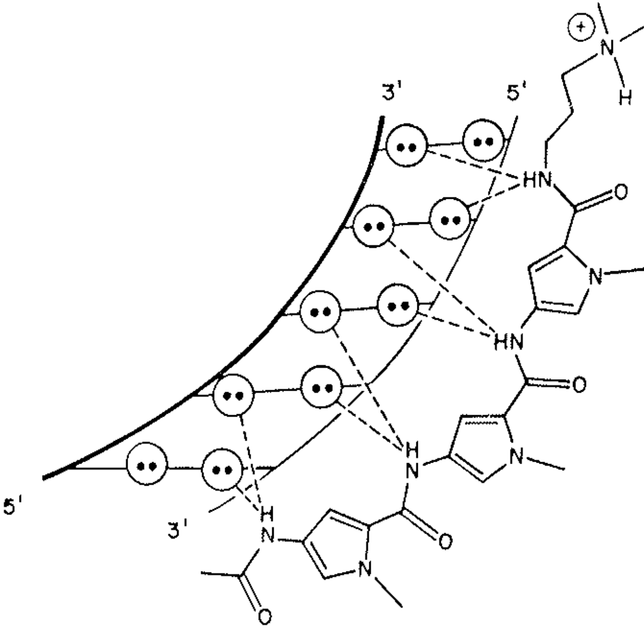

Fig. 2. Model for *tris*-N-methylpyrrolecarboxamide binding in the minor groove of DNA at A,T rich sequences 5 bp in size. (See Kopka *et al.*, 1985) Circles with two dots represent lone pairs of electrons on N3 of adenine and 02 of thymine. Dotted lines are hydrogen bonds.

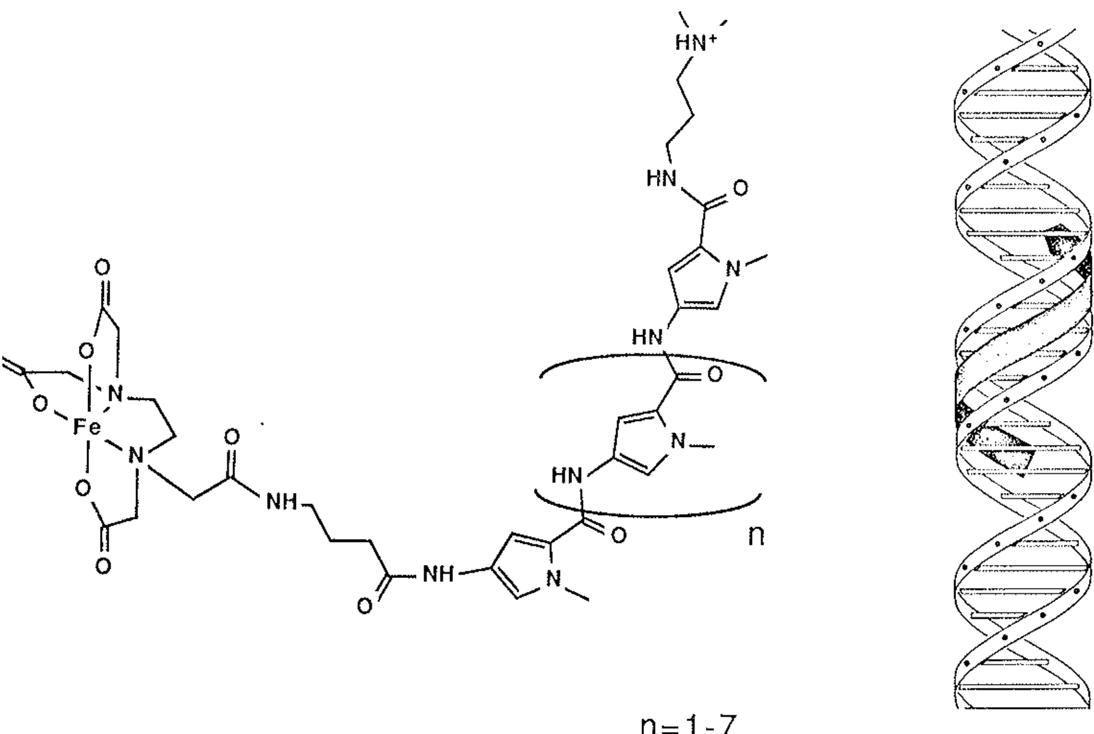

FIG. 3. *Oligo*-N-methylpyrrolecarboxamides equipped with iron-EDTA at the amino end (Youngquist and Dervan, 1985, 1986).

binding site, the orientation preference of the oligopeptide at each binding site can be estimated as a function of local sequence, flanking sequences, and number of N-methylpyrrolecarboxamide units. Dickerson has shown that, although netropsin binding neither unwinds nor elongates the dodecamer, it does force open the minor groove by 0.5 to 2.0 Å and bends back the helix axis by 8° across the region of attachment. One explanation for nonequivalent binding orientation on an AT-rich binding site that lacks twofold symmetry is that the narrowness of the minor groove in B-DNA differs with local DNA sequence. For example, DE has an orientation preference for the amino end of the tripeptide to the 5′ side of the sequence 5′-agAAATTgc-3′. DE has no orientation preference for the sequence 5′-ttAAATTgc-3′ (Taylor *et al.*, 1984; Youngquist and Dervan, 1985). Since the binding sites are the same but occur in different locations

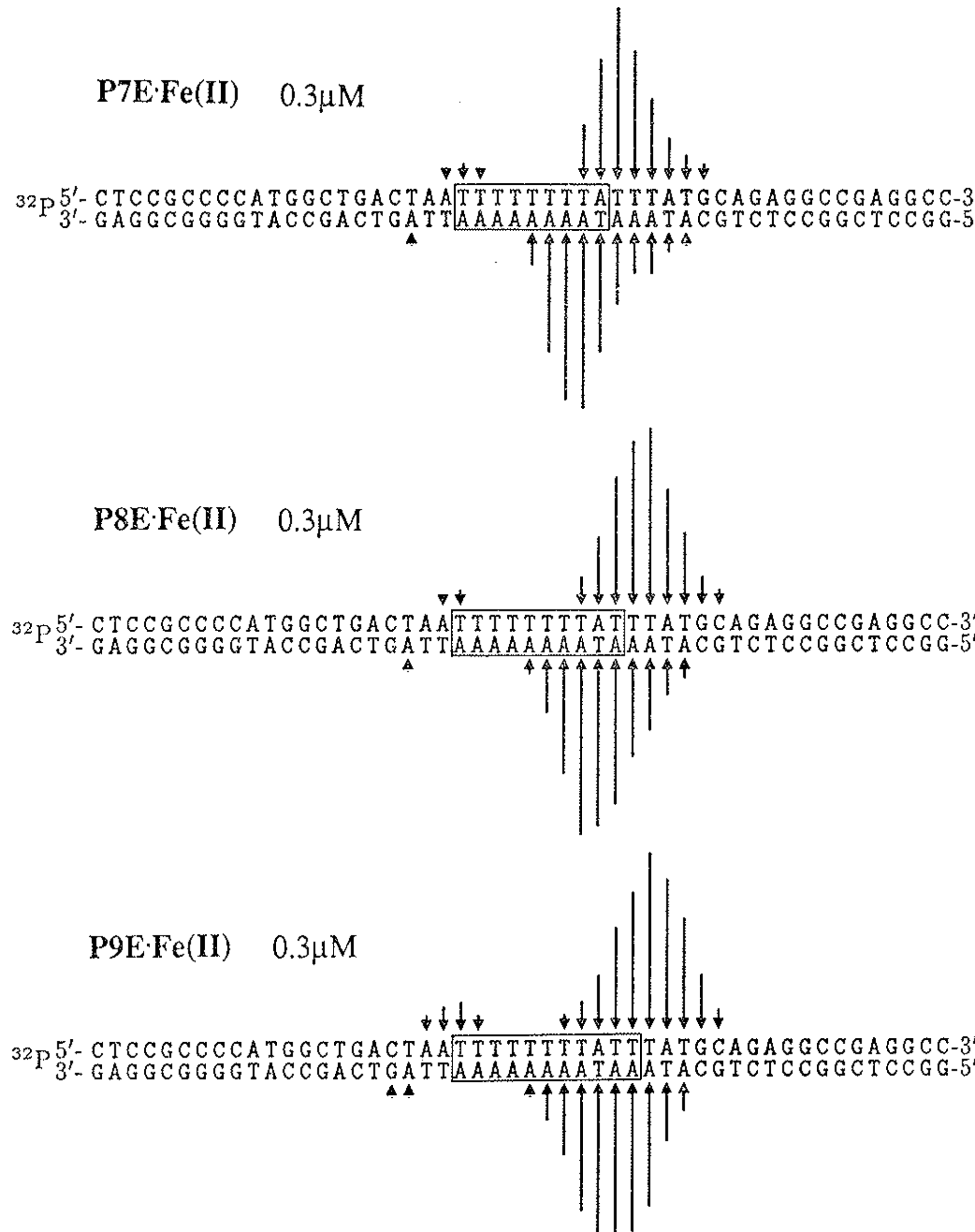

FIG. 4. Histograms of the DNA cleavage patterns on a DNA restriction fragment by P7E·Fe(II), P8E·Fe(II) and P9E·Fe(II). (Youngquist and Dervan, 1986). Boxes define binding site location and size based on model described in Taylor, *et al.*, 1984; Youngquist and Dervan, 1985; Dervan, 1986.

on DNA, we conclude that different local structures can be conferred on identical binding sites by flanking sequences.

Netropsin and distamycin have been characterized as molecules that bind preferably to A,T-rich regions of DNA. It is known from equilibrium binding studies of distamycin analogs that homopolymer dA·dT sequences are preferred over alternating d(A-T)·d(A-T) copolymer sequences. From footprinting and affinity cleaving data we find that G,C base pairs are sometimes permissible in the preferred binding sites of netropsin, distamycin, and higher oligo(N-methylpyrrolecarboxamide) homologs on DNA. (Youngquist and Dervan, 1985) Perhaps the close van der Waals nonbonded contacts between the pyrrole CH and the $-NH_2$ group of guanine are adjustable or are not identical for every pyrrole position on the bound crescent-shaped oligopeptide. Moreover, there are several pure A,T sites that are not strong binding sites for the oligo(N-methylpyrrolecarboxamide)s.

According to the $n+1$ rule, the minimum recognition unit for the N-methylpyrrolecarboxamide on B-DNA is 2 bp. If the recognition elements for the carboxamide NH are on adjacent residues on opposite helix strands, there are ten bridged base possibilities: AA, AT, AC, AG, TT, TC, TG, CC, GG, and CG. The data from affinity cleaving studies reveal that the preferences of bridge hydrogen bonds between adjacent bases on opposite helix strands decrease in the following order: AT ≫ AA > TT > AC,TC,TG ≫ AG,CG,CC,GG.

4. Coupled DNA Binding Units of Similar Specificity in the Minor Groove

Undoubtedly there is an upper limit where oligo(N-methylpyrrolecarboxamide)s will no longer fit the natural twist of the B helix. For sequence-specific DNA-binding molecules that read large sequences of double-helical DNA, there will be a need to *couple* DNA-binding units derived from natural products of similar (or mixed) base-pair specificities. For the success of this coupled DNA-binding unit approach, the base-specific recognition elements of each subunit and the linkers connecting them must be compatible with the same groove and conformational state of the DNA.

The initial design attempts for large sequences of A,T-rich DNA involved the construction of dimers of di- and tripeptides connected by flexible hydrocarbons tethers (Khorlin *et al.*, 1980; Schultz and Dervan, 1983). From affinity cleaving experiments, a tripeptide dimer with the

C_7 linker, bis(EDTA-distamycin)Fe(II) [BED·Fe(II)], binds a 9-bp A,T-rich site (5'-ATTTTTATA-3'), a result consistent with simultaneous binding. However, this dimer also binds a 5-bp site (5'-AATAA-3'), suggesting that the C_7 hydrocarbon tether allows both dimeric and monomeric binding modes (Schultz and Dervan, 1983).

A shorter linker, such as the diamide of fumaric acid, more closely mimics the N-methylpyrrolecarboxamide DNA-binding unit with regard to shape and curvature between the amide NH's. Bis(EDTA-distamycin) fumaramide (BEDF), which is a crescent-shaped octamide containing two N-methylpyrrole tripeptide units coupled at the amino termini via a C_4 tether, fumaric acid, reveals major cleavage sites flanking two AT-rich sequences, 5'-ATTTTTATA-3' and 5'-ATAATAAT-3' (Youngquist and Dervan, 1985). The observation of 8- to 9-bp binding in the absence of 5-bp binding for BEDF suggests that the tripeptides are binding exclusively simultaneously on double-helical DNA.

5. Coupled DNA Binding Units of Mixed Specificity in the Minor Groove

Several natural products that bind double-helical DNA have the structural-binding feature of being both an intercalator and groove binder. The natural product actinomycin D consists of an aromatic chromophore, phenoxazone, coupled to two identical cyclic pentapeptide lactones (Jain and Sobell, 1972; Takusagawa *et al.*, 1982). Actinomycin is an intercalator-groove binder that unwinds DNA by 26°. The cyclic pentapeptides fit snugly above and below the intercalating ring in the minor groove of DNA. Actinomycin binds 4 bp with a preference for 5'NGCN-3' sequences (Van Dyke *et al.*, 1982; Van Dyke and Dervan, 1982; Scamrov and Beabealashvilli, 1983; Lane *et al.*, 1983).

We tested whether the intercalator portion of actinomycin, phenoxazone, can be covalently linked to a different minor groove binder, distamycin, in such a way that the sequence specificity of *both* moieties is retained. The two cyclic pentapeptides of actinomycin were replaced with the tripeptide from distamycin and the postulated key carbonyl amide guanine NH recognition element of actinomycin was retained (Dervan and Sluka, 1986). As judged from distance and steric considerations from model building studies, the distamycin tripeptide was connected to the phenoxazone by a glycine tether (Dervan and Sluka, 1986). The resulting bis(distamycin)phenoxazone is a synthetic hybrid groove binder-intercalator

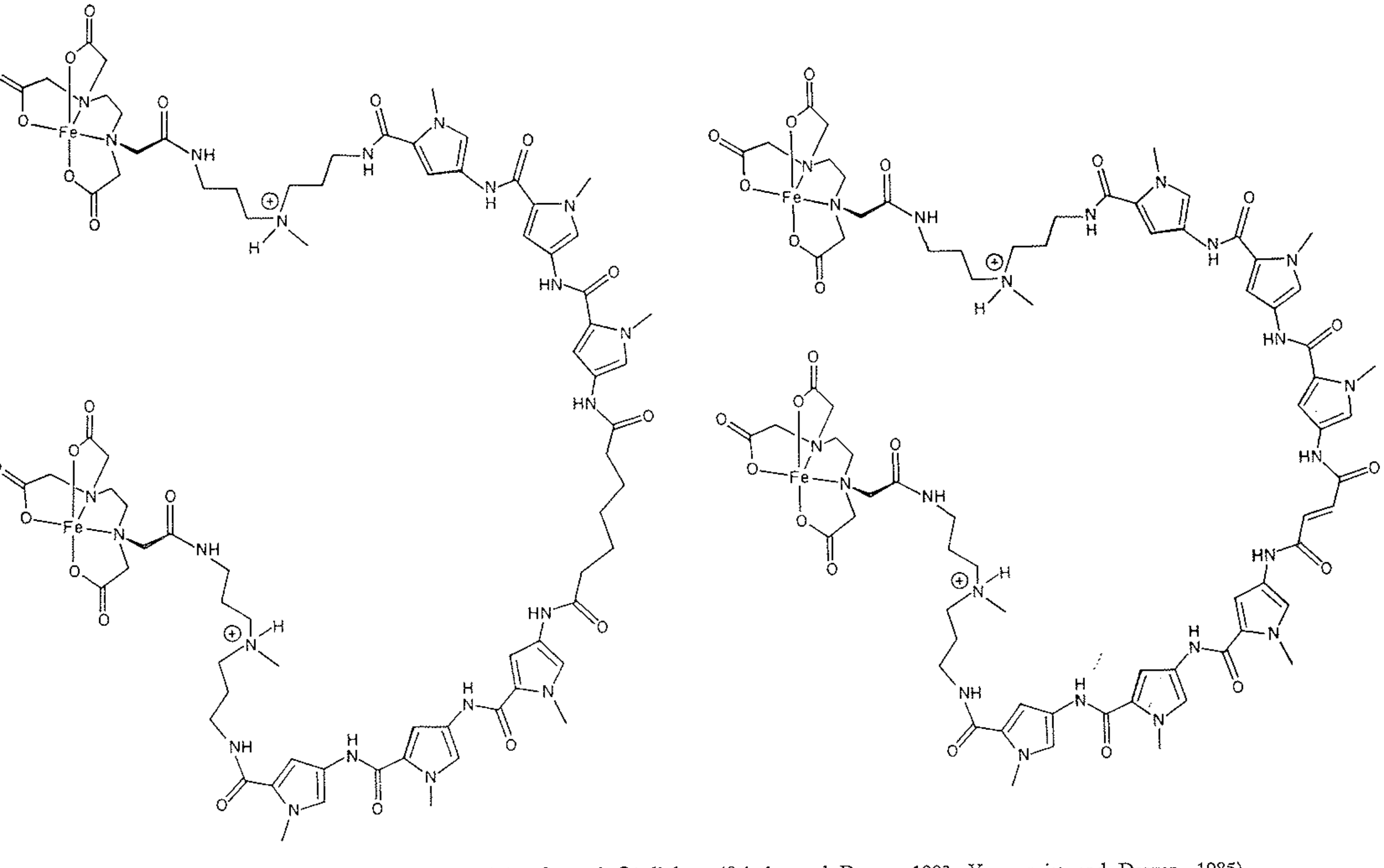

FIG. 5. Dimers of distamycin connected by C7 and C4 linkers (Schultz and Dervan 1983, Youngquist and Dervan, 1985).

that in a formal sense might bind 10 bp of DNA having the sequence $(A,T)_4(G,C)_2(A,T)_4$. The sequence specificity of bis(distamycin)phenoxazone on DNA restriction fragments was tested by the affinity cleaving method (Dervan and Sluka, 1986).

The DNA cleavage patterns reveal a major cleavage site flanking the 10-bp sequence, 5'-TATAGGTTAA-3' (Dervan and Sluka, 1986). One interpretation of the data is that the tripeptides are binding simultaneously at A,T-rich sequences 4 bp in size flanking the central 5'GG-3' phenoxazone binding site. Although not a proof, this would be consistent with the groove binder-intercalator-groove binder mode. However, at two other sites, single-cleavage loci were observed consistent with one tripeptide or possibly a tripeptide-phenoxazone binding at these sites (Dervan and Sluka, 1986). Perhaps at these sites there is intercalation of the phenoxazone with sequence dependent and unequal local distortion on both sides of the intercalation site which would make one site incompatible with the tripeptide groove binder. Because there are 524,800 unique sequences of doublehelical DNA that are 10 bp in size, it is likely that the optimal 10-bp recognition site for bis(distamycin)phenoxazone has not yet been identified.

This limited success with a DNA-binding molecule that is a hybrid of two natural products makes it appear possible that groove binders can be mixed and matched with intercalators and that A,T words can be coupled with G,C words. The choice of a linker connecting the recognition elements may be a critical design feature with regard to simultaneous binding of all moieties. Perhaps further efforts should focus on the synthesis of "mixed specificity" molecules that are *exclusively* groove binders. This might minimize distortion on the DNA partly responsible for the incompatibility of coupled DNA binding units such as intercalators and groove binders. Because our understanding of G,C recognition in the minor groove is not as well advanced as A,T recognition, a challenge for the future with regard to minor groove recognition is the design of G,C minor groove binding moieties.

6. Alteration of the Specificity of Distamycin from Pure (A,T) to Mixed (G,C/A,T)

Recently, we found that replacement of one N-methylpyrrolecarboxamide unit in the tripeptide portion from distamycin with pyridine-2-carboxamide affords a new synthetic DNA minor groove binding molecule,

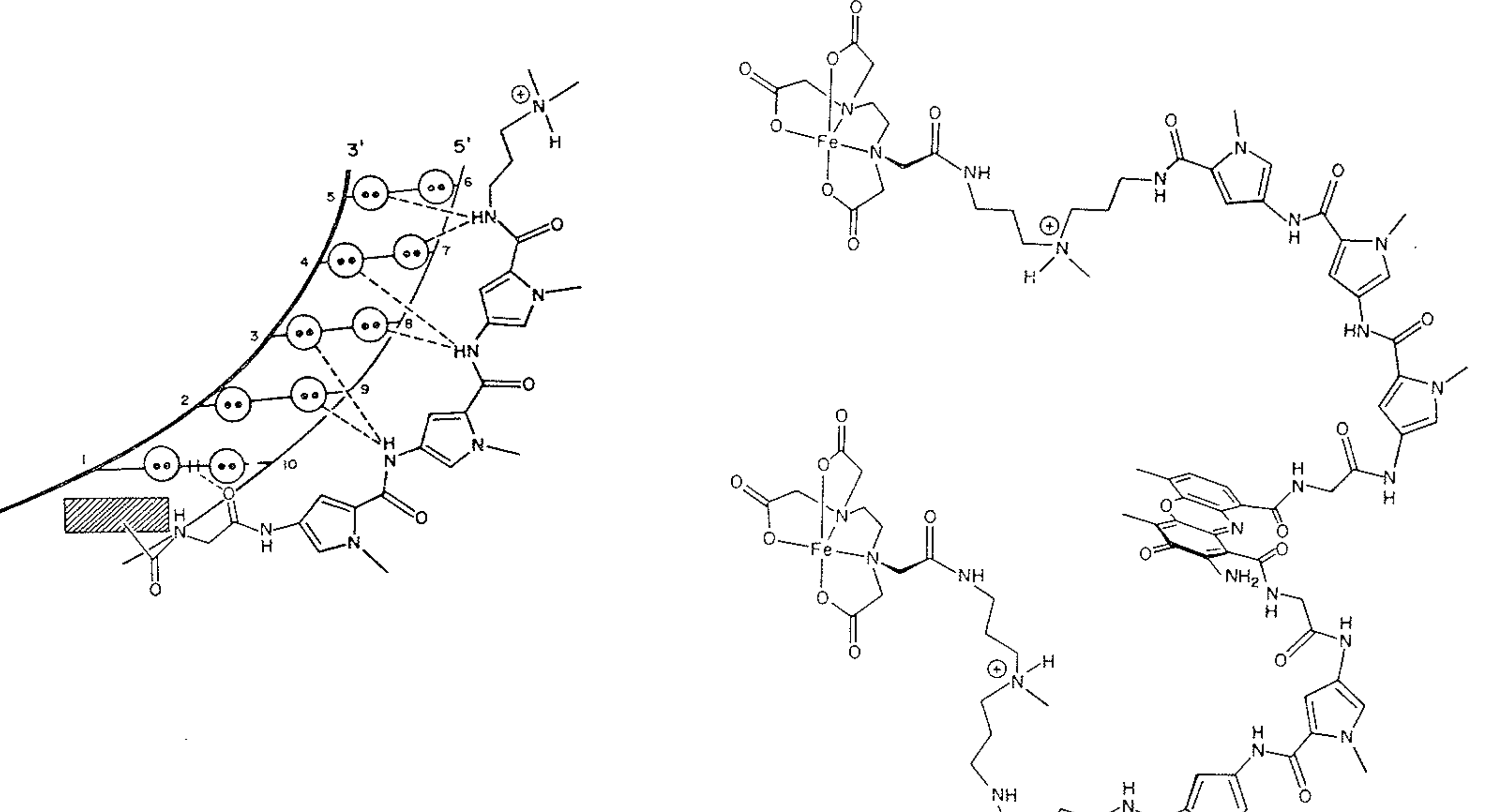

FIG. 6. (*left*) Model for a hybrid intercalator-groove binder, distamycinphenoxazone. Circles with two dots represent lone pairs of electrons on N3 of adenine and 02 of thymine. Circles with two dots and H interposed represent lone pairs of electrons on N3 of guanine and 02 of cytosine flanking the NH_2 of guanine at the edges of the base pairs on the floor of the minor groove of righ-handed DNA. Dotted lines are hydrogen bonds. (*right*) Bis [Fe(II)·EDTA-distamycin]phenoxazone BEDP·Fe(II) (Dervan and Sluka, 1986).

pyridine-2-carboxamidenetropsin (2PyN), that now accepts G,C/A,T base pairs *in preference* to pure A,T stretches of DNA (Wade and Dervan, 1986). Pyridine-3-(or pyridine-4)-carboxamidenetropsin (3-PyN and 4-PyN) do *not* show this effect supporting a model where the lone pair of electrons on the pyridine nitrogen is responsible, at least in part, for the G,C recognition (Wade and Dervan, 1986).

D

	X	Y	Z
2-PyN	N	CH	CH
3-PyN	CH	N	CH
4-PyN	CH	CH	N

FIG. 7. (*left*) Analog of distamycin. (*right*) Synthetic groove binders, pyridinecarboxamidenetropsin.

7. Polypeptide Recognition in the Major and Minor Groove of DNA

In recent years x-ray crystal structures of a number of sequence specific DNA binding repressor proteins have become available (Ohlendorf *et al.*, 1982; Takeda *et al.*, 1983; Pabo and Lewis, 1982; Pabo and Sauer, 1984; Lewis *et al.*, 1983; Steitz *et al.*, 1983; McKoy *et al.*, 1982; McKoy and Steitz, 1981). Comparison of the three-dimensional structures has led to the postulate that a conserved helix-turn-helix motif is intimately involved in sequence-specific DNA recognition. The "solvent exposed" face of the second helix is believed to lie on the floor of the major groove of B-DNA and make sequence-specific contacts with the exposed edges of the bases. Comparison of the amino acid sequence of other DNA binding proteins to the postulated binding domains of repressor proteins suggests that the helix-turn-helix motif interacting with the major groove of B-DNA may be a common structural feature employed by some DNA binding proteins.

To apply the affinity cleaving method to the protein: DNA recognition area, we have developed a general method to attach EDTA to a specific amino acid residue of a synthetic polypeptide fragment. Because the polypeptide-EDTA·Fe(II) should be oriented in one direction on the DNA helix, the affinity cleaving data would reveal the binding location and the orientation of the peptide on the DNA. Importantly, the asymmetry of the cleavage pattern would also reveal the location of that particular amino acid residue equipped with EDTA·Fe(II). For cleavage in the minor groove the pattern is asymmetric to the 3′ side. For cleavage in the major groove the pattern is asymmetric to the 5′ side.

We have synthesized a polypeptide, 52 amino acid residues in length, derived from the DNA binding domain of the Hin site-specific recombinase protein, that is equipped with EDTA at the N-terminal end (Sluka *et al.*, 1986). The experimental work will not be documented here, but early indications from affinity cleaving experiments reveal that the synthetic polypeptide-EDTA with two structural and functional domains (DNA recognition/DNA cleavage) binds 12 base pairs of DNA, most likely both in the major and minor groove (Sluka *et al.*, 1986).

8. Summary

The design of synthetic sequence-specific binding molecules for double helical DNA has advanced in recent years due in part to the development

of analytical techniques such as footprinting and affinity cleaving for analyzing rapidly and precisely on sequencing gels hundreds of potential DNA binding sites. In the minor groove, the synthetic *nona*-N-methylpyrrolecarboxamide binds 11 contiguous base pairs of A,T DNA. This is comparable to the specificity of a restriction enzyme which recognizes uniquely 6 bp of DNA. Corresponding success in the major groove with synthetic polypeptides puts unique specificity at the 12 base pair recognition level within reach. This is an encouraging first step toward defining a set of rules for the three-dimensional read-out of double helical DNA. A long range goal is *unique* specificity at the 15 base pair level which would make possible the design of artificial repressors for the inactivation of oncogenes.

Acknowledgements

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STEREOCHEMICAL AND SEQUENCE SELECTIVITY OF COVALENT BINDING OF THE PYRROLO (1,4) BENZODIAZEPINES AND CC-1065 TO DNA

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ABSTRACT

The pyrrolo(1,4)benzodiazepines and CC-1065 are potent antitumor agents that bind covalently in the minor groove of DNA through N_2 of guanine and N3 of adenine respectively. The stereochemical and sequence selective interactions of these antibiotics with oligodeoxynucleotides and restriction enzyme fragments have been studied by NMR and DNA sequencing technology. The stereochemistry at the covalent linkage site between the pyrrolo(1,4)benzodiazepines and N_2 of guanine has been found to be dependent upon both the structure of the drug and the binding sequence. A comparison of the experimental results of DNA sequence specificity studies with those predicted through theoretical studies is presented, together with evidence for a drug induced asymmetric effect on DNA conformation. For two analogs of CC-1065 examined covalent adduct formation and biological activity is dependent upon the stereochemistry of the DNA reactive cyclopropane ring.

1. INTRODUCTION

Elucidating the molecular basis for the potent antitumor activity of the pyrrolo(1,4)benzodiazepines [P(1,4)Bs] and CC-1065, which are drugs that bind covalently within the minor groove of DNA, has been the subject

of research in this laboratory for a number of years. Our approach has been to tackle this problem simultaneously at two levels: to characterize the structures of the covalent DNA adducts in a three dimensional sense, and to determine the biochemical and biological consequences of DNA modification by these agents. Ultimately we hope to be able to relate the biochemical and biological consequences of DNA damage to the structural modification of DNA produced by the P(1,4)Bs (Figure 1A) and CC-1065 (Figure 1B) and use this information to aid in the design of clinical, more useful antitumor agents.

Several classes of DNA-reactive agents interact in some manner with the minor groove of B-form DNA. Adriamycin and actinomycin are stabilized upon intercalation by secondary noncovalent association within the minor groove (Quigley *et al.*, 1980; Takasagawa *et al.*, 1982). Non-covalent DNA ligands such as distamycin and netropsin bind entirely within the minor groove (Kopka *et al.*, 1985). A third class of anti-neoplastics bind covalently within the minor groove of B-form DNA. Members of this latter group of compounds include CC-1065, the safra-mycins (Lown *et al.*, 1982), naphthyridinomycin (Zmijewski *et al.*, 1985), and the P(1,4)Bs. The latter three groups contain the DNA-reactive carbinolamine or its chemical equivalent. The covalent minor groove binders have been observed to be 10^2 and 10^3 times more potent as antitumor agents than those of the intercalating class, while the latter agents possess significantly greater potency than the noncovalent minor groove binders (Hurley and Needham-VanDevanter, 1986).

In the article we will discuss some of the structural features of the interaction of the P(1,4)Bs and CC-1065 with DNA. Specifically evidence leading to the elucidation of the structures of the drug-DNA adducts will be reviewed and then aspects of the stereochemistry of covalent linkage formation and sequence specificity of these drugs will be described. The significance of recently published theoretical modeling of P(1,4)B with DNA will be discussed in reference to experimental data which suggests that these drugs cause an asymmetric effect on DNA structure. For a list of reviews and recent articles on other aspects of the P(1,4)Bs and CC-1065 see Hurley and Needham-VanDevanter (1986).

2. Elucidation of the Covalent Drug-DNA Adduct Structures

A vigorous demonstration of the points of covalent attachment between both the P(1,4)Bs and CC-1065 and DNA was an essential first

A

I

II

III

IV

B

Subunits

A

B

C

FIG. 1. Structures of the P(1,4)Bs; sibiromycin (I), anthramycin (II), tomaymycin (III), and the neothramycin A (R_1 = H, R_2 = OH) (IV) and B (R_1 = OH, R_2 = H) (IV) (A) and CC-1065 (B).

step in investigating the structural and biological consequences of DNA modification. For both groups of drugs, a series of steps was undertaken to first establish that DNA was the primary target and then to gain circumstantial and finally unambiguous structural information on the DNA adduct structures.

A) *Structures of the P(1,4)B-DNA Adducts*

Covalent binding of the P(1,4)Bs to duplex DNA was first suggested by the stability of drug-DNA complexes to ethanol precipitation or dialysis (Hurley *et al.*, 1977; Kohn and Spears, 1970). In contrast, similar stability experiments following drug incubations with RNA, protein, or denaturated DNA did not show evidence of covalent binding (Hurley *et al.*, 1977; Kohn and Spears, 1970). Subsequent experiments revealed that the P(1,4)Bs form DNA adducts which significantly increase the DNA helix melting temperature, do not cause unwinding of supercoiled DNA, produce shifts in a circular dichroism spectra, and induce bathochromic shifts in the absorbance maxima of the drug chromophores (Hurley *et al.*, 1977; Kohn *et al.*, 1974).

Indirect evidence for the structures of the P(1,4)B-DNA adducts was obtained by reaction of these agents with synthetic DNA polymers (Kohn *et al.*, 1974; Petrusek *et al.*, 1981). In particular, the inability of anthramycin to form a covalent adduct with poly(dI)-poly(dC) contrasted with its avid binding to poly(dG)-poly(dC) (Kohn *et al.*, 1974). Denaturing alkaline cesium sulfate gradient centrifugation of the latter species demonstrated that tritiated drug chromophore was only associated with the poly(dG) strand (Petrusek *et al.*, 1981). Taken together these results suggested that anthramycin binds covalently in the minor groove of DNA through N_2 of guanine.

Further evidence for covalent minor groove DNA binding was obtained from experiments in which it was demonstrated that anthramycin binds covalently to coliphage T7 DNA, which has extensive glucosylation in the major groove (Hurley and Petrusek, 1979). The possibility of covalent binding through N-7 or C-8 of guanine was further eliminated by experiments in which incubation of (8-^{3}H)-guanine-labeled DNA with anthramycin did not show loss of tritium (Petrusek *et al.*, 1981). Finally, melting of the DNA strands in P(1,4)B-DNA adducts by heat (100°C for 5 min) released chemically unchanged drug from the DNA (Hurley *et al.*, 1977).

Collectively, these results suggest that the P(1,4)Bs bind covalently

to guanine, and furthermore specifically through the exocyclic 2-amino group, which resides within the minor groove of B-form DNA (Hurley and Petrusek, 1979; Petrusek *et al.*, 1981). The stability of the N-glycosidic linkage of the covalent modified guanine to acid and alkaline hydrolysis is consistent with alkylation at N_2 of guanine, which does not disturb the purine aromaticity. DNA-binding experiments using structural analogues of the P(1,4)Bs which lack the carbinolamine or its chemical equivalent (N10, C11 imine or 11-alkyl ethers) failed to yield covalent DNA adducts (Horwitz *et al.*, 1971), providing the first evidence that this functionality is the DNA-reactive moiety. Several reaction mechanisms which would lead to formation of an aminal linkage between C11 of the P(1,4)B and N_2 of guanine were proposed, including nucleophilic attack on the carbinolamine or the imine. The imine intermediate is favored as the ultimate DNA-reactive species (Hurley *et al.*, 1977; Barkley *et al.*, 1986; Figure 2). Last, the facile hydrolysis of P(1,4)B-DNA adducts following DNA melting under neutral or acidic conditions, but not under basic conditions, is consistent with an aminal linkage (N-C-N) from the drug carbinolamine carbon (C-11) to N_2 of guanine.

^{1}H and ^{13}C-NMR experiments provided direct evidence for the covalent linkage sites between the P(1,4)Bs and DNA. Anthramycin specifically enriched with carbon-13 at C-11 was bound to calf thymus DNA and analyzed by ^{13}C-NMR (Graves *et al.*, 1984). Upon covalent binding to DNA, a 16 ppm upfield shift was observed for C-11 of anthramycin relative to anthramycin-11-methyl ether, which is consistent

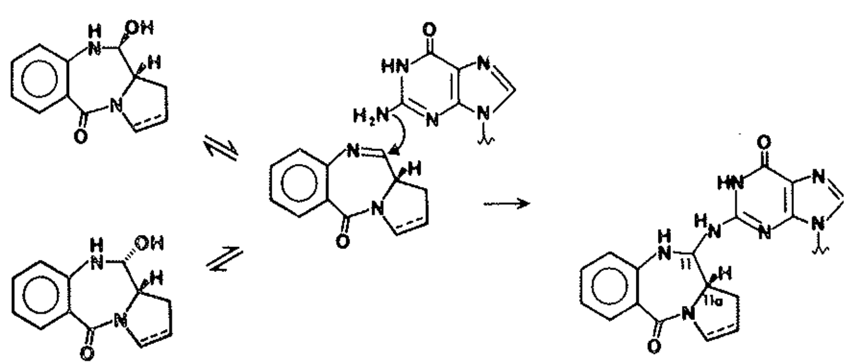

FIG. 2. Proposed mechanism for alkylation of DNA by the P(1,4)Bs to form the P(1,4)B-N_2-guanine)DNA adduct.

with the carbinolamine carbon of anthramycin being the point of covalent attachment to DNA. Additionally, the carbon-13 chemical shift observed for C-11 upon binding to DNA agreed with model compounds for the formation of an aminal linkage at C-11 from the drug carbinolamine to N_2 of guanine. Confirmation that N_2 of guanine was the point at which DNA was covalently bound to the drug was obtained from ^{1}H-NMR studies on the anthramycin-$(ATGCAT)_2$ duplex adduct (Graves *et al.*, 1984).

B) *Structure of the CC-1065-DNA Adduct*

Similar studies to those described for the P(1,4)Bs employing various cellular macromolecules such as RNA, protein, and synthetic or natural DNAs were used to demonstrate that covalent binding occurred exclusively to DNA and subsequently to pinpoint the location of CC-1065 binding to DNA (Li *et al.*, 1982). CC-1065 was shown to bind most avidly to synthetic poly(dA)-poly(dT), less avidly to poly(dA-dT)-poly(dA-dT), and did not bind covalently to poly(dG)-poly(dC) or poly(dG-dC)-poly(dG-dC) (Swenson *et al.*, 1982). CC-1065 was able to prevent binding of netropsin to calf thymus DNA and could also displace prebound netropsin from DNA (Swenson *et al.*, 1982). CC-1065, like anthramycin, binds strongly to phage T7 DNA. These results suggested that CC-1065 binds covalently in the minor groove of B-form DNA to AT-rich regions (Swenson *et al.*, 1982). When CC-1065 calf thymus DNA adducts were heated and butanol extracted, the organic phase was found to contain a chromophore similar to CC-1065 which could be isolated by RP-HPLC, but which did not comigrate with CC-1065 (Hurley *et al.*, 1984). In similar experiments, but using DNA which was prelabeled with tritiated adenine, guanine, cytosine, or thymine, only butanol extraction of the CC-1065-[(^{3}H)-adenine] DNA produced fractions containing excess tritum. The excess radioactivity was found to comigrate with modified CC-1065 chromophore in the RP-HPLC system (Hurley *et al.*, 1984). Thus adenine seemed a likely candidate for the base modified by CC-1065. The sole adenine nucleophile in the minor groove is N-3. Since alkylation of N-3 of adenine alters the aromaticity of the base, this would explain the base loss observed upon thermal treatment of CC-1065-DNA adducts. These observations allowed tentative identification of N-3 of adenine as the CC-1065 binding site on DNA. The identity of the CC-1065-DNA adduct linkage was determined by ^{1}H and ^{13}C-NMR studies on the CC-1065-adenine adduct, which was

isolated as described before (Hurley *et al.*, 1984). The isolated species proved to be adenine alkylated at the N-3 position by the methylene carbon (C-4) of the cyclopropyl ring of the "A-subunit" of CC-1065 (Figure 3).

3. Stereochemistry at the Drug-DNA Linkage Site and Orientation of the Pyrrolo(1,4)Benzodiazepines in the Minor Groove of DNA

In principle, the P(1,4)Bs can bind to DNA via either an 11R or 11S geometry with each enantiomer in one of two possible orientations in the minor groove. The four possibilities can be easily reduced to two alternatives since the steric interaction between the drug molecules and DNA sterically disallows those two options where the right-handed twists of the drug and B form DNA are sterically opposed. Of the remaining options (11S with the aromatic ring positioned to the 3′ side of the modified guanine, and 11R with aromatic ring to the 5′ side of the modified guanine) theoretical calculations both using a force field method (Rao *et al.*, 1986) and "SIR" methodology (Zakrzewska and Pullman, 1986) strongly favor the 11S option for anthramycin. The situation is more complex for tomaymycin since the overall twist of this drug molecule is less than for anthramycin and CPK and molecular modeling studies (Remers *et al.*, 1986) suggest both linkage site geometries are possible.

Experimental results on the stereochemistry of binding of the tomaymycin and anthramycin to DNA are available from fluorescence and ^{1}H-NMR studies. For anthramycin one-dimensional (1D)-^{1}H-NMR studies on the anthramycin-$(ATGCAT)_2$ duplex show that only one species is bound to this oligomer (Graves *et al.*, 1984). More recent studies using two-dimensional (2D)-^{1}H-NMR predict the stereochemistry of binding to be 11S (figure 4) (Graves *et al.*, 1985). This conclusion is based primarily upon 2D-NOE experiments in which an NOE cross-peak between the anthramycin aromatic methyl protons and $A^{5}H_2$ was tentatively assigned. More recently, we have been able to unequivocally assign a cross-peak in the 2D-NOE spectrum of the anthramycin-$(ATGCAT)_2$ duplex adduct between the proton at C-13 of anthramycin (figure 1A) and $A^{11}H_2$ which confirms the assignment previously made by the Krugh laboratory.

For tomaymycin, fluorescence studies (Barkley *et al.*, 1986) strongly suggested that tomaymycin can bind in two orientations on calf thymus DNA. We have recently obtained direct evidence for this from 1D and

FIG. 3. Reaction of CC-1065 to form the CC-1065-(N3-adenine)DNA adduct and the products of thermal cleavage of the CC-1065-DNA adduct.

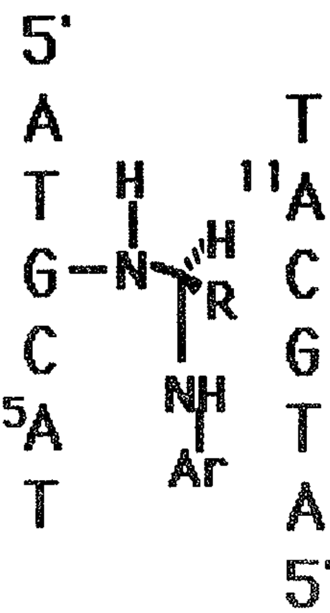

FIG. 4. Schematic representation of the numbering system for anthramycin-(ATGCAT)$_2$ duplex adduct.

2D-NMR studies on tomaymycin-(ATGCAT)$_2$ duplex adduct (Cheatham *et al.*, 1986). Two distinct sets of signals for the tomaymycin molecule are present in the proton NMR spectrum of the duplex adduct (figure 5) and in addition these studies also show connectivities from four cytosine H_5-H_6 and eight thymine methyl-H_6 protons and clearly establish the presence of two distinct species of tomaymycin-(ATGCAT)$_2$ adducts in solution. In a NOESY experiment, cross-peaks between both the aromatic H9 proton and the ethylidine methyl protons of tomaymycin and adenine H_2 protons of (ATGCAT)$_2$ were identified which provide direct evidence for two orientations of tomaymycin in the duplex adduct (Cheatham *et al.*, 1986). These results together with modeling studies show that the aromatic ring of tomaymycin can lie to either the 3′ or 5′ side of the covalently modified guanine in the two diastereomeric tomaymycin-(ATGCAT)$_2$ adducts.

4. Sequence Specificity of the Pyrrolo(1,4)Benzodiazepines

A) *Experimental Results*

The DNA sequence specificity of the P(1,4)B antibiotics has been determined by a footprinting method using methidiumpropyl-EDTA-iron(II) [MPE·Fe(II)], and the results show that each of the drugs has a two to three base-pair sequence specificity that includes the covalently modified guanine residue. 5'PuGPu is the most preferred sequence for the P(1,4)Bs, and 5'PyGPy is the least preferred sequence, with mixed Pu/Py flanking bases of intermediate preference (Hertzberg *et al.*, 1986). Footprinting analysis by MPE·Fe(II) reveals a minimum of three to four base-pair footprint size for each of the drugs on DNA with a larger than expected offset (two to three base pairs) on opposite strands to that observed in previous analyses of noncovalently bound small molecules. There is an extremely large enhancement of MPE·Fe(II) cleavage be-

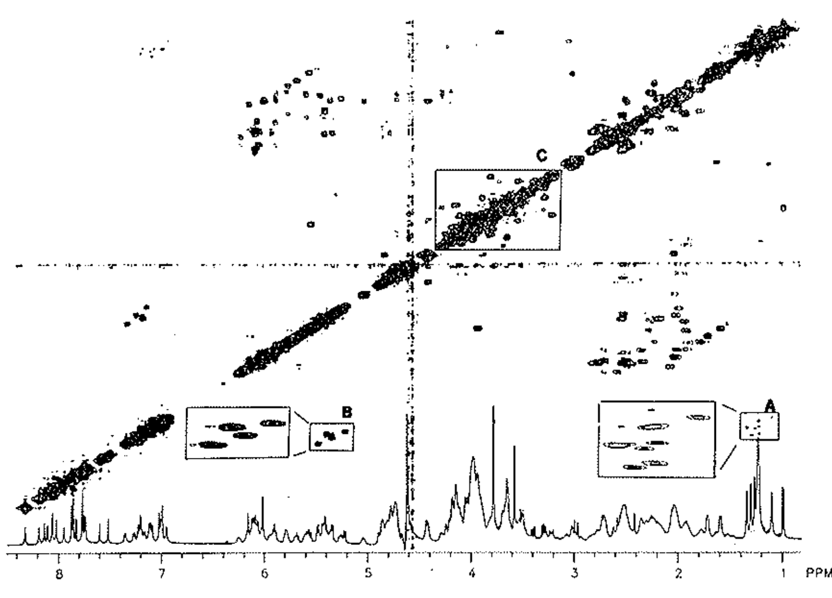

Fig. 5. COSY spectrum of the tomaymycin-$(ATGCAT)_2$ adducts in D_2O at 23°. Box A shows the region containing the thymine methyl-H_6 proton cross-peaks and its expansion, box B shows the cytosine H_5-H_6 proton cross-peaks and its expansion, and box C shows the region containing cross-peaks of the $H_{3'}$, $H_{4'}$, $H_{5'}$, and $H_{5''}$ protons of the duplex.

tween drug binding sites in AT rich regions, probably indicating a drug-induced change in the conformational features of DNA which encourages interaction with MPE·Fe(II).

B) *Theoretical Investigation of the Sequence Specificity*

Two theoretical studies on the sequence specificity of P(1,4)B binding to DNA have been reported. The UCSF group (Rao *et al.*, 1986) have used molecular mechanics methods for energy calculations with the program AMBER-UCSF (Assisted Model Building with Energy Refinement) to examine the effect of sequence variation around the covalent binding site. These studies did not predict that the neighboring sequence would have a marked effect on the binding energies of anthramycin with DNA, although, as previously noted, this method was predictive of the experimentally determined linkage site stereochemistry.

The Pullman group have used SIR (Successive Infinitesimal Rotations) methodology (Sklenar *et al.*, 1986; Lavery *et al.*, 1986a,b) to study the sequence specificity of anthramycin (Zakrzewska and Pullman, 1986). A comparison of these results with those obtained experimentally demonstrates the predictive power of the SIR methodology. These results predict that the deformation energy (E^{def}) appears to be the critical factor which constitutes the major difference between PuGPu sequences, which are the most favored, over PyGPy sequences, which are the least favored. Consequently, these authors conclude that DNA flexibility is the critical function which controls the specificity of binding of anthramycin.

5. Models for the Pyrrolo(1,4)Benzodiazepine-DNA Adducts

Two models have been proposed for P(1,4)B-DNA adducts (Hurley and Petrusek, 1979; Petrusek *et al.*, 1981; Zakrzewska and Pullman, 1986). The original model proposed by the Hurley group assumed that DNA remained in the B-form upon adduct formation, while the more recent model, which is based on theoretical studies, favors a change in nucleic acid conformation which produces an opening in the minor groove of the duplex which is similar to that existing in A-DNA. As a consequence of covalent binding, the B-DNA undergoes a conformation change to an A-DNA type structure and facilitates the steric accommodation of the twisted anthramycin molecule by eliminating the close contacts found

between anthramycin and B-DNA. This latter model is shown in figure 6. The characteristic features of this model are a low (2.99 Å) displacement between base-pairs along the helical axis, a movement of base-pairs along the local dyadic axis towards the minor groove, a strong negative tilt of the base-pairs and a negative propeller twist. What experimental evidence is there to support the model proposed by the Pullman group which shows a drug induced conformational change in DNA structure? DNA sequencing experiments have been performed which provide some information on the effect of drug binding on DNA conformation. The relative susceptibility to cleavage of the backbone of DNA around P(1,4) B-DNA lesions has been probed by two different reagents: MPE and methylene blue. MPE·Fe(II) shows a large enhancement of cleavage adjacent to P(1,4)B binding sites, particularly at AT rich regions. The pattern of enhancements on either side is fairly uniform, but it is difficult to make definitive statements because of the multiplicity of adducts on the restriction enzyme fragment examined. However, in the case of light activated methylene blue with sibiromycin and tomamycin modified DNA aberrant "G" reaction sites for cleavage are found at defined positions relative to the drug binding sites. These always occur two or

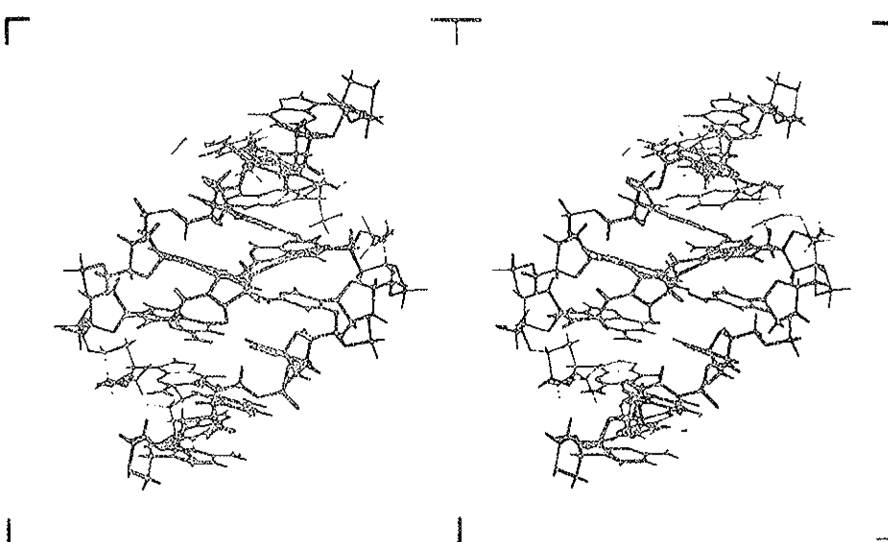

Fig. 6. Minimized conformation of the anthramycin-(dG dC)-adduct obtained with a regular helical distortion of DNA (from Zakrzewska and Pullman, 1986).

three bases removed from a guanine binding site, but on the opposite strand (Hertzberg *et al.*, 1986). Based upon our limited knowledge of sequence dependent orientations for tomaymycin, these are predicted to occur on the same side as the aromatic ring of tomaymycin. Significantly, no drug directed induced breakage occurs at any other position relative to the bound drug molecule. This is suggestive of an asymmetric effect on local DNA conformation around the drug binding-site with a unique effect at the aberrant "G" reaction site. The SIR methodology limits the helical parameters to symmetry constraints. In view of our experimental results, which predict loss of helical symmetry, it would be of considerable interest to apply extensions of the methodology to drug-DNA adducts with heteronomous strands.

6. Stereochemistry of Binding of CC-1065 and Analogs to DNA

CC-1065 has two chiral centers which are located at the cyclopropyl ring junctions, C-3b and C-4a. Only the relative configurations at C-3b and C-4a of CC-1065 were determined by X-ray crystallographic analysis. CPK modeling studies showed that nucleophilic attack by N-3 of adenine upon the methylene carbon (C-4) of the CC-1065 cyclopropyl ring must result in a drug-DNA adduct with the bulk of the CC-1065 molecule lying either to the 5′ side or the 3′ side of the covalently modified adenine, dependent upon the absolute stereochemistry at C-3b. It was proposed that if CC-1065 exhibits a sequence selectivity in binding, the directionality of this specificity would reveal the absolute configuration of the adduct, and by inference, the absolute stereochemistry of CC-1065.

Since the thermal treatment of CC-1065-DNA adducts produces strand breakage immediately to the 3′ side of covalently bound adenine (Figure 3), this led to a convenient means to determine the location of CC-1065 binding sites on DNA (Reynolds *et al.*, 1985). CC-1065-DNA binding sequences were determined on Maxam-Gilbert sequencing gels by locating CC-1065 thermally induced DNA strand breaks on single 5′-^{32}P end-labeled DNA restriction enzyme fragments isolated from the early promoter region of SV40 DNA. Analysis of this data as well as fragments from T7 DNA, led to identification of two subsets of sequences, 5′PuNTTA (Pu = Purine, N = any base) and 5′AAAAA, which are preferred CC-1065 binding regions, with the 3′ terminal adenine of each of the sequences covalently

modified by CC-1065. Since this marked sequence specificity lies solely to the 5′ side of the covalently modified adenine, the absolute stereochemistry of CC-1065 must be 3b-R, 4a-S (Reynolds *et al.*, 1985).

Molecular modeling studies suggested that the natural configuration of the cyclopropane ring of CC-1065 and its analogs might be important for covalent binding to DNA if alkylation of N3 of adenine was dependent upon a proximity effect. This is because the long axis of the drug molecule does not lie perpendicular to the helix axis and consequently, reversal of the stereochemistry of the cyclopropane ring leads to inaccurate positioning of the cyclopropane ring relative to N3 of adenine. To test this hypothesis, the binding of U-71,184 (Warpehoski, 1986) and its enantiomer U-71,185 (Figure 7) to DNA were evaluated using the thermal strand breakage assay. The results (Figure 8) show that U-71,185 only binds to DNA at 100 fold and higher concentrations than U-71,184. Since U-71,185 contains a small amount of impurity of U-71,184 (1-2%), the residual binding of U-71,185 can be fully accounted for by the U-71,184 contaminant and consequently we conclude that U-71,185 is unable to bind covalently to DNA (Lee and Hurley, 1986). This result establishes the importance of the stereochemistry of the cyclopropane ring of U-71,184 for covalent binding to DNA.

It is of considerable importance that while U-71,184 is equipotent with CC-1065 both *in vitro* and *in vivo*, and has greatly superior activity

U-71184 - 3bR,4aS

U-71185 - 3bS,4aR

FIG. 7. Structures of U-71,184 and U-71,185.

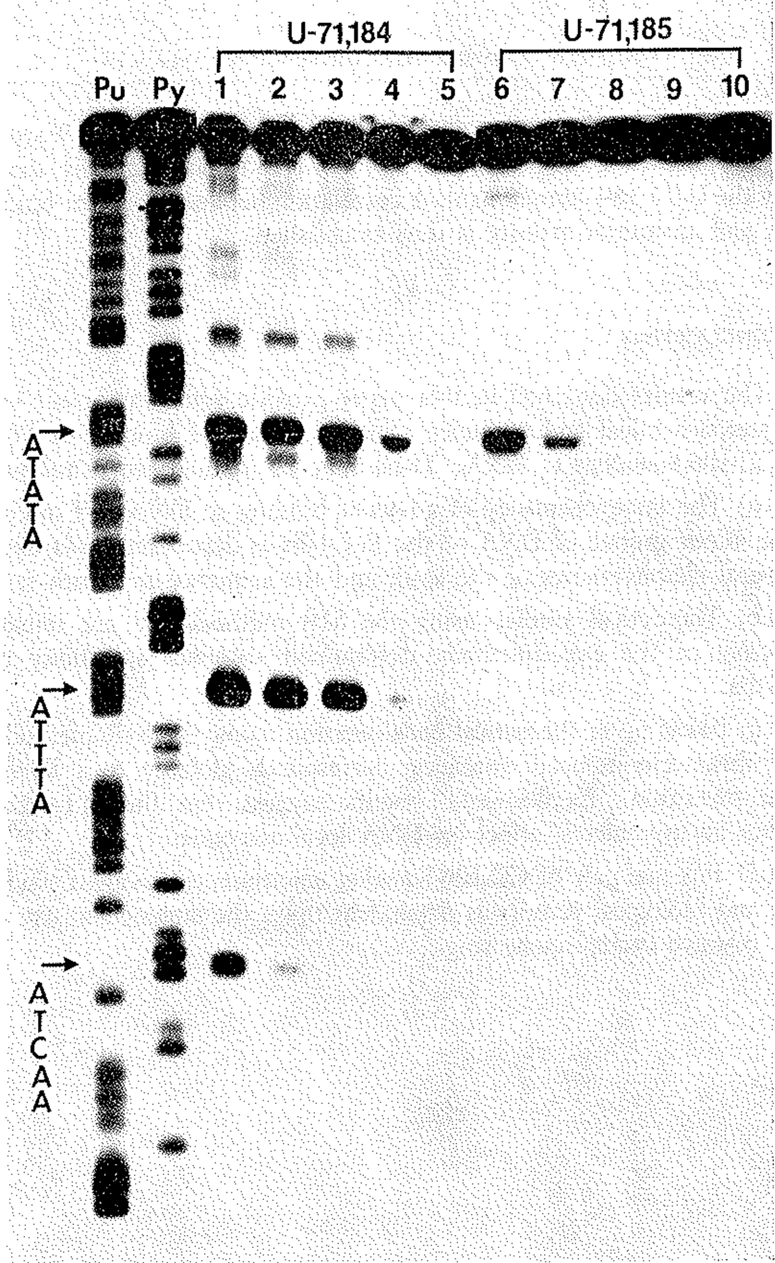

Fig. 8. Effect of U-71,184 and U-71,185 dose on DNA strand breakage produced by thermal treatment of drug-DNA adducts. A 118 bp fragment from SV40 DNA was isolated by MboI-HinfI digestions and 5′-end-labeled with ^{32}P. Far left two lanes are purine and pyrimidine Maxam-Gilbert sequencing reaction. Lanes 1-5 and 6-10 are 1:10, 1:10^2, 1:10^3, 1:10^4 and 1:10^5 dilutions of stock solutions (280µM) of U-71,184 and U-71,185 respectively. The adducts were prepared by incubating the DNA for 1 hr at 37°C with the appropriate concentration of U-71,184 or U-71,185 and then the DNA was precipitated, and electrophoresed as described before (Reynolds *et al.*, 1985). The arrows indicate the thermal cleavage product which corresponds to the binding sequence listed below the arrow. The relationship between the electrophoretic mobility of the thermal cleavage product and the

against a variety of murine tumors and human xenografts (Warpehoski *et al.*, 1986), the biological activity of U-71,185 could be fully accounted for by the U-71,184 contaminant. A correlation between covalent binding to DNA and antitumor activity is thus established.

7. Conclusions

The results described in this paper provide some important insights into stereochemical and sequence selective effects of binding of P(1,4)Bs and CC-1065 analogs to DNA. These may be summarized as follows:

(a) The stereochemistry of covalent binding to DNA and orientation in the minor groove of DNA of the P(1,4)Bs is dependent both upon the structural characteristics of the drug and the sequence in which it binds.

(b) Theoretical studies using the SIR methodology are remarkably predictive of the experimentally determined sequence specificity of the P(1,4)Bs.

(c) Based upon theoretical consideration using the SIR methodology, the anthramycin induced distortion corresponds globally to a B towards A-like transition. Experimental results suggest that the P(1,4)Bs may produce an asymmetric effect on DNA local structure.

(d) For one pair of CC-1065 analog enantiomers, covalent binding to DNA and biological activity is dependent upon the stereochemistry of the DNA reactive cyclopropane ring.

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EFFECTS OF ANTITUMOR DRUGS ON TRANSCRIPTION

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ABSTRACT

We describe the effects of six DNA-binding drugs on the separate stages of transcription initiation and propagation at the *E. coli lac* UV5 promoter by *E. coli* RNA polymerase. We have examined the influence of added drug on open complex formation and dissociation, initiation from the open complex, dissociation of the initiated complex, and blockage of transcription elongation at sites occupied by drugs. The narrow groove binding drugs distamycin and DAPI were highly efficient at destabilization and inhibiting formation of the open complex, and in preventing initiation from the open complex. The three intercalators daunomycin, actinomycin and ethidium bromide were relatively ineffective against these stages of initiation. The bis-intercalator bis-daunomycin inhibited open complex formation better than its parent daunomycin. With the possible exception of actinomycin, the drugs tested were not able to disrupt preformed open complex. The two intercalating compounds actinomycin and bis-daunomycin were much more effective at inhibiting the elongation step than were distamycin and daunomycin. The blockage sites are more specific than would be expected from the drug DNA sequence preference in binding; strong pausing was observed at GpC for actinomycin and CACACA for bis-daunomycin. We generalize from our results to conclude that

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halting elongation requires a long-lived drug-DNA complex in the presence of RNA polymerase, a feature that is consistent with the kinetically stable actinomycin and bis-daunomycin DNA complexes. However, the lifetime of the drug at the specific blockage sites detected by our measurements is about fivefold longer than measured by the detergent sequestration method. Inhibition at the level of the open complex, on the other hand, is preferentially carried out by relatively rapidly binding and dissociation drugs. We surmise that they are able to bind to DNA at multiple sites in the presence of polymerase and cause its stepwise dissociation.

Introduction

The molecular mechanism of drug-DNA interaction has been the subject of intensive study for more than a quarter of a century; indeed, 1986 marks the 25th anniversary of Lerman's proposal of the intercalation model for the complex of acridines with DNA (Lerman, 1961). In view of all the work on drug-DNA complexes done since that time, it is sobering to acknowledge how little is known about the mechanism of biological action of these compounds. The urgent medical need for better cancer therapy agents dictates that special attention be paid to deciphering how DNA-binding antitumor drugs exert their special effects on malignant cells. In some cases it may be that DNA binding is an incidental property of the drug, but in most instances it is likely that DNA in fact constitutes a primary target of drug action, given the central role of DNA in controlling cell function and growth.

A major gap remains between the demonstration of drug-DNA binding and understanding the basis of biological action. Drug attachment by itself is only a passive influence, since there can be an effect on the cell only when DNA function is activated. Hence the research direction needed is examination of specific functional complexes of DNA containing the relevant proteins, interacting with antitumor drugs. Such experiments are more challenging, but potentially much more informative, than continued study of drug binding to naked DNA.

Several DNA functions are possible targets for drug action, obviously including replication and transcription. Recent work (Tewey *et al.*, 1984; Pommier *et al.*, 1985) opens the exciting possibility that topoisomerase-DNA complexes may provide a general target for drug action. Our laboratory has focussed on transcription from bacterial promoters (Straney and Crothers, 1985, 1987a,b), and we have used the techniques developed

in the course of that effort to investigate the influence of several DNA binding drugs on the specific stages of transcription (Phillips and Crothers, 1986; Straney and Crothers, 1987c). Eventually it should be possible to do similar experiments on specific eukaryotic promoters, but the difficulties that accompany specific *in vitro* transcription in such systems have so far made it advisable to restrict the work to bacterial systems.

STAGES OF TRANSCRIPTION

Open and closed complexes. In 1974 Chamberlin suggested a two step model for transcription initiation, involving an initial attachment of RNA polymerase to DNA in a "closed" complex, followed by an isomerization step in which the DNA helix unwinds, producing the "open" complex, which then catalyzes initiation of transcription:

$$\text{RNA polymerase (R)} + \text{promoter (P)} \overset{K_B}{\rightleftharpoons} \text{closed} \overset{k_2}{\rightarrow} \text{open} \rightarrow \text{initiated complex}$$

Additional intermediates in this basic model, sure to exist in such a complex selection and synthesis process, have been proposed, based largely on kinetic evidence. An intermediate "pre-closed" complex preceding the closed complex has been hypothesized by Hawley *et al.* (1982), Rosenberg *et al.* (1982), and Hofer *et al.* (1985). Further intermediates preceding or within the open complex state have also been suggested. Rosenberg *et al.* (1982) proposed a rate-limiting step above 20°C in the T7 A1 promoter for which base pair opening is not required, based on the lack of salt or temperature dependence of the rate in this temperature range. Hawley *et al.* (1982) reached a similar conclusion from the greater temperature dependence seen for poly(dA-s^4T) hyperchromicity than observed for the isomerization rate k_2 for the *lac* UV5 promoter. Roe *et al.* (1985) reported a temperature dependence of nitrocellulose filter binding kinetics, using the λ P_R promoter, consistent with the scheme $R + P \rightarrow I_1 \rightarrow I_2 \rightarrow RP_{open}$. Arguing from the activation energies, they assigned $I_1 \rightarrow I_2$ to a conformational change in the polymerase, and further associated the step $I_2 \rightarrow RP_{open}$ with base-pair unwinding. The results from all three groups point to an additional step in open complex formation which does not involve a temperature-dependent base pair unwinding. Our results, described below, are in agreement with this conclusion. Additionally, Buc and Mc Clure (1985) proposed an intermediate RP_i in the *lac* UV5

promoter, between closed and open complexes, reasoning from the observation that transcriptional activity is lost faster than is the stable promoter complex when the temperature is dropped. In probing RP_i at low temperature, Spassky *et al.* (1985) found the same DNAase I footprint as seen for the open complex at higher temperature, but did not observe protection of the DNA from chemical methylation at the lower temperature comparable to that seen at higher temperature.

Our effort on this problem has focussed on exploiting the ability of non-denaturing gel electrophoresis to separate intermediates on the transcription pathway (Straney and Crothers, 1985, 1987a,b). As shown in Figure 1, gel electrophoretic analysis of a transcription complex in which polymerase and *lac* UV5 promoter are mixed and incubated in the absence of nucleotide triphosphates reveals a closed complex and two heparin-stable open complexes. The ratio and yield of the latter is highly temperature-dependent, as shown in Figure 2, with the upper complex on the gel, O_u, becoming dominant at high temperature over the lower complex, O_ℓ, which is favored at low temperature. It seems probable that the electrophoretically distinct O_ℓ complex corresponds to one or more of the additional intermediates along the initiation pathway hypothesized by other groups. Enzymatic and chemical footprinting studies (Straney and Crothers, 1985, 1987a,b) reveal that O_u and O_ℓ are very similar, and are characterized by significant opening of the double helix in both cases. The subtle differences between the two include a better ability by O_u to accommodate extensive promoter unwinding as the temperature proportion of O_u found at higher temperature (Straney and Crothers, 1987b).

The stable initiated complex. Gel electrophoresis of an open complex transcription mixture to which has been added GpA as a primer, GTP, ATP, UTP, and 3′-0-methyl CTP reveals a stable polymerase-DNA-RNA complex, again with a distinctive gel electrophoretic mobility (Straney and Crothers, 1985). The nascent RNA chain is blocked from further growth by incorporation of 3′-0-methyl C at the 11th nucleotide, corresponding to the first C in the growing mRNA; no smaller RNAs, which are nonetheless present in the reaction mixture, are found to be stably enough bound to withstand gel electrophoresis. We also showed that incorporation of a stably bound RNA is accompanied by loss of footprinting protection in the upstream region and expulsion of the polymerase σ subunit from the complex (Straney and Crothers, 1985, 1987a,b).

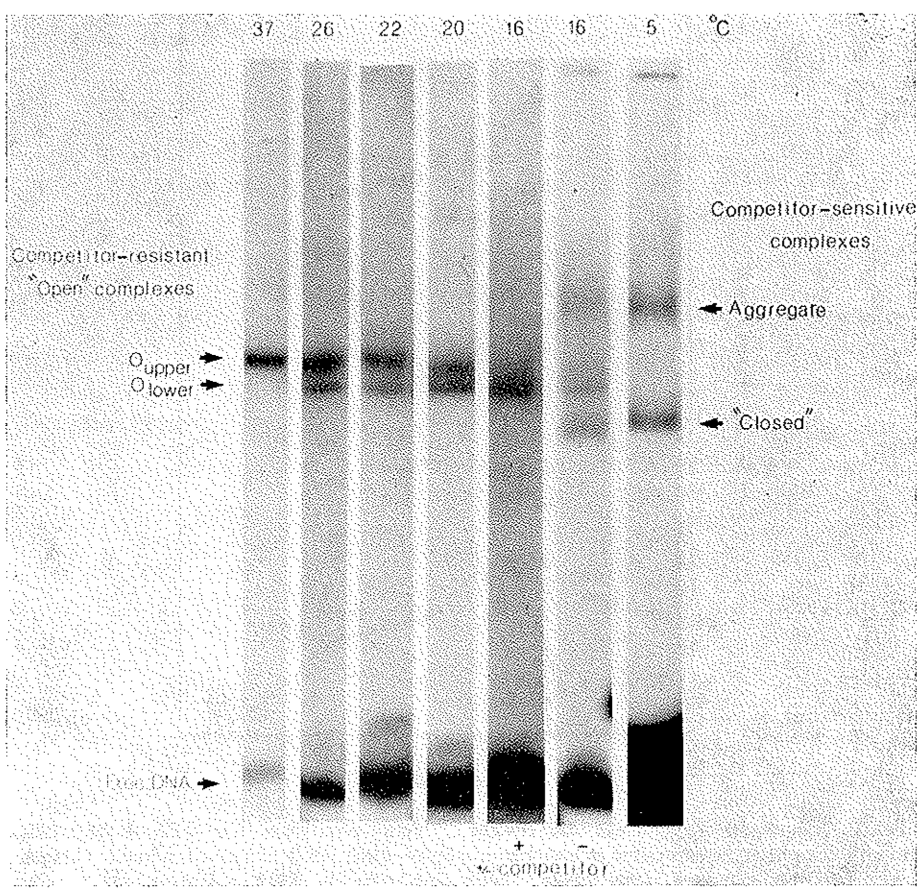

FIG. 1. Binary Gel Complexes. RNA polymerase-DNA binary complexes were formed with the *lac*-UV5 203 bp fragment; after incubating at the specified temperature (top of lanes) for 15-30 minutes, the reactions were run on separate 4% polyacrylamide gels, each maintained at the constant temperature at which the reaction was incubated. The marker dyes were run to the same distance on each gel, and the relative mobilities of the complexes on different gels determined by aligning marker DNA fragment bands (not shown). The 37°, 26°, 22°, 20° and 16°C (– competitor) lanes contain a two-fold excess of non-specific calf thymus DNA, added immediately before loading, to remove the small amounts of aggregate band formed under these conditions; the 5°C lane does not contain any of this non-specific DNA. The 16°C (+ competitor) lane represents the addition of a ten-fold excess of poly d(A-T) · d(A-T), measured in bp, immediately before loading onto the gel. The poly d(A-T) · d(A-T) competes with the labeled 203 fragment for RNA polymerase binding and so produces net dissociation of complexes which are in rapid equilibrium with free DNA.

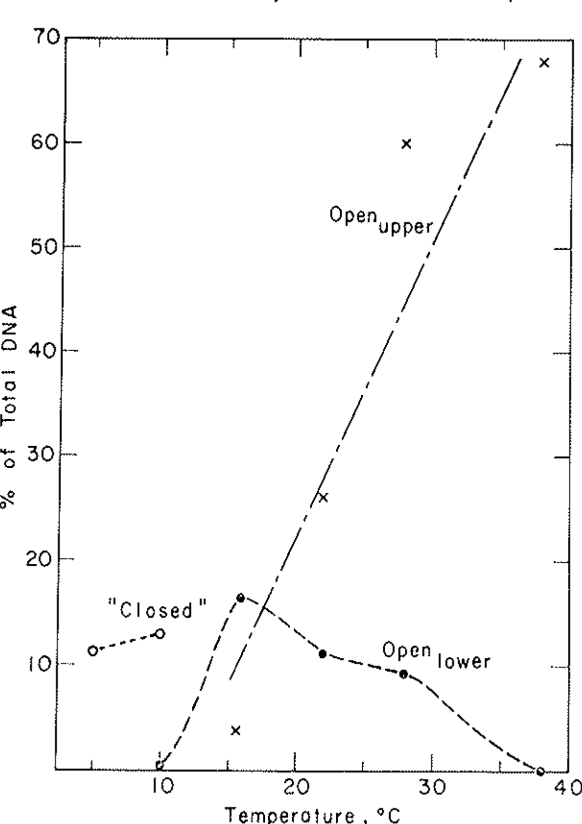

FIG. 2. Temperature dependence of the yield of binary complexes. The yield of the various binary complexes is expressed as the percentage of total DNA contained in each complex. Although the values of these yields change with different RNA polymerase-DNA ratios, the relative yields do not.

Abortive initiation. Natural cycling of RNA polymerase at the start of RNA synthesis to produce abortive transcripts 2 to 9 bases long has been observed on various promoters (Carpousis and Gralla, 1980; Reznikoff *et al.*, 1982; Grachev and Zaychikov, 1980). McClure and colleagues have exploited the abortive initiation process, in which RNA chains are initiated but ejected by the polymerase before they can form a stable initiation complex, to assay the equilibrium binding constant K_B and the isomerization rate k_2 in the initiation reaction scheme shown above. In the *lac* promoter, either entry into abortive transcription or escape into

productive initiation appears to be the rate-limiting step (Stefano and Gralla, 1979; S.B. Straney and Crothers, 1987).

The role of gene activating proteins. A view to the future of studies of drug-DNA action affirms the special role to be expected for specific gene activating proteins, for it is such interactions that help to distinguish one gene from another, thus offering especially tempting targets for DNA-mediated antitumor agents. The *E. coli lac* promoter is not lacking in this regard, for the cAMP-binding CAP protein represents the prototypical gene activating protein, whose presence is required for efficient expression of the *lac* operon *in vivo*. Wu and Crothers (1984) showed that the DNA bending induced by CAP protein is reduced by distamycin binding. How this will affect the function of the protein is not known at this time. Unfortunately, little systematic work has been reported on the ability of DNA-binding drugs to interfere with the action of gene activating proteins; this is clearly a fruitful subject for future research.

A revised initiation mechanism. Of the two open complexes identified by our experiments, O_u was found to be much more effective in supporting stable initiation (Straney and Crothers, 1985). In addition, the pattern of chemical methylation found in the initiated complex is found to parallel closely that of O_u rather than O_ℓ (Straney and Crothers, 1987b), suggesting again that the initiated complex derives from O_u. The results can be summarized in the revised mechanism shown in Figure 3:

$$R + P \underset{}{\overset{K_B}{\rightleftarrows}} RP_{closed} \overset{k_2}{\rightarrow} O_\ell \overset{K_{\ell u}}{\rightleftarrows} O_u + rNTPs \overset{k_3}{\rightarrow} \text{stable initiation}$$

$O_\ell \downarrow$, $O_u \downarrow$: abortive initiation

FIG. 3. Proposed mechanism of transcription initiation at the *lac* UV5 promoter.

It should be noted that O_u and O_ℓ in this mechanism are in relatively rapid (< 1 min) equilibrium. O_u is clearly the primary source of the initiated complex, but it cannot now be stated that O_ℓ is an obligatory intermediate along the pathway, especially at high temperature, where that form is at best barely detectable. A modified mechanism in which the closed complex can also convert to O_u directly would, given our present state of knowledge, be equally acceptable.

Effect of Drugs on Initiation

Drugs studied. We chose for this work a selection of intercalating drugs (ethidium, daunomycin, and actinomycin), two narrow-groove binding drugs (distamycin and DAPI), and a bis-intercalating derivative of daunomycin (daunomycin-glycyl-succinyl-glycyl-daunomycin).

Rate of open complex formation. We measured the ability of several DNA binding drugs to interfere with formation of the open complex, using promoter DNA pre-equilibrated with the drug. After incubation of the drug-DNA complex with excess RNA polymerase for varying lengths of time, heparin was added to dissociate weak complexes, and the mixture was subjected to gel electrophoresis. Figure 4 shows typical results from the kinetic experiments, with the fraction of the total DNA which is found in the open complex plotted against time. Since RNA polymerase is in substantial excess, the reaction conditions are pseudo-first order, and, as expected, the time course follows an exponential approach to a plateau value. Both the time constants and the plateau values depend on the nature of the drug; the results are collected in Table I. The two minor groove-binding drugs distamycin and DAPI were found to be much more effective in reducing both the formation rate and the plateau level of open complex than were the monointercalators daunomycin and actinomycin. Clearly the most effective drug in this regard is distamycin, which reduces the on-rate to 1.5% of the control value, and the plateau extent of complex formation to 23% of the control. The bis-intercalator bis-daunomycin is significantly more active in this assay than either of the mono-intercalators, reducing the formation rate to 6.9% of control.

We also examined the relative importance of kinetic and equilibrium factors in the influence of distamycin on open complex formation, by adding an excess of unlabeled free DNA to the drug-DNA complex just before adding the polymerase. Under these conditions, in which the excess DNA sequesters the drug as it dissociates from the labeled DNA, we found that the rate of complex formation approached that of the control. This means that the rate of complex formation is not governed by slow kinetics of dissociation from DNA, but must rather reflect a strong drug-DNA binding constant for important sequences in the promoter. The strong ability of distamycin and DAPI to reduce open complex formation must reflect their A·T specificity and relatively strong binding constants (see Table I).

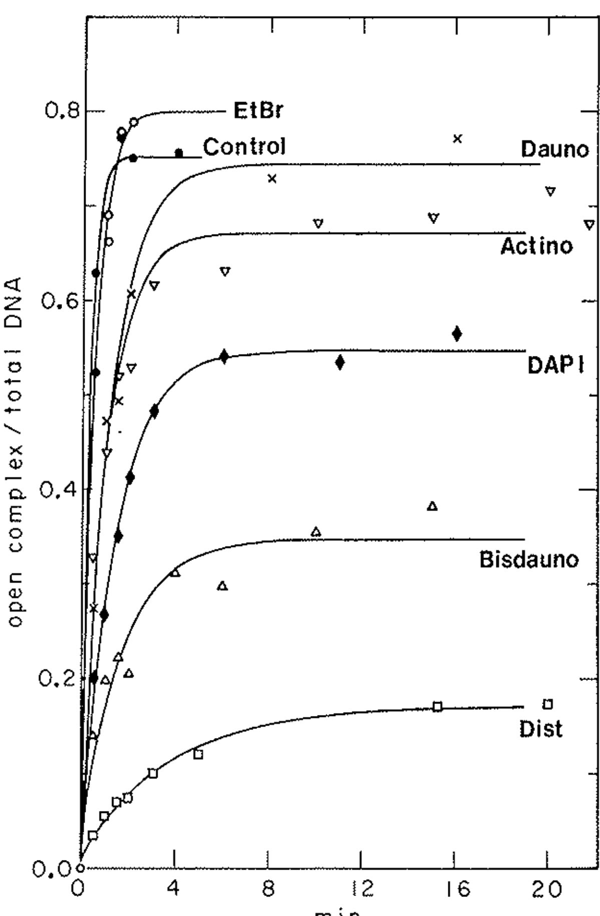

FIG. 4. Time course of the formation of open complex. The fraction of open complex over total DNA (open and free DNA) is plotted over time of sampling after polymerase addition. Exponential curves fitted to the points are used to determine the initial rate (slope at t = 0) and the equilibrium level of open complex (plateau) in table 1. The drugs present during the open complex formation are: ● Control; ○ Ethidium Bromide; × Daunomycin; ▽ Actinomycin; ♦ DAPI; △ Bis-daunomycin; □ Distamycin.

TABLE I - *Effect of Drug upon Formation of Open Complex.*

Drug	Relative Initial Rate of formation	Open Complex[a] Equilibrium Level	$t_{1/2}$ Drug Dissociation	$K_{equ.}$ Drug-DNA
[Control]	2.6	.75	—	—
Ethidium Bromide	1.6	.80	0.006 sec.[b]	$2x10^4$ [b]
Daunomycin	0.62	.75	0.69 sec.[c]	$2x10^4$ [c]
Actinomycin	0.62	.67	375 sec.[d]	$2x10^6$ [d]
DAPI	0.37	.55	not known	$5x10^6$ [e]
Bisdaunomycin	0.18	.35	38 sec.[f]	10^8-10^9 [f]
Distamycin	0.04	.18	not known	$1x10^6$ [g]

[a] expressed as fraction of total DNA
[b] Bresloff and Crothers, 1975
[c] Chaires *et al.*, 1985
[d] Müller and Crothers, 1968
[e] Manzini *et al.*, 1983
[f] Phillips and Crothers, 1986;
[g] Luck *et al.*, 1974.

Drug effects on stability of the open complex. The amount of open complex decays with time when heparin is added to prevent recycling of the polymerase. By adding drug simultaneously with heparin, the ability of the drug to perturb the kinetics of dissociation of the open complex can be measured. Table II summarizes these results, and shows that the ability of the drugs to destabilize an existing open complex increases in the order actinomycin < ethidium < daunomycin < bis-daunomycin < DAPI = distamycin. Again it is seen that the two narrow-groove binders have the greatest influence on the open complex.

A drug's ability to force dissociation of the polymerase-DNA open complex implies more than a simple steric competition for binding sites. One possibility is that the drug enters the complex at a DNA locus not directly attached to the protein, distorting a section of the nucleic acid in the process, so that the affinity of the protein for its attachment sites is reduced. For example, intercalators could function this way by unwinding the DNA and altering the phasing between two protein-DNA interaction sites. The minor groove binders could attach to DNA at a point where the protein occupies the major groove, bending the DNA or propagating some other conformational effect into adjacent regions. A

second possibility is that the drug binds to regions of the DNA which are more weakly or transiently bound by the protein. A succession of such steps could lead to progressive weakening of the complex; this explanation clearly requires significant protein flexibility in the complex so that it can surrender its contacts step-wise. Whatever the final explanation, these experiments make it clear that drugs can interact with polymerase-DNA complexes, with important functional consequences.

Drug effects on initiation from the open complex. These experiments were done by allowing formation of open complex in the presence of drug, then adding primer GpA, GTP, ATP, UTP, and 3′-0-methyl CTP. This combination allows formation of the stable initiated complex containing an 11-mer RNA product. The rate of production of the initiated complex was normalized to the amount of open complex present before addition of NTPs, thus correcting for the ability of the drug to decrease the amount of starting material in the conversion from open to initiated complex. As shown by Figure 5, in the control (drug-free) samples about 70% of the open complex was converted to initiated complex. Daunomycin, ethidium, and bis-daunomycin-containing samples deviated only slightly from the control values. Distamycin and DAPI, on the other hand, inhibited the conversion to 50% and 70% respectively of the values seen in the control.

In the presence of actinomycin, the initiated complex content peaked

TABLE II - *Effect of Drugs upon the Dissociation of Polymerase from Open Complex.*

Drug	$t_{1/2}$ Dissoc. min. Open Complex	Rate of Formation[a] Drug-DNA Complex (reference
[Control]	127	
Actinomycin	91	1×10^4 $M^{-1}s^{-1}$ (Müller and Crothers, 1968) .081 s^{-1} [a]
Ethidium Bromide	72	1×10^6 $M^{-1}s^{-1}$ (Bresloff and Crothers, 1975)
Daunomycin	64	7×10^6 $M^{-1}s^{1-}$ (Chaires *et al.*, 1985) 4.2 s^{-1} [a]
Bis-daunomycin	52	similar to daunomycin but not known
DAPI	17	not known
Distamycin	17	not known

[a] slowest step in forming the most stable drug-DNA interaction

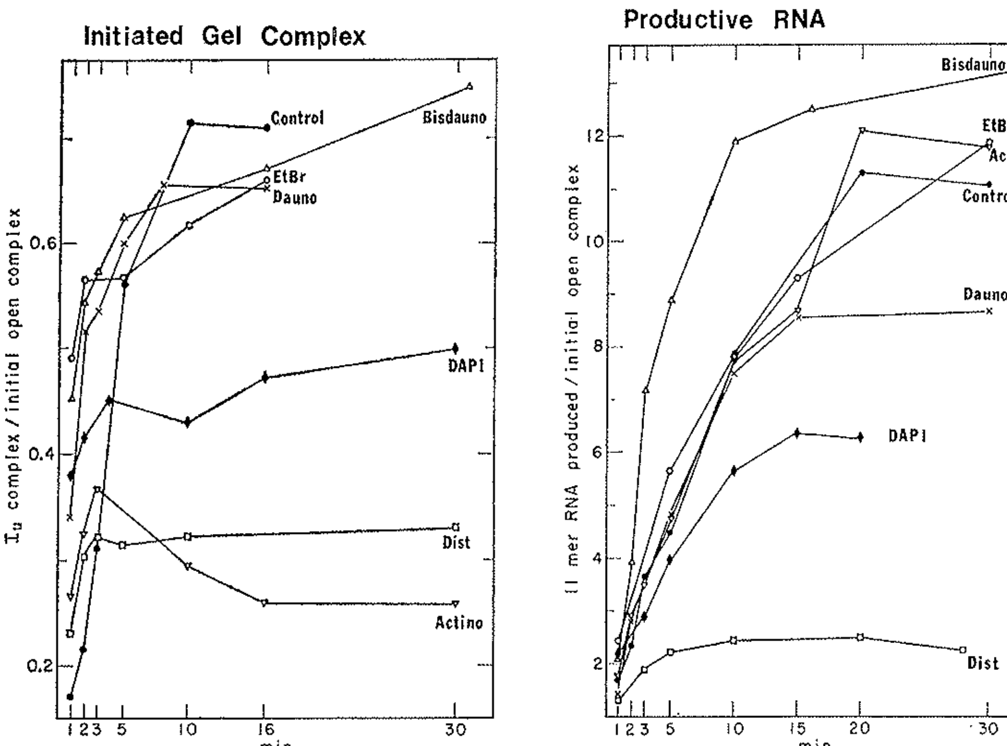

FIG. 5. Quantitation of initiation from open complex in the presence of drugs. The effect of drug upon the ability of open complex to initiate transcription is assayed by formation of stable initiated gel complex (left) and formation of RNA (right). The level of I_u complex is represented as the fraction of the initial open complex present with each drug at the time after ribonucleotide addition; the relative amount of RNA produced is similarly normalized to the initial amount of open complex. The final amount of initiation in the presence of Ethidium Bromide (○), Daunomycin (×), and bis-daunomycin (△), are similar to control (●). Actinomycin (▽) allows RNA production but limits I_u formation. Distamycin (□) and DAPI (♦) inhibit initiation in both assays.

initially, followed by a slower decay of the complex already formed. Experiments measuring the amount of RNA present (Figure 5) showed more 11-mer than could be accounted for on the basis of the amount of initiated complex on the gel. We conclude that the initiated complex formed in the presence of actinomycin is unstable. This contrasts with the behavior of an initiated complex formed in the absence of the drug, which is not dissociated by drug addition. Again it is clear that the protein-DNA complex has special properties not reflected in the simple drug-DNA interaction effects.

Drug effects on stability of the initiated complex. In these experiments, drugs were added to preformed initiated complexes, and the content of initiated complex was assayed as a function of time by raising the salt from 100 μM to 350 μM, thus dissociating any open complex, but leaving initiated complexes intact (Straney and Crothers, 1985). None of the six drugs tested showed any deviation from the off-rate observed for the drug-free control. The relative instability of the open complex to drug addition was further amplified by the use of high concentrations of drug. For example, at ethidium to DNA bp ratios of 1:1 and 70:1, we observed a rapid dissociation of the open complex within 5 min, whereas there was no influence on the stability of the initiated complex.

Drug Effects on Transcriptional Elongation

Once transcription initiation is complete, RNA polymerase loses its σ subunit and occupies a much smaller region of the *lac* DNA. We explored the ability of a series of bound drugs to inhibit passage of the polymerase in the RNA chain elongation mode. The experiments (Phillips and Crothers, 1986) were done by incubating polymerase and promoter with GpA, ATP, GTP, and {α-^{32}P}UTP, followed by addition of drug and a further 10 min incubation. Transcription elongation was begun by addition of CTP, and the ^{32}P-labeled RNA chains were assayed by gel electrophoresis, taking aliquots at various times after the reaction was started. The presence of bound drugs was signaled by the appearance of "pausing" in RNA chain synthesis, as indicated by the transient appearance of RNA chains less than the run-off length in samples containing drugs.

Figure 6 shows a comparison of the gel patterns for a transcription mixture with and without added actinomycin. Significant amounts of natural pausing are seen without the drug, but a strong new pause region

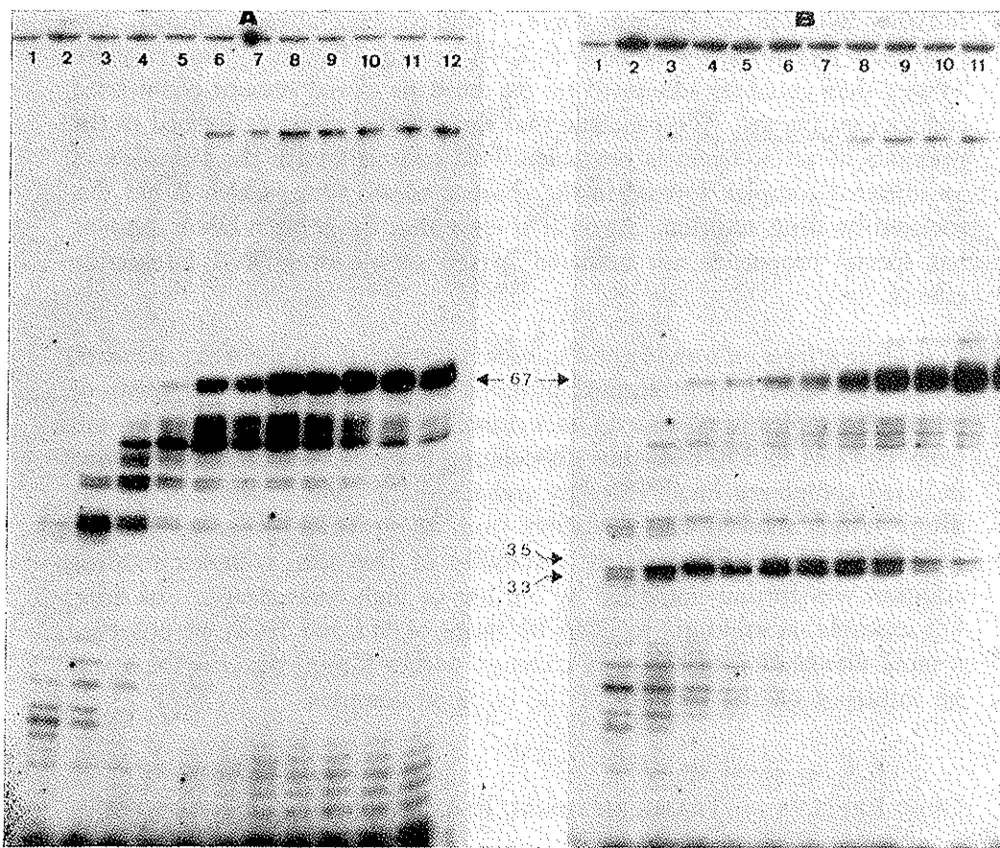

Fig. 6. Sequencing gel of Actinomycin D blocked transcripts. Lanes 2-12 show a time course (0.25, 0.5, 1, 1.5, 3, 5, 8, 12, 20, 30, and 50 min. respectively) for elongation of the transcripts subsequent to addition of CTP, in the absence (A) and presence (B) of Actinomycin D after the initiated complex had been formed. Lane 1 is a control which lacked CTP. Several time points were rerun in a sequencing gel (see Experimental) to assign sequence lengths.

appears at nucleotides $+ 33 \rightarrow 35$ from the natural transcription start site. In addition, natural pausing at $+ 52 \rightarrow 57$ is significantly reduced in the presence of the drug. Figure 7 shows the RNA sequence in the region of interest. The nearest downstream actinomycin binding site to the blockage site is the GpC sequence at $+ 36 \rightarrow 37$; similar results for GpC actinomycin blockage sites were found by Aivashahvilli and Beabeahashvilli (1983) in T7 DNA. Analysis of the strength of DNA binding by actinomycin for DNAs of varying G·C content indicates that

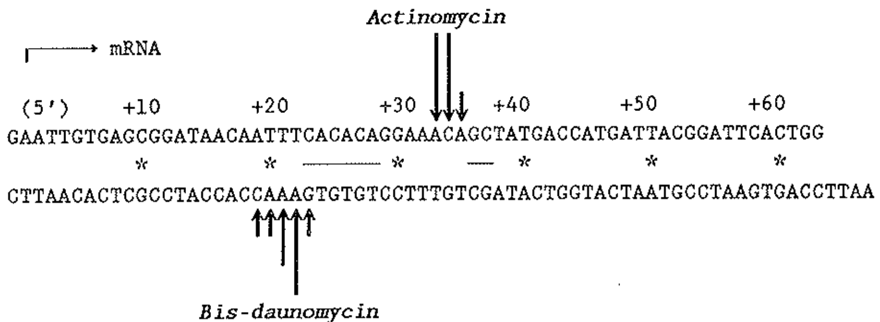

FIG. 7. Transcriptional blockage points for actinomycin and bis-daunomycin denoted by arrows whose length is an indication of the strength of the pause observed at each nucleotide. The lower strand is the coding strand. Horizontal lines between the two strands indicate the probable site of drug binding which is responsible for blockage.

the primary requirement for a binding site is a guanine base (Müller and Crothers, 1968). Under the conditions of the experiment, 1 actinomycin was added for each 10 bp, at concentrations such that most of the drug is bound. Hence there should be on the order of 4-5 drug molecules bound in the DNA region traversed by the polymerase during elongation. In spite of this, only one strong blockage site was found, indicating a special character for the complex of actinomycin bound to GpC in the presence of RNA polymerase.

The results of a similar experiment on bis-daunomycin are also summarized in Figure 7. In that case, blockage occurs in the $+19 \rightarrow 23$ region, and essentially nowhere else. The repeating CACACA sequence at $+23 \rightarrow 28$ is thought to be responsible for transcription blockage in this case. Monomeric daunomycin elicited only a small delay ($\sim$ 30 sec) in the appearance of full length transcripts, without the appearance of any discrete blockage sites. Distamycin produced no detectable effect on transcription elongation.

The decay of the amount of paused transcripts at $+33 \rightarrow 35$ must reflect release of the actinomycin-induced blockage, presumably by dissociation of the drug. The time dependence of the transcript populations is shown in Figure 8, and Table III reports the measured first order time constants for release of blockage, both by actinomycin and bis-daunomycin.

Detergent sequestration assay of drug-DNA complex dissociation kinetics. The rate of dissociation of drugs from naked DNA can be measured by the addition of SDS, which sequesters the drug as it is released,

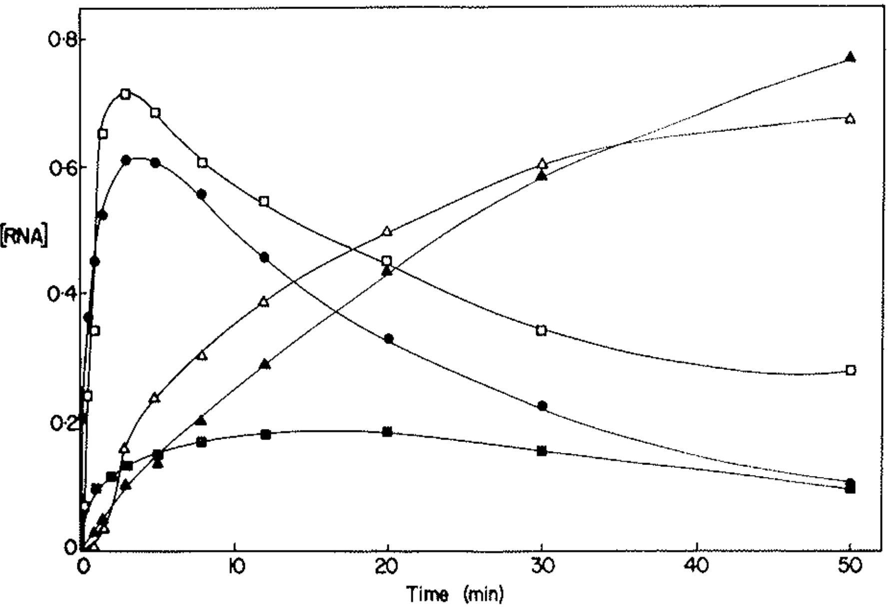

FIG. 8. Kinetic profile of transcript formation. The kinetics of the elongation process are shown as the mole fraction of each discrete transcript, as a function of elongation time in the absence (open symbols) and presence (filled symbols) of Actinomycin D. Transcript lengths are + 33 to + 35 (○), + 52 to + 57 (□) and full length transcripts (△).

TABLE III - *Time constants for dissociation of drug-DNA complexes.**

Drug	transcription assay	SDS assay
Actinomycin D	1500 sec	300 sec
Bis-daunomycin	190 sec	38 sec

* Transcription buffer, 37°C.

preventing rebinding (Müller and Crothers, 1968). We carried out this assay on actinomycin and bis-daunomycin, under ionic conditions equivalent to those of the transcription assay. The results, collected in Table III, show that the longest measured time constant detected by the SDS sequestration assay is about 5 times shorter than the time constant for release of blockage of transcription. These results indicate that an especially stable drug binding site is formed in the ternary complex containing DNA, nascent mRNA, and RNA polymerase, specifically at the DNA sequence GpC for actinomycin, and CACACA for bis-daunomycin. Once again it is evident that the protein-DNA complex has special properties for drug binding. Clearly the ability to block transcription is more sequence specific than is simple binding.

Drug Properties

Kinetic effects versus equilibrium binding strength. DNA binding drugs differ substantially in their equilibrium binding strength, by roughly two to three orders of magnitude if the bivalent bis-intercalators are excluded from the comparison. Differences in the kinetic properties are even more pronounced, with dissociation time constants varying from milliseconds for the acridines to as much as a thousand seconds for the actinomycins. Slow dissociation kinetics appears to be essential for efficient blockage of transcription, as illustrated by actinomycin and bis-daunomycin. Drugs that dissociate rapidly from the ternary RNA-DNA-polymerase complex require the polymerase to pause only briefly in its progression along the DNA template. The tightly binding distamycin has a relatively short complex lifetime, and is completely ineffective in blocking transcription at the concentrations ($\sim$ 1 μM) studied. However, the effectiveness of these drugs has the reverse order when inhibition of transcription initiation is considered. Distamycin is highly effective at destabilizing and preventing formation of the open complex, as is also the small groove binder DAPI. It seems inescapable that these drugs can bind to the protein-DNA complex, probably producing progressive destabilization by successive binding events. In this instance it is the ability of the drug to bind tightly and rapidly to sites that may be only transiently open that appears to be the essential feature.

Plausible targets of drug action . Our work provides some guides in thinking about the central question of the site of biological action of

antitumor drugs, although the results are by no means decisive on that issue. Anthracyclines, represented in our sample by daunomycin and bis-daunomycin, are among the most clinically effective antitumor drugs. Daunomycin, in our assay, is comparatively quite ineffective in blocking either transcription initiation or propagation. It seems likely that the actual biological action lies elsewhere, perhaps at the level of topoisomerase function. It should always be recognized, however, that the specific protein involved in a protein-DNA complex can confer distinctive properties on drug interactions, and it remains possible that daunomycin can have pronounced effects on specific eukaryotic promoter function.

Actinomycin is a slowly equilibrating drug, and relatively ineffective at inhibiting transcription initiation. It is, however, remarkably effective at blocking transcription propagation when bound to a GpC sequence. This general feature may be responsible both for the antitumor action and the highly toxic character of the actinomycins, which have had relatively little clinical utility.

The rapidly equilibrating narrow groove binding drugs distamycin and DAPI have a dramatic ability to block transcription initiation, which provides a plausible site of biological action for those compounds. In this case it may be possible to target individual promoters by building greater DNA sequence specificity into their binding interactions.

Enhancing Drug-DNA Sequence Specificity

DNA is now one of the best characterized potential receptors for pharmaceutical agents, providing a great challenge both to the chemist in designing sequence-specific compounds, and to the pharmacologist in devising methods to deliver the sequence-specific agents to the cell nucleus, which is of necessity the site of action. In 1979 our laboratory (Kosturko *et al.*, 1979) reported a pilot study of this kind in which we used a combination of G·C and A·T specific agents to create by template polymerization DNA binding agents able to rebind with specificity to their original templates. The G·C selective agents were acryl derivatives of the intercalating phenyl neutral red, and the A·T specific compounds were acryl derivatives of the outside binding triphenyl methane dye malachite green (Figure 9). The selection of these compounds grew out of the extensive work of Müller and his colleagues (Müller and Crothers, 1975; Müller and Gautier, 1975; Müller *et al.*, 1975) on the DNA base

FIG. 9. Chemical structures of the monomers used to create sequence-specific DNA binding polymers (Kosturko *et al.*, 1979). APNR is acryl phenyl neutral red (G·C-specific), AMG is acryl malachite green (A·T) specific) and MA, methylacrylamine, is used as a spacer.

and sequence specificity of small molecule binding. Polymerization of the substituted ligands occurred through the acryl groups by a radical chain mechanism, initiated by ammonium persulfate, in the presence of the DNA which served as template. DNA templates were T7 or λ virus preparations, respectively, and specificity was assayed by measuring the ratio R of transcription from T7 to transcription from λ, as a function of added drug polymer concentration. The properties of these complex materials were somewhat erratic, but as shown in Figure 10, polymers made on λ DNA showed a significant ability to increase R by selectively inhibiting T7 transcription.

Knowledge of DNA sequences essential for promoter function has

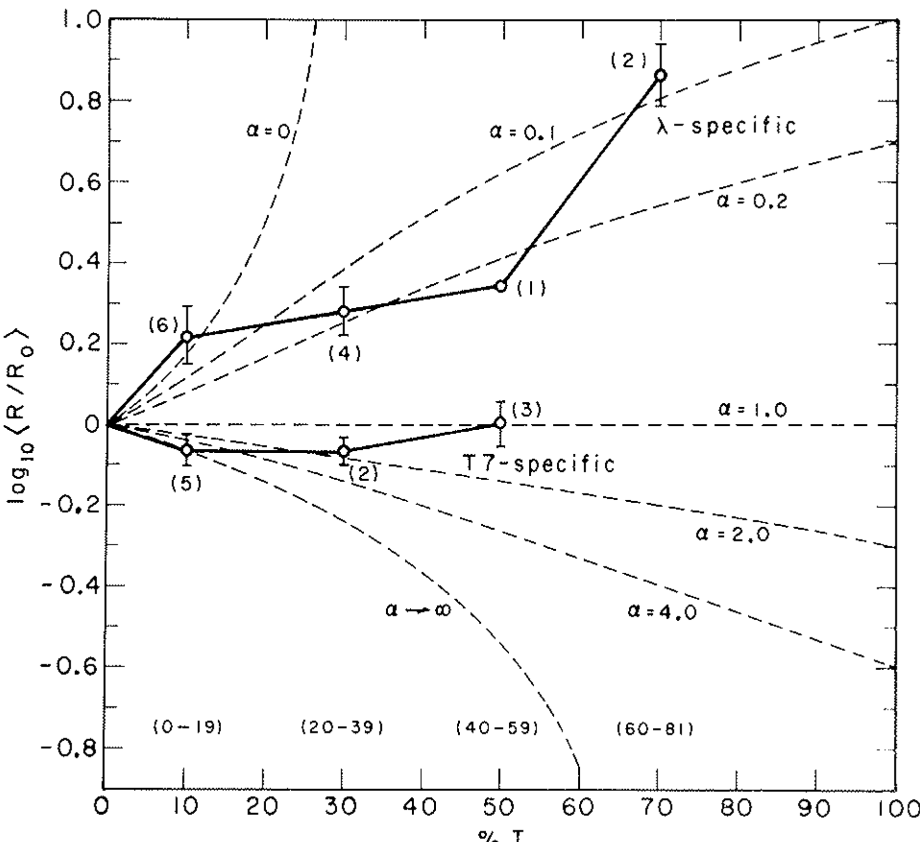

FIG. 10. Percent inhibition (%I) dependence of average values of the ratio R (transcription from T7 DNA divided by transcription from λ DNA) divided by R_0, the value of R found when no polymer is added. All data are shown as $\log\langle R/R_0\rangle$, where R/R_0 was averaged for percent inhibition values falling within the ranges 0-19%, 20-39%, etc. λ-specific polymers were synthesized on λ-DNA, and T-7 specific polymers were synthesized on T7-DNA. The dashed lines are theoretical curves for a model in which transcription is proportional to the fraction of DNA not covered by polymer. The quantity α is the ratio of binding constant for T7 DNA divided by the constant for λ DNA, ranging from 0 (totally λ-specific) to ∞ (totally T7-specific). The numbers in parentheses adjacent to each point represent the number of data points averaged to calculate the value graphed. The error bars correspond to the estimated standard deviation of the mean of each set of averaged values.

greatly increased since 1979, as has the ability to prepare large amounts of specific sequences to serve as template for polymerization. A renewed effort at inhibition of the function of specific DNA sequences seems warranted at this time.

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MOLECULAR MECHANISMS OF DNA SUGAR DAMAGE BY ANTITUMOR ANTIBIOTICS

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ABSTRACT

The molecular mechanisms by which antitumor antibiotics damage DNA deoxyribose are compared. The nonprotein chromophore of the antibiotic neocarzinostatin intercalates between DNA base pairs in the minor groove of DNA, and, when activated by thiol, abstracts a hydrogen atom from C-5′ of deoxyribose to produce strand breaks, base release and covalent drug-DNA adducts, depending on the availability of oxygen. Activated drug itself appears to function as a targeted radical in causing DNA damage. By contrast, the antibiotic bleomycin and ionizing radiation attack DNA deoxyribose via an activated reduced form of oxygen, that abstracts a hydrogen atom from C-4′ (as well as other deoxyribose carbons for ionizing radiation) to generate strand breaks and base release. The DNA damage products produced by the three agents have been characterized and can be rationalized according to proposed mechanisms of formation.

DNA is a target for a number of biologically active agents, including natural products, environmental carcinogens, cancer chemotherapeutic drugs, and ionizing radiation. The interaction between DNA and such agents can have profound effects on the structure of DNA and result in interference with its normal function in replication and transcription. Such effects may be the consequence of physical or covalent binding of the compound to DNA or result from specific chemical damage to its base or sugar components. The changes in DNA structure can account for many of the mutagenic and carcinogenic properties of these agents.

Whereas a number of antitumor antibiotics interact with DNA to form reversible complexes or covalent adducts with the bases, relatively few damage DNA by specifically interacting with the deoxyribose moiety (Goldberg and Friedman, 1971; Goldberg *et al.*, 1977, 1981; Povirk, 1983). Neocarzinostatin (NCS) and bleomycin (BLM) are agents that damage DNA primarily via attack on the DNA sugar, resulting in strand breakage, base release and other related lesions. Unlike BLM or ionizing radiation, which damage DNA through the intermediacy of an activated reduced form of oxygen (see Povirk, 1983; von Sonntag *et al.*, 1981; Hutchinson, 1985), NCS-induced DNA damage results from the direct attack by an activated form of the drug on the deoxyribose (Kappen and Goldberg, 1985). In fact, with NCS it appears that activated drug itself acts as a targeted radical in producing DNA damage. This paper will describe current information on the nature of the interaction between the active form of NCS and DNA, on the molecular mechanism of the ensuing deoxyribose damage, and on some of the biological consequences of these actions. These actions will be contrasted with those of BLM and ionizing radiation.

1) NCS STRUCTURE

NCS is a member of a family of macromolecular ($M_r > 10{,}000$) antitumor antibiotics obtained from culture filtrates of *Streptomyces* (Ishida *et al.*, 1965). All the biological activity resides in a methanol-extractable nonprotein chromophore ($M_r = 659$) (Napier *et al.*, 1979; Kappen *et al.*, 1980; Ohtsuki *et al.*, 1980; Suzuki *et al.*, 1980) that is tightly ($K_D \sim 10^{-10}$ M) and specifically bound to an apoprotein ($M_r = 11{,}000$) (Povirk and Goldberg, 1980). The latter acts as a carrier and protects the labile chromophore (NCS-Chrom) from degradation. The X-ray crystallographic structure at 2.5Å resolution of the related antibiotic, actinoxanthin, shows that the protein molecule is kidney-shaped and has a well defined cavity into which a chromophore is proposed to fit (Pletnev *et al.*, 1982). A similar structure has been described for NCS (Sieker, 1981). In aqueous solution at neutral pH the isolated chromophore is totally degraded in a few seconds, whereas it is stable for hours when complexed with its apoprotein (Povirk and Goldberg, 1980). Circular dichroism and absorption spectroscopy of titrations of isolated chromophore and apoprotein show that the final complex consists of a one-to-one mixture of the two components (Napier *et al.*, 1981a).

NCS-Chrom has been purified by high-pressure liquid chromatography, and a structure has been proposed for its major form (A) (Hensens *et al.*, 1983; Shibuya *et al.*, 1984; Edo *et al.*, 1985) (Fig. 1). It consists of three subunits: a 5-methyl-7-methoxy naphthoate, a 2,6-dideoxy-2-methyl-amino-galactose moiety and an interconnecting highly unsaturated C_{12}-subunit bearing a cyclic carbonate moiety and an epoxide. The C_{12}-subunit consists of a novel, highly strained bicyclo (7, 3, 0) dodecadiyne system. Mercaptan treatment in methanol results in the addition of one molecule of mercaptan as well as in the reduction of the C_{12}-substructure, consuming a total of three sulfhydryls (or four hydrogens from $NaBH_4$) (Hensens *et al.*, 1983).

Two other forms of NCS-Chrom (A) resulting from alteration in the cyclic carbonate (1,3-dioxolan-2-one) moiety have been identified (Napier *et al.*, 1981a,b): Form C, the methyl ester, results from storage of NCS-Chrom in methanol, whereas form B, the open diol decarboxylation product, is present in the material used clinically at a level of 10% of form A. Form C is active both *in vitro* and *in vivo*, whereas form B is active only *in vitro*. These results show that the cyclic carbonate moiety is not essential for DNA damage, but may be required for entry of the drug into mammalian cells. Both B and C were somewhat more stable than A. Thus, it is possible that by modification or substitution of the cyclic carbonate moiety the selectivity of the drug for malignant and normal cells can be altered.

FIG. 1. Structure of NCS-Chrom.

2) Reversible Binding of NCS-Chrom to DNA

NCS-Chrom binds tightly to DNA ($K_D = \sim 10^{-6}$ M) as shown by (1) quenching by DNA of the 440 nm fluorescence and shifting of the emission peak to 420 nm, (2) protection by DNA against spontaneous loss of activity in aqueous solution, and (3) inhibition by DNA of the spontaneous generation of 490 nm fluorescence due to degradation of NCS-Chrom to a highly fluorescent product (Povirk and Goldberg, 1980; Dasgupta and Goldberg, 1986). Chromophore has a preference for DNA high in T and A residues (Povirk and Goldberg, 1980), especially in alternating sequence, in agreement with earlier studies showing that DNAs rich in these nucleotides are better targets for attack by NCS (Poon *et al.*, 1977). Spectroscopic titrations suggest at least two types of chromophore binding sites on DNA: the tight binding one is saturated at $r_b = 0.125$ chromophore molecules/nucleotide (4 base pairs), and the other at $r_b = 0.25$ (2 base pairs) (Povirk *et al.*, 1981; Dasgupta and Goldberg, 1985. Only the latter site is found in synthetic polynucleotides such as poly(dA-dT)·poly(dA-dT) (Dasgupta and Goldberg, 1985, 1986). Since there is no physical evidence for interaction of native NCS or its apoprotein with DNA, it appears that dissociation of the chromophore from the protein and association with DNA are steps in the degradation of DNA by NCS (Kappen and Goldberg, 1980; Povirk and Goldberg, 1980; Jung and Kohnlein, 1981). Thiol-treated (and inactivated) NCS-Chrom has a much lower affinity for the apoprotein than does untreated chromophore, but both forms of NCS-Chrom bind equally well to DNA (Povirk and Goldberg, 1980).

NCS-Chrom shares many of the characteristics of classical intercalators in its interaction with DNA. Thus, viscosity studies indicate that the DNA helix unwinding induced by the chromophore is 21°, and electric dichroism measurements show that each bound chromophore molecule lengthens DNA by 3.3Å (Povirk *et al.*, 1981). Further, absorbance transitions of the chromophore at 315-385 nm (range over which the naphthoate moiety absorbs (Napier and Goldberg, 1983)) are oriented approximately parallel to the DNA bases (Fig. 2), as expected for an intercalated aromatic ring such as the naphthoate subunit of NCS-Chrom. Also, supercoiled DNA is nearly twice as effective as relaxed DNA in protecting the chromophore from degradation. Equilibrium and stopped-flow kinetic studies on the reversible interaction between NCS-Chrom and nucleic acids show that NCS-chrom binds in the minor groove of B-DNA,

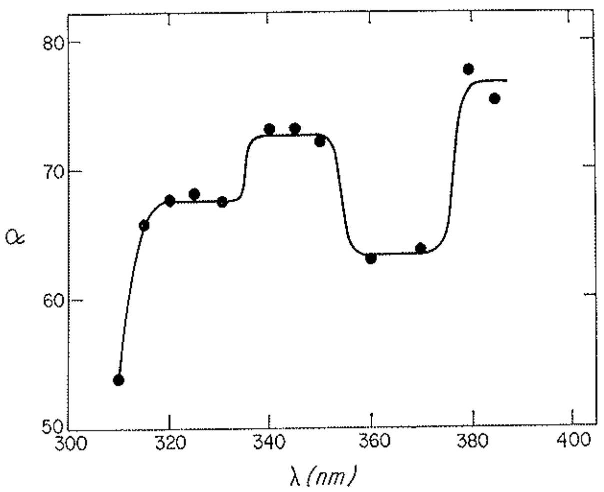


FIG. 2. Electric dichroism spectrum of DNA-NCS-Chrom complex plotted as apparent angle α between chromophore transition moments and DNA helix axis.

as revealed by the lack of effect of bulky moieties in the major groove of DNA and the inhibition of binding by minor groove specific agents, such as the antibiotics netropsin and distamycin (Dasgupta and Goldberg, 1958). Spectroscopic, thermodynamic and ^{31}P-NMR studies (Dasgupta and Goldberg, 1986) show that binding of NCS-chrom to polynucleotides differs qualitatively as well quantitatively depending on base sequence (Table I), leading to the conclusion that microheterogeneity in B-DNA structure due to sequence determines the nature of NCS-Chrom binding. In particular, binding of NCS-Chrom to poly(dA-dT) appears to be enthalpy-driven, whereas that to poly(dG-dC) is entropy-driven. Also, differences in binding parameters and in enthalpy of binding of NCS-Chrom to poly(dG-dC) and poly (dI-dC) suggest that the 2-NH_2 group of guanine in the minor groove of DNA interferes with chromophore binding. Furthermore, we have recently found that NCS-Chrom induces Z→B transition in poly(dG-5-methyl dC) (D. Dasgupta and I.H. Goldberg, unpublished data).

The spectra of NCS-Chrom have been recorded during its reversible association with poly(dA-dT) (at intervals of 17 ms) by a cryospectroscopic method (Fig. 3) (Dasgupta *et al.*, 1985). Binding of NCS-Chrom to poly(dA-dT) is a two-step process in which the spectral properties of

TABLE I - *Dissociation Constant for the Chromophore-Polynucleotide Interaction.*[a]

Polynucleotide	Methanol (% v/v)	K_{as} (= r_b/K_d)[b] ($10^5 M^{-1}$)	K_d (μM)
poly(dA-dT)	5	3.02	0.83
	20	2.5	0.98
polydA · polydT	5	0.26	5.6
poly(dG-dC)	5	0.35	3.6
	20	0.21	6.0
polydG · polydC	5	0.23	4.5
poly(dI-dC)	5	1.15	1.1
	20	0.53	2.4
polyA · polyU	5	0.038	

[a] All measurements were done at 14°C in 20 mM sodium acetate buffer, pH 5.0, containing the specified percentage of methanol.

[b] K_{as} denotes the apparent binding constant and includes the binding stoichiometry.

the intermediate poly(dA-dT)·NCS-Chrom species closely resemble those of the final equilibrium species. On the basis of cryokinetic studies (at single wavelengths) carried out at low temperature (2°C) the mechanism shown below was quantitated:

$$\text{poly(dA-dT)} + \text{chromophore} \underset{21\ s^{-1}}{\overset{7.8 \times 10^5\ M^{-1}s^{-1}}{\rightleftharpoons}} (PD)_I \underset{0.75\ s^{-1}}{\overset{9.2\ s^{-1}}{\rightleftharpoons}} (PD)_{II}$$

Since the spectra of $(PD)_I$ and $(PD)_{II}$ are closely similar, it is possible that they represent two forms of an intercalated complex, although the classical view is that they are externally bound and intercalated complexes, respectively. Of additional interest, is the use of apoprotein, instead of detergent, to follow the kinetics of the reverse reaction. The addition of apoprotein at an equimolar concentration to the chromophore to a NCS-Chrom-poly(dA-dT) mixture results in the abstraction of the chromophore from the poly(dA-dT), so as to allow measurement of the dis-

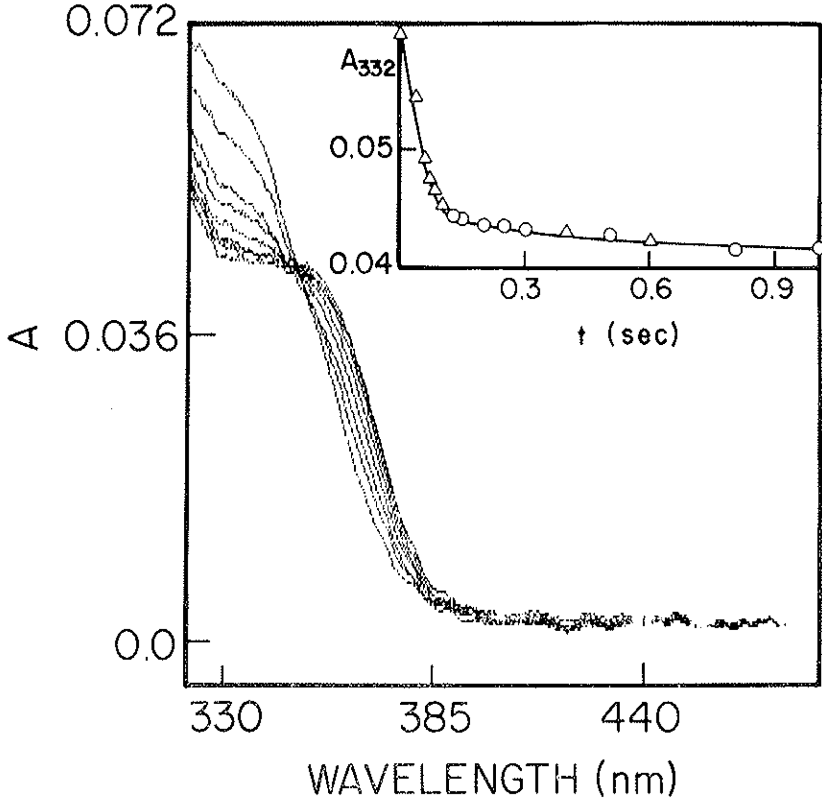


FIG. 3. Absorption spectra of the intermediates in the reversible binding of NCS-Chrom to poly(dA-dT) at 1.6°C. The spectra were recorded at 0 (uppermost), 33, 50, 67, 84, 101, 400, and 600 ms after mixing. Inset: Absorbance change at 332 nm as a function of time after mixing.

sociation constant (0.75 s^{-1}). This slow off rate for the second step might place the chromophore on the DNA for a sufficient length of time to be activated by thiol.

3) DNA DAMAGE BY NCS

Low doses of NCS selectively inhibit DNA replicon initiation in mammalian cells, while having little or no effect on chain elongation. NCS or NCS-Chrom treatment of mammalian cells induces single-strand

breaks in the DNA, that can be repaired to a considerable extent (see Goldberg *et al.*, 1981; Hatayama and Goldberg, 1979; Kuo *et al.*, 1984). Experiments using the nucleoid sedimentation technique show a quantitative correlation between NCS-induced inhibition of replicon initiation and generation of sufficient strand breakage to relax domains of supercoiling in DNA (target size of about 10^9 daltons) of mammalian cells (Povirk and Goldberg, 1982a). Double-strand breaks appear to result from the coincidence of single-strand breaks on opposite strands within a few base pairs of one another.

DNA damage can be reproduced in a system devoid of cells or cellular components, provided that a reducing agent, such as a thiol, DNA and drug are simultaneously present (see Goldberg *et al.*, 1981; Beerman and Goldberg, 1974). With NCS-Chrom the reaction is fast even at 0°C, but higher temperatures are required in the reaction with the holoantibiotic, since the apoprotein must first unfold in order to release the chromophore (Kappen *et al.*, 1980). Not only does NCS degrade naked DNA, it also solubilizes chromatin from mammalian cells and isolated nuclei by attacking primarily the linker DNA between nucleosomes (Kuo and Samy, 1978; Kappen and Goldberg, 1987).

At least four types of lesions are formed in DNA *in vitro* by NCS-Chrom in the presence of sulfhydryl cofactors. The predominant lesion is a strand break (T>A≫C>G), resulting from selective, oxygen-dependent oxidation of deoxyribose to a 5'-aldehyde (Kappen *et al.*, 1982; Kappen and Goldberg, 1983). The next most frequent lesion is release of free base (T>A≫C>G), presumably associated with some as yet undetermined form of sugar oxidation. In addition, at least two types of adducts between DNA and NCS-Chrom have been detected. In the presence of 2-mercapto-ethanol and oxygen, a labile adduct containing an oxidized deoxyribose is formed at a very low yield (Povirk and Goldberg, 1982b,c). In the presence of dithiothreitol, a much more stable adduct species is produced at a somewhat higher yield (Povirk and Goldberg, 1984, 1985a). Under anaerobic conditions, the yield of this type of adduct is further increased (while strand breakage and base release are suppressed), so that stable adducts become the predominant DNA lesion. This stable adduct species, and probably the labile adduct species as well, involves covalent linkage of the chromophore to deoxyribose in DNA.

DNA strand breakage, base release and the formation of nucleoside 5'-aldehyde by NCS require O_2 (see Goldberg *et al.*, 1981). Kinetic studies have indicated that, in the presence of DNA, O_2 and sulfhydryls, the

chromophore undergoes a single oxidation-reduction cycle, which results in efficient DNA damage and leaves an inactive product devoid of further redox activity (Povirk and Goldberg, 1983). The process can be resolved into three steps. The first step, which is accompanied by marked changes in chromophore spectral properties, but which does not require O_2, is a reaction between the chromophore and a single sulfhydryl, probably an addition reaction. Formation of this activated chromophore species is rapidly followed (within a few seconds) by the uptake of one mole of O_2 per mole of chromophore. O_2 uptake is then followed by uptake of at least an additional sulfhydryl group. Since consumption of the second sulfhydryl does not occur in the absence of O_2, it may represent a one-electron reduction of a reaction intermediate which includes some form of reduced oxygen (see below). The oxygen that ends up in the 5′-aldehyde at the strand break is derived entirely from O_2 and not H_2O, as shown by 0^{18} incorporation experiments (Chin *et al.*, 1984). Furthermore, there is no evidence that a reduced form of oxygen, such as superoxide, peroxide or hydroxyl free radical, is involved in NCS-induced DNA damage, although NCS-Chrom can undergo autoxidation with the production of superoxide free radical (Chin and Goldberg, 1986).

Nitroaromatic radiation sensitizers such as misonidazole, nitrofurazone and metronidazole substitute for O_2 in NCS-induced DNA strand breaks (Fig. 4) (Kappen and Goldberg, 1984). Compounds with higher one-electron reduction potentials are more effective in stimulating the anaerobic reaction. It is of interest, however, that while the DNA base attack site specificity for NCS remains the same as in the aerated reaction, the DNA damage products are different. Spontaneous base release increases and is associated with spontaneous (and alkali-labile) breaks with phosphates at both the 3′- and 5′-ends (i.e., gaps) and no nucleoside 5′-aldehyde.

A) In vitro *strand breakage, base release, and deoxyribose damage.*

The identification of T and A residues as targets in double-stranded DNA in the scission reaction was first revealed by experiments in which various synthetic and natural DNA's of different base composition were tested for their ability to protect against the degradation of a radioactive DNA by NCS (Poon *et al.*, 1977). Subsequent studies using the DNA sequencing technique of Maxam and Gilbert (1980), where $^{32}PO_4$ is attached to the 5′-end of the DNA, also showed that NCS cleaves double-stranded DNA restriction fragments almost exclusively at T and A residues (Hata-

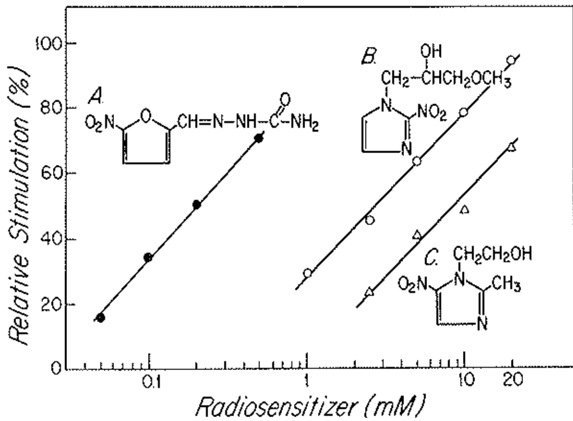


FIG. 4. Comparison of the ability of different nitroaromatic radiation sensitizers to substitute for oxygen in the stimulation of NCS-dependent DNA damage in nitrogen. (A) Nitrofurazone, E^1_7/mv = −257; (B) Misonidazole, E^1_7/mv = −389; and (C) Metronidazole, E^1_7 = −486.

yama *et al.*, 1978; D'Andrea and Haseltine, 1978). Overall, T residues are attacked much more frequently than are A residues, although there is variability in the attack rate for both at different locations in the DNA molecule, presumably because of tertiary structural constraints (see above). While all T residues are sites of scission, not all A residues are cleavage sites. These studies also confirm earlier studies that there is a phosphate moiety at the 3′-end of the break. In addition to spontaneous breaks there are alkali-labile breaks (Bose *et al.*, 1980; Kappen and Goldberg, 1983; Boye *et al.*, 1984; Povirk and Goldberg, 1985b), the exact chemistry of which is yet to be elucidated (see below).

As noted above, binding studies with synthetic polynucleotides (Dasgupta and Goldberg, 1986) suggest that base sequence-dependent minor variations in structure (Dickerson, 1983; Levene and Crothers, 1983) account for the different NCS-Chrom binding properties. Such alterations in the microstructure of DNA also appear to be responsible for the attack site specificities of nucleases and intercalating drugs (Jessell *et al.*, 1982; Cartwright and Elgin, 1982; Drew and Travers, 1984). The base sequence-dependent alteration in the binding affinity, as recorded by the polynucleotide-chromophore binding studies, is clearly reflected in the observation that not all T-residues in DNA restriction fragments are attacked with equal efficiency by NCS (Takeshita *et al.*, 1981). Further-

more, an occasional A-residue will be attacked much more frequently than adjoining T-residues. Similarly, there is considerable heterogeneity in the formation of alkali-labile apurinic/apyrimidinic (AP) sites in NCS-treated DNA. This is most clearly seen in the increased frequency of AP site formation at the C-residue in AGC (see below) (Povirk and Goldberg, 1985b). Although there is no clear-cut base sequence specificity for NCS-induced strand breakage, adenine is more frequently found on the 5′-side of the thymine attacked by NCS-Chrom (Takeshita *et al.*, 1981). This same preference was also found in binding studies with the dinucleoside monophosphates, dApT and dTpA (Dasgupta and Goldberg, 1986). Of particular interest is the finding that T and A residues that are part of a pyrimidine-purine step were more frequently attacked and that this can be correlated with positive roll angles and relatively small helical twist angles (A. Galat and I.H. Goldberg, unpublished data).

Earlier studies with DNA's labeled with different forms of radioactive thymidine indicated that DNA strand breakage is accompanied by damage to the deoxyribose moiety (Hatayama and Goldberg, 1980). These findings were confirmed by DNA sequencing experiments, using DNA restriction fragments with $^{32}PO_4$ at the 3′-end, that showed that the 5′-end of the strand-break possessed a modified nucleoside not an exposed phosphate group (Kappen and Goldberg, 1983). In these studies the radioactive band at the point of a break moved about two nucleotides more slowly on the electrophoresis gel than the marker produced by chemical treatment in the Maxam-Gilbert procedure, which has a 5′-phosphate end. The modified nucleoside could be removed by alkali so as to generate a phosphate-ended fragment. The modified nucleoside in thymidine-labeled DNA was liberated by nuclease digestion and was characterized as thymidine 5′-aldehyde (see *3* in Scheme II) (Kappen *et al.*, 1982). Since nucleoside 5′-aldehyde would be expected to release its base (and sugar) via β-elimination reactions upon alkaline treatment, the finding that over 80% of base-release is alkali-dependent indicates that most of the breaks result from oxidation at the C-5′ of deoxyribose in DNA. Similarly, DNA sequencing experiments show that strand breaks (gaps) with phosphate moieties at both ends account for less than 20% of the spontaneous breakage.

Four types of evidence suggest that similar DNA damage products and mechanisms exist in intact HeLa cells treated with NCS (Kappen and Goldberg, 1987): 1) nucleoside 5′-aldehyde (measured as borohydride-dependent incorporation of ^{32}P from γ-labeled $AT^{32}P$ by poly-

T A T
pH 7-8.5
T + A T + Chromophore
Chromophore
pH 12 or 1 N HCl
Venom Phosphodiesterase
T + Ade + T
(+Chromophore and Deoxyribose Products)
A + T

FIG. 5. Labile adduct hydrolysis patterns. Incubation of intact adduct at pH 12 or in 1 M HCl releases adenine as well as both 3'dTMP and 5'dTMP. Incubation at pH 7 or pH 8.5 releases, simultaneously, chromophore, 3'-dTMP, and a modified (*) d(ApT) that is a substrate for venom phosphodiesterase.

nucleotide kinase) accounts for at least 30 to 45% of the drug-generated 5'-ends, 2) seventy-seven percent of the ^{32}P representing nucleoside aldehyde is in TMP; the rest is in AMP≫CMP>GMP, and 3) sequencing experiments, using the highly reiterative 340 base pair alphoid DNA fragment isolated from NCS-treated cells, show a cleavage pattern similar to that produced in the *in vitro* reaction, and 4) in cells depleted of glutathione, DNA strand breakage is greatly decreased. The latter finding agrees with the earlier results of deGraff *et al.* (1985a,b) that NCS is much less toxic and mutagenic in glutathione-depleted mammalian cells.

B) *Labile NCS-Chrom-deoxyribose adducts.*

In addition to causing DNA strand breaks with nucleoside 5'-aldehyde and 5'-phosphate termini, NCS-Chrom forms novel labile covalent adducts with DNA (Povirk and Goldberg, 1982b,c). The predominant adduct recovered from nuclease digests of NCS-Chrom-treated poly(dA-dT)·poly(dA-dT) in the presence of 2-mercaptoethanol and dioxygen is a compound with the structure chromophore-d(TpApT) in which the attached chromophore rendered both phosphodiester bonds refractory to endonuclease S1. This adduct fragment was completely hydrolyzed at pH 12, releasing adenine, 3'-dTMP, and 5'-dTMP (Fig. 5). At pH 7 the adduct fragment slowly released chromophore and 3'-dTMP with parallel kinetics, leaving a modified d(ApT), which was cleaved by snake venom phosphodiesterase

to yield 5′-dTMP and a modified deoxyadenosine. These hydrolyses patterns are unlike those of any previously characterized base or phosphotriester DNA adduct but indicate the presence of an altered deoxyadenosine sugar with chemical properties similar to those of thymidine 5′-aldehyde. The modified deoxyadenosine was identified as deoxyadenosine 5′-aldehyde by a series of chemical derivatizations. The formation of adducts containing a modified deoxyribose suggests that deoxyribose is the site of covalent chromophore attachment. Support for such a possibility comes from experiments in which this compound was subjected to mild acid hydrolysis to release free adenine and to snake venom exonuclease (pH 6.5) treatment to release 5′-TMP so as to leave, in both cases, adducts of slightly altered chromatographic mobility. These results eliminate adenine and 5′-dTMP as possible sites of covalent chromophore attachment. In addition, electrophoresis data indicate that the adduct is not a phosphotriester (Povirk and Goldberg, 1982b). These data suggest that NCS-Chrom is covalently attached to the C-5′ of deoxyadenosine in some labile structure that breaks down at neutral pH to deoxyadenosine 5′-aldehyde and free chromophore.

C) *Stable NCS-Chrom-deoxyribose adducts.*

A similar but much more stable adduct species has also been isolated, again with structure chromophore-d(TpApT) (Povirk and Goldberg, 1984; S. Lee and I.H. Goldberg, unpublished data). Acid hydrolysis of this adduct released free adenine, as well as both 3′-dTMP and 5′-dTMP, leaving a compound which contained only chromophore and the deoxyadenosine sugar moiety (Fig. 6). These results clearly indicate the formation of a covalent linkage between chromophore and deoxyribose in DNA. Formation of this adduct shows an oxygen dependence which is the inverse of that seen for nucleoside 5′-aldehyde formation, i.e., the yield of adduct is maximal under anaerobic conditions. This apparent competition between DNA sugar oxidation and adduct formation suggests that the two lesions share a common precursor. A chromophore-induced carbon-centered radical on C-5′ of deoxyribose, that could react by addition with the same chromophore molecule to form the stable covalent adduct or with O_2 to form the 5′-aldehyde would be the most logical candidate for such a precursor (see below). Recently, a minor stable adduct with the structure chromophore-d(ApTpA) has been isolated (S. Lee and I.H. Goldberg, unpublished data).

The resistance of both types of chromophore-oligonucleotide adducts to further nuclease digestion suggested that the adducts block exonucleolytic digestion of the DNA and that the sequence specificity of adduct formation might then be determined by mapping the adducts as exonuclease termination sites in defined-sequence DNA. A 5′-end-labeled DNA restriction fragment was treated with NCS-Chrom under anoxia in the presence of dithiothreitol, conditions known to maximize formation of chromophore-deoxyribose adducts. Under conditions where unmodified DNA was digested to completion, chromophore-treated DNA was highly resistant to digestion by exonuclease III plus the 3′→5′ exonucleolytic activity of T4 DNA polymerase, and partially resistant to digestion by exonuclease III plus snake venom exonuclease (Povirk and Goldberg, 1985a). The electrophoretic mobilities of the products of exonucleolytic digestion suggested that (i) digestion by exonuclease III or T4 polymerase terminated one nucleotide before the nucleotide containing the adduct, (ii) the remaining nucleotide directly adjacent to the adduct (3′ side) could be removed by snake venom phosphodiesterase, but at a slow rate, (iii) the covalently linked chromophore decreased the electrophoretic mobilities of the digestion products by the equivalent of approximately 3 nucleotides, and (iv) adducts formed under anaerobic conditions occurred at the same nucleotide positions as the strand breaks formed under aerobic conditions (primarily at T, and to a lesser extent, A residues). The site of adduct formation was further identified by isolating the adduct containing fragment on a sequencing gel and then subjecting it to the chemical cleavage reactions

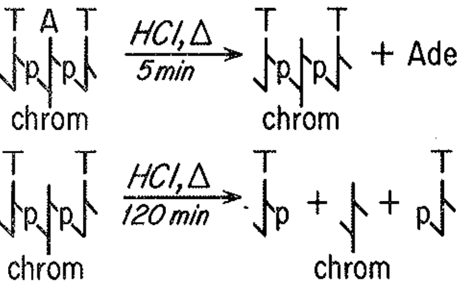


Fig. 6. Acid hydrolysis of stable adduct. Treatment of chromophore-d(TpApT) with hot acid results in release of adenine free base, followed by hydrolysis of both phosphoester bonds of the depurinated sugar to release 3′dTMP and 5′dTMP. The compound that remains contains only chromophore and the deoxyadenosine sugar moiety, implying a covalent linkage of chromophore to deoxyribose.

described by Maxam and Gilbert (1980). The close similarity in sequence specificity of adducts and strand breaks suggests that a common form of nascent DNA damage may be a precursor to both lesions. Again, a chromophore-induced free radical on C-5′ of deoxyribose, subject to competitive fixation by addition reactions with either oxygen or chromophore, is the most likely candidate for such a precursor.

Adducts on the sugar in DNA have not been described before. Most adducts of nucleic acids are on the base or phosphate (Singer and Kusmierek, 1982). In the case of RNA only, an adduct of dimethylbenzanthracene is found on the 2′-hydroxyl of the ribose (Frenkel *et al.*, 1981). It is clear that in the case of NCS a chemical reaction on C-5′ in DNA is necessary before a suitable site is generated for adduct formation. Any agent capable of inducing carbon-centered deoxyribose radicals could, in principle, also induce deoxyribose adducts as a result of addition reactions between these radicals and other molecules (or intramolecularly with its base, as with ionizing radiation — see below). Bulky adducts on DNA sugars obviously could not be removed by repair glycosylases, and it seems unlikely, though not impossible, that the DNA would be recognized by polymerases as a normal substrate. Furthermore, the phosphodiester bonds of adduct-containing DNA sugars appear to be relatively resistant to a variety of nucleases.

4) Molecular Mechanism of NCS-Induced DNA Damage

As noted above, ^{18}O incorporation studies showed that dioxygen, not water, is the source of the oxygen in the 5′-aldehyde generated at the 5′-end of an NCS-induced DNA break. These results are compatible with a mechanism in which dioxygen adds to a carbon-centered radical at C-5′ (the addition mechanism in Scheme I) to form a peroxyl radical adduct (see below for modified version). The carbon-centered radical on the deoxyribose forms when the thiol-activated NCS-Chrom abstracts a hydrogen atom from the C-5′ position. Using DNA in which the hydrogens of the deoxyribose were replaced by tritium in the 1′, 2′ or 5′ positions, it was found that the activated NCS-Chrom selectively abstracted tritium from the 5′-position and incorporated it into a stable, non-exchangeable form of the chromophore (Fig. 7) (Charnas and Goldberg, 1984; Kappen and Goldberg, 1985). The abstracted tritium remained covalently attached to the chromophore despite various chemical treatments. Drug activation and the subsequent hydrogen abstraction reaction do not require molecular

oxygen. DNA damage is dependent on thiol, as is also 5′[^{3}H] abstraction into the chromophore. Fig. 8 shows that in the aerobic reaction there is a good correlation between the amount of 5′[^{3}H] abstracted into the chromophore and the extent of DNA damage over a wide range of thiol concentrations; at low levels of thiol there is a nearly linear increase both in 5′[^{3}H] abstraction and DNA damage. At the highest level of thiol used, there is a small but significant reduction in both DNA damage and

SCHEME I. Possible roles of O_2 in NCS-induced DNA scission.

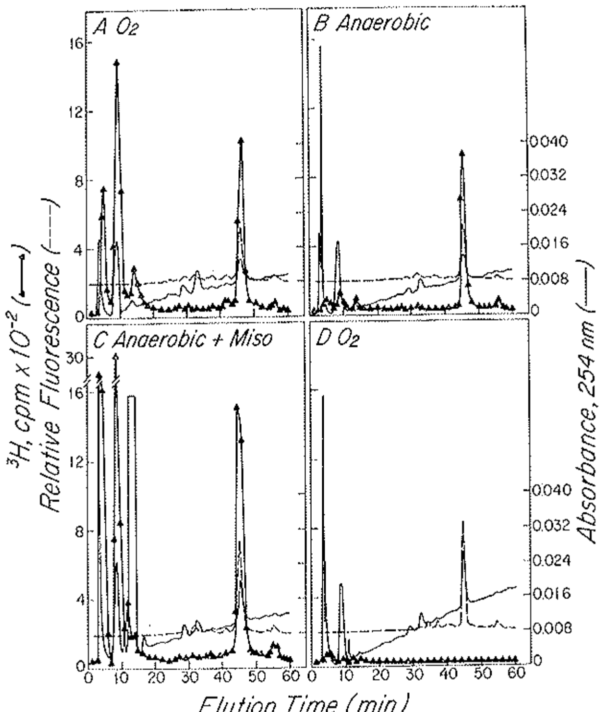


FIG. 7. Abstraction of 5′[^{3}H] from DNA into NCS-Chrom. An equimolar mixture of 5′[^{3}H]- and [methyl-^{3}H]thymidine-labeled λ DNA was reacted with NCS-Chrom. After removal of DNA, the chromophore was analyzed by HPLC on a μBondapak C_{18} column. A, aerobic; B, anaerobic; C, anaerobic containing 40 mM misonidazole; D, NCS-Chrom inactivated by preincubation with thiol replaced the active drug in an otherwise complete aerobic reaction. The absorbance peak at 8 min is due to the nonradioactive thymine marker, and that at 14 min in C represents misonidazole. The peak of radioactivity associated with the main UV-absorbing and fluorescent peak of the chromophore has a retention time of 45 min.

^{3}H-abstraction, probably due to a faster rate of inactivation of the drug prior to its interaction with DNA (Kappen and Goldberg, 1978).

Studies on the kinetics and stoichiometry of uptake of thiol and O_2 showed that one mole of O_2 and at least two moles of thiol per chromophore were taken up under conditions of efficient DNA degradation (Povirk and Goldberg, 1983). Under anaerobic conditions only one mole of thiol was consumed, but the activation of the drug as judged by fluorescence changes was as complete as in O_2 despite the nearly complete

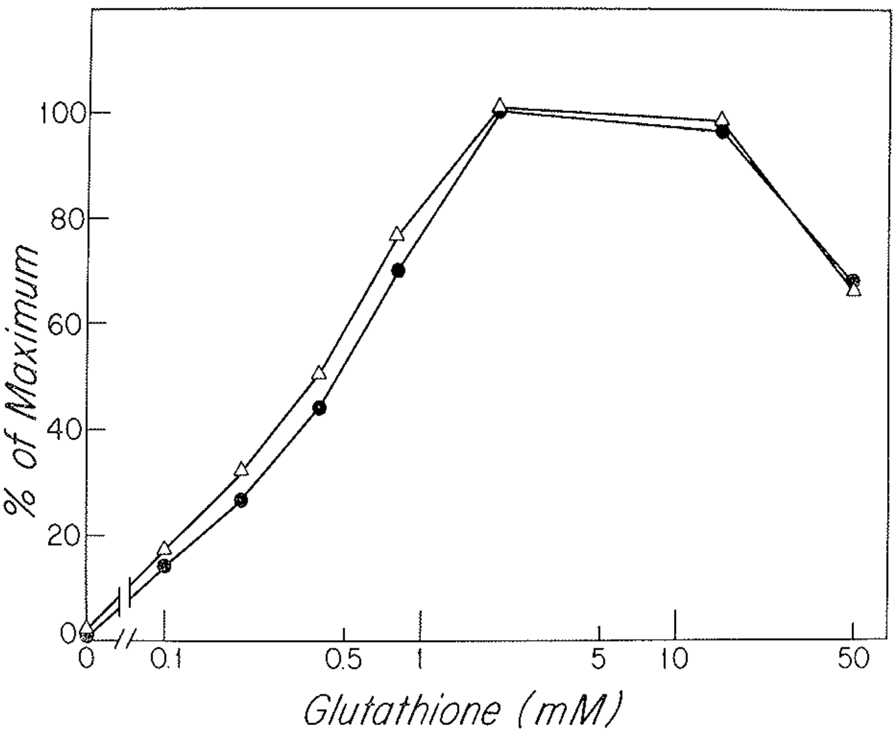


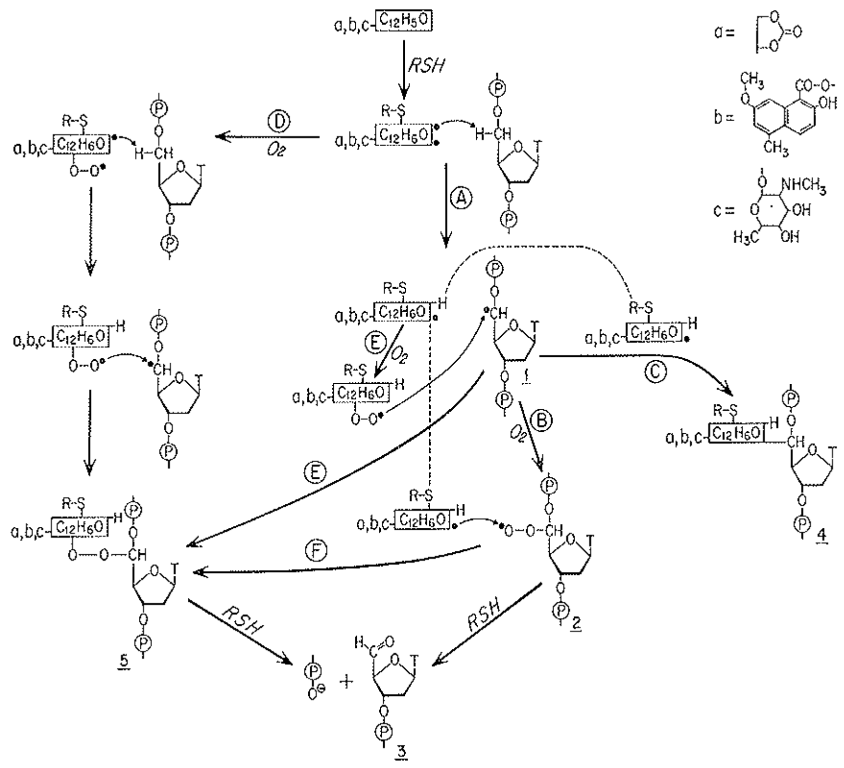
FIG. 8. Dependence of 5′[^{3}H] abstraction from DNA into NCS-Chrom and DNA damage on thiol. In an aerobic reaction containing DNA and NCS-Chrom, glutathione levels were varied. One portion of the reaction mixture (after removal of DNA) was analyzed by HPLC to determine the chromophore-bound ^{3}H. A second aliquot was used to estimate the total thymine released (spontaneous and alkali-labile). One-hundred percent activity found at the optimal glutathione level (2 mM) is 186 pmoles 5′[^{3}H] abstracted into chromophore and 628 pmoles total released thymine. ●-●, 5′[^{3}H] abstracted; Δ-Δ, total thymine released.

inhibition of DNA degradation as measured by total thymine release. Whether the thiol-activated NCS can directly induce nascent damage on the DNA, or the thiol-activated intermediate has to further interact with O_2 to form a reactive species prior to the attack on the DNA, however, was not clear from these studies. The finding that thiol-activated NCS chromophore, under strict anaerobic conditions, abstracts from the deoxyribose of DNA as much 5′[^{3}H] as in the presence of O_2 (Kappen and Goldberg, 1985) clearly shows that O_2 is not a necessary participant in its primary attack on the DNA. It is very unlikely that the tritium abstraction

found under anaerobic conditions is due to trace contaminant O_2. We have calculated that at least 10 times as much DNA damage products are formed under aerobic conditions as there is O_2 potentially available under the anaerobic (10^{-3} torr) conditions used. Since tritium abstraction is the same under both conditions, the aerobic damage should be a quantitative reflection of the hydrogen abstraction reaction. Since about 3-fold more nucleoside aldehyde is produced than tritium is covalently incorporated into NCS-Chrom, this value provides an approximate measure of the observed isotope effect in the hydrogen abstraction reaction.

Although chromophore, inactivated by preincubation with thiol, failed to abstract any 5′[^{3}H] from DNA or to produce DNA damage, it produced the same amount of UV-absorbing and fluorescing material eluting at 45 min (Fig. 7) as in a parallel reaction containing active drug. This is not surprising since activation of the drug precedes its inactivation. Whatever be the mechanism of inactivation, it is possible that the species that has abstracted the hydrogen atom from DNA and the species representing the inactivated molecules are either structurally the same (e.g., if it accepts a hydrogen from thiol instead of from DNA) or too close to give a different elution and absorbance profile.

Based on the accumulated data, we have formulated a reaction scheme in which a carbon-centered radical at C-5′ is an intermediate in the formation of various DNA damage products formed under either aerobic or anaerobic conditions (Scheme II) (Kappen and Goldberg, 1985). The first step involves the generation of an active species of the drug (shown as a free radical) upon thiol addition to the highly unsaturated C_{12} subunit of the chromophore. The activated drug abstracts a hydrogen atom from the 5′-carbon of deoxyribose (pathway A) to generate a carbon-centered radical (*1*) on the DNA, the fate of which depends on whether or not O_2 is available. Addition of O_2 to the 5′-carbon-centered radical (pathway B) gives rise to a peroxyl radical (*2*) (see von Sonntag *et al.*, 1981 and Isildar *et al.*, 1982 for discussion of peroxyl radical degradative reactions) which degrades in the presence of thiol (via formation of a hemiacetal) to produce DNA strand breaks with 5′-terminal nucleoside aldehyde (*3*). The involvement of thiol in the degradation of a peroxyl structure to an aldehyde is compatible with our experiments in which at least a second molecule of thiol is consumed in the reaction beyond the one used in the initial activation of the drug. In the absence of O_2 the carbon-centered radical on the deoxyribose (*1*) may instead react with the chromophore containing the abstracted hydrogen (pathway C) to form a stable covalent chromophore-



SCHEME II. Proposed mechanism of DNA damage by NCS.

DNA adduct (*4*), possibly by addition of the C-5′ radical across an unsaturated bond in the chromophore, or be repaired by abstraction of a hydrogen from thiol. This analysis, involving a common DNA intermediate, is consistent with studies (Povirk and Goldberg, 1984) showing that there is a competition between stable chromophore-DNA adduct formation under anoxia and DNA strand breakage and base release in O_2.

Although drug activation and hydrogen atom abstraction from the 5′-position of deoxyribose in DNA can proceed in the absence of O_2, they do not rule out the possibility that in the presence of O_2 the activated chromophore can add O_2 to form a peroxyl derivative of the activated chromophore (pathway D). The peroxyl radical on the drug cannot itself be responsible for hydrogen atom abstraction, since the latter is incorporated into the

chromophore in a non-exchangeable form. Nevertheless, a radical elsewhere in the peroxyl form of the drug could abstract the hydrogen atom and then react (possibly as part of a concerted reaction) with the carbon-centered radical on the DNA by way of its peroxyl radical to form a labile adduct, possibly with the structure shown in (*5*), as an intermediate in strand break formation. Again, this peroxyl adduct may be cleaved by thiol to form *3*. Consistent with such a second role for thiol in the formation of the 5′-aldehyde are experiments showing that 2-mercaptoethanol accelerates the decomposition of the labile adduct (L.F. Povirk and I.H. Goldberg, unpublished results) and that high thiol concentrations increase both the absolute yield of 5′-aldehyde and the ratio of aldehyde to released free base (L.S. Kappen and I.H. Goldberg, unpublished results).

It is also possible that the activated chromophore adds O_2 after it abstracts the hydrogen atom from DNA (pathway E) and then reacts with *1* to form the labile adduct, *5*; or it may instead react with the peroxyl form of the deoxyribose (*2*) to form (*5*) via pathway F. Since the chromophore is anchored on the DNA by intercalation and electrostatic interactions, it would not be surprising if it participated in such reactions after the initial hydrogen abstraction. Reaction pathway E, involving a peroxyl form of the drug and a labile chromophore-DNA adduct intermediate, best fits data showing that one molecule of O_2 is consumed for each molecule of chromophore, whereas DNA damage is somewhat less than stoichiometric. Furthermore, pathway E is more attractive than D, since it does not require postulating a different hydrogen-abstracting form of the chromophore under aerobic and anaerobic conditions. Finally, it should be noted that it is possible to combine pathways A and E in a concerted mechanism involving a ternary complex of activated chromophore, O_2 and C-5′ of deoxyribose.

The proposed scheme focuses on the 5′-carbon of deoxyribose as the predominant site of attack. It is consistent with the findings that over 80% of the DNA strand breaks (major lesion) have nucleoside 5′-aldehyde at their 5′-termini and that C-5′ oxidation also occurs in the formation of the labile chromophore-DNA adducts. The absence of any ^3H abstraction from the C-1′ and C-2′ of thymidine in DNA eliminates these positions as targets. It should be noted, however, that C-1′ appears to be the primary site of hydrogen abstraction when radiolytically-activated NCS reacts with DNA (Favaudon *et al.*, 1985). DNA damage under these circumstances is associated with base release and the formation of alkaly-labile sites. Furthermore, since the mechanism of spontaneous base

release and the chemistry of the alkali-labile lesions in the thiol-dependent reaction are unknown, it is not possible at this stage to invoke attack at C-5′ in the generation of these lesions.

When misonidazole substitutes for O_2 in the NCS-DNA reaction, the attack site specificity is the same as in O_2, but in the presence of misonidazole base release and gap formation (with 3′- and 5′-phosphoryl termini) predominate (Kappen and Goldberg, 1984). Despite the difference in final DNA products, abstraction of 5′-hydrogen by thiol-activated drug to form a carbon-centered radical on C-5′ of deoxyribose appears to be a common initial step in both the O_2- and misonidazole-dependent reactions; in fact, there appears to be a competition between the misonidazole-dependent and the oxygen-dependent pathways. It is likely that after the induction of the same nascent lesion on the DNA participation of misonidazole in the subsequent steps, by a mechanism different from that of O_2, leads to different products. Since the abilities of the nitroaromatic sensitizers to substitute for O_2 depend on their one-electron reduction potentials, it is possible that they oxidize the carbon-centered radical at C-5′ to a radical cation. It is not clear, however, how this reactive species degrades to produce DNA strand gaps with sugar release. Using ^{14}C-labeled misonidazole, we have obtained preliminary evidence that this compound undergoes reduction in the reaction. Further, we have found that under anaerobic conditions with misonidazole NCS causes the release of substantial amounts of a 5′[^{3}H]-labeled sugar fragment (from 5′[^{3}H]thymidine-labeled DNA) that elutes from HPLC at 4 min (L.S. Kappen, D.-H. Chin and I.H. Goldberg, unpublished data). This substance is uncharged but when treated with either acid or alkali is converted into formic acid (identified by its volatility and mobilities on thin-layer chromatography and paper electrophoresis).

Based on spectroscopic, electric dichroic and hydrodynamic data we have proposed a model for NCS binding to DNA in which the naphthoic acid part of the chromophore intercalates via the minor groove of DNA between the DNA base pairs, and the positively charged amino sugar binds electrostatistically to the minor groove of DNA with its deep negative potential. These two attachment sites are viewed as serving as anchors for the proper placement in the minor groove of the highly strained bicyclo(7,3,0)dodecadiyne system (with its epoxide moiety) close to C-5′ of deoxyribose. Upon reaction with only one mole of thiol (Povirk and Goldberg, 1983), the highly strained unsaturated part of the molecule undergoes a marked rearrangement, perhaps forming a radical species capable

of abstracting a hydrogen from C-5′ of deoxyribose. ESR studies show that free radical signals are generated during the thiol reaction with NCS (Edo *et al.*, 1980; Sheridan and Gupta, 1981) but their relationship to "activated" drug is not yet clear. Further, it is not known whether the thiol reacts with one of the acetylenic bonds or with the epoxide to activate the molecule in the DNA damage reaction. On the other hand, reaction of thiol with NCS-Chrom in methanol (in the absence of DNA) leads to the consumption of three thiols — one forming an adduct and the other two presumably reducing an acetylenic bond (Hensens *et al.*, 1983. This reduction of an acetylenic bond by thiol (or sodium borohydride) is unexpected and is probably a reflection of the highly strained nature of the ring systems. To clarify the specific sites on the NCS chromophore involved in thiol activation and hydrogen abstraction, additional information on the chemical reactivity of the chromophore is essential.

In order to understand the chemical properties of the NCS chromophore molecule further, derivatives with altered reactivities have been prepared (S. Lee and I.H. Goldberg, unpublished data). By reaction of NCS-Chrom with HCl the chlorohydrin derivative (by reaction at the epoxide moiety) has been prepared. Similarly, treatment of chromophore with perchloric acid converts the epoxide to the diol derivative. Both diol and chlorohydrin bind to DNA, as determined by spectroscopy, but only the chlorohydrin has activity in cleaving DNA (about half that of the epoxide). Furthermore, the diol is completely stable in aqueous solution, whereas the chlorohydrin's stability is intermediate between that of the epoxide and the diol. These studies emphasize the importance of the epoxide moiety in NCS chromophore action. It is not surprising that the chlorohydrin is active (perhaps not as active as the epoxide because it is less strained), since its chemical reactivity — due to the ability of the chlorine atom to act as a leaving group to form a radical — is analogous to that of the epoxide.

5) Basis of NCS-Induced Mutagenicity

NCS is an SOS-dependent mutagen that induces base substitutions and, to a lesser extent, frameshifts (Eisenstadt *et al.*, 1980). NCS and a number of other "oxidative mutagens" have been found to be mutagenic for a *Salmonella* tester strain, TA 102, that contains A-T base pairs at the site of mutation in a multicopy plasmid (Levin *et al.*, 1982), consistent with the *in vitro* base specificity studies. On the other hand, studies of

NCS-induced mutations in the *lacI* gene revealed that all types of monitorable base substitutions were induced, with some preference for G:C to A:T transitions (A:T to G:C transitions, however, are not monitorable in this system) (Foster and Eisenstadt, 1983). The spectrum was dominated by a G:C to A:T transition hotspot at the ochre 21 locus. Several less prominent hotspots were also present, while no mutations at all were detected at nearly half the monitorable sites. Since the predominant lesions induced in DNA by NCS or NCS-Chrom are strand breaks or covalent NCS-Chrom-deoxyribose adducts occurring mainly at T and A residues, it appears that many of the mutations, particularly those occurring at G:C base pairs, result from other, minor lesions, which are formed with a different base specificity.

Since free bases from DNA are released by NCS, it seemed likely that apurinic/apyrimidinic (AP) sites with an intact sugar-phosphate backbone are formed. In fact, AP endonuclease-sensitive sites have been detected in NCS-treated supercoiled DNA and DNA restriction fragments (Bose *et al.*, 1980; Kappen and Goldberg, 1983). When defined-sequence DNA from the *lacI* region of plasmid pMCl was treated with NCS-Chrom in the presence of glutathione, alkali-dependent strand breaks, occurring most prominently at certain cytosine residues, were detected in addition to the direct strand breaks at T and A residues (Povirk and Goldberg, 1985b) (Fig. 9). Although these sites were substrates for endonuclease IV, a five-fold greater concentration of enzyme was required for their cleavage than was required for cleavage of apurinic sites in depurinated DNA. These sites were also at least five-fold less sensitive to *E. coli* endonuclease VI (exonuclease III) and at least ten-fold less sensitive to *E. coli* endonuclease III. The correlation between alkali-dependent cleavage at C residues and release of free cytosine base, as well as the susceptibility of the lesions to endonuclease IV of *E. coli*, strongly suggest that they are AP sites. It seems reasonable to propose that the NCS-induced sites are resistant because the sugar residue has a chemical structure different from that of apurinic sites generated by acid treatment. In fact, when NCS-induced AP sites at C residues in 5′-end-labeled DNA are cleaved by heating at pH 8, the resulting fragments comigrate on sequencing gels with the 3′ phosphate-ended marker; however, fragments corresponding to heat-induced cleavage of acid-induced AP (apurinic) sites migrate one nucleotide more slowly, suggesting that the sugar remains attached to the 3′-end. Thus, the 5′ sugar-phosphate linkage is apparently more labile in the NCS-induced site than in the authentic acid-induced AP site.

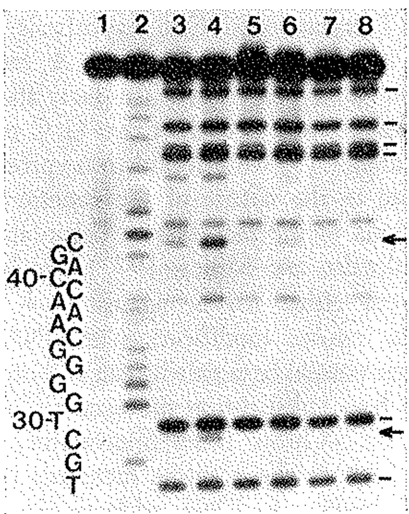


FIG. 9. Formation of apyrimidinic sites at C residues. 5′-end-labeled *lacI* DNA fragment was either depurinated (lanes 1 and 2), or treated with NCS-Chrom in presence of glutathione (lanes 3 and 4), dithiothreitol (lanes 5 and 6) or 2-mercaptoethanol (lanes 7 and 8). Samples in even lanes were heated in alkali. Arrows show C residues at which alkali-dependent cleavage occurs.

At this time we do not know if the NCS-produced abasic lesion is due to drug attack at C-5′ of deoxyribose, as is true for strand breaks, or is due to attack elsewhere on the deoxyribose, such as C-1′ as appears to occur with radiolytically activated drug (Favaudon *et al.*, 1985).

When chromophore-induced apyrimidinic sites were quantitated as alkali-dependent breaks at eleven specific sites in the *lacI* gene, a correlation (coefficient of 0.94) was found between occurrence of these lesions (Foster and Eisenstadt, 1983; Povirk and Goldberg, 1985b) (Fig. 10) and the reported frequencies of G:C to A:T transitions at the same sites. All occurrences of the trinucleotide sequence AGC, including the ochre 21 mutational hotspot, were particularly prominent sites. The selective formation of endonuclease-resistant apyrimidinic sites at specific C residues may explain the high frequency of G:C to A:T transitions in the mutational spectrum of NCS, especially since it has been shown that there is a selective incorporation of A residues opposite AP sites (Loeb, 1985).

The observed occurrence of adducts almost exclusively at T and A residues (Povirk and Goldberg, 1985a), does not suggest a role for adducts

in generating GC:AT transitions. One hypothesis which could not be excluded, however, is that adducts on T or A residues may result in mutations at adjacent G:C base pairs. In fact, the T residues directly preceding the G residues associated with two G:C to A:T transition hot-spots (ochre 21 and amber 19) were found to be prominent sites of adduct formation. Since, due to the nature of the genetic code, all monitorable G:C to A:T transitions in the *lacI* system occur at G residues preceded by a T residue (Coulondre and Miller, 1977), the NCS spectrum in *lacI* provides little evidence either for or against such a mechanism. Since chromophore adducts involve modification of DNA sugars, rather than bases, it is possible that normal base-pairing can still occur during replication, and that little or no base substitution mutagenesis occurs at adduct sites. On the other hand, NCS-Chrom is an intercalator, and other covalently bound intercalators, such as the acridine mustards (Creech *et al.*, 1972), are potent frameshift mutagens. Thus, adducts may be involved in NCS-induced frameshift mutagenesis, rather than base substitution mutagenesis. The sequence specificity of NCS-induced frameshifts is unknown.

As an alternative approach for studying the biological consequences of various NCS-induced lesions, we have also examined the ability of NCS-

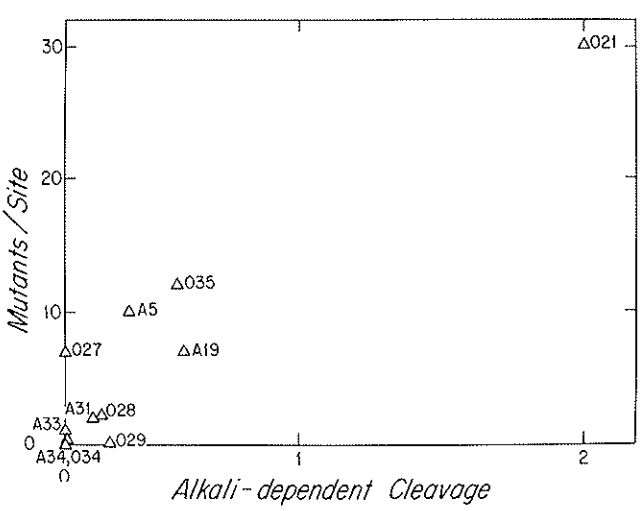


Fig. 10. Occurrence of G:C to A:T transition *in vivo* vs. alkali-dependent cleavage *in vitro* at sites in the lacI gene. Values represent relative probability of alkali-dependent cleavage at each site. Data on mutation frequency are those of Foster and Eisenstadt (1983). A = amber sites, O = ochre sites.

Chrom to induce forward mutations in the *c*I gene of lambda phage (Povirk and Goldberg, 1986). Results show that chromophore treatment is about two-fold less lethal and five-fold less mutagenic under anaerobic conditions, suggesting that adducts are less lethal and less mutagenic than oxygen-dependent lesions (making it less likely that stable adducts are mutagenic), and are probably repaired in *E. coli.* Treatment of intact lambda phage with NCS-Chrom resulted in the generation of clear-plaque mutants. This effect required a preincubation at low pH to allow diffusion of chromophore into the phage head. Chromophore activation was then effected by addition of a sulfhydryl cofactor, followed by a shift to neutral pH. Sequence analysis of mutations mapped to the DNA-binding region of the *c*I gene revealed that nearly all were single base substitutions. Significant numbers of all possible base changes were found, with A:T to G:C transitions being the most frequents events (Table II). Of 11 G:C to A:T transitions, 7 were found at C residues in the trinucleotide sequence

TABLE II - *NCS-Chrom-Induced Mutations in the* cI *Gene.*

Single-base Substitutions:

Change	Occurrences	Positions in Gene**
A:T to G:C	16	2, 2, 7, 89, 101, 134, 152, 157, 173, 173, 175, 175, 185, 185, 242
A:T to C:G	6	2, 60, 95, 110, 185, 185
A:T to T:A	10	58, 136, 153, 163, 166, 166, 167, 168, 173, 182
G:C to A:T	10	5, 34*, 34*, 37*, 46, 92*, 92*, 148*, 149, 214
G:C to T:A	8	78, 92*, 92*, 149, 161, 218, 235, 235
G:C to C:G	4	3, 61, 92*, 214

Other:

-1 frameshift at base 129-132

A:T to G:C at base 25 plus G:C to A:T at base 36*

A:T to C:G at base 11 and at base 76

A:T to G:C at base 136 plus -1 frameshift at base 139-140

* C residue in an AGC sequence.

** For the complete *c*I sequence see Sauer (1978).

AGC, which we had previously shown to be a hotspot for chromophore-induced depyrimidination in the *lacI* system (Povirk and Goldberg, 1985b); of 58 mutants sequenced only two frame-shifts were detected. Overall, in the *c*I gene AGC sites were eight-fold more mutable than other G:C base pairs. This result, as well as the SOS dependence of mutagenesis and the overall distribution of various types of base substitutions, is consistent with the hypothesis that AP sites are important mutagenic lesions and rules out the possibility that the mutations are due to lesions at adjacent T residues.

6) Contrasting Mechanisms of Action of NCS, BLM and Ionizing Radiation

The bleomycins (Fig. 11), a group of glycopeptide antitumor antibiotics, are believed to act by degrading cellular DNA (for reviews see Hecht, 1979; Povirk, 1983). Their ability to degrade DNA *in vitro* has been extensively studied and shown to require transition metals, such as Fe(II), and O_2, to produce single and double strand breaks and alkali-labile breaks, and to release free nucleic acid bases and base propenals. Base propenals have been found to be as cytotoxic as BLM, raising the possibility they are responsible for the cytotoxic properties of BLM (Grollman *et al.*, 1985).

BLM forms one-to-one chelates with a number of transition metals but it is its iron complex that is believed to be involved in the oxidative cleavage of DNA (Sausville *et al.*, 1976), although recently question has been raised as to whether metal-binding is essential for the biological effects (Lyman *et al.*, 1986). Evidence in support of the role of BLM-iron complexes in cells has recently been published (Takahashi *et al.*, 1986). BLM binds Fe(II) or Fe(III) to produce a complex that is activated by O_2 or peroxides, respectively, to a form of the drug termed "activated BLM" (Burger *et al.*, 1981; Kuramochi *et al.*, 1981). The X-ray crystallographic structure of either complex has not yet been reported, although the crystallographic structure of a complex of Cu(II) with a biosynthetic intermediate of BLM (P3A) has been published (Itaka *et al.*, 1978). In this structure there is a square-pyramidal chelation site with five nitrogen ligands. The secondary amine nitrogen, pyrimidine ring nitrogen, deprotonated peptide nitrogen of the histidine residue and the histidine imidazole nitrogen coordinate as the planar donor, and the α-amino nitrogen of β-aminoalanine coordinates as the axial donor. A similar

FIG. 11. Structure of BLM. 1) Double-bond which is replaced by a single-bond in phleomycin, 2) site of attachment of an additional talose sugar derivative in tallysomycin, and 3) amide which is deaminated in the inactive metabolite, deamidobleomycin. Enclosed area is the DNA-binding region, tripeptide S, when it contains the terminal amine of bleomycin A_2 (taken from Povirk, 1983).

structure, except for the carbamoyl group at the sixth coordination site, has been proposed for iron complexes of BLM (Itaka *et al.*, 1978, Dabrowiak *et al.*, 1978; Dabrowiak, 1980; Umezawa and Takita, 1980), although other possibilities based on NMR analysis have also been proposed (Oppenheimer *et al.*, 1979a,b, 1981).

BLM binds to duplex DNA; the bithiazole and terminal amine are the most important moieties in complex formation. It remains uncertain, however, whether BLM, in particular the bithiazole, intercalates between DNA bases. BLM and its tripeptide S (Fig. 11) induce changes in the hydrodynamic properties of DNA characteristic of an intercalating agent (Povirk *et al.*, 1979, 1981); however, early NMR studies were equivocal for significant intercalation (Chen *et al.*, 1980; Glickson *et al.*, 1981). More recent investigations of this question are also conflicting, some being most compatible with only partial insertion of the bithiazole ring between the bases (Henichart *et al.*, 1985) and others with classical intercalation (Fisher *et al.*, 1985). In any case, it appears that intercalation of the bithiazole may not be essential in determining the DNA binding (and thus cleavage) pattern, since the related phleomycin, which lacks one double bond in the bithiazole moiety and thus planarity, probably does not intercalate and yet has basically the same base sequence specificity as BLM, with preferential cleavage at GC and GT sequences (Takeshita *et al.*, 1981; Kross *et al.*, 1982).

"Activated BLM", formed from Fe(III)·BLM either in a direct reaction with peroxide (Kuramochi *et al.*, 1981; Burger *et al.*, 1981) or from Fe(II)·BLM and O_2 (Suguira and Kikuchi, 1978; Burger *et al.*, 1979), followed by a reduction, is an oxygen·Fe(III)·BLM complex in which the iron is low spin ferric (Burger *et al.*, 1983). Many of the features of iron·BLM action resemble those of ferric cytochrome P-450, a protein that catalyzes a similar oxygen-dependent reaction (Burger *et al.*, 1983). Evidence has been obtained for a cyclical mechanism of iron·BLM action in which a series of one-electron reductions occurs. Fe(III)·BLM is first reduced by a single electron reaction to form Fe(II)·BLM. The latter reacts rapidly with O_2 to form O_2·Fe(II)·BLM, which reacts to form a 1:1 mixture of "activated BLM" and Fe(III)·BLM by a one-electron reduction. It has been proposed (Burger *et al.*, 1985) that "activated BLM" participates in two successive one-electron reactions: the drug is first reduced to a one-electron oxidizing species by hydrogen atom abstraction to form a C-4′ radical on the deoxyribose; the drug then reacts with the sugar radical by radical recombination to yield Fe(III)·BLM and a 4′-hydroxylated

SCHEME III. Proposed mechanism of degradation of poly(dA-dU) by activated BLM to form free base, base propenal and terminal 3′-glycolate. The intermediacy of a 4′-ketone is shown for the production of free base (taken from Wu *et al.*, 1985b).

deoxyribose. The latter reacts to form an alkali-labile 4′-ketone on the deoxyribose and free base. In the presence of O_2 the carbon centered radical at C-4′ can react with the O_2 to form an unstable peroxy species, leading to DNA cleavage with a glycolate moiety at the 3′-terminus and base propenal release and a phosphate at the 5′-terminus (Giloni *et al.*, 1981; Murugesan *et al.*, 1985). Such proposals are compatible with the finding that free base production does not require O_2 once "activated BLM" has been formed, but that O_2 is essential for the formation of base propenal (Burger *et al.*, 1981, 1982).

Recently, in a series of experiments using poly(dA-dU), specifically tritiated at defined positions of the deoxyribose of the deoxyruidine, Wu *et al.* (1983, 1985a,b) have obtained convincing evidence that the formation of both free base and base propenal results from a rate-determining 4′-carbon-hydrogen bond cleavage and an O_2-dependent partitioning of the intermediate radical (Scheme III), as proposed above. From the identification of an alkali-derived product of the abasic DNA lesion Sugiyama *et al.* (1985) have proposed that the alkali-labile lesion is a 4′-hydroxyl sugar derivative. Although the precise molecular species

of oxygen in "activated BLM" is not yet known, it is clear that activated reduced oxygen is involved in hydrogen abstraction. Further, it has been found that BLM is capable of multiple cycles of oxidation-reduction and of DNA damage (Povirk, 1979; Burger *et al.*, 1979; Ekimoto *et al.*, 1979; Sugiyama *et al.*, 1986).

Hydroxyl radicals, formed by water radiolysis, are the principal agents causing base and sugar damage when DNA is irradiated in aqueous solution (Ward, 1975). While measurements of the yield of base destruction in nucleosides and nucleotides indicate that only a small proportion of the hydroxyl radicals (10-20%) react with the sugar moiety, sugar damage is more significant in duplex DNA due to shielding of the bases in the double helical structure (Ward, 1975; Hutchinson, 1985). Some of the DNA sugar damage may be caused by base hydroxyl adduct radicals that react with oxygen to form peroxyl radicals which abstract a hydrogen atom from the deoxyribose of an adjacent nucleotide (Deeble and von Sonntag, 1986). Diffusible hydroxyl radicals directly abstract hydrogen atoms from any of the five carbons comprising the sugar backbone to produce corresponding carbon-centered radicals (von Sonntag *et al.*, 1981). This is followed by the diffusion-controlled addition of oxygen to form the five possible peroxyl radicals. Degradation of these radicals generates a variety of deoxyribose lesions with associated spontaneous or alkali-labile breaks. DNA sequence analysis of irradiated DNA shows that base attack rate is random, that C-4′ is a common attack site, and that breaks have both phosphate and glycolate termini at the 3′-end (Henner *et al.*, 1983). Further, base propenal has been identified as a product of the reaction (Janicek *et al.*, 1985). Thus, ionizing radiation and BLM have some DNA damage products in common, although the extent of their formation may be different. While DNA sugar degradation products have been identified that are presumed to have followed hydrogen atom abstraction from C-5′ (von Sonntag *et al.*, 1981), more direct evidence for such a reaction has been wanting, although deoxyguanosine 5′-aldehyde is formed when deoxyguanosine is irradiated (Berger and Cadet, 1983; Langfinger and von Sonntag, 1985). Recently, however, 8,5′-cyclo-2′-deoxyadenosine (Fuciarelli *et al.*, 1985) and 8,5′-cyclo-2′-deoxyguanosine (Dizdaroglu *et al.*, 1986) have been identified in γ-irradiated DNA. These compounds are believed to result from the intramolecular cyclization between C-5′ of deoxyribose and C-8 of the purine base, following hydrogen atom abstraction from C-5′ by radiation generated hydroxyl radicals. These cycloaddition products may be considered to be analogous to the stable drug-DNA adducts produced by

NCS. In the latter case, instead of the radical at C-5′ reacting with its base, it reacts with the well-positioned drug. As was found with the NCS-DNA adduct, formation of the cycloaddition product produced by radiation is enhanced by the exclusion of oxygen (M. Dizdaroglu, personal communication).

In summary, NCS, BLM and ionizing radiation share the ability to damage DNA by hydrogen atom abstraction from one of the carbons of deoxyribose. The action of the first two agents appears to be restricted to the DNA sugar moiety. Depending on which carbon is attacked, the sugar degradation product and the chemistry of the ensuing DNA strand break will vary. NCS appears to attack mainly C-5′, BLM mainly C-4′, and ionizing radiation probably all carbons, as would be expected of an agent that is not restricted in its interaction with DNA by a specific type of binding. Covalent adduct formation between drug and DNA has been described only for NCS, and this occurs only between the DNA deoxyribose, the C-5′ of which has been "activated", and NCS-Chrom. Ionizing radiation produces an analogous lesion in which "activated" C-5′ reacts intramolecularly with C-8 of its own base to form a cyclized product. Although a reduced form of oxygen is involved in both BLM and ionizing radiation action, molecular oxygen is the precursor for BLM, whereas water is the source of hydroxyl radicals for ionizing radiation. Further, BLM can function catalytically in producing DNA damage, whereas the others are single cycle agents. Finally, while ionizing radiation attacks DNA in a random fashion, the base sequence specificities of attack by NCS and BLM are unique and, presumably, determined by specific DNA binding requirements. All three agents have biological effects in common (see Goldberg *et al.*, 1985).

It should be pointed out that other antitumor antibiotics possessing quinone structures, such as the anthracycline adriamycin, have been shown to engage in oxidation-reduction reactions with the generation of reduced forms of oxygen (Bachur *et al.*, 1978; Berlin and Haseltine, 1981; Doroshow, 1983, 1986; Eliot *et al.*, 1984). While it is less clear that DNA is the primary target of such action in intact cells, DNA cleavage *in vitro* has been related to hydroxyl radical formation (Berlin and Haseltine, 1981; Eliot *et al.*, 1984).

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MECHANISM OF ACTION OF ANTI-TUMOUR PLATINUM COMPOUNDS

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ABSTRACT

Many of the biological properties of the neutral platinum coordination complexes, including their cytotoxic effects on cells, result from modifications to the genetic material. More specifically, selective inhibition of DNA replication leads in turn to chromosomal damage and cell death. The antitumor effects of platinum complexes in both rodents and man also result from modifications to DNA. Repair of platinum-induced damage to cellular DNA can reduce cytotoxicity, although mechanisms other than excision repair also determine the response of cells to DNA-bound platinum. One such process is amenable to inhibition by caffeine.

1. INTRODUCTION

Studies of the biological activities of platinum coordination complexes and of their possible use in cancer chemotherapy began in the 1960s when Rosenberg and co-workers discovered that certain electrolysis products generated from platinum electrodes passing current through growth medium selectively inhibited cell division in bacteria (Rosenberg *et al.*, 1965, 1967). Subsequently it was shown that photochemical changes had led to the formation of a neutral platinum coordination complex and that these complexes displayed significant anti-tumor activity against rodent tumours (Rosenberg *et al.*, 1969). One particular compound, *cis*-diamminedichloroplatinum(II) (Cisplatin, *cis*-DDP) has now been

shown to be a useful antitumour drug in man particularly effective against testicular cancer, both alone and in combination with other drugs such as vinblastine and bleomycin, and active against ovarian carcinoma and squamous cell carcinoma of the head and neck (for references see Prestayko *et al.*, 1980). There is now much current evidence to indicate that these platinum drugs exert both their cytotoxic and antitumour effects through an interaction with DNA (for reviews, see Roberts and Thomson, 1979; Roberts and Pera, 1983; Pinto and Lippard, 1985a). In this review I will present more recent evidence supporting this hypothesis and discuss the evidence that some DNA interactions may be more critical than others in inducing cytotoxic effects. Studies of the levels of binding of platinum to cellular DNA for given effects on cell survival were designed to determine whether some cells are inherently more sensitive than others to platinum bound to their DNA and, if so, whether this is due to differences in their abilities to repair or tolerate DNA damage. The DNA repair characteristics of rodent cells sensitive or resistant to platinum drugs have been examined, and by methods designed to answer the question "Is the fidelity of DNA repair the same in sensitive and resistant cells?". In addition I will consider whether the responses of human tumour cells to DNA-bound platinum resemble those of the sensitive or the resistant rodent tumours and hence the relevance of the latter for studies of drug resistance in cancer chemotherapy. Finally I will present some recent comparative studies on the reactions of Cisplatin and the second generation compound, carboplatin (Paraplatin), with DNA *in vitro*, with the DNA of cells in culture and with the DNA of rodent and human cells *in vivo* which further indicate that both the cytotoxic and antitumour actions of the platinum compounds are likely to be the result of reactions with cellular DNA.

2. Biological effects of platinum compounds indicative of interactions with DNA

Some of the properties of platinum drugs, apart from antitumour activity, that are shared with a variety of physical and chemical agents that react with DNA, including induction of lysogeny, inactivation of viruses and transforming DNA, mutagenicity in prokaryotic and eukaryotic systems and cellular transformation and carcinogenic activity have been reviewed previously (Roberts and Pera, 1983) and will not be discussed

further in this review. Likewise a number of alternative hypotheses for the mechanism of action of platinum antitumour drugs have been considered previously (Roberts and Pera, 1983).

3. Biochemical effects of platinum compounds indicative of DNA interactions

The significance of the interaction of platinum compounds with cellular DNA became apparent in early studies of their effects on macromolecular synthesis. *Cis*-DDP selectively and persistently, even after removal of the drug, inhibits the rate of DNA synthesis with little or no effect on RNA or protein synthesis in cells in culture (Harder and Rosenberg, 1970; Roberts and Pascoe, 1972a; van den Berg and Roberts, 1976; Fraval and Roberts, 1978a and b; Johnson *et al.*, 1980) and cells *in vivo* (Howle and Gale, 1970). Under conditions of low cell killing, the selective inhibition of DNA synthesis leads to the formation of giant cells, a feature observed in cells treated with a variety of agents also known to block DNA replication selectively. The association between effects on DNA replication and cytotoxicity and the uniqueness of reactions induced by *cis*-DDP but not *trans*-DDP has emerged from a study of the effect of both isomers on DNA synthesis and cell cycle progression in cells in culture as a function of the dose of the agent and of the amount of platinum actually bound to the DNA (Roberts and Friedlos, 1987). It was found that *trans*-DDP had little effect on overall DNA synthesis whether administered as a 2h pulse of 100 μM to Chinese hamster cells or as a continuous exposure of 10 μM to Chinese hamster or African Green monkey kidney cells during periods of up to 40 hours. By contrast continuous treatment with 10 μM *cis*-DDP dramatically inhibited DNA synthesis in both cell lines. There was no difference in the final extent of binding of platinum to the DNA of Chinese hamster cells treated continuously with a 10 μM dose of either agent. Therefore the observed differential effect on DNA synthesis at early times after treatment by *cis*- or *trans*-DDP is not a function of different cellular uptake or reaction with DNA but due to an intrinsic difference in the consequences of the different types of DNA adducts formed by the two isomers on DNA replication. The markedly greater effect of *cis*-DDP on DNA synthesis seen at late times after treatment is due to a combination of this effect and to the subsequent block to cell cycle progression induced by the *cis* but not by the *trans* isomer. Moreover these studies did not reveal any

differences in the rates of loss of platinum from the DNA of *cis*- or *trans*-DDP-treated cells (indicative of different rates of repair of *cis*- or *trans*-DDP-induced damage—see later) and such as could account for the different effects on DNA replication and cell killing by the two compounds.

It may however be noted that quantitative analysis of the effects of these platinum compounds on DNA replication in model systems has not always indicated the greater inhibitory effect of *cis*-DDP induced adducts. Thus both *cis*-DDP and *trans*-DDP can inactivate primed templates as substrates for DNA polymerase α and β (Harder *et al.*, 1976) and polymerase I (Pinto and Lippard, 1985b) and were equally effective at inhibiting replication of SV40 DNA when bound to the same extent (Ciccarelli *et al.*, 1985). On the other hand, *cis*-DDP adducts were five times more effective than those induced by *trans*-DDP at inhibiting DNA replication on templates of T7 DNA (Johnson *et al.*, 1978). RNA polymerase is similarly inhibited by the binding of both *cis*- and *trans*-DDP to DNA but the *cis* isomer inhibits much more strongly (Srivasta *et al.*, 1978).

The alternative possibility that DNA synthesis is inhibited because of the inactivation of enzymes involved in DNA replication seems unlikely, not only because of the failure of *cis*-DDP to block protein synthesis, but also because of the failure to inactivate DNA polymerase *in vitro* except with very high concentrations (Harder *et al.*, 1976). Moreover, as discussed in detail later, the levels of reaction occurring with cellular macromolecules at doses of *cis*-DDP that kill cells is such that only one protein molecule in several hundreds would have been reacted with platinum, which, in the absence of a selective attack on any given protein, would be inadequate to inactivate any enzyme. Effects of *cis*-DDP on DNA replication have been more precisely defined in studies of synchronously-growing Chinese hamster (fig. 1) (Fraval and Roberts, 1978a) or HeLa (fig. 2) (Fraval and Roberts, 1978b) cells treated during the GI phase of the cell cycle. *Cis*-DDP causes chromosomal aberrations in Chinese hamster cells (van den Berg and Roberts, 1975a) and these are the likely cause of death induced by platinum compounds as with a variety of other DNA modifying agents (Bender *et al.*, 1974; Grote and Revell, 1972). The fact that chromosome aberrations do not occur until some 4h after treatment suggests that treatment during G2 does not lead to chromosome damage at the following mitosis, and that DNA replication on a damaged template is required for their formation. In this respect therefore *cis*-DDP resembles UV-irradiation (Griggs and Bender, 1973),

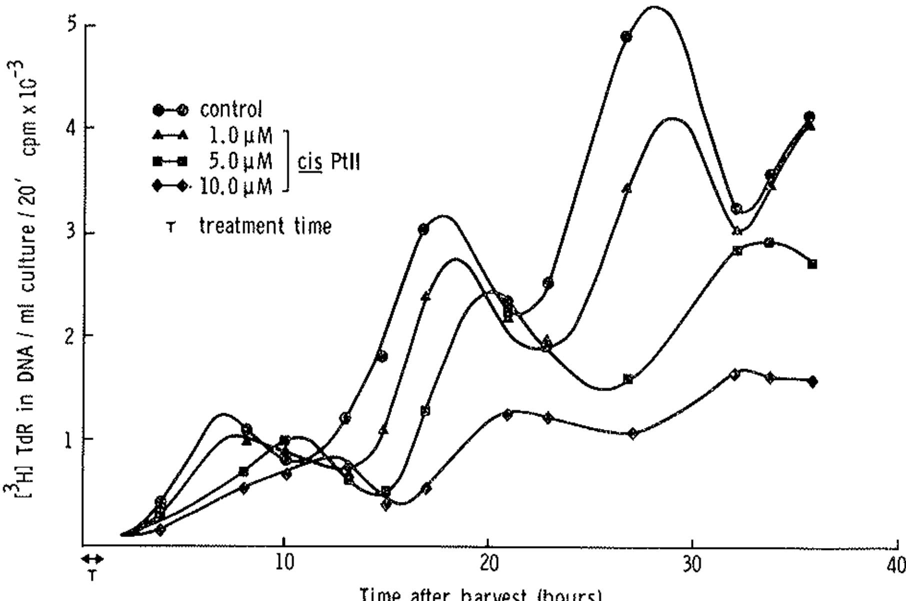

FIG 1. The rate of DNA synthesis in synchronously growing Chinese hamster V79 cells after *cis*-DDP treatment compared with an untreated control. Cells were treated with 1μM (▲—▲), 5μM (■—■), and 10μM (◆—◆) *cis*-DDP approx. 0.5h after mitotic selection. Control cells (●—●) were treated with DMSO to a final concentration of 0.1%. The cells were removed from *cis*-DDP containing medium by gentle centrifugation at 37°C after a 1-h treatment (↔) and resuspended in fresh medium. One ml. aliquots of cell suspension were withdrawn at intervals and the uptake of [^{3}H]TdR (0.5 μCi/ml) into DNA during a 20-min incubation at 37°C was determined.

bifunctional (Evans and Scott, 1969) or monofunctional agents (Kihlman *et al.*, 1973; Roberts *et al.*, 1974) rather than X-irradiation (Scott and Evans, 1967). The association between cell death and chromosome damage arising as a consequence of DNA replication on a damaged template was further indicated by studies of the effects of post treatment with non-toxic doses of caffeine on both these processes. Post-treatment incubation of cells in medium containing 0.75 mM caffeine dramatically potentiated the toxicity of *cis*-DDP and increased the number of cells containing chromosome damage. Caffeine not only increased the number of cells containing aberrations but also enhanced the severity of the damage

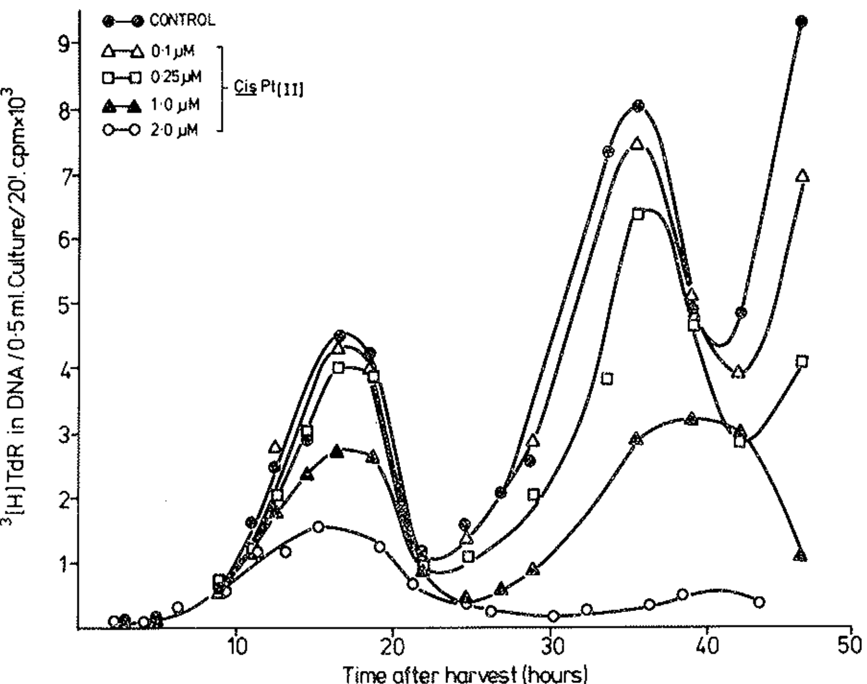

FIG. 2. The rate of DNA synthesis in synchronously growing HeLa cells after *cis*-DDP treatment compared with an untreated control. Cells were treated with 0.1μM, (△—△); 0.25μM, (□—□); 1μM, (▲—▲); and 2μM, (○—○), *cis*-DDP approx. 0.5h after mitotic selection. Control cells (●—●) were treated with DMSO to a final concentration of 0.1%. The cells were removed from *cis*-DDP containing medium by gentle centrifugation at 37°C after a 1-h treatment and were resuspended in fresh medium. Aliquots of 0.5 ml of cell suspension were withdrawn at intervals and the uptake of [^{3}H]TdR (0.5μCi/ml) into DNA during a 20 minute incubation at 37°C was determined.

observed (van den Berg and Roberts, 1975a). That these effects are likely to result from modifications to DNA emerged from studies of the rate and mode of DNA synthesis in cells treated with a combination of *cis*-DDP and caffeine. The immediate, dose-dependent, selective and persistent inhibition of DNA synthesis induced by *cis*-DDP leads, for G1-phase treated Chinese hamster cells, to a dose-dependent extension of the time they take to pass through S-phase and a corresponding delay in the time they take to undergo cell division (Fraval and Roberts, 1978a). Post treatment incubation in medium containing caffeine reverses the *cis*-DDP-induced inhibition of DNA synthesis and the associated delay in

mitosis (van den Berg and Roberts, 1976). The reduced rate of DNA synthesis in *cis*-DDP-treated cells, as measured by a reduced uptake of [^{3}H]TdR into DNA during a short pulse, can also be visualized as a dose-dependent decrease in the size of pulse-labelled, newly-synthesised DNA in treated cells (van den Berg and Roberts, 1976). If, however, compensation is made for the reduction in the rate of synthesis by increasing the labelling period in *cis*-DDP-treated cells then the alkaline sucrose gradient sedimentation profile of labelled DNA in treated cells is nearly the same as that of DNA in control cells. It was concluded from such findings that the replicating machinery is delayed at the site of platinum-induced lesions in DNA in the template strand but, given sufficient time, it can circumvent the lesions without forming discontinuities (gaps) in the newly-synthesised DNA. Alternatively, if gaps are first formed opposite platinum reaction sites in DNA then they are rapidly filled and are too transitory for detection.

The size of newly-synthesised DNA in *cis*-DDP-treated cells may be contrasted with the size of such DNA in cells treated similarly with *cis*-DDP and labelled with [^{3}H]TdR in the presence of non-toxic concentrations of caffeine. Under these conditions the size of nascent DNA is markedly reduced as compared with that in untreated control or *cis*-DDP-treated cells and in a dose-dependent manner. The distance between platinum atoms on one strand of the DNA was calculated from atomic absorption measurements of the platinum bound to DNA isolated from *cis*-DDP-treated cells and this was found to correspond to the size of the newly-synthesised DNA. It was concluded, therefore, that in Chinese hamster cells all platinum adducts in DNA are circumvented during DNA replication by a caffeine-sensitive process.

Subsequent studies of cells treated with other DNA-damaging agents revealed that post-treatment with caffeine, while decreasing the size of nascent DNA, also induced concomitant changes to the size of template DNA. In cells treated with sulphur mustard the template DNA, by 24 h after treatment, approximated to the size of the small molecular weight nascent DNA, also induced concomitant changes to the size of template that template DNA was being incised at the site of caffeine-induced gaps in nascent DNA, possibly by the action of single-stranded endonucleases. Such a possibility is depicted by the model shown in fig. 3. The consequence of such effects on both nascent and template DNA would be to produce, effectively, double strand breaks in DNA. The recently

developed technique of neutral elution has been used to demonstrate the formation of double strand breaks in X-irradiated DNA (Bradley and Kohn, 1979). As can be seen in fig. 4 this technique revealed the existence of double strand breaks in the DNA of cells that had been treated with *cis*-DDP and then incubated in the presence of caffeine for 24 h. No comparable formation of double strand breaks was seen in *cis*-

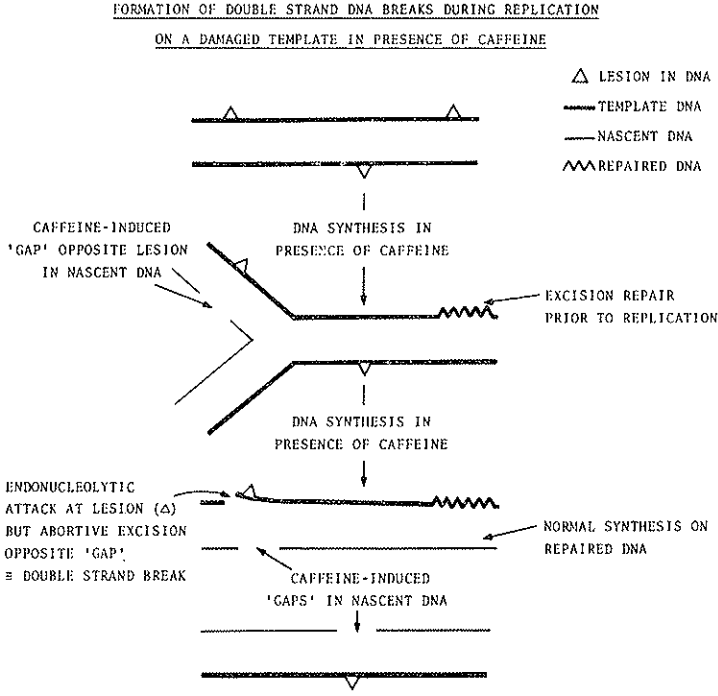

FIG. 3. Scheme showing how replication of DNA in the presence of caffeine and on a template containing excisable damage could lead to DNA-template degradation and DNA double-strand breaks. Upper: template DNA containing lesions (△). Middle: both excision repair and replication occur on the damaged template. Excision repair in a non-replicating region proceeds successfully, although possibly slowed by the presence of caffeine, while in the replicating region, the influence of caffeine is to disrupt the ability of the replication machinery to circumvent lesions, leading to gaps in the nascent DNA. Lower: replication proceeds, but before the caffeine-induced gaps in the nascent DNA are sealed, incision of the template strand at the site of the adduct occurs, producing a DNA double-strand break, which, if persistent into mitosis, could lead to the observed chromosomal effects.

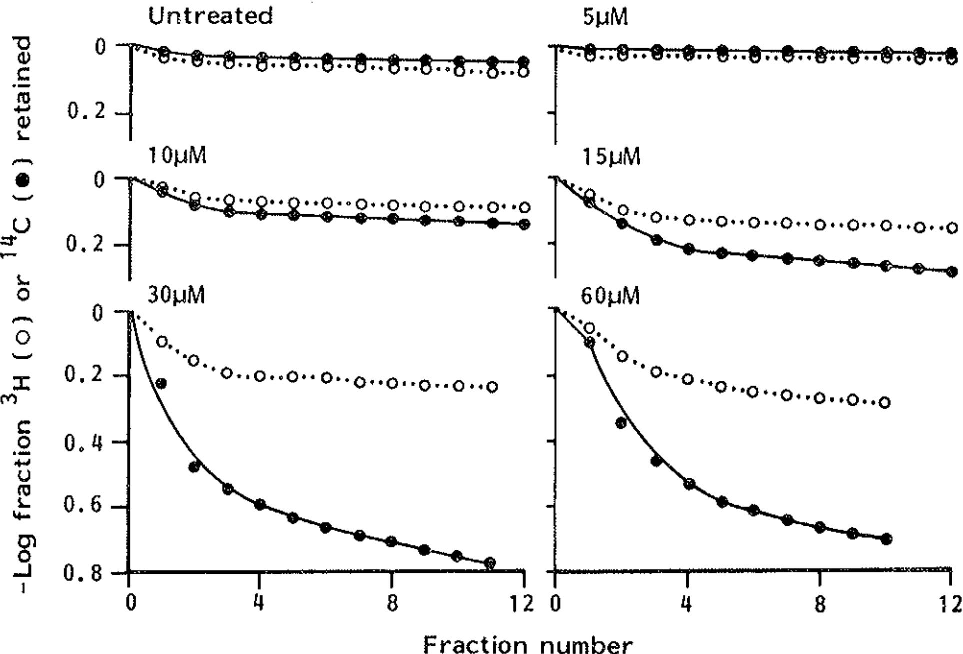

FIG. 4. Neutral elution of DNA of Chinese hamster cells treated with *cis*-DDP and incubated in the presence (●) or absence (○) of caffeine for 24h. Monolayer cultures were labelled overnight with [2-^{14}C]thymidine [0.02µCi/ml; 50-60 mCi/mmole] or [6-^{3}H]thymidine [0.5 µCi/ml; 50-60 mCi/mmole], split into 6 replicates and treated with either 5, 10, 15, 30 or 60µM *cis*-DDP or left untreated. After 15 min caffeine (1 mM) was added to the *cis*-DDP-treated and the untreated ^{14}C-labelled cells and both ^{3}H- and ^{14}C-labelled cells were incubated for 24 h at 37°C. Cells were detached from the plastic with trypsin and ^{3}H- and ^{14}C-labelled cells (approx. 2×10^5 of each) that had been treated with a given dose of *cis*-DDP or untreated with *cis*-DDP were mixed in cold phosphate-buffered saline (PBS) (15 ml) prior to addition on polycarbonate filters (22 mm; 2.0 µ pore size) by gentle suction. Cells were washed with cold PBS (10 ml) and subjected to lysis and neutral elution exactly as described by Bradley and Kohn (1979). Fractions of approx. 2.5ml were collected every 90 min.

DDP-only treated cells except after very high doses of *cis*-DDP (Roberts and Kotsaki-Kovatsi, 1986). These findings provide a satisfactory explanation for the enhancement of chromosome damage by caffeine in *cis*-DDP-treated cells since such damage has been thought to require the formation of double strand breaks in DNA.

4. Reactions of platinum drugs with DNA *in vitro*

A) *Sites of reaction*

The above biochemical consequences of platinum drug-cell interactions have therefore stimulated extensive studies of the adducts formed in DNA by both active (*cis*) and inactive (*trans*) platinum compounds and of which ones could be responsible for the above discussed perturbations of DNA synthesis. Early studies of the reactions of platinum compounds with nucleic acid components (see reviews by Roberts and Pera, 1983; Reedijk and Lohman, 1985; Pinto and Lippard, 1985a) indicated that the platinum compounds reacted with the nitrogen atoms of the nucleic acid bases guanine, adenine, and cytosine. Of the various possible binding modes it seemed that those at the N-7 positions of adenine and guanine were the most likely ones to be involved in reactions with DNA. Moreover many studies pointed to the probability of reactions occurring with adjacent guanines in DNA with *cis*- but not *trans*-DDP (Kelman and Buckbinder, 1978; Cohen *et al.*, 1980). Recent studies of the products of the reaction of DNA with platinum compounds have confirmed these predictions (Eastman, 1983; Fichtinger-Schepman *et al.*, 1985). Following enzyme digestion of *cis*-DDP-treated salmon sperm DNA to (oligo) nucleotides four platinum-containing products comprising approximately 90% of the total platinum on the DNA were separated by preparative chromatography on a diethylaminoethyl-Sephacel column at pH 8.8 and characterised by ^1H NMR. The two major adducts were *cis*-$Pt(NH_3)_2$d(pGpG) and *cis*-$Pt(NH_3)_2$d(pApG), both derived from intrastrand cross-links of *cis*-DDP on neighbouring nucleobases. Only the d(pApG) but not the d(pGpA) adduct could be detected. Two minor adducts were $Pt(NH_3)_3$dGMP, resulting from monofunctionally-bound *cis*-DDP to guanine and *cis*-$Pt(NH_3)_2d(GMP)_2$ originating from interstrand crosslinks on two guanines as well as from intrastrand crosslinks on two guanines separated by one or more bases (Fichtinger-Schepman *et al.*, 1985).

B. *Formation of DNA interstrand crosslinks*

Cis-platinum compounds can induce the formation of interstrand crosslinks in isolated DNA (Horacek and Drobnik, 1971; Pascoe and Roberts, 1974; Harder, 1975; Roberts and Friedlos, 1981, 1982) or in the DNA of whole cells (Roberts and Pascoe, 1972b; Zwelling *et al.*, 1978). Trans com-

pounds are as effective as *cis* compounds at inducing crosslinks in isolated DNA (Pascoe and Roberts, 1974) but induce far fewer crosslinks, for the same amount of platinum binding, in the DNA of mammalian cells (Pascoe and Roberts, 1974; Zwelling *et al.*, 1978). The frequency of interstrand crosslinks was originally estimated by Pascoe and Roberts (1974), from a combination of measurements of the amount of platinum bound to HeLa cell crosslinked DNA of estimated size. Crosslinking was shown to be a relatively rare event, accounting for about one per cent of the total number of reactions with cellular DNA. In order to estimate crosslinks in the DNA, one strand of DNA was given a density and radioactive label by growing cells in the presence of 5-bromo-2′-deoxyuridine and tritiated thymidine. Crosslinking between a "labelled heavy" strand and a "light unlabelled" strand produced a "labelled hybrid" strand and these species could be separated in an alkaline caesium chloride gradient (fig. 5) (Pascoe and Roberts, 1974; Roberts and Friedlos, 1981). Recent quantitative studies of crosslinking of Chinese hamster cell DNA, using a variety of techniques, confirmed the rarity of crosslinks in whole cells at the time of their maximum development (Roberts and Friedlos, 1981, 1982; Pera *et al.*, 1981b).

5. Reactions of platinum compounds with the DNA of cultured cells

The relevance to cytotoxicity of platinum binding to DNA, rather than to RNA and/or protein, emerged from studies of the extent of reaction with these macromolecules after treatment of cells with doses of platinum compounds that permitted measurements of their subsequent survival (Pascoe and Roberts, 1974; van den Berg and Roberts, 1976; Fraval *et al.*, 1978; Fraval and Roberts, 1979a,b; Pera *et al.*, 1982). From such data it was possible to construct curves showing the relationship between the log of cell survival and the amount of reaction with these particular macromolecules and thence the number of platinum atoms bound to each of these (B_0) following treatment of cells with a dose (D_0) of *cis*-DDP that reduced cell survival from a fraction f to 0.37 f (this is theoretically the concentration at which, on the average, one inactivating event occurs in each cell). When expressed in this manner there were no major differences in the levels of reaction with these three macromolecules. However this estimate of the reactions occurring in the cell takes no account of the very great differences in the molecular weights of RNA and protein molecules

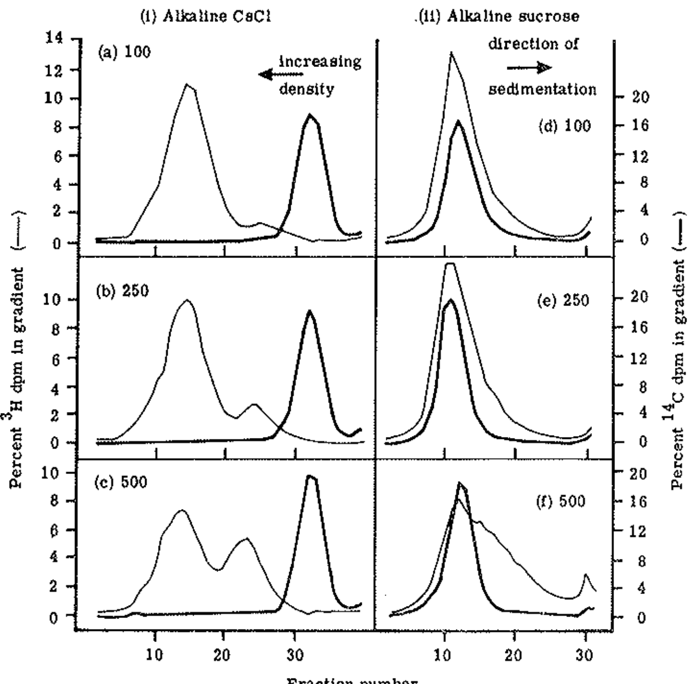

Fig. 5. The effect of cisplatin-induced DNA-DNA interstrand crosslinks on the sedimentation patterns in alkaline CsCl or alkaline sucrose gradients of density-labelled DNA from cisplatin-treated Chinese hamster V79 cells. Cells were prepared as detailed in the text, such that one of the complementary strands of their DNA was labelled with respect to both buoyant density and ^{3}H radioactivity, whilst the other strand remained unlabelled. An otherwise untreated, marker culture with ^{14}C-labelled DNA provided an indication of the molecular weight and normal buoyant density of control, untreated DNA. Experimental and marker cultures were mixed and their DNA was co-harvested and subjected to alkaline CsCl(i) or alkaline sucrose (ii), gradient sedimentation analysis. The cisplatin produces in the CsCl profiles (a-c), in a dose-responsive fashion, a novel peak, midway between the 'heavy' (^{3}H, —) and 'light' (^{14}C, ▬) peaks, indicative of a crosslinking of the two, whilst in the alkaline sucrose gradients (d-f), a corresponding increase in molecular weight is seen. The number average molecular weights of the unlabelled alkaline sucrose gradient peaks were all around 2.10^7 daltons. The numbers on the figures (100, 250, 500) refer to the doses (μM/2h) of the platinum compound used in these experiments.

as compared with that of a DNA molecule. When these B_0 binding values were expressed as molecules of platinum bound to molecules of DNA, RNA and protein, then it became apparent that on a molar basis there was far more reaction with DNA than with RNA or protein. Moreover it could be deduced that the levels of reaction with RNA or protein would be too low to produce their inactivation within the cell, presuming, that is, that there is no selective reaction with any particular RNA or protein molecule. On the other hand the level of reaction with DNA was such that every DNA molecule contained many platinum atoms making it by far the most probable target for the cytotoxic action of platinum compounds. Furthermore these calculations continue to support the notion, discussed above, that the biochemical effect of platinum compounds which is most relevant to their cytotoxic action, namely the selective inhibition of DNA synthesis, is the result of these reactions with DNA which inactivate it as a template for further replication.

DNA binding measurement at determined levels of cell killing have been carried out in Chinese hamster cells with a number of other platinum compounds which showed useful antitumour activity (Roberts and Fraval, 1978). The B_0 values were similar for three such compounds despite large differences in the doses required to achieve equal toxicity (D_0) (Table 1). Again, for all compounds it could be argued that only in the case of DNA was there sufficient reaction with platinum to induce a

TABLE 1 - *Binding of platinum compounds to the DNA of tumour cells* in vitro *and Chinese hamster cells* in vitro.

Compound	ADJ/PC6 tumour ([a])		Chinese Hamster V79 cells ([b])	
	ID_{90} (mg/Kg)	Binding to tumour DNA (max) nmoles/g	Do (μM)	Binding to DNA (nmoles/g)
Carboplatin	14.5	1.8	120	3.0
CHIP	4.2	2.0	48	2.5
Cisplatin	1.6	3.5	15	8.5

([a]) From Roberts (1981).
([b]) From Roberts and Fraval (1978).

cytotoxic effect, although clearly the kinetics of the reaction differed as between the three compounds (see below). Since the non-leaving groups of cisplatin and carboplatin are the same then they would be expected to give rise to the same chemical adducts on DNA. If, therefore, their mechanisms of action are the same, one would expect to observe the same amount of cell killing for a given extent of DNA reaction. That this is so emerged from a more extensive study of DNA binding and cell killing in a resistant (see later) line of Walker tumour cells as shown in fig. 6 (Roberts *et al.*, 1986b; Knox *et al.*, 1986a).

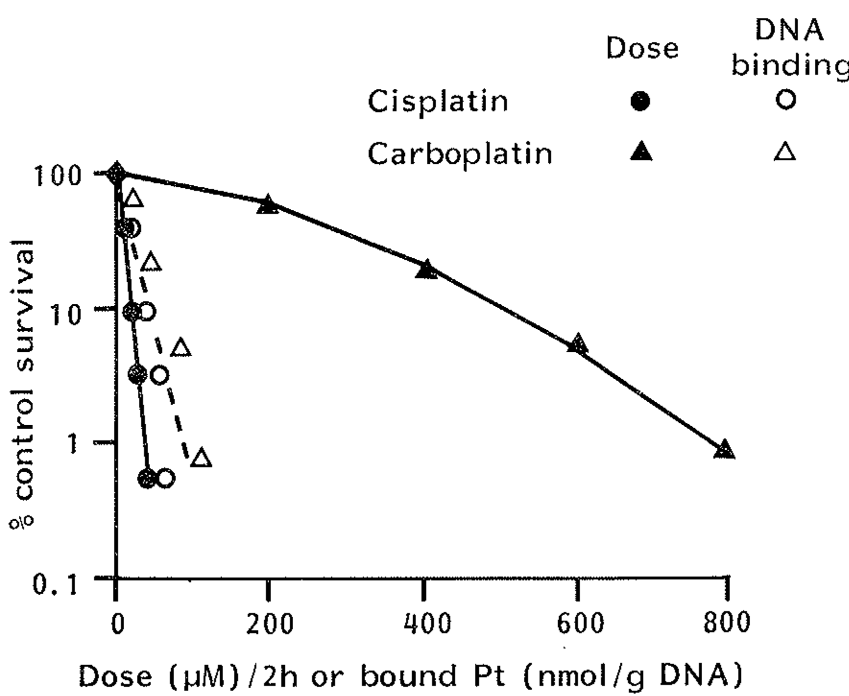

FIG. 6. The effect of dose or the resulting DNA bound cisplatin or carboplatin on the survival of resistant Walker cells. Cells 2×10^5 were treated for 2 h at 37°C and survival assayed by colony forming ability in semi-solid agar or their DNA extracted and bound platinum measured by atomic absorption spectrometry.

6. Reactions of platinum compounds with the DNA of cells *in vivo*

A) *Rodents*

For DNA to be the target for the antitumour effect of platinum compounds as well as for their cytotoxic action on cells in culture a similar relationship to that discussed above should exist for cells *in vivo* with respect to the reaction of platinum with tumour cell macromolecules and measured effects on their survival. Pera *et al.* (1982) studied the reactions of *cis*-DDP and hydroxymalonatodiammine platinum(II) ($Pt[OHmal(NH_3)_2]$) with DNA of B16 melanoma and bone marrow in C57Bl mice and carried out colony forming assays to quantitative toxicity to melanoma and bone marrow cells. The binding of both compounds to the DNA of bone marrow cells was comparable to that found for the binding of platinum to the DNA of cells in culture at equitoxic doses, a finding that again strengthens arguments concerning the mechanism of action of these compounds based on *in vitro* work. The greater selective toxicity of $Pt[OHmal(NH_3)_2]$ towards the B16 melanoma was associated with increased binding of platinum to tumour DNA, relative to *cis*-DDP and probably results from pharmacological factors that enhance delivery of active drug to tumour cells.

The maximum levels of binding of platinum to the DNA of cells isolated from the ADJ/PC6 tumour in mice, at various times after injection of platinum compounds that induced 90% tumour regression (ID_{90}), were also of the same order as those to cells in culture at what may be regarded as approximately similar levels of cell killing (Table 1). Thus for three platinum compounds, *cis*-DDP (cisplatin), *cis*-diammine (1:1-cyclobutanedicarboxylato)platinum(II) (carboplatin Paraplatin), and *cis*-dichloro-*trans*-dihydroxy-*cis*-*bis*-(isopropylamine)platinum(IV) (CHIP) the maximum levels of binding to DNA were within a factor of two of each other and all were similar to the amount of platinum reacting with the DNA of Chinese hamster cells in culture at doses corresponding to their D_0 values.

The maximum amount of platinum bound to a human pancreatic tumour, growing as a xenograft in nude mice, following injection of a dose of *cis*-DDP (10 mg/kg) that reduced the survival of the tumour cells to 10% when these were plated out 24 h later (Courtenay *et al.*, 1982) was 6 nmoles/g, a value in accord with the other *in vivo* binding studies.

B) *Humans*

It has recently been possible to measure the extent of reaction with the DNA of cells isolated from patients who had received Cisplatin or carboplatin chemotherapy. The levels of reaction of platinum with the DNA of cells obtained from ascitic fluid removed from various patients at certain times after they had received Cisplatin were all remarkably similar (Table 2) (Knox *et al.*, 1986a). The value of platinum binding of around 10 nmoles/g is clearly of the same order as that of binding to the DNA of cells in culture or to the DNA of normal or tumour mouse cells *in vivo* following *cis*-DDP treatment and again indicates that the antitumour action of *cis*-DDP, like its cytotoxic effects, is also likely to be due to reactions with DNA.

Some indication of the possible amount of cell killing which would result from the platinum DNA binding observed in patients can be gained by a comparison of the anticipated killing of various cell types if their DNAs were reacted to this or a similar level. As shown in table 3 it is apparent that for a number of cell types, treated either *in vitro* or *in vivo*, 10 nmoles/g of platinum bound to their DNA would reduce cell survival to not lower than about 20%, except where cells have been shown to be particularly sensitive to DNA-bound platinum, as in the case of *xeroderma pigmentosum* fibroblasts or the *cis*-DDP-sensitive Walker

TABLE 2 - *Relationship between dose of cisplatin and binding to cellular DNA* in vivo.

Cell type	Dose of cisplatin (mg/m^2)	Time of Biopsy (hr)	Binding to DNA (nmoles/g)
ovarian	100 mg/m^2	62	11.2
carcinoma —	4×20 mg/m^2/day	24	10.4
ascites cells		(after last dose)	
ovarian	30 mg/m^2	24	10.3
carcinoma — pleural effusion cells	50 mg/m^2	28	28.8

tumour cells. Successive treatment of initially-sensitive Walker cells with further doses of *cis*-DDP leads to the selection of increasingly resistant cells. There appeared to be a limit to the degree of resistance that could be achieved by this procedure. Thus Walker tumour cells did not become more resistant than most of the cultured cell lines or than the normal rodent or normal human cells discussed above.

Parallel studies to the above were carried out with the second generation platinum compound carboplatin. DNA that was isolated from a metastatic nodule (from an OAT cell tumour) 36 hours after ad-

TABLE 3 - *Relationship between the binding of cisplatin to the DNA of cultured cells or normal or tumour cells* in vivo *and their subsequent survivals.*

Cells	Dose	Survival %	Binding to DNA (nmoles/g)
Chinese hamster ([a])	20 μM/1h	60	10
HeLa ([a])	10 μM/1h	20	10
Human foetal ([b]) lung	10 μM/1h	20	10
XP ([b])	20 μM/1h	0.2	10
Walker resistant ([c])	5 μM/1h	60	10
Walker sensitive ([c])	5 μM/1h	0.01	10
Mouse bone ([d]) marrow	10 mg/Kg	20 spleen colonies	10
Mouse B16 ([d]) melanoma	4 mg/Kg	20 lung colonies	10
Human pancreatic tumour Xenograft	10 mg/Kg	10 (in vitro) ([e])	6 ([f])

([a]) Fraval and Roberts (1979a).
([b]) Fraval *et al.* (1978).
([c]) Roberts *et al.* (1986b).
([d]) Pera *et al.* (1982).
([e]) Courtenay *et al.* (1982).
([f]) Roberts (1981).
XP = xeroderma pigmentosum.

TABLE 4 - *Relationship between binding of carboplatin to cellular DNA* in vivo *and* in vitro *and cell survival.*

Cell	Status	Dose	Survival	Binding to DNA (in nmoles/gm)	
Human tumour nodule (OAT cell)		400 mg/m^2		2.2	38 h after jniection
Chinese hamster (in culture)	Exponential	120 μM/4h	10%	4	
Mouse ADJ/PC6 plasmacytoma		14.5 mg/Kg	ID_{90} dose	1.8	24 h after injection
Mouse pancreatic tumor xenograft (in mice)		100 mg/Kg	30	15.5	24 h after injection
Walker resistant		200 μM	58	17	
Walker sensitive		60 μM	0.4	8	

([a]) Roberts *et al.* (1986b).

ministration of carboplatin (400 mg/m^2) had a level of bound platinum of 2.2 nmoles/g. An ID_{90} dose of carboplatin for the ADJ/PC6 tumour (14.5 mg/kg) resulted in the comparable binding level of 1.8 nmoles/g, while a higher dose of 100 mg/kg resulted in a commensurately higher level of binding to the DNA of cells of a human tumour xenograft growing in an immune suppressed mouse (Table 4) (Knox *et al.*, 1986a). These various DNA binding studies further support the notion that DNA is the target for the cytotoxic and the antitumour effects of platinum compounds.

7. NATURE OF DNA DAMAGE ASSOCIATED WITH CYTOTOXICITY

Earlier comparative studies of the reactions of *cis*-DDP and the corresponding *trans* isomer, *trans*-DDP, which is not an antitumour agent and which is far less toxic to cells, with the DNA of mammalian cells pointed to the likely importance of DNA interstrand crosslinking in

inducing cytotoxicity. A correlation between interstrand crosslinking and cytotoxicity has been demonstrated in the case of *cis*- and *trans*-DDP-treated HeLa (Pascoe and Roberts, 1974), mouse (Zwelling *et al.*, 1978; Zwelling *et al.*, 1979a) and Chinese hamster (Zwelling *et al.*, 1979b; Plooy *et al.*, 1984) cells. Again, investigations in mouse leukemia cells (Zwelling *et al.*, 1981) and normal or transformed human cells (Erickson *et al.*, 1978; Laurent *et al.*, 1981) of varying sensitivity to *cis*-DDP showed that sensitivity generally correlates well with interstrand cross-link formation. No similar correlation was found between DNA-protein crosslinking and cytotoxicity in Chinese hamster (Zwelling *et al.*, 1979b) or mouse cells (Zwelling *et al.*, 1979a). Ducore *et al.*, (1982) found that of three Burkitt lymphoma cell lines only two showed a correlation between cytotoxicity and crosslinking, while the other underwent a unique and marked cell lysis 12 to 14 hours after treatment.

It has been found that cells can be protected from the toxic effects of *cis*-DDP by preventing the formation of crosslinks by incubating cells in the presence of thiourea immediately after treatment (Zwelling *et al.*, 1979c). Conversely potentiation of cell killing accompanied by increased crosslinking can be achieved by post treatment with 1-β-D-arabinofuranosylcytosine (ara-C) (Bergerat *et al.*, 1981), nucleosides (Drewinko *et al.*, 1985) or hyperthermia (Meyn *et al.*, 1980). These various observations all support the notion that an ability to form an interstrand crosslink is critical for the cytotoxic activity of the *cis* orientated platinum compounds.

On the other hand, as discussed above, reactions resulting in DNA interstrand crosslinks only account for a small proportion of the total platinum adducts on cellular DNA and the major product of reaction is a crosslink between the N-7 positions of adjacent guanines on one strand of DNA. Since *trans*-DDP is stereochemically unable to form such an adduct it has been proposed that the ability or inability to form such 1,2-d(GpG) crosslinks adequately accounts for the difference in the antitumour activities of the *cis* and *trans* platinum compounds (Pinto and Lippard, 1985a). However an indication of the possible importance of any specific lesion in DNA is only likely to emerge from studies of the repair of DNA damage in cells of varying sensitivity to platinum compounds.

8. Repair of platinum-induced DNA damage

A) *General studies*

Early studies established that bacterial strains defective in DNA repair are less able to survive exposure to *cis*-DDP than wild type cells (Drobnik *et al.*, 1973; Beck and Brubaker, 1973). Recently Sancar and Rupp (1983) have used purified proteins coded by the *uvr* genes to reconstitute the UVRABC nuclease to study the incision step of excision repair of DNA damaged by a variety of agents. The UVRABC nuclease cut plasmid DNA containing damage caused by either *cis*-DDP or *trans*-DDP. On the other hand adducts caused by the two compounds were recognized differently by the nuclease. A specific cutting pattern involving incisions at the 8th phosphodiester bond to the 5′ and the 4th phosphodiester bond to the 3′ of the adjacent GGs was observed for damage induced by *cis*-DDP but not for that induced by *trans*-DDP (Beck *et al.*, 1985).

In mammalian cells the techniques of alkaline sucrose gradient sedimentation, alkaline elution and DNA renaturation (van den Berg and Roberts, 1975b; Zwelling *et al.*, 1979a; Pera *et al.*, 1981b) have clearly demonstrated repair of DNA-DNA and DNA-protein crosslinks. Interstrand crosslinking was indicated by a shift in alkaline sucrose gradients of DNA molecules towards the high molecular weight end of the gradient, by a decrease in the rate of filter elution of DNA from x-irradiated cells or by increase in the rapidly renaturing fraction of DNA in cell lysates. These various drug-induced phenomena reached a maximum 6-12 hours after drug treatment and then declined. Since only minimal degradation of DNA occurs during this time and since crosslinks are stable in isolated DNA under physiological conditions it may be presumed that this reversal represents DNA repair. When measured by any of these methods the half-life of DNA interstrand crosslinks appeared to be between 12 and 24 hours.

Platinum bound to the DNA of *cis*-DDP-treated exponentially growing Chinese hamster cells is lost with a half-life of approximately 24 hours (Fraval and Roberts, 1979b). Again, since such DNA-bound platinum is stable chemically under physiological conditions this loss can be attributed to the operation of a DNA excision repair process. A recent report (Ciccarelli *et al.*, 1985) claimed that the different kinetics of accumulation of platinum on the DNA of African Green monkey kidney cells treated continuously with either *cis*-DDP or *trans*-DDP was due to

a difference in the repair of their respective DNA-bound products and this difference would provide an appealing rationale for the enhanced biological activity of the *cis* compound. However we have failed to confirm the more rapid loss of *trans*-DDP-induced adducts, as compared with *cis*-DDP-induced adducts, in either Chinese hamster or African Green monkey kidney cells (Roberts and Friedlos, 1987). Moreover it was found that in Chinese hamster cells the different kinetics of accumulation of platinum on DNA could be adequately explained by the different effects produced by the two isomers on DNA replication and cell cycle progression.

B) *Relationship between DNA excision repair and cytotoxicity*

The level of binding of platinum to cellular DNA induced by *cis*-DDP resulting in a defined degree of cell killing (B_0) is indicative of a cell's inherent sensitivity to DNA-bound platinum and is likely to reflect its ability to repair or tolerate such damage. Thus the B_0 value for the repair-defective *xeroderma pigmentosum* cells is appreciably less than that for various other cultured cells (Fraval *et al.*, 1978). Chinese hamster (Fraval and Roberts, 1979b) or human foetal lung (Pera *et al.*, 1981a) cells in stationary phase are more sensitive to treatment with *cis*-DDP (when they are plated out immediately after treatment for assessment of colony-forming ability) than are these same cells growing exponentially. The greater sensitivity of stationary-phase treated cells is not due to an increased uptake of drug but rather to a decreased rate of loss of platinum from DNA resulting probably from their decreased excision repair capability. It was further found that the relationship between the number of platinum molecules *not* excised from the DNA and the logarithm of the survival of stationary phase cells, when plated out for estimation of cell survival, was the same as that for cells treated with several doses of *cis*-DDP and plated out immediately. This relationship strongly supported the hypothesis that damage present on the DNA template at the time of entry into the proliferative cycle was responsible for cellular toxicity. Moreover the results showed that the loss of platinum from the DNA was actually effective in achieving biological recovery.

On the other hand attempts to relate the inherent sensitivity of a cell to DNA-bound platinum to its repair capacity have not generally supported this thesis. Thus sensitive and resistant Walker tumour cells, which reacted to the same extent with *cis*-DDP, removed total DNA-bound

platinum, DNA interstrand crosslinks and DNA-protein crosslinks from their DNA at essentially equal rates (Rawlings and Roberts, 1986). No DNA strand breaks accumulated in the DNA during the period lesions were lost from DNA. It can therefore be presumed that the repair process(es) operate with equal efficiency in sensitive and resistant cells and furthermore restore the *integrity* of DNA. However the *fidelity* of repair may be defective in sensitive cells. This possibility can be studied by assaying for expression of bacterial genes that have been damaged *in vitro* prior to transfection into recipient cells. The recombinant plasmid pSV2gpt contains the bacterial gene gpt coding for xanthine guanine phosphoribosyl transferase (XGPRT) and when transfected into a mammalian cell enables the cell to utilize xanthine. It was found that sensitive or resistant cells when transfected with *cis*-DDP-treated pSV2gpt expressed XGPRT to similar extents. This would imply similar levels of repair of damaged plasmid by the two cell lines, a result consistent with the above findings in whole cells. More importantly it could imply comparable fidelity of repair in the two cell lines. However it was apparent that about 10 platinations on the gpt gene were required to produce, on average, one inactivating event (B_0). Since DNA interstrand crosslinks occur with a frequency of only about one per cent it follows that these are not the lesions in plasmid DNA responsible for the inactivation of this gene. The inactivating lesion would therefore appear to be the major product of reaction with DNA, namely an intrastrand crosslink. Therefore this particular transfection system cannot be used to compare sensitive and resistant Walker tumour cells for their abilities to repair DNA interstrand crosslinks. On the other hand it should be noted that much other evidence indicates that it is the decreased ability of sensitive Walker cells to tolerate DNA interstrand crosslinks that is the basis for their unique sensitivity to difunctional agents (Roberts *et al.*, 1986a).

The above considerations recall earlier observations which showed that bacteriophage T7 (Shooter *et al.*, 1972) or phage λ (Filipski *et al.*, 1980) could be inactivated by levels of platination that did not produce, on average, one interstrand crosslinking event in their DNAs. Again, therefore these molecules were probably inactivated by the major product present on the DNA, namely an intrastrand crosslink. Clearly the conclusions drawn with respect to inactivating lesions for exogenous DNA molecules in these various *in vitro* systems may not always be extrapolated to indicate the nature of inactivating lesions for mammalian cells. However despite these reservations the above transfection system did indicate

the relative effectiveness (in inactivating mammalian cells) of lesions introduced into DNA by different platinum compounds. Thus it was found that for the monofunctional platinum compound, Pt(dien), the level of reaction with pSV2gpt which was required to produce one inactivating event was approximately ten times that required for *cis*-DDP and this reflects the much higher level of reaction of the former compound with cellular DNA at equitoxic doses of the two compounds (Knox *et al.*, 1986b).

It can be presumed that these levels of reaction with cellulat DNA at equitoxic doses reflect a cell's ability to repair or tolerate the different types of damage to its DNA. Some support for the concept that Walker tumour cells sensitive or resistant to difunctional agents do differ in their abilities to tolerate lesions in their DNA has emerged from studies of the rate and mode of DNA synthesis in these cells following treatment with sulphur mustard or *cis*-DDP. Both cell lines exhibited the same dose-dependent and progressive depression in the rate of DNA synthesis for up to 4 hours after treatment. Thereafter the depression in rate of synthesis was partially reversed in the resistant cells but the rate of synthesis continued to decrease in sensitive cells resulting in a slower transit through the S phase and a persistent block in the G_2M phase of the cell cycle. Sensitive cells which finally escaped the block in G_2 carried more chromosome aberrations than the corresponding resistant cells. Neither cell line was defective in daughter strand gap repair. In their sensitivity to difunctional but not to monofunctional compounds, their failure to recover from the early depression of DNA synthesis, their apparent lack of a defect in excision repair and their sensitivity to chromosome aberration induction, the Walker cell phenothype closely resembles that of the human Franconi's anaemia cell (Roberts *et al.*, 1986a).

9. Conclusions

This review of recent as well as past evidence concerning the mechanism of action of platinum antitumour drugs continues to support the view that many of their biological properties could result from interaction with the cellular genetic material. *In vitro* work has now elucidated the nature of the majority of the interactions of platinum compounds with DNA. The earlier indications that reaction with adjacent bases on one strand of DNA occurred with *cis-DDP*, but not with *trans*-DDP, have

now been confirmed by the isolation and characterisation of the resulting product in enzyme digests of platinated DNA.

Further studies of the reaction of platinum compounds with the DNA, RNA and protein of cultured cells at measured levels of cell survival again indicate that only in the case of DNA is the reaction adequate to account for cytotoxicity. It is now clear that reaction with tumour or host DNA *in vivo* is similar to that observed in cells in culture at comparable levels of cell survival. Increased reaction of platinum with tumour DNA has been shown to be associated with improved antitumour selectivity in one model. However at present there is little indication that the mechanism of action of platinum analogues is markedly different from that of *cis*-DDP. In cultured cells it is often the case that DNA interstrand crosslinking reactions correlate with cytotoxicity. In the situations when this does not apply as in the case of cells that are inherently sensitive to *cis*-DDP-induced DNA damage, but which are not defective in excision repair processes, it seems that such cells are likely to be defective in the mechanism(s) that circumvent DNA damage during DNA replication. At the present time however there is no evidence to suggest that intrastrand crosslinking reactions are more cytotoxic than those resulting in interstrand crosslinks.

Key questions which emerged from these various *in vitro* and *in vivo* studies are whether some human tumour cells are, like certain rodent cells in culture, uniquely sensitive to DNA bound platinum and, if so, is this due to some defect in the mechanism which repairs such damage or in that which permits cells to tolerate damage during DNA replication. Cloning of genes responsible for such repair or tolerance mechanisms may make it possible to identify such drug-sensitive tumours.

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