THE EFFECTS OF MECHANICAL TENSION AND THE STEFFIMYCIN ANTIBIOTICS ON D.N.A. CONFORMATION

by

PHILIP JOHN BLAKELEY

A Thesis submitted for the Degree of
Doctor of Philosophy
in the
University of Keele

September 1976

Physics Department,
University of Keele,
England.

"Thus says the Lord, your Redeemer,
and the one who formed you from the womb,
"I, the Lord, am the maker of all things,
Stretching out the heavens by Myself,
And spreading out the earth all alone, ...""

Isaiah 44:24

Acknowledgements

I wish to thank Professor W. Fuller for supervision of the projects and provision of the research facilities and apparatus used in this work.

I am indebted to Dr. W.J. Pigram for discussion and encouragement beyond the call of duty, to Dr. C. Nave for suggestions on the construction of the models of stretched D.N.A. and practical assistance in the production of X-ray diffraction patterns, and to Mr. D.C. Goodwin for help in the use of computer programs.

I am grateful, too, for the assistance of Mr. G. Dudley and co-workers in the Physics workshop, to Mr. M. Daniels whose darkroom labours produced the photographs for this thesis, and to many in the Physics Department at Keele who I have called on for help from time to time. Thanks also to Mr. D. Emley of the Geology Department for technical assistance in the use of the flame emission spectrophotometer.

I appreciate with gratitude the work of Miss H.O. Henry for typing this thesis.

I am fortunate to have a wife who has been patient in allowing me a student's life for these three years.

The Science Research Council provided me with a three year award for this Ph.D. course.

CONTENTS

Abstract

The conditions necessary to produce stable specimens of D.N.A. under mechanical tension were investigated. This involved the examination of the sodium chloride content of D.N.A. fibres and a study of the relative humidity range within which the stretched form is maintained.

X-ray and optical methods were used to obtain helical parameters with sufficient resolution to construct a series of models of the structure. These models were built so that the destructive interference of scattered X-rays would result in calculated intensity data which closely resembles the observed diffracted intensities.

The resulting models present various advantages in the replicative and transcriptional roles of the molecules.

Steffimycin and steffimycin B are two antibiotics which bind to nucleic acids and an investigation was carried out on the conformation of the resulting complex when formed with natural and synthetic D.N.A.'s. Spectroscopic studies were made to examine the extent of drug binding to D.N.A. and any base pair specificity of the interaction.

X-ray diffraction methods revealed that the helix pitch is extended in such a complex with the results best explained as the effect of intercalation of the drug chromophore between base pairs.

Model building studies of the D.N.A.-steffimycin conformation were carried out and a comparison of the mode and strength of binding of the two steffimycins and some other antibiotics was made.

Chapter 1. INTRODUCTION

1:1:a D.N.A. : Structure and Function

Deoxyribonucleic acid, (D.N.A.), is an important constituent of all replicating living cells since the genetic code of the organism is contained within its structure. The molecule is a long chain polymer and the monomeric unit, a nucleotide, consists of a phosphate group covalently bonded to a sugar ring similarly linked to a flat aromatic base. This base can be a purine, either adenine or guanine, or a pyrimidine, either thymine or cytosine. All four bases are found in D.N.A. so that adenine and thymine are present in equal molar proportions as are guanine and cytosine. A consideration of the stereochemical features of these bases led Watson and Crick to propose that they were hydrogen bonded together in pairs so that the two nucleotide chains formed a double helical structure [1]. Wilkins and his collaborators prepared fibres of oriented D.N.A. molecules and found that the structure of the polymers was sufficiently regular to obtain X-ray diffraction patterns [2] [3]. An analysis of the X-ray data showed the molecule had a helical shape supporting the existence of the D.N.A. double helix. In recent years, extensive analysis of well defined diffraction data and detailed model building has shown with increasing resolution that the double helix is not confined to a particular conformation.

The relevance of the D.N.A. base sequence in the living cell is seen in its function to provide a code to direct the combination of amino acids required to form proteins. Groups of three base pairs code for the insertion of a particular amino acid into a protein molecule. Separation of the complementary strands of D.N.A. exposes

the hydrogen bonding sequence of the bases. This could provide single stranded templates for the semi-conservative replication of two new double helices where complementary bases are hydrogen bonded in pairs once more. Thus identical code carrying molecules are produced which provide complete genetic information for all new cells formed. This close connection between D.N.A. structure and its important function has continued to stimulate the investigation of its conformation.

D.N.A. is just one of a large number of complex macromolecules present in the living cell and the stability and conformation of the helix is dependent upon the effects of this local environment. The nucleic acid is extracted from the host organism and purified to allow a careful study to be made of its physical properties free from the presence of other cell components. Any structural information about the polymer obtained on pure D.N.A. must then be considered in the context of the molecular environment in vivo.

1:1:b D.N.A. conformations

Fig. (1:1) shows a schematic representation of the D.N.A. molecule. It is a long polymer with a molecular weight of several million depending upon the host organism. Monomers are linked by a covalent bond between the phosphate O1 of one nucleotide and the sugar C3 of the next. The two polynucleotide chains are joined by hydrogen bonds between each pair of bases and the resulting double helix is stabilised with the hydrophobic bases in the centre and the negatively charged phosphate groups arranged externally. This leaves two helical grooves along the polymer between the sugar-phosphate chains. The molecular structure results in an approximate dyad axis through the plane of each base pair with all glycosidic

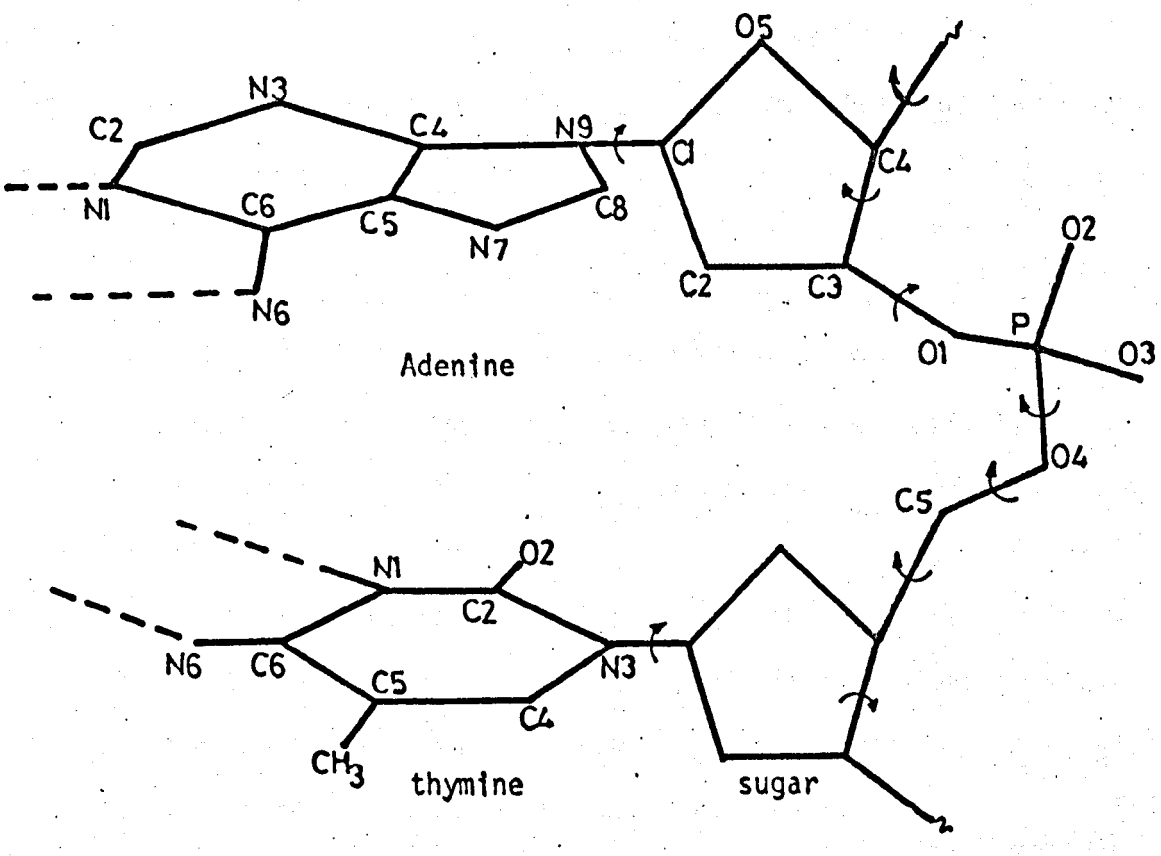


Fig. (1:1) Schematic representation of D.N.A. structure showing the nomenclature for pyrimidine and purine bases and the conformational angles that define the structure. Sugar pucker also needs to be defined.

links being equivalent and the two nucleotide strands being antiparallel.

The conformational freedom, studied in this thesis, is of particular interest since D.N.A. responds with specific structural changes to physical and chemical influences. The effect of changing the counter ion associated with the phosphate groups and also the humidity of D.N.A. specimens is to stabilise forms of the double helix with different helical parameters and crystal lattices.

These structural variants arise from a combination of sources of conformational freedom. The base pairs, which are effectively rigid planar groups with only slight flexibility of the hydrogen bonds, can not only tilt as a whole, relative to the helix axis, but also one base can twist relative to the other. Twist and tilt axes can be defined for these base pairs. One such definition is shown in fig. (1:2) after Arnott [4]. The pucker of the deoxyribose sugar ring also affects the overall conformation and a survey by Arnott and Hukins [5] reveals that C2 and C3 endo or C3 exo rings can be observed in nucleic acids whilst C2 exo has not been observed. Various bonds are free to rotate (shown in fig. (1:1)) and change the sugar-phosphate chain conformation. When these components are combined in the nucleic acid, the base tilt, sugar pucker and stereochemistry of the sugar-phosphate strands are interdependant, resulting in several distinct forms of D.N.A., each having a well defined conformation.

Three main conformations of naturally occurring D.N.A. have been observed where the helix parameters and atomic coordinates are known. Details of these molecular structures are given in fig. (1:3). The

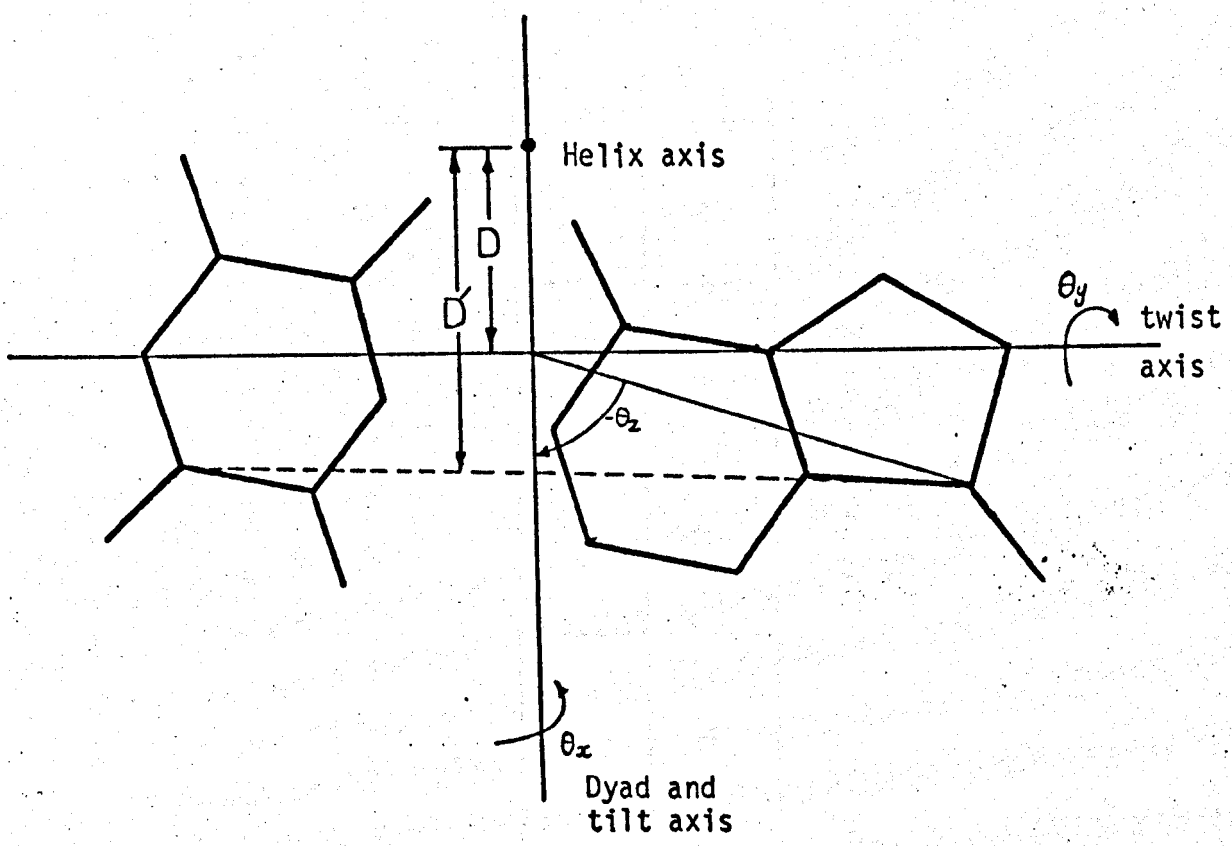


Fig. (1:2) Tilt, θ_x, twist θ_y and displacement D after Arnott et al [4]. D' is the displacement referred to in the construction of stretched models in chapter 8.

Form	sugar pucker	pitch (A)	residues per turn	rise per nucleotide (A)	distance D (A)	tilt (°)	twist (°)	ref.
A	C3 endo	28.15	11	2.56	4.72	20	-1.2	6
B	C3 exo	33.8	10	3.4	-0.16	-5.9	-2.1	6,4
C	C3 exo	30.9	9.3	3.22	-2.13	-6.0	5	7

Fig. (1:3) Helical parameters of three main conformations of D.N.A.

different forms[1] of D.N.A. occur in the presence of different cations and/or humidity [9]. The molecular environment effects not only the helix parameters but also its stability. If the D.N.A. poly-anion is dissolved in water with insufficient cations to neutralise the phosphate groups then the molecule denatures; the hydrogen bonds being weaker than the resulting electrostatic forces. The stability of D.N.A. in solution and the preparation of dried specimens with a controlled salt content was examined and is reported in Chapter 3.

Slight variations of major conformations have been reported. Bram reports some differences of the B conformation of D.N.A. [8] relating to the guanine - cytosine content of the molecule but the effect is not great and the work is so far only qualitative. Premilat and Albiser [10] report that the base pair composition of D.N.A. has a negligible effect on the diffracted intensities and they could account for slight variation of the B form diffraction patterns by slightly altering the base tilt. An earlier study of Hamilton et al [11] had also previously shown that D.N.A. from a wide range of species and diverse tissues, including cells with a high mitotic rate, gave identical X-ray diffraction patterns. So far, no variations of the A form of pure D.N.A. have been reported although in dried specimens, the intermolecular separation is dependent upon the relative humidity. Thus, previous workers have shown that there are distinct conformations of the D.N.A. molecule

---

1 Bram points out [8] that the term "form" should only be used for crystalline D.N.A. structures. The word is used here to mean any D.N.A. conformation.

with the possibility of only slight deviations occurring in the B form.

D.N.A. structure is so closely related to its function that it is possible that the different conformations are involved in the cell mechanisms. It is generally accepted that the B form of D.N.A. which is most stable at high humidities is the most probable conformation in solution in the living cell. Milman et al [12] have shown that a D.N.A. - R.N.A. hybrid which occurs during transcription takes the D.N.A. A form even at high humidities. Crick and Klug [13] conclude, from model building studies, that the B form of D.N.A. can have a kink in the helix and suggest that this conformational change is important for D.N.A. packing in chromatin. Thus the understanding of any new conformation of D.N.A. could also throw light on D.N.A. function. The subject of this study was to examine the conformation of D.N.A. firstly under the effects of tension applied along the molecule, and secondly as a result of the presence of two related antibiotics - Steffimycin and Steffimycin B. Both studies involved the investigation of changes in D.N.A. structure.

It is possible to stretch many long chain polymers and hence there is nothing unusual in the fact that D.N.A. should also show this property. This is particularly so when one considers that even though the B form is more extended than the A, the molecule is still in the form of a tightly wound helix with a large area of contact between base pairs. It is hoped that a study of the stretching of the D.N.A. molecule could increase the general understanding of its conformational possibilities. This is useful in considering its function as an information carrying molecule in the cell where

structure plays a crucial role in transcription and replication.

The effect of antibiotics on D.N.A. is an important area of research. Many such drugs are used to treat malignant tumours [14, 15] where the replication of cells proceeds in a seemingly uncontrolled way at many times the normal rate. Some antibiotics have been successful in the inhibition of the replicative process. When used to treat various forms of cancer, the rapid reproduction of cells has been slowed down or arrested at least for a time although a side effect is also to interfere with normal cell replication. One mechanism by which this is achieved is for the drug to render D.N.A. useless as a template for protein production and cell division [16]. Knowledge of the interaction of the drugs throws light on the nature of the replicative process, examines any specificity of the antibiotic action and provides guidelines for the development of further anti-tumour drugs.

1:2 Stretched D.N.A.

The investigation of D.N.A. by X-ray diffraction studies has involved the preparation of fibres of oriented molecules. Early attempts were made to improve the orientation of the helices by holding the fibre under tension. This was done by preparing it between glass rods attached to a spring which maintained the tension. During these experiments, Wilkins et al [17] discovered that if a sudden increase was made in the tension along the fibre, the D.N.A. molecule underwent a conformational change. The effect was observed by a sudden narrowing of the fibre with the formation of a neck the diameter of which was some 20% less than the original fibre. If the tension was maintained, the narrow region continued to extend along

the length of the fibre which elongated until the overall length was up to 2 times that of the original. Wilkins and his colleagues also noted that the extension of fibres on stretching was reversible and that the fibre dimensions varied with changes in humidity. (In this thesis, the term "stretching" refers to this process where the tension has caused a conformational change and a "stretched" fibre is the result.)

An oriented fibre of D.N.A. exhibits different refractive indices for polarized light depending upon whether the polarisation is perpendicular or parallel to the fibre axis. This birefringence is negative for well aligned fibres in the unstretched form but slightly positive for stretched D.N.A. Seeds reports that thin films of positively birefringent nucleic acid could also be prepared by shearing a D.N.A. gel between polished microscope slides [18]. This change in the birefringence was attributed to a variation in the tilt of the bases and these measurements, together with the changes in fibre dimensions, were used to postulate that the base pairs were tilted at ~45° to the helix axis as opposed to ~90° in B D.N.A.

X-ray diffraction studies of the stretched fibres performed by H.R. Wilson were not conclusive but showed that there was a strong reflection near the meridional at 5.4A with little or no 3.4 A meridional reflection [19]. This X-ray work was used to suggest that the stretched molecule was a greatly extended helix resembling a twisted step ladder with base pairs 5.4 A apart [20].

Later X-ray studies were carried out by W. Fuller who produced stretched fibres which gave good diffraction patterns. Such photographs not only gave a clear 5.7A spacing but also a layer line spacing of 30.4A. The 3.4A spacing associated with the base pair

separation in unstretched D.N.A. was still evident [21]. Equatorial reflections were indexed upon the basis of a hexagonal unit cell with a = 23.0A. There was, however, a general paucity of diffracted radiation with an absence of any intensities on layer lines other than those mentioned.

This X-ray data from stretched D.N.A. was very different from other forms of helical D.N.A. where diffracted intensities appear on well defined layer lines with a separation between them related to the helix pitch. The combined data from these previous studies of stretched D.N.A. indicated the possibility that the double helix had untwisted causing a tilting of the bases. At the same time, the molecular diameter decreased to give a smaller hexagonal lattice than non-stretched D.N.A. Attempts to build a model along these lines failed due to steric hinderances arising mainly from the compressed sugar-phosphate chain which resulted from the unwinding of the helix.

This thesis seeks to examine further the structure of extended D.N.A. molecules. In particular, studies were carried out to characterise the formation of this stretched form and its stability by varying such conditions as salt content and humidity. Birefringence measurements were used to investigate the extent of stretching and tilting of base pairs and an attempt was made to correlate the values of measurements made with the degree of alignment observed in X-ray diffraction patterns. Further X-ray work was carried out to establish the molecular spacings involved and to distinguish between reflections produced by stretched D.N.A. and those associated with any residual unstretched molecules.

The work on this novel form is interesting because of the unusual nature of the X-ray patterns and the possibility of a large unwinding effect which could give new evidence on the conformational flexibility of the molecule. It is suspected that D.N.A. is able to untwist to facilitate the replication process in the living cell, but the mechanism is not understood. Such a conformational contortion of the polymer could suitably position the code carrying base pairs for transcription. Conformational changes produced by tension applied to D.N.A. have consequences for any experiments which involve the application of mechanical forces to the double helix. The preparation of D.N.A. fibres for X-ray work and samples for electron microscope grids, for example, could distort naturally occurring conformations.

Increased base pair separation suggested by stretched D.N.A. occurs in the interaction of some antibiotics which intercalate into the D.N.A. helix. This particular method of lengthening the nucleic acid molecule is discussed in the next section.

1:3:a The Interaction of Antibiotics with Nucleic Acids

It has been noted for some years that many antibiotics are able to bind to nucleic acids. The aminoacridines were amongst the first to be intensively studied [22]. The molecules under investigation in this thesis, steffimycin and steffimycin B, are both antibiotics and have been found to interact with double stranded D.N.A. [23]. The result is to change the replicative and transcriptional template properties of D.N.A. This suggests that the antibiotics either block the transcription of the genetic code, possibly by forming an obstruction in the major or minor groove of the double helix, prevent

the separation of the double helical strands thus inhibiting replication, or interfere with the base sequence of the D.N.A. producing a frameshift mutation of the base pair code. The latter effect would result in a mutant variety of the original species or render the D.N.A. code meaningless preventing further transcription. This leads to the possibilities of the drugs being mutagenic as well as being chemotherapeutic. The tissues most susceptible to interference by these antibiotics are those which proliferate rapidly.

It is the purpose of this work to examine the effect in vitro and in the solid state of two antibiotics on both naturally occurring and synthetic D.N.A. The physical studies were specifically carried out to look at changes in the nucleic acid conformation as a result of the binding of drug molecules.

1:3:b Previous Studies of Drug Binding to D.N.A.

Much has been studied of the binding of drug molecules to the nucleic acid double helix. Previous work can be divided into:-

1. Experiments in solution, utilising the effects of absorption spectroscopy, [24,25,26,27,28,29], sedimentation coefficients, [30,31], viscosity [32,33], melting temperature [30,34], biochemical reactions [30, 35,36], fluorescent emission [25], polorographic behaviour [25], dichroism, polarised fluorescence, birefringence and electric dichroism all of molecules oriented by flow [37,38], kinetics of binding [39], X-ray diffraction of solutions [32], and
2. Experiments in the solid state on oriented specimens of the drug/nucleic acid complexes by X-ray diffraction

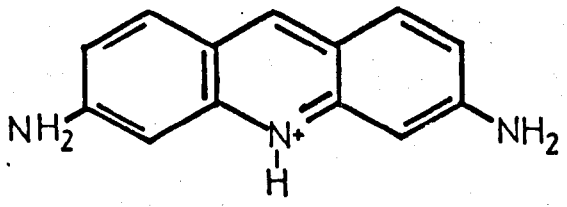
[28,30,40] and various optical studies such as birefringence and dichroism for example.

The results of all this work, although varied in details for each drug, suggest that there are often two types of interaction with D.N.A. Peacocke and Skerret called these two types weak and strong binding [24,41]. Lerman proposed that the strong binding was due to the flat drug molecule integrating itself between base pairs of the double helix: a process called intercalation [42].

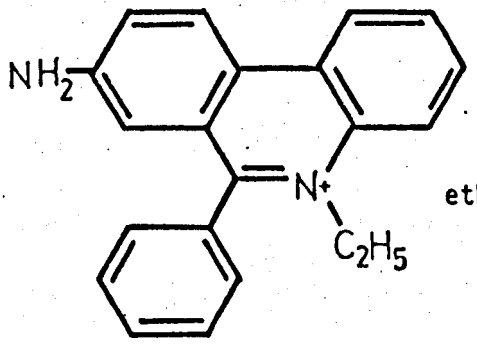
Strong binding was found to reach a maximum for 0.20 to 0.25 drug molecules per phosphate group, i.e. with a phosphate to drug ratio, P/D, of 4 or 5. It is suggested that the electrostatic and hydrophobic forces between D.N.A. and drug give rise to the weak binding which becomes important when the intercalation sites are saturated. This involves the drugs binding externally to the D.N.A. helix and includes the possibility of drugs aggregating together [28] [41]. This behaviour is not true for all drugs which bind to D.N.A. but summarises types of interaction which need to be considered in the examination of a new antibiotic.

Those drugs that inhibit D.N.A. dependent R.N.A. synthesis can do so by intercalating into the double helix, but many are cytotoxic through some other mechanism [31] [36]. If a molecule intercalates into D.N.A. then it appears to require a flat grouping of three or sometimes two aromatic rings [29]. Examples of such intercalating chromophores are the acridines e.g. proflavine, and ethidium bromide. Examples of non intercalating drugs that interfere with D.N.A. are spermine with no ring system and Irehdiamine where the ring system is puckered. See fig. (1:4).

As a result of intercalation there is an extension of the D.N.A.

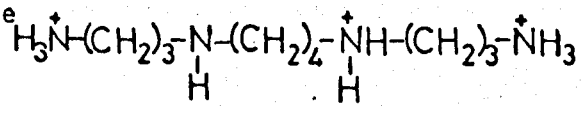


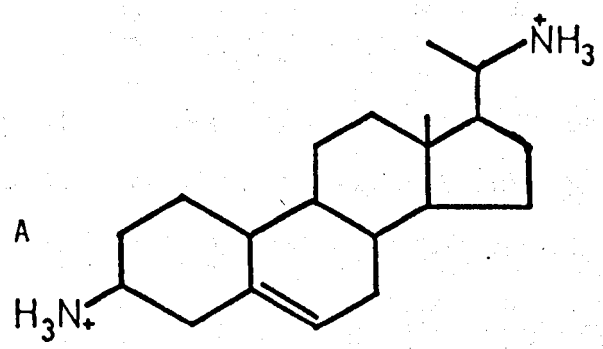
proflavine



ethidium

spermine





Irehdiamine A

Fig. (1:4) Structural formulae of some compounds which bind to D.N.A.

molecule due to the antibiotic being positioned between base pairs [43,44]; and a change in mass per unit length which depends on the molecular weight of the drug [32]. X-ray diffraction studies have shown a retention of the base separation of 3.4A, an increase in helical pitch and a decrease in intermolecular separation of the D.N.A. molecules [45]. Models proposed for intercalation and intended to account for this evidence suggest an unwinding of the D.N.A. helix by 12° [30,46] although other values have also been suggested. In these models, the interbase separation remains constant and a conformational change in the sugar-phosphate chain allows for a maximum of one drug intercalated for every two base pairs [47]. From energy and stereochemical considerations [29,40] it is believed that several of these D.N.A./drug complexes are stabilised by hydrophobic interactions between D.N.A. base pairs and the drug aromatic ring system [30], and by hydrogen bonds.

Factors influencing the extent of intercalation are pH, presence of other ionic species and relative concentrations of the drug and nucleic acid. Humidity is found to influence the helical parameters and intermolecular separation in dried specimens. Even for the strongly bound drug where intercalation is evident, it is likely that not all the drug molecules are intercalated [28,39].

Physical methods of investigation of the two anthracyclines in this work were U.V. and visible spectroscopy and X-ray diffraction. Spectroscopic methods were used to determine the relative numbers of drugs and base pairs involved in complex formation. X-ray diffraction yielded information on the helical parameters when the drug was bound to the double helix. This information was then used to propose a model for the D.N.A./drug complex.

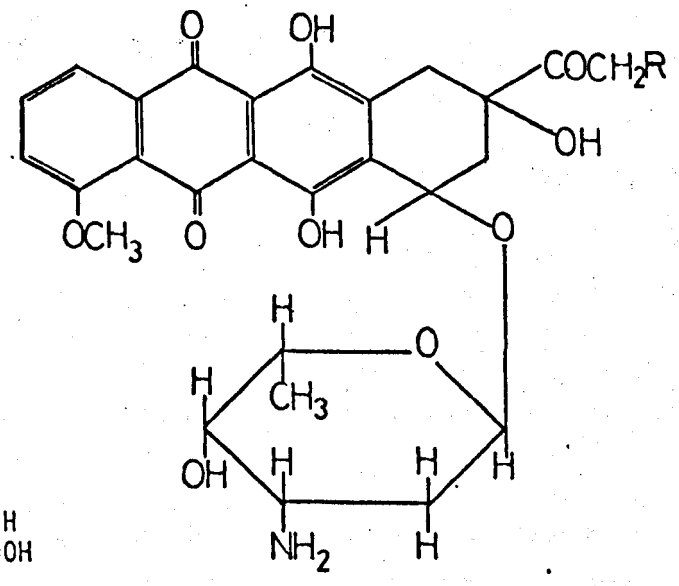
1:3:c Steffimycin and Steffimycin B

These two antibiotics were synthesised in the culture broth of a strain of Streptomyces. Steffimycin was isolated from Streptomyces steffisburgensis, in 1967 [48], and both Steffimycin and Steffimycin B from Streptomyces elgreteus, in 1974 [49].

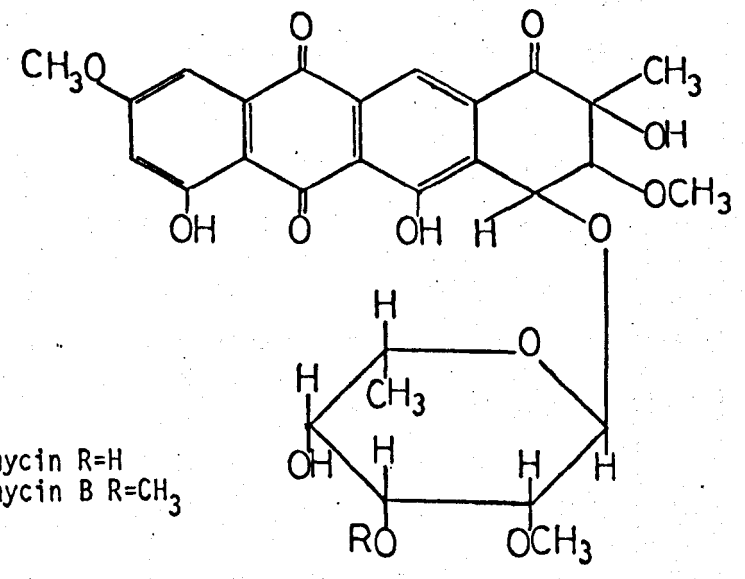
Steffimycin inhibits gram positive bacteria in vitro and is cytotoxic against mammalian cells grown in vitro. It appears to have little in vivo activity. Steffimycin B shows similar effects with bacteria. In vivo it has not demonstrated antibacterial activity in experimentally infected animals but has significantly inhibited the growth of mouse leukemia cells in vitro.

The steffimycins are anthracyclines consisting of an anthraquinone chromophore substituted with a sugar. Steffimycin B differs from steffimycin in that it possesses an additional methoxy group on the sugar. The antibiotics are similar to daunomycin and adriamycin, the main difference being that these two drugs have an amino group in the 3^{1} position on the sugar. Structures of these molecules are shown in fig. (1:5). The steffimycins possess the flat aromatic groups which have been found to allow intercalation with D.N.A. in studies on previous drugs. Specimens of both drugs are in the form of minute orange crystals and are more soluble in non polar solvents. The alcoholic or aqueous solutions are orange when neutral or acidic and become violet upon addition of base.

Steffimycin was found to bind to double stranded D.N.A. as indicated by its inhibition of D.N.A. directed R.N.A. synthesis when the drug was added to the D.N.A. template. Inhibition was not total but was more evident when the template used was poly d(A-T). This data, together with the results from difference spectroscopy



Daunomycin R=H
Adriamycin R=OH



Steffimycin R=H
Steffimycin B R=CH_3

Fig. (1:5) Structures of anthracyclines used in these studies

experiments, was used to suggest that steffimycin bound specifically to adenine-thymine base pairs [50]. Reusser makes two qualifications on this work: firstly, that no information was obtained as to which particular base of these two was involved and secondly, that the experiments do not exclude the possibility of interactions also with the guanine or cytosine residues in the D.N.A. helix.

Base specificity has also been reported for steffimycin B binding to D.N.A. [23]. This was shown by its inhibition of D.N.A. polymerase when the antibiotic was added to the D.N.A. template. The reactions were only inhibited when poly d(A-T) was used as template. Poly(dG) poly(dC) - primed R.N.A. and D.N.A. polymerase reactions were shown to remain unaffected by steffimycin B. Reusser reports that reactions primed with natural D.N.A. were insensitive to steffimycin B inhibition and suspects that the complexes formed between the antibiotic and natural D.N.A. significantly differ in some way from complexes with poly d(A-T) as regards their transcriptional properties. Steffimycin B, therefore, showed preferential binding to poly d(A-T) compared with poly(dG) poly(dC) but any specificity of interaction with natural D.N.A.'s was not clear. Base specificity has been noted for other intercalating drugs. Actinomycin, for example, requires guanine to allow binding to the double helix [51].

In this work the binding of the steffimycins to D.N.A. was examined by U.V. and visible spectral studies of drug/D.N.A. solutions. Base specificity of drug interaction was investigated in synthetic polynucleotides and also in various naturally occurring D.N.A.'s with differing A-T content. The experiments were extended

to compare the steffimycins with daunomycin, adriamycin, actinomycin and nogalamycin. Oriented steffimycin/D.N.A. specimens were then prepared with various ratios of the phosphate to drug ratio which were used to obtain X-ray diffraction patterns of the complex. Again, the complexes were made using D.N.A.'s of a range of A-T contents. Analysis of the results showed that there was an increase in the helix pitch of D.N.A. as found with intercalation. Helix parameters were found to be a function of P/D ratio and humidity. The steffimycins were also observed to leave intercalation sites and crystallise out in the presence of oriented D.N.A. This work is reported in detail in Chapters 4 and 5. A model for the D.N.A./ steffimycin complex is proposed in Chapter 6.

Chapter 2 MATERIALS AND EXPERIMENTAL TECHNIQUES

2:1 Materials

2:1:a Deoxyribonucleic Acids

The nucleic acids used were Calf Thymus D.N.A. from Sigma Chemical Co. Type V and Miles Research Laboratories, batch (36-155-2), both were repurified; Cℓ Perfringens D.N.A., Sigma Type XII, batch 104C-6870 and M Lysodeikticus D.N.A., Miles research Lab. batch 23-338-17. Poly d(A-T) and poly d(G-C) were obtained from Boehringer, Germany. The molar extinction coefficient was taken as E = 6600 at 258 n.m. in all cases; a value midway between Peacocke and Walker [52] and Rusconi [53]. The dependence of this value on salt concentration is considered in chapter 3:1. D.N.A. solutions were prepared at 0.003 molar in phosphate, 0.003 molar sodium chloride. Tris (Tri(Hydroxymethyl) methylamine)/HCℓ maintained pH7.

2:1:b Steffimycin and Steffimycin B

Samples of these antimetabolites were a gift to Dr. W.J. Pigram from Dr. F. Reusser, Upjohn Co., U.S.A. They consist of small orange crystals with M.W. of 574 for steffimycin and 588 for steffimycin B [49]. The antibiotics had been purified from the culture broth by chromatographic methods and crystals were prepared by precipitation from isopropanol. The samples supplied were used without further purification.

Both antibiotics were only slightly soluble in water but more so in alcohol. Reusser noted that steffimycin B was less polar than

steffimycin from its behaviour in chromatographic separation. Solubilities are given in fig. (2:1). Saturated aqueous solutions of these antibiotics were not sufficiently concentrated to prepare D.N.A./drug complexes with P/D less than 10. P/D's in the range 4 to 10 were made by using solutions of the drug in 20% ethanol, 80% H_2O. This resulted in an ethanol content of the complexes below that which would cause precipitation of D.N.A.

Typical absorption spectra of the drugs in the U.V. and visible regions of the spectrum are shown in fig. (2:2). The drugs show two absorption spectra - for pH < 8.5 where the solutions are orange, and for alkaline solutions, pH > 8.5 which are purple. The work on binding of drugs to nucleic acids was performed at pH 6.7-7.0.

Spectra of the drugs differ according to the solvent used. The extinction coefficients given by Reusser were for steffimycin in methanol and steffimycin B in ethanol. In order to determine concentrations of the drugs in the aqueous solutions used in these experiments, the variations of the extinction coefficients as a function of the water content of the solvent was calculated. This was achieved by preparing a solution of the drug in alcohol and determining its concentration using the known extinction coefficient, diluting aliquots of this solution with known volumes of water and measuring their U.V./visible spectra. Since the drug concentrations were known, new extinction coefficients could be calculated for the complete range of alcohol/water mixtures. Extinction coefficients for the absorption peaks at different alcohol contents are shown in fig. (2:3) and (2:4).

Drug	solvent			ref.
	H_2O	methanol	20% ethanol	
steffimycin	<870μM	>870μM	-	48
	50μM	-	190μM	this work
steffimycin B	12μM	1500μM	25μM	this work

Fig. (2:1) Table showing the solubility of steffimycin and steffimycin B.

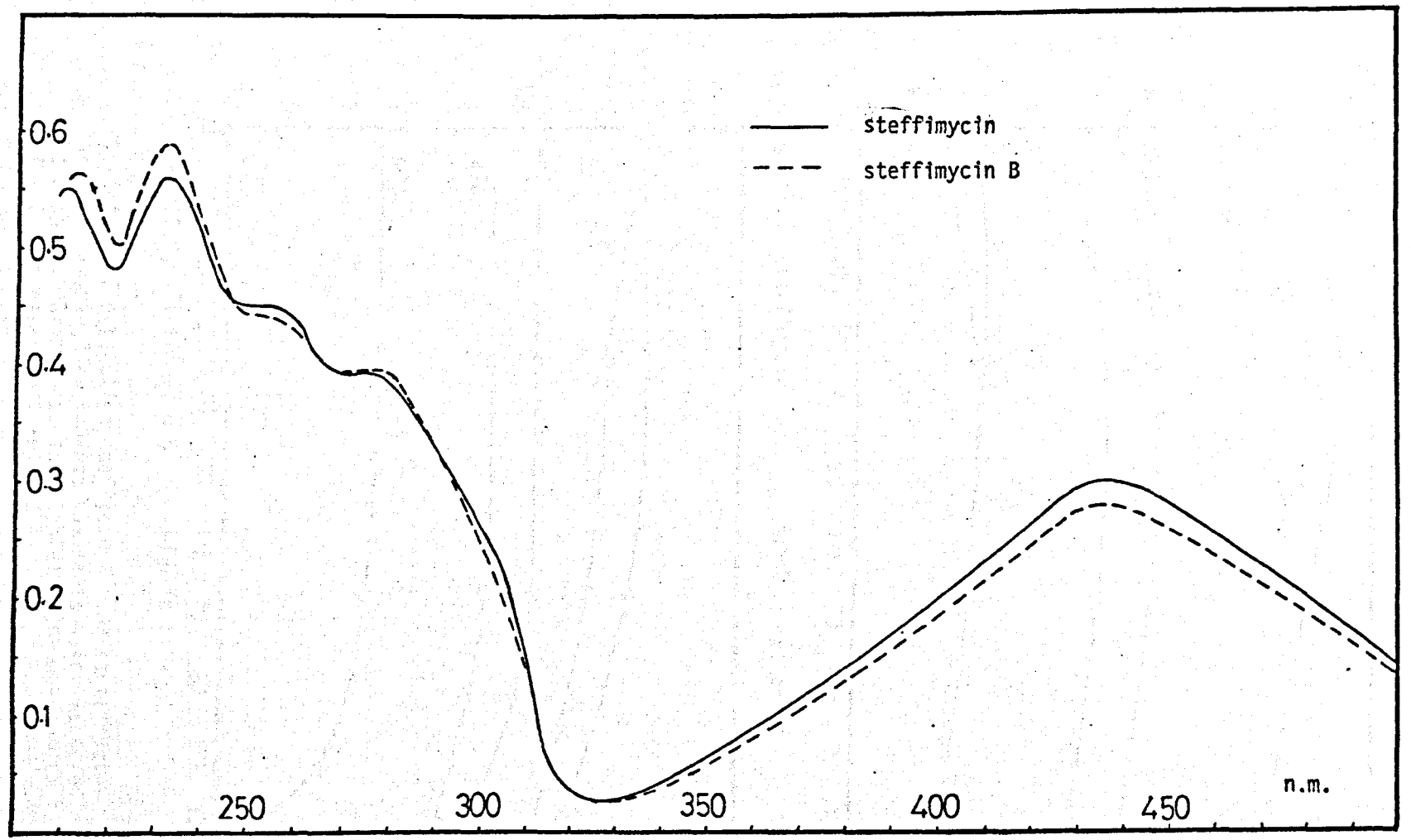


Fig. (2:2) Absorption spectra of steffimycin and steffimycin B in methanol at 20μM

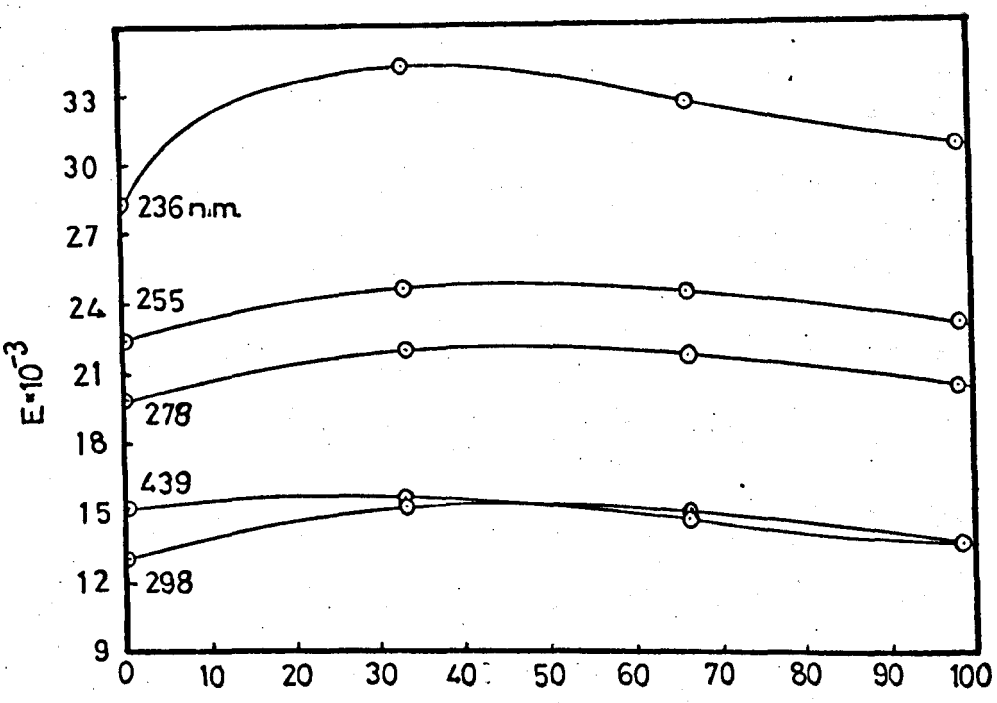


Fig. (2:3) steffimycin %H_2O in methanol

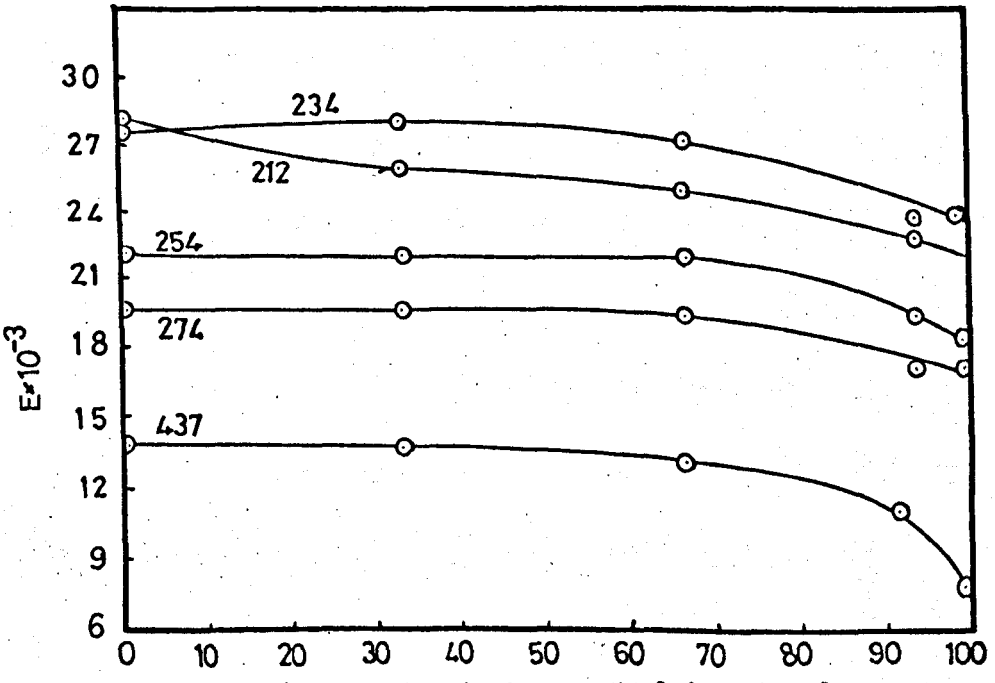


Fig. (2:4) Steffimycin B %H_2O in ethanol

Variation of extinction coefficients for steffimycin and steffimycin B absorbance maxima as water is added to alcohol. Graphs are labelled with the wavelength of the absorbance peak in n.m.

2:1:c Other drugs used

1. Daunomycin hydrochloride ($C_{27}H_{29}NO_{10}HCl.\ H_2O$) was a gift to Dr. Pigram from Farmitalia, S.A. Farmaceutice, Italia. Lot 11/A. The extinction coefficient in the visible region at 475 n.m. is quoted by Rusconi [53] as 9860.
2. Adriamycin, ($C_{27}H_{29}NO_{11}HCl.\ H_2O$) also a gift to Dr. Pigram from Farmitalia. Lot 2020. Extinction coefficient used was 9860 for visible absorption peak.
3. Actinomycin D was supplied by Sigma Chemical Co. Lot 52C-0951-9. Anhydrous molecular wt. 1247.5. Extinction coefficient used was 25000 [53,54].
4. Nogalamycin (U-15, 167) was a gift from the Upjohn Co., Kalamazoo, Michigan, to Dr. Pigram. Lot no. 6741-THP-129-18. Extinction coefficient was not known and hence the concentration could only be estimated and the results are qualitative.

Solutions of the drugs were prepared using glass distilled water and were stored in the dark at 4°C. It was necessary to use 10% ethanol to obtain solutions of nogalamycin of sufficient concentrations for spectroscopic work. The steffimycin solutions were stored at room temperature since crystals formed from the saturated solutions if the temperature was lowered.

2:2 Spectroscopic analysis of solutions

The single absorption peak of the deoxyribonucleic acids at around 260 n.m. was used to determine concentrations in solution. This absorption is in the U.V. region of the spectrum and becomes negligible at wavelengths of 350 n.m. and above. It is in this visible region of the spectrum, 350-500 n.m., that the spectroscopic

investigation of the binding of the steffimycins and other drugs to D.N.A. was carried out.

The spectroscopic investigation used a method developed by Peacocke and Skerrett [24] who studied the binding of proflavine to herring sperm D.N.A. These workers followed the change in the visible spectrum of a solution of proflavine as the amount of D.N.A. was increased. It was essential that Beers law held for both free and bound drug in the concentrations, ionic strength and pH used. The procedure was to dissolve the drug in phosphate buffer containing NaCℓ. A series of samples of this drug solution was prepared, each sample with a different concentration of D.N.A. Distilled water was used to give an identical final volume to all the samples. The spectra of these complexes were then plotted showing the change in absorbancy between the free and bound drug.

Waring [26] prepared his samples by mixing D.N.A. and drug solutions of the maximum P/D ratio to be examined. Lower P/D ratios were then prepared by the addition of more drug solution at the same concentration as the drug in the original mixture. In this way, the absorption spectra were plotted to follow the change from bound to free drug whilst keeping the concentration of the drug constant and diluting the D.N.A.

The method used in this work was to plot the spectrum firstly of the pure drug solution. Aliquots of D.N.A. solution were then added and successive spectra obtained as more and more drug was bound. A correction to the absorbance was made to allow for the increase in volume of the mixture. Spectra can then be obtained of the drug, both free in solution and bound to D.N.A. Providing these two are the only conditions in which the drug can exist then

an analysis of the spectra of any P/D ratio in solution can be made. The theory is developed in chapter 4.

Spectral studies of the binding to D.N.A.'s were carried out for all the drugs mentioned in sections 2:1:b and 2:1:c. Visible and U.V. spectra of drugs and D.N.A.-drug complexes in solution were produced on a Cary 118 spectrophotometer using 1cm. path length cuvettes. The studies of complexes formed in solution were performed by placing stock solutions of drug in a cuvette with 0.004 M NaCℓ, pH 6.7. Aliquots of D.N.A. solution of identical Na+ content and pH were added to the drug solution. The average time between mixing the components and plotting the spectra was two minutes. Binding reactions were found to be completed well within this time period. No further change in the spectrum was found after ten minutes or after one hour in selected samples. No precipitation of drug or complex was observed except for Daunomycin. All spectra of the complexes were adjusted for the slight change in volume due to the addition of the nucleic acid. Drug concentrations were used between 10μm and 50μm and D.N.A. concentrations between 1000μm and 5000μm.

2:3 Preparation of D.N.A./drug complex fibres

2:3:a Preparation of Gels

Gels of steffimycin and steffimycin B complexes with various D.N.A.'s were made with a known P/D ratio so that samples could be dried as oriented fibres for the X-ray studies. The required ratios were prepared as solutions and placed in 10ml centrifuge tubes. The

nucleic acid content was calculated to be 1-2mg to ensure the formation of sufficient gel, and the drug content was adjusted to give the desired P/D. Distilled water and NaCℓ solution were added to give a volume of 9.5mℓ and Na+ concentrations of between 0.001 and 0.01M. In order to obtain steffimycin/D.N.A. complexes of P/D < 10 and steffimycin B/D.N.A. of P/D < 20 it was found necessary to use 20% ethamolic solution of the drugs. This resulted in a maximum ethanol concentration of 13% by volume in the centrifuge tube when diluted by the other components. Extracts from centrifuge tubes were taken before centrifugation for spectroscopic analysis to verify the D.N.A. and drug contents for some runs. The tubes were then centrifuged on an M.S.E. Superspeed 50 TC centrifuge with a 10 x 10 mℓ fixed angle rotor at 40000 r.p.m., ∿ 150000g.

After centrifugation, the supernatant was decanted and kept for spectral analysis to determine the D.N.A. and drug concentrations remaining in solution. This facilitated the calculation of the P/D of the complex forming the gel. The sediment was drained and the last drops of supernatant liquor were removed by Pasteur pipette. The viscosity of the resulting gel was greatest for the higher salt and drug concentrations.

2:3:b Orientation of samples

In order to obtain the maximum information from X-ray diffraction studies of D.N.A. and the complexes, it is necessary to orient the molecular helices along one direction in an arrangement referred to as a fibre. The fibres were formed by placing a drop of gel between the rounded ends of two glass rods as described by Fuller et al [55]. The rods were supported in a cell

which could be sealed to allow control of humidity and also permitted the separation of the rod tips which was normal practice during drying to improve the uniformity of the fibre and orientation of the helices.

It was found to be detrimental to dry the gels too quickly thus allowing the outer surface to dry first. The interior then dries onto the outer skin which causes the fibre to collapse from its ideal cylindrical shape. This problem was avoided by drying at 92% relative humidity or by sealing the cell which allows the humidity to remain high. Fibres were prepared at 20°C although some were formed at 4°C for very slow drying. In this way, oriented specimens of D.N.A. and the complexes were prepared with a diameter of about 100 microns and length of one to two millimetres.

As a result of the alignment of the helical polymers, the fibres are birefringent. Birefringence was measured with Zeiss rotary compensators; Quartz for path differences up to 7λ and Calcite up to 140λ.

2:4:a Stretched D.N.A. fibres

It is possible, whilst in the process of drying a gel of D.N.A. between the tips of glass rods, to separate the rods and increase the length of the resulting fibre. In this way, elongated specimens of a centimetre or more in length can be formed. In order to produce a fibre of D.N.A. in the "stretched" molecular conformation; it is not sufficient merely to draw out the gel during drying. Stretched fibres were prepared by allowing the D.N.A. gel to dry in the normal way but just before drying was complete, a sudden but

smooth extension was performed. The conformational change was identified by a narrowing of the fibre commencing at one point referred to as a "neck". This narrow region then extended along the fibre usually in both directions as the D.N.A. took up the stretched conformation.

It was necessary to design a way to prepare a stretched fibre and maintain its tension so that it remained stable for the 48 hours required to obtain an X-ray diffraction pattern. Thus a "stretching frame" was designed for this purpose and is shown in fig. (2:5). This stretching frame provided a means of preparing a stretched fibre, taking X-ray photographs and storing the specimen with the fibre still attached to the tips of two glass rods and under longitudinal tension.

2:4:b Operation of the Stretching Frame

A description of the use of the apparatus shown in fig. (2:5) and fig. (2:6).

The stretching frame consists of two brass blocks. One (1) is free to slide along two parallel steel rods which are fixed in the other block (2). Block (2) was secured in a stationary position by the cheese head screw (3) through the fibre pulling cell (4) shown in section. Block (1) is secured to brass rod (5) by screw (6). This rod can be pushed in or out of the cell controlled by the knurled screw (7). The glass rods (8) used to prepare the fibre from the gel are secured on the brass blocks by Araldite, and mounted along one axis so that when screw (7) is turned, the rod on block (1) moves in the line of the rod on block (2).

The gel was placed between the glass rods in the usual way and

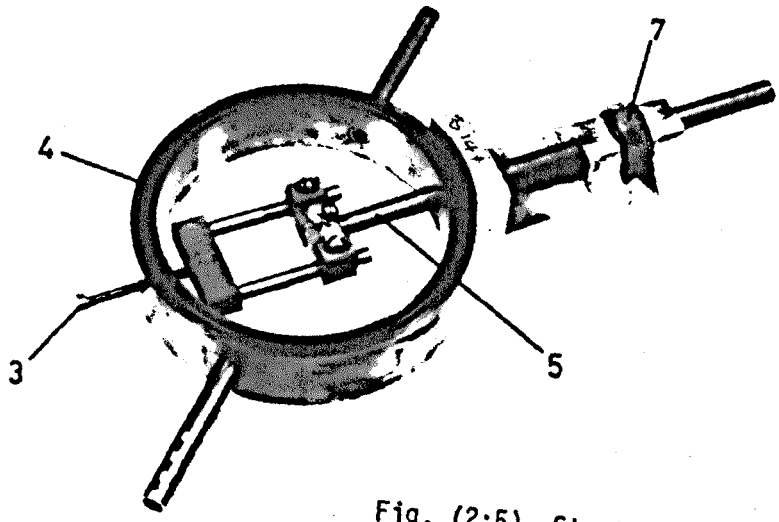


Fig. (2:5) Stretching frame in fibre pulling cell

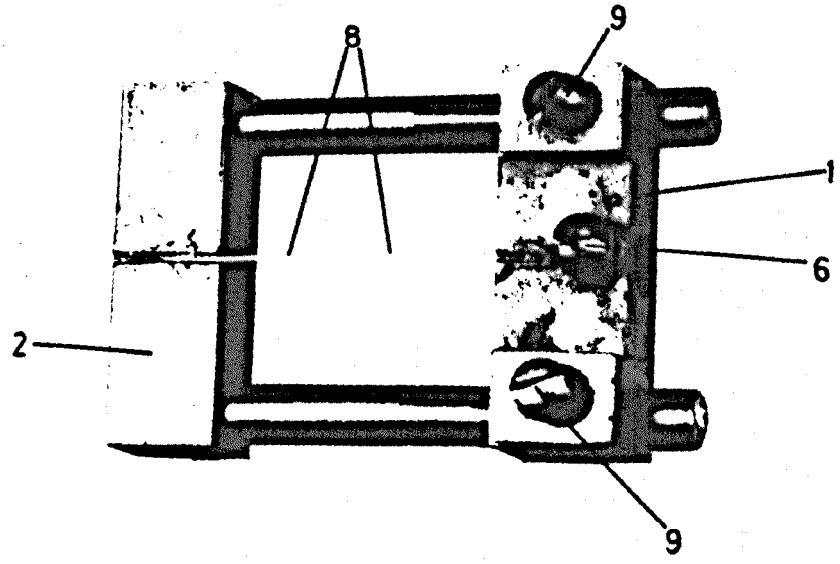


Fig. (2:6) Stretching frame

stretched when almost dry. The fibre was kept under tension and the stretching frame was released from the fibre pulling cell by clamping the sliding block onto the steel rods using the clamping screws (9), and releasing screws (3) and (6) which hold the frame in position. The frame was then used to mount the fibre in the X-ray cameras. The holder was designed so that it would not interfere with the X-ray beam, and could fit into either the pinhole or the Searle cameras used in this work. Fibres mounted in this way were found to remain in the stretched form after several months of storage and could be used to produce several X-ray patterns.

2:5 X-ray Diffraction

2:5:a Cameras

Two types of camera were found suitable for recording the X-ray diffraction from D.N.A. fibres out to spacings of 3A. Microcameras, with a set of three gold apertures to collimate the X-ray beam, were used following the original design of Chesley [56]. The specimen to film distance in these cameras was about 3cm. Searle cameras using toroidal or Franks optics with specimen to film distance set at around 4cm were also used. The toroidal optics produced a well collimated beam which was annular in cross section. The segment of the annulus used to produce diffraction patterns was of the same area of cross section as the fibre dimensions and was focussed to a point on the X-ray recording film. The Franks optics produced a fine monochromatic beam with a diameter less than that of the fibre although the intensity is low necessitating exposure times longer than the toroidal optics.

Both types of camera were sealed from the atmosphere and were fitted with inlet and outlet orifices to allow the continual passage of helium gas. The atmosphere of the camera scatters X-rays proportionally to the square of the number of electrons per atom or molecule. Scattering is at a minimum by hydrogen molecules since the two electrons are shared by two nucleii, whereas helium scatters to a slightly greater extent because the two electrons are confined to one nucleus. Nevertheless, helium was chosen in preference to hydrogen for safety reasons despite the higher cost.

Use of helium also enabled a constant relative humidity to be maintained by previously passing the gas through an appropriate salt solution. In order to ensure a known relative humidity of the atmosphere surrounding the fibre, a pot of the same solution was also placed in the camera. Conditions inside the camera were allowed to stabilise by flushing with humidified helium for half an hour before irradiating.

2:5:b X-ray Generators

Two types of X-ray sets were used. Hilger and Watts microfocus sets with nickel filtered CuKα radiation of 1.54A, operated at 35kV, with a tube current of 3mA. The exposure time on these sets for a 100μ fibre with a 3cm specimen to film distance was 48 hours. The other set used was an Elliott rotating anode, again with CuKα radiation with a 60mA tube current at 35kV. Exposure time with a 4cm specimen to film distance was commonly about 4 hours using toroidal optics and 48 hours with Franks optics in a Searle camera.

Stretched fibres presented some difficulties in obtaining

satisfactory X-ray photographs. Firstly the fibres tended to be about 50μ diameter compared with ∿ 100μ for an unstretched fibre necessitating a four fold increase in exposure time. Secondly, only part of a D.N.A. fibre could be obtained in the stretched conformation and the size of the beam produced by toroidal optics was often too large to select out this section of the specimen. In this case either pinhole cameras or Franks optics in the Searle camera had to be used.

Vaterite powder ($CaCO_3$) was used to calibrate the D.N.A. fibres. This particular crystalline form was chosen instead of calcite because it is easily obtained as a fine powder being prepared by precipitation. The powder is inert and anhydrous, and because it has a smaller particle size than calcite, is easily sprayed onto a fibre using a wash bottle adapted for the purpose. It gives a uniform ring at 3.40A as opposed to the irregular circle of reflexions that calcite often produces. The disadvantage is that this diffraction ring coincides with the 3.4A meridional reflexion of D.N.A. However, the former is so sharp and the latter so intense that there was no interference with essential data.

2:5:c Measurement of diffraction patterns

The reflexions on the X-ray pattern were measured with a two dimensional travelling microscope. A Joyce-Loebel microdensitometer 3CS was utilised to measure intensities and this could also provide the radial positions of some reflexions.

2:6 Model Building

Two types of model were used in the examination of the D.N.A.

structures. Firstly C.P.K. atomic models [57], providing a space filling representation of a molecule with dimensions of the component atoms corresponding to a scale of 1.25cm per A. They were used to examine the stereochemical feasibility of the binding of the steffimycins to D.N.A. A model of the D.N.A. double helix was built where the central rod holding the bases in position was made from two sections. These could be separated and clamped to allow the double helix to reveal an intercalation gap with the appropriate degree of unwinding [45]. Various possibilities for intercalation and external binding could be quickly examined and possible hydrogen bonds, Van der Waals contacts, and electronic interactions between drug and D.N.A. could be considered.

Secondly, rigid skeletal models with a scale of 4cm per A were used when an accurate representation of interatomic distances and bond angles was required. Cylindrical polar coordinates were measured using a pointer attached to a vertical vernier scale for the z direction, r and ϕ being marked on the baseboard.

Computer programs were used to construct, compare and refine atomic models.

2:7 Use of Computer

Several different computer programs were used during the course of this work and a brief description of their functions is given because of the essential part they played in processing the data. All were concerned with model building except "Film" which processes diffraction pattern data.

"Bonds and Angles"

This program calculates bond lengths and angles between atoms along a chain, given the cylindrical polar coordinates, r, ø, and z. A quick check could then be made of the conformation of known molecular groups. Written by the author, it was used on an H.P. 9100B Calculator.

"Bonang"

Given the coordinates of atoms on a model, this program computes all covalent bond lengths and angles, dihedral angles and distances between "non bonded" atoms. This enabled an analysis to be made of the conformation of a proposed model. The program was written by the author for use on the Elliot 4130 computer at Keele based on an original version by W. Fuller.

"Model Build"

This program refines a proposed molecular model to give a more favourable conformation. A molecule is considered as a single chain with side branches for this procedure. The aim of the refinement is to minimise Van der Waals interactions and to conform the molecule to a set of weighted constraint equations which relate to known fixed parameters such as helical repeat distance. A set of least squares equations are solved to produce shifts in the dihedral angles of the atom chain. The modified coordinates can then be used as data for successive refinements until the energy of atomic interactions and the deviation from constraint conditions reaches a minimum. The program was rewritten by Mr. D.C. Goodwin from the work of W.J. Pigram [45] and run on the C.D.C. 7600

computer at the Manchester Computer Centre.

"Data Prep"

The input data for this program are the atomic coordinates of a trial model, distinguishing between main chain and pendent atoms. Covalent bond lengths, dihedral angles and direction cosines, which define the positions of pendent atoms, are all calculated. The molecule is related to a set of axis. This data is used to provide the information required to run "Model Build" and was written by the same authors again for the Manchester computer.

"Film"

Written by W.J. Pigram, this program processes the measurements taken from diffraction patterns. The coordinates of the centre of the pattern are determined by a least squares method from measurements of the 3.40A vaterite ring and the positions of the reflexions are corrected to allow for the use of a flat recording film. The print out gives the coordinates of the centre, specimen to film distance, X and Y coordinates of reflexions, ξ, ζ, ρ and the Bragg spacing, d. The program was written in Algol and run on the Elliot 4130 at Keele University.

"Helix 1"

This program calculates the value of the cylindrically averaged Fourier transform for a single molecule. This is generated from the relationship:-

$$G(R, n, \ell/c) = \sum_j f_j J_n (2\pi R r_j) \exp i (2\pi \ell Z_j/c - n\phi_j)$$

where r_j, ϕ_j, z_j; are the cylindrical polar coordinates of the jth atom, f_j is its structure factor, R is the reciprocal space radius, ℓ the layer line separation and c the crystal repeat in the z direction. Jn is an nth order Bessel function where n is an integral solution of $n = (\ell - mN)/K$, N/K is the ratio in whole numbers of the translation per residue divided by the helix pitch. m is any integer and its range is defined in the program so that the Bessel functions considered are those contributing significantly to the Fourier transform. The transform is summed over all atoms in the nucleotide with suitable weightings introduced so that all four bases can be taken into account.

The program was rewritten by D.C. Goodwin [58] from an original by W. Fuller [59], and run on the C.D.C. 7600 at the Manchester Computer Centre.

Chapter 3.

THE PRESENCE OF SODIUM CHLORIDE IN D.N.A. SAMPLES

At pH values greater than 2, the phosphate groups on the D.N.A. molecule are completely singly ionized so that the salt form of the acid is studied in this work. Sodium D.N.A. was used in the studies of stretched D.N.A. and in the formation of complexes with the steffimycins. Thus, sodium chloride was often involved in the purification and preparation of D.N.A. solutions, gels and fibres and it is the effect of sodium and chloride ions upon the nucleic acid which is the subject of this chapter. The studies are concerned with the concentration of sodium chloride in D.N.A. solutions which prevents denaturation of the double helix, and the determination of the relative amount of salt and D.N.A. in sedimented gels and fibres together with the effect this has on D.N.A. conformation.

3:1 Purification of D.N.A.

The different D.N.A.'s used in this work had been extracted from the host organism and purified commercially. The Miles calf thymus D.N.A., for example, is claimed to be free of excess salt and to be 95% pure with a molecular weight in excess of a million. Other components present in the sample are mainly R.N.A., <2%, and protein, <1%. All the D.N.A.'s used, however, were repurified with the exception of the synthetic polynucleotides. The method devised by Langridge et al [60] was adapted for this purpose with the intention of improving the quality of the X-ray diffraction patterns

and the reliability of the D.N.A. interactions with antibiotics. The D.N.A. was not assayed subsequent to purification but the improvement in the quality of the X-ray photographs was taken as an indication of increased purity.

The purification procedure was carried out at ~4°C and pH~7 and involved:-

1. Dissolving D.N.A. in 0.003M sodium chloride solution to a concentration of 1mg/ml - a stable environment for D.N.A. in solution.
2. Centrifuging at 3000 r.p.m. in an M.S.E. bench centrifuge for about 15 minutes to remove any contamination by macroscopic particles.
3. Gently agitating the decanted solution for 20 minutes with an equal volume of freshly distilled phenol which was saturated with 0.1N NaCℓ. Protein precipitates out at the interface between the aqueous and phenolic phases [61].
4. Centrifuging again at around 3000 r.p.m. for 15 minutes to separate the phases and pipetting off the aqueous layer containing the D.N.A. from the phenolic layer.
5. Reprecipitating D.N.A. from the aqueous phase by adding the solution to an equal volume of propan-2-ol. The D.N.A. immediately precipitates whereas if the alcohol was added to the D.N.A. solution, the nucleic acid precipitates gradually. R.N.A. impurities remain in solution at this stage [62].
6. Collecting the white fibrous precipitate on a glass stirring rod, washing in 80% ethanol to remove excess

salt; in 95% ethanol to remove water; and finally in acetone to remove ethanol and facilitate drying due to its high volatility.

7. Drying the D.N.A. in a dessicator at 1°C either over P_2O_5 or under vacuum.

3:2 Effect of salt on D.N.A.

The D.N.A. poly anion in solution is highly charged along its length due to ionisation of the two phosphate-sugar chains. The molecule is stable when in the form of a salt with monovalent cations e.g. Li, K, Na [63]. Multivalent cations produce complexes probably due to cross linking between phosphate groups on adjacent helices. Water molecules serve to stabilise the double helix in vivo and in vitro. This is very possibly due to some interstitial arrangement of the solvent in the D.N.A. lattice. The interactions are likely to be electrostatic and involve hydrogen bonding due to the polarity of water molecules and phosphate groups, but the arrangement of the solvent molecules in the hydrated double helix is not fully understood. Various workers have suggested the stabilisation of D.N.A. is due to the formation of an "ice-like" structure around the helix or the formation of a "water bridge" between phosphate groups and cations. [64] [65] [66]. I.R. absorbance spectral studies on the hydration of D.N.A. films by Falk et al [67] have revealed that the existance of ice like layers of H_2O surrounding D.N.A. is improbable and in their review, Bloomfield et al. [68] suggest it is most likely that there is only one layer of partially ordered water around D.N.A.

The presence of salts in a dried specimen of D.N.A. plays an

important role in the hydration of the molecular environment probably by fixing water around the polymer due to deliquescent properties. Several workers have shown that the salt content of D.N.A. and the humidity of its environment are major factors determining the conformation of the macromolecules [69] [70] [71]. Briefly summarised, there are three major forms, A, B and C, in which naturally occurring D.N.A.'s can be observed. The C form occurs with Lithium salts while the A form is found with low sodium chloride concentrations of less than 5% by weight. With the salt concentration around 5% then an A to B transition occurs in the same fibre as the relative humidity is raised from 66 to 92%. The B form persists at all relative humidities with higher salt concentrations. These forms have different molecular conformations and crystal structures [70] [72]. When there is excess salt (i.e. $[Na^+]>[PO_4^-]$) present in a dried specimen of D.N.A., the B conformation is stabilised with the bases perpendicular to the helix axis [69].

The ease with which a fibre can be made to take up the stretched conformation and the birefringence of the resulting specimen is influenced by its sodium chloride content. These effects will be discussed later in Chapter 7 but they were important enough to necessitate an examination of the salt content of oriented fibres. Experiments were carried out to determine salt concentrations in D.N.A. solutions, in D.N.A. samples used and in D.N.A. sedimented gels. The studies are confined to sodium chloride since this was the only salt associated with D.N.A. throughout this work.

3;3 A method of determining the salt content of D.N.A. specimens

The purpose of these studies was to be able to predict the

conformation (A or B) of D.N.A. fibres given the salt concentration of the solution used to centrifuge the gel. Cooper and Hamilton [70] had shown that the conformation of D.N.A. in a fibre was dependent upon its salt content. The question now asked is - What is the relationship of salt concentration in the centrifuge tube to salt content in the resulting gel and hence in the fibre?

In their analysis, Cooper and Hamilton determined the chloride concentration of many D.N.A. fibres which provided a direct method of determining how much sodium chloride was present in the D.N.A. fibre. Since chloride analysis of samples as small as D.N.A. fibres requires techniques that are tedious, it was decided to develop an alternative method to investigate salt contents. The intention was to measure the sodium ion concentration in the gels and solutions using flame emission spectroscopy. This is not as direct as chloride analysis since the sodium present is associated with both chloride and phosphate groups. Sodium chloride estimation can only be made by assuming that the difference between total Na^+ concentration and the Na^+ concentration associated with the phosphate is due to the presence of salt.

Details of the technique are discussed below where appropriate. It must be pointed out however that the aim was to develop a rule of thumb technique so that a gel could be prepared of which one could be reasonably confident about the molecular conformation when dried in fibres. Are sodium chloride contents of gels reproducible? Does the volume of gel, D.N.A. concentration or pH effect the imbibation of salt? These are questions on which this analytical method could throw some light.

A full analysis of the physical chemistry of D.N.A. gels in

ionic solutions is beyond the scope of this work. No special techniques were used other than those normally involved in the preparation of D.N.A. samples. It is these standard procedures that the experiments of this chapter are aiming to calibrate. Only those aspects of the results that are pertinant to the immediate projects are analysed in detail.

3:4 Effect of ionic strength on solutions of D.N.A.

3:4:a Denaturation and change in extinction coefficient

Under various conditions it is possible to denature or degrade the D.N.A. molecule. The hydrogen bonds linking the two polynucleotide chains break under conditions of denaturation giving regions of single stranded molecules. The molecule is said to degrade when covalent bonds are broken and it fragments into shorter sections. Both the denaturation and degradion processes can be followed by observing the absorption peak of nucleic acid which occurs near 260 n.m. The absorption coefficient is around 40% greater for single nucleotides than for the intact double helix [73]. Denaturation will take place in solutions with pH>9 [68] or with low ionic strength [63]. A low ionic strength does not lead to any fragmentation of the molecule but an increase in the absorption coefficient at 260 n.m. can be seen.

It is important to determine this variation in the extinction coefficient of D.N.A. at different ionic strengths of NaCℓ in order to measure the concentration of D.N.A. in any sample solution. In experiments to determine this variation, a series of samples was prepared in which the D.N.A. concentration was the same in each and

of known value, but where the sodium chloride concentration differed. This was achieved by dialysing a 100ml. D.N.A. solution (1.2mg/ml) in 0.02M NaCℓ against distilled water at 4°C. The dialysis tubing was impermeable to D.N.A. molecules but freely allowed the passage of sodium and chloride ions and water molecules. Three ml. samples of the Na. D.N.A. solution, pipetted from the tubing after various time intervals, had gradually decreasing salt contents.

The absorption spectrum of the solution outside the tubing showed that there was no significant transfer of D.N.A. through the semi-permeable membrane. This bath was replaced with fresh distilled water after every sample was taken. The change in volume of the D.N.A./NaCℓ solution inside the tubing was less than 0.5ml. indicating that there was a negligible passage of water molecules either way through the membrane due to osmotic pressure. Thus concentrations of the D.N.A. in solution remained constant.

The samples extracted from the dialysis tubing were analysed for apparent phosphate and actual sodium cation concentrations. Apparent phosphate concentrations were measured on a Cary Spectrophotometer using E = 6600 for D.N.A. at 260 n.m. This PO_4^- concentration measured agrees with the real, known concentration only when no denaturation has taken place. The absorption coefficient for D.N.A. in each sample was then determined.

If the D.N.A. solution obeys the Beer-Lambert Law:-

$$E_i = \frac{\text{absorbance of D.N.A. solution} \times E}{\text{absorbance of native D.N.A. solution}}$$

where E_i = extinction coefficient at ionic strength i.

E = extinction coefficient of native D.N.A.

The sodium ion concentration was measured on a Unicam SP 1900

Flame emission spectrophotometer (F.E.S.). The F.E.S. was first calibrated by measuring the intensity of the sodium D line when standard solutions of NaCℓ were injected into a gas flame. A typical calibration curve, needed for each separate series of test solutions, is shown in fig. (3:1). When samples from dialysis were passed into the flame, the intensity of the radiation at 589 n.m. indicated the absolute concentration of sodium. Any other ions present emit radiation at other wavelengths and do not interfere with these measurements.

Sodium ion concentrations can be measured to a greater accuracy than 0.00005 N; around 1 to 2% of the concentrations measured. The spectrophotometric determination of D.N.A. concentration is accurate to around 4%. The maximum error of a stated sodium to phosphate ratio is therefore about 6%. The error in the calculated extinction coefficient also around 6% since it is dependent upon the accuracy of sodium and phosphate concentration measurements.

The extinction coefficient for D.N.A. as a function of ionic strength is shown in fig. (3:2). These results show that in order to measure the concentration of D.N.A. it is important to use the appropriate coefficient. If the Na^+ concentration is lower than one per phosphate, then the extinction coefficient should be increased accordingly.

3:4:b Effect of increasing the ionic strength

Denaturation of D.N.A. due to low ionic strengths involves the separation of the two sugar-phosphate chains. Complementary bases are able to hydrogen bond together once more if the ionic strength of such denatured D.N.A. is increased but do so in a random and

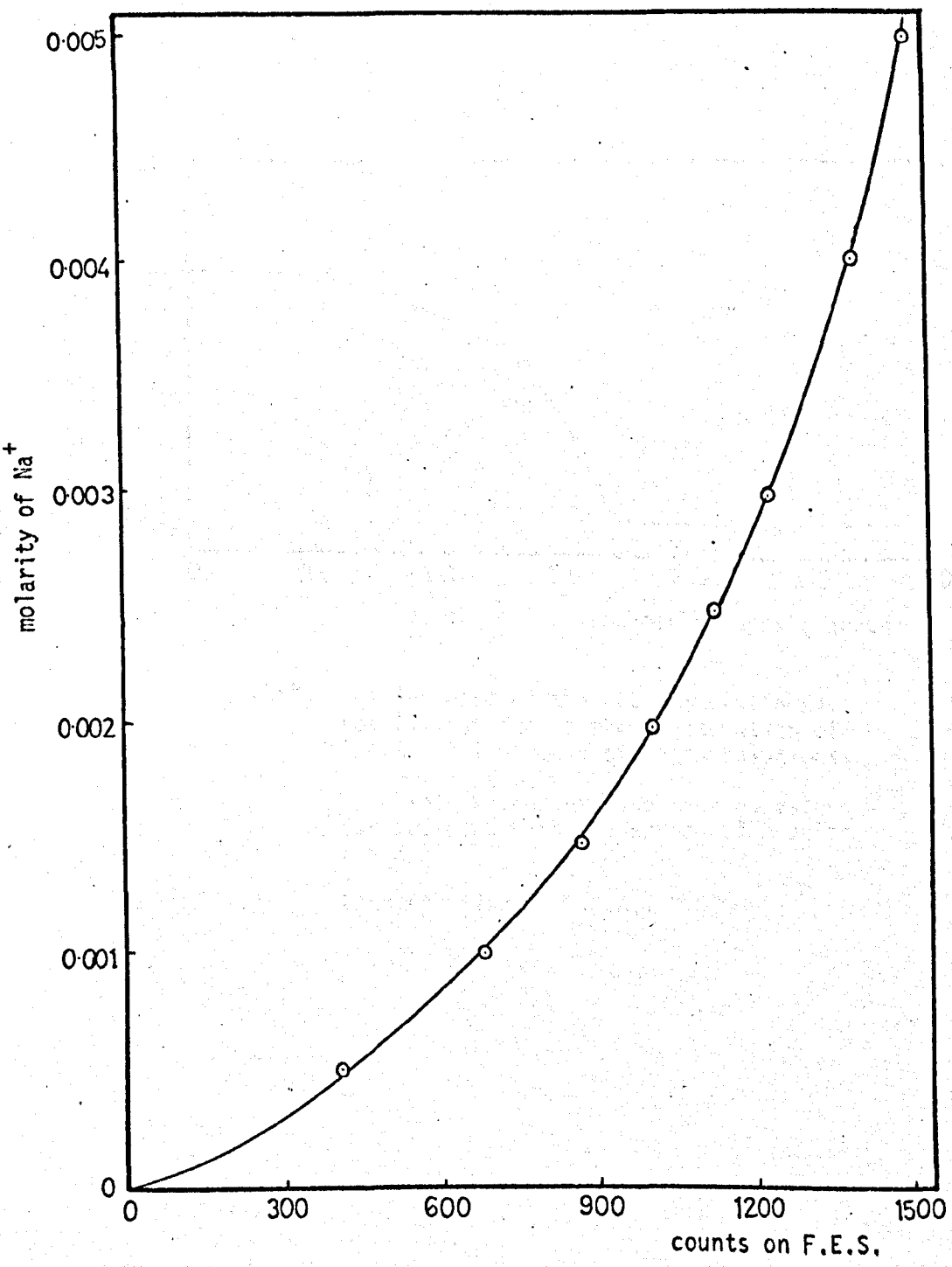


Fig. (3:1) Typical calibration curve for the concentration of sodium cations in solution against the count on the flame emission spectrophotometer.

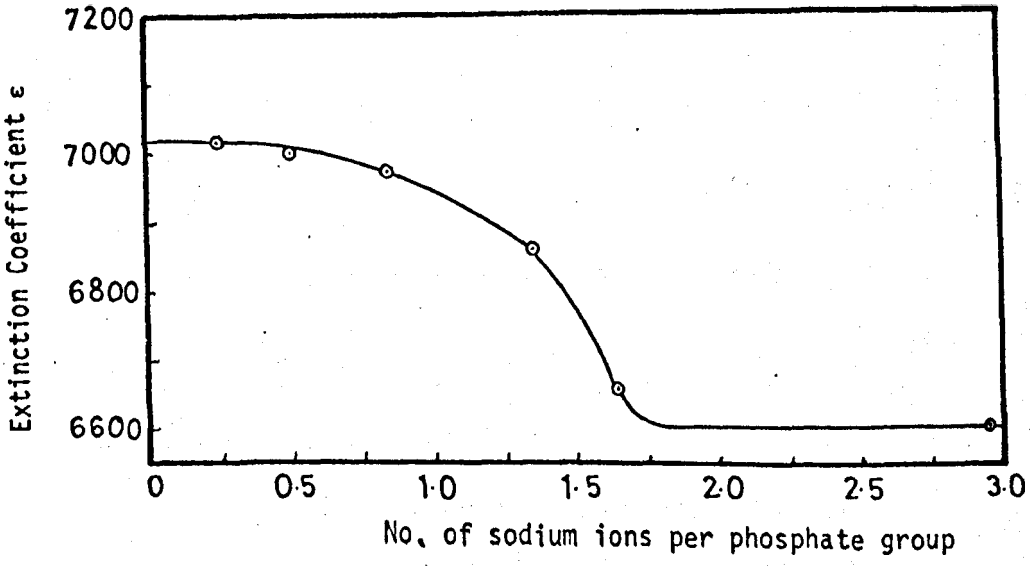


Fig. (3:2) The increase of the 260 n.m. extinction coefficient due to the denaturation of D.N.A. at low ionic strength solutions.

This represents the average values for two runs with the spread of results $\varepsilon = \pm 300$

Concentration of D.N.A., 0.006M.

incomplete way. This partial renaturation was observed by following the change of the extinction coefficient for a solution of D.N.A. as the sodium chloride concentration was increased. Fig. (3:2) shows that the value of the extinction coefficient for D.N.A. with Na/PO_4^- ratio less than one is 7000. When the ionic strength was raised to 0.0065M with 2 N_a^+ for every PO_4^- then the extinction coefficient was reduced to a value of ~6800 compared with 6600 for intact double stranded D.N.A.

This indicated that D.N.A., originally dissolved in distilled water and subsequently having its salt concentration raised to >0.003M, has a coefficient slightly higher than that of the undenatured molecules. If experiments are to be performed on D.N.A., therefore, it is important to dissolve it in sodium chloride solution of at least 0.003M to be certain that the helix is not denatured.

3:5 Examination of D.N.A. used in this work

The Na : phosphate ratio was measured for several unpurified commercial samples of sodium D.N.A., and for samples of purified D.N.A.. Results are shown in fig. (3:3). The ratio of sodium ions to phosphate is less than unity indicating that other cations perhaps including H^+ were present. The Ca^{2+} concentration, the most likely contaminant, was measured by F.E.S. and found to have a concentration in glass distilled water of less than 0.00001M, an amount which is negligible. Purification of D.N.A. decreases the Na^+/PO_4^- ratio still further possibly because sodium ions may be removed by the 80% ethanol used to wash the precipitate. Saline ethanol made using 0.003M sodium chloride would be a better washing

Sample	pH	molarity D.N.A.	molarity Na^+	na^+ per PO_4^-
B.D.H.	4.3	0.0066	0.0050	0.76
Sigma V	6.4	0.0059	0.0045	0.76
Miles VI	6.4	0.0027	0.00275	1.02
purified C.T.	6.2	0.0025	0.00150	0.60
purified C.T.	6.3	0.0016	0.00096	0.60
purified C.T.	6.1	0.0014	0.00091	0.65

Fig. (3:3) Na^+ to PO_4^- of some D.N.A. samples

agent.

3:6 Salt content of D.N.A. sediments

3:6:a Examination of Na^+ content of gels

Oriented fibres of sodium D.N.A. were made by allowing a gel to dry between the tips of two glass rods. This gel was prepared by ultracentrifugation of a D.N.A./NaCℓ solution. Under some conditions, not all of the D.N.A. would sediment and the percentage of D.N.A. recovered as a pellet is shown in fig. (3:4) as a function of the NaCℓ concentration in the centrifuge tube. The role of sodium chloride in the solution is to mask the electrostatic repulsion between the D.N.A. helices thus preventing denaturation and permitting sedimentation.

Salt concentrations of between 0.002M and 0.05M are required to sediment sodium D.N.A. from a solution of around 0.001M in phosphates. At lower concentrations of salt, the D.N.A. may produce a thin unworkable gel or none at all, whilst at higher concentrations sodium chloride crystals tend to form when the gel dries. Knowledge of salt and D.N.A. concentrations in the solution used for centrifugation does not give any information about the salt concentration in the gel itself. Because of this, an examination was made to determine whether there was any change in the relative concentrations of sodium and phosphate in the gel after centrifugation compared with their concentrations in the original solution.

Rupprecht et al. has developed a method of making D.N.A. fibres by a wet spinning process [74] in which D.N.A. solution was ejected through a nozzle into an aqueous ethanolic bath. This caused the

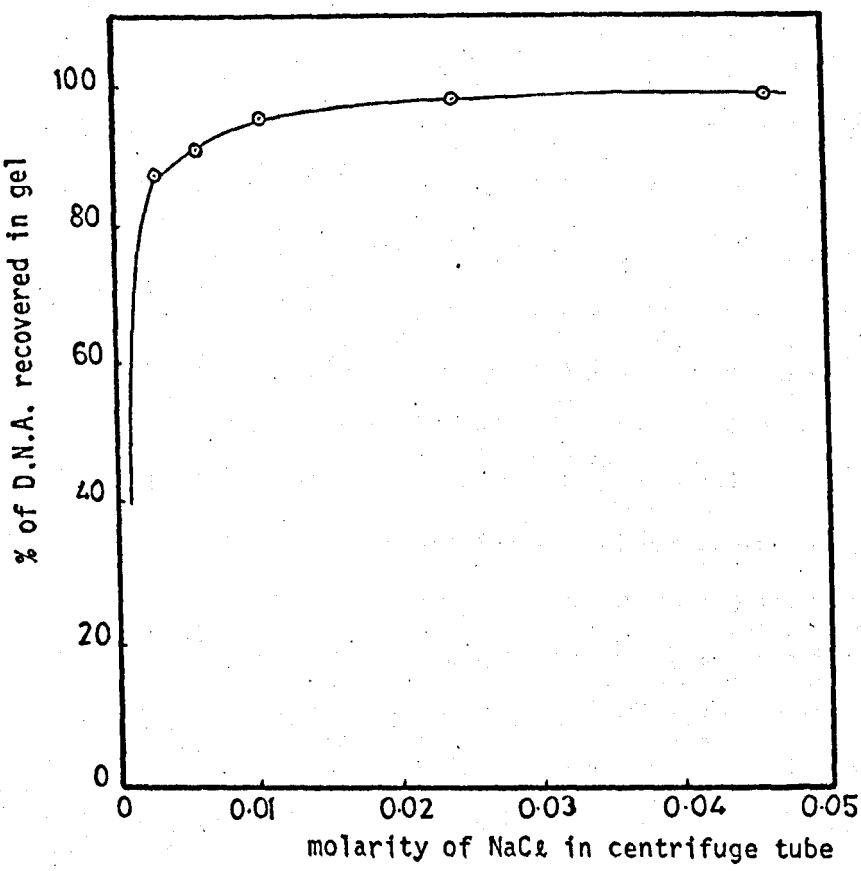


Fig. (3:4) Percentage of 2 m.g. sample of D.N.A. recovered from a 10 ml centrifuge tube after sedimentation as a function of the sodium chloride concentration Centrifugation for 4hrs at ∿ 50000 r.p.m.

immediate precipitation of a long fibre which was wound on a teflon cylinder, and provided a simple way of controlling salt concentration in the precipitate. The D.N.A. was cut off the cylinder, washed and analysed for salt and phosphate content.

The method chosen to investigate salt content in fibres in this work differed from that of Rupprecht in order to obtain information about the components of fibres prepared from gels. Gel preparations were carried out in the presence of a range of sodium chloride concentrations. The supernatant liquor was poured off and the last traces were removed either by Pasteur pipette or by washing briefly in 80% ethanol. The gel was then redissolved in distilled water where the phosphate and sodium ion contents were determined. Analysis of the supernatant liquor showed that the sodium chloride concentration was unchanged and revealed the extent of D.N.A. precipitation. Na^+ concentration was again measured by flame emission spectroscopy of the sodium D line and phosphate concentration by the 260 n.m. absorption.

Firstly the $[Na^+]:[PO_4^-]$ ratio was determined for the D.N.A. sample used in the experiment. Let this ratio be a:b where a and b are the respective concentrations of sodium and phosphate. Upon centrifugation, sodium and chloride ions from solution will be present in the D.N.A. gel. If we call this NaCℓ concentration c, then the ratio of $[Na^+]:[PO_4^-]$ in the gel is (a + c):b. When the gel was assayed then the total sodium content measured was (a + c). This excess sodium concentration, c, was calculated simply by subtracting the concentration of sodium present in the D.N.A. sample used, from the concentration found in the gel, (a + c) - a.

Graphs (3:5) (3:6) (3:7) (3:8) show that the ratio of sodium

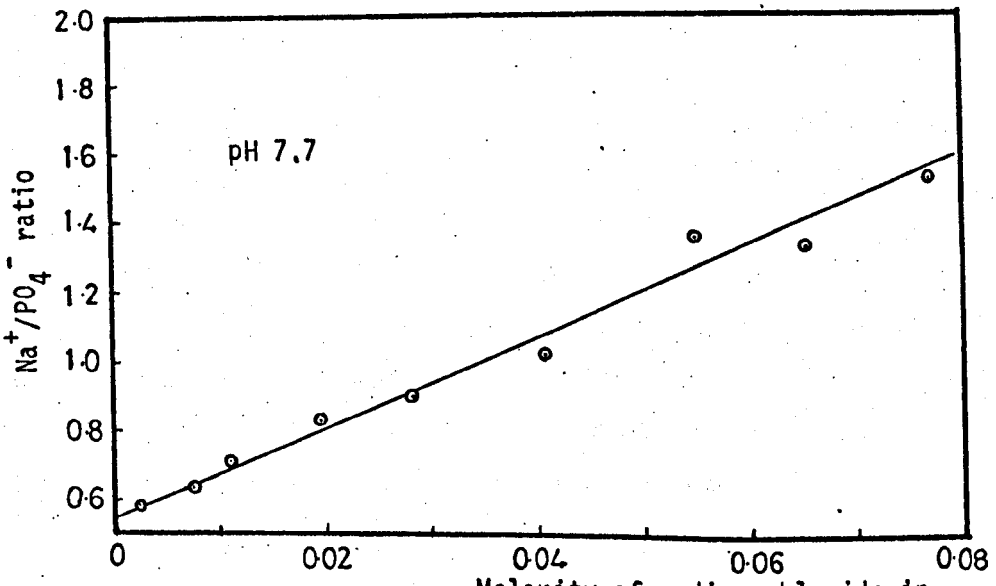


Fig. (3:5) Run 1

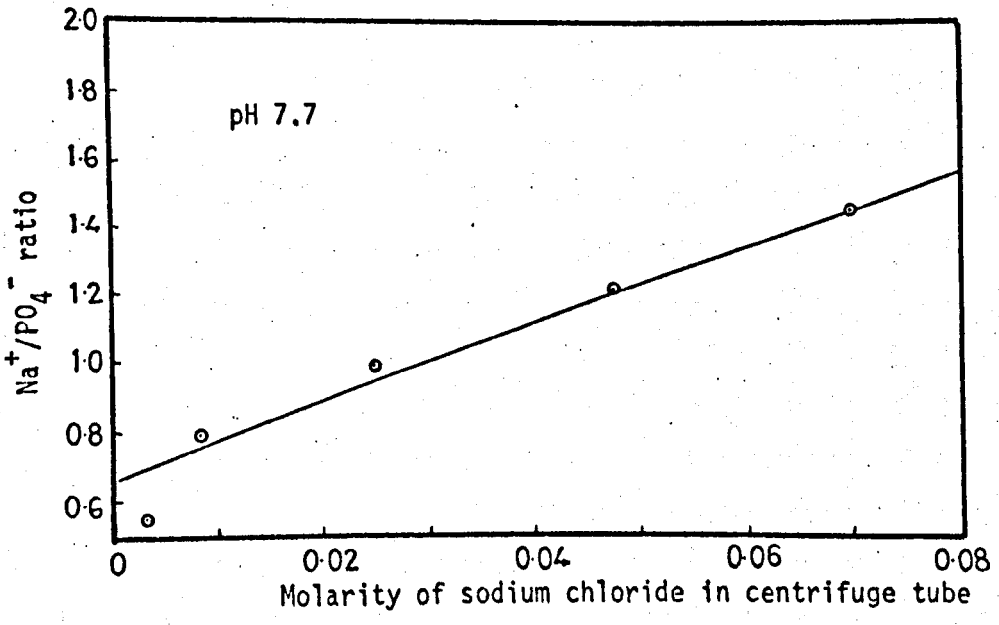


Fig. (3:6) Run 2

Na^+/PO_4^- ratio in sedimented gel as a function of the sodium chloride concentration in the original solution

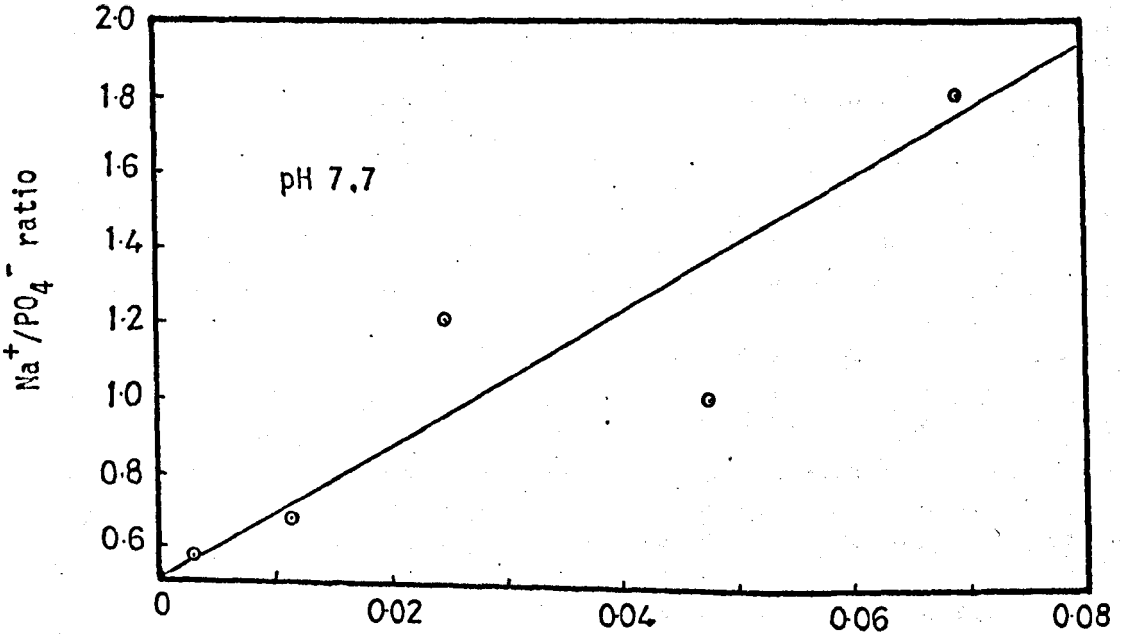


Fig. (3:7) Molarity of sodium chloride in centrifuge tube Run 3

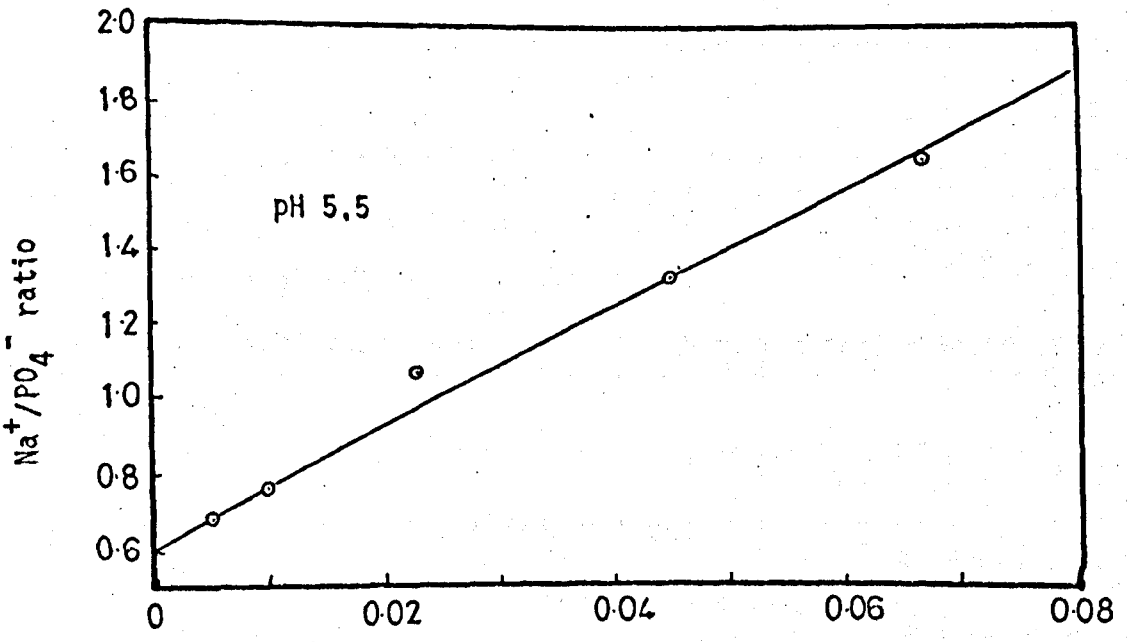


Fig. (3:8) Molarity of sodium chloride in centrifuge tube Run 4

Runs 3 and 4. Na^+/PO_4^- ratio in sedimented gel as a function of the sodium chloride concentration in the original solution

ions present for each phosphate group in the gel increases linearly with the NaCℓ concentration of the supernatant liquor. The graphs show that for four separate runs, the uptake of salt occurs to a slightly different extent and that the Na^+ to PO_4^- ratio in the original samples was slightly variable being between 0.5 and 0.6. Graph (3:9) restates the information from these four runs in terms of the NaCℓ content in the final gel as a function of salt concentration in the centrifuge tube. The result is a linear relationship with a slight spread of results. This trend was also obtained by Rupprecht who obtained straight lines for the % of NaCℓ in the fibre against NaCℓ concentration of the bathing solution, yet also with differing slopes. In general, Rupprecht used a higher range of salt concentrations. The range of salt concentrations used in these experiments coincided with those commonly used to prepare gels.

The experiment set out to determine the concentration of sodium chloride in the gel. Hence graph (3:9) shows NaCℓ content at zero for a theoretical gel produced with no salt in the centrifuge tube. This salt content then increases with the increasing molarity of the supernatant liquor.

3:6:b Dependence upon D.N.A. concentration

The possibility that the volume of the gel produced upon centrifugation influenced the NaCℓ content was examined. Gels which were prepared with identical NaCℓ molarity in the centrifuge tube but different D.N.A. concentrations were then analysed for Na^+ and PO_4^- content. Results are shown in fig. (3:10). The effect of gel volume was found to be negligible within experimental errors and is

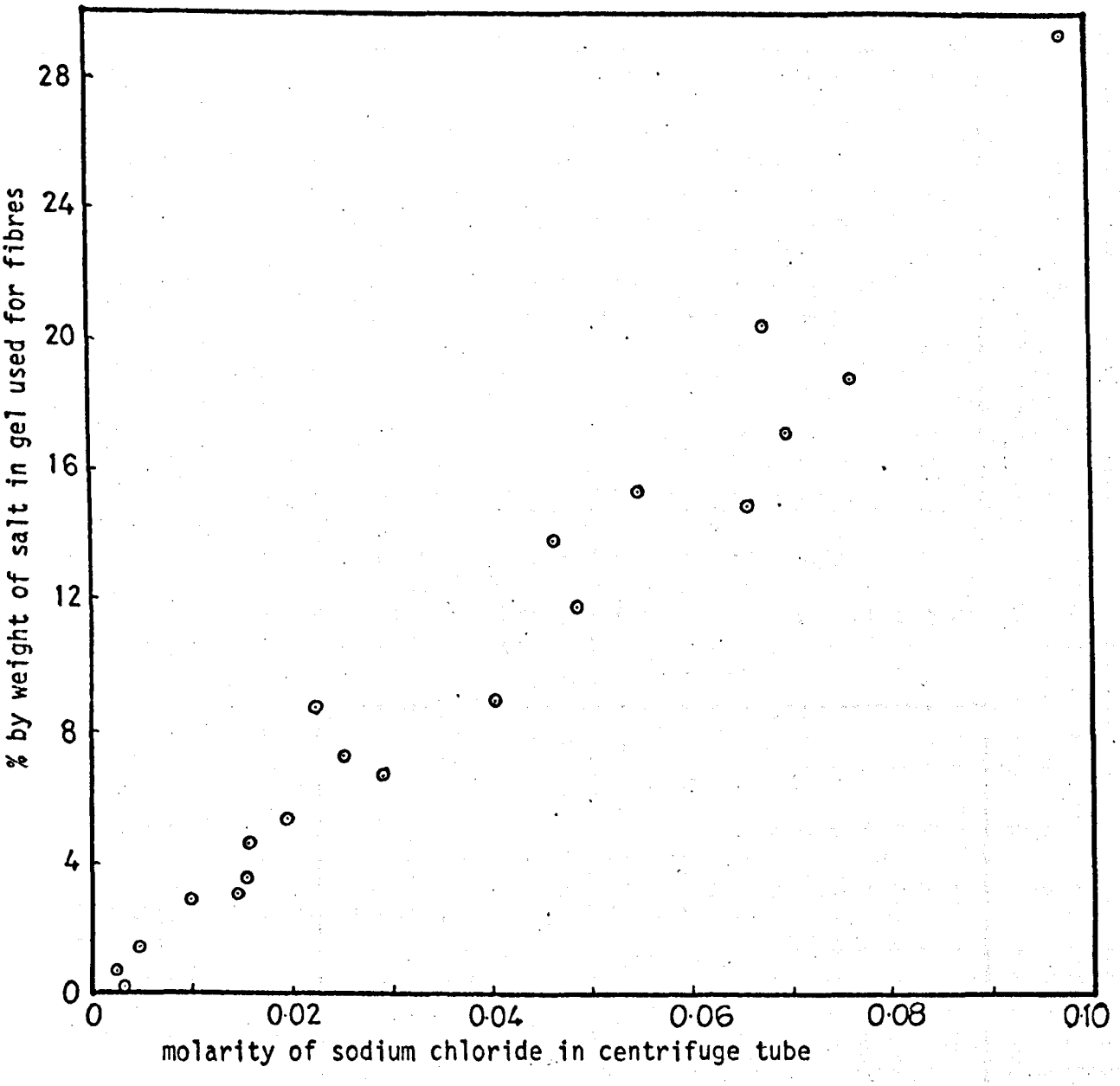


Fig. (3:9) Percentage weight of sodium chloride in a sedimented gel as a function of salt concentration in centrifuge tube.
(Points taken from four runs, pH 5.5 and 7.7)

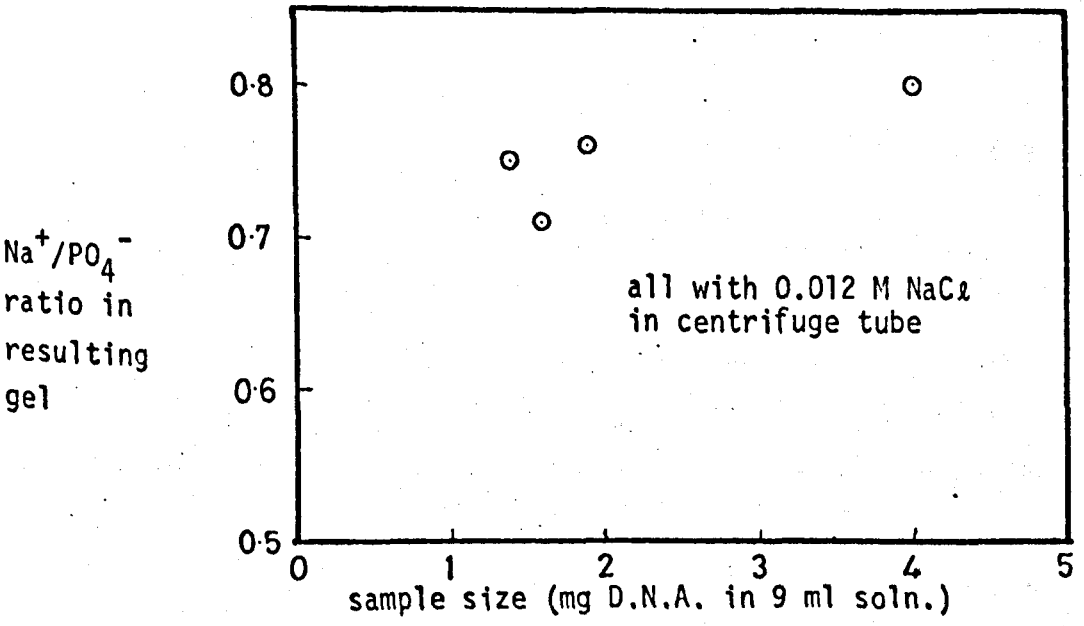


Fig. (3:10) Effect of gel volume on imbibation of salt

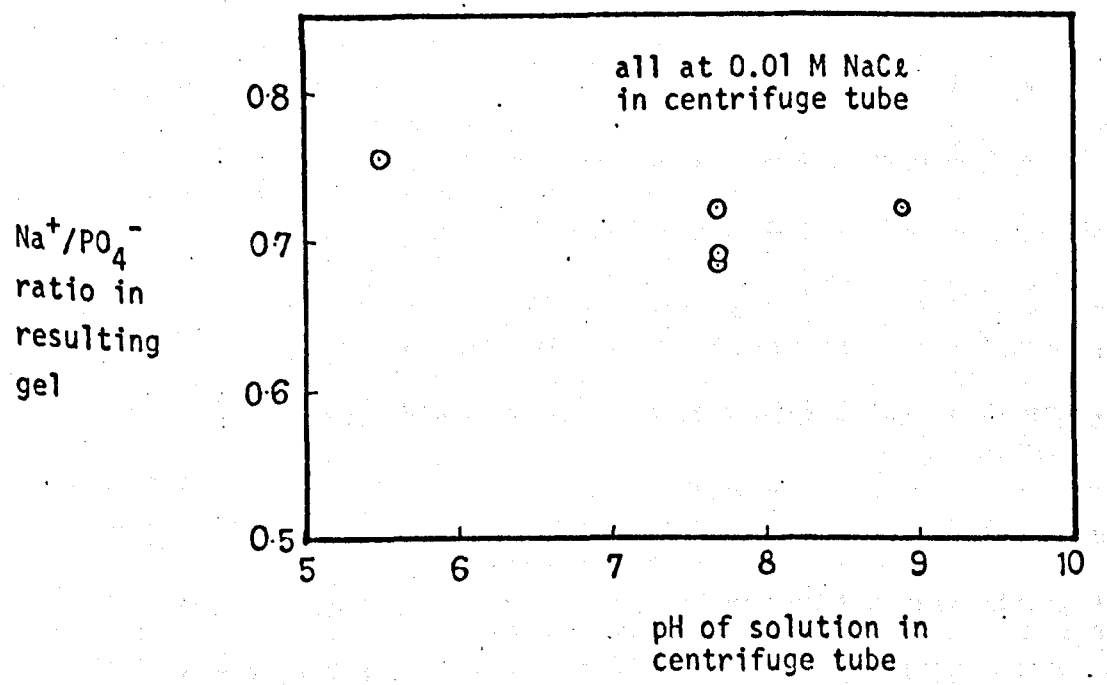


Fig. (3:11) Effect of pH upon Na^+/PO_4^- ratio of the solution in the centrifuge tube

explained by the large volume of NaCℓ solution compared with the gel size. Gel size would be expected to be important only if it occupied a significant volume of the centrifuge tube. Two m.g. of D.N.A. in a 10ml. centrifuge tube will sediment to give a pellet of 0.1 to 0.2 ml which is 1 to 2% of the volume of the tube.

3:6:c Dependence upon pH of centrifugation

Gels were precipitated in buffered solution with the pH of each experiment given on the respective graph. A comparison of the Na^+/ PO_4^- ratio as a function of pH is shown in fig. (3:11). The pH was found not to significantly effect the salt content of the gels over the range where D.N.A. is stable.

3:7 Conclusions

Thus, the experiments show that the salt contents of gels and hence of the resulting fibres depend upon the molarity of supernatant. Little dependence upon pH and concentration of D.N.A. in the centrifuge tube was found. It is, therefore, possible to predict empirically the salt content of a gel from the molarity in solution with reasonable accuracy and without lengthy preliminary experiments.

Cooper and Hamilton [70] analysed D.N.A. fibres and found that up to about 5% sodium chloride could be present and the A form of D.N.A. would persist. Above 5% salt, the B form is found with photographs taken at 92% relative humidity. The A-B transition can occur for NaCℓ contents less than 5% depending upon humidity.

Collecting the data of Cooper and Hamilton, Rupprecht and these experiments together, the conformation of D.N.A. from an

oriented fibre expected from a gel preparation is shown in fig. (3:12). The effects of the NaCℓ molarity of gel preparation, and relative humidity of the dried sample upon D.N.A. conformation are shown. Other experimental details make these conformational regions approximate but they represent useful starting conditions when preparing samples.

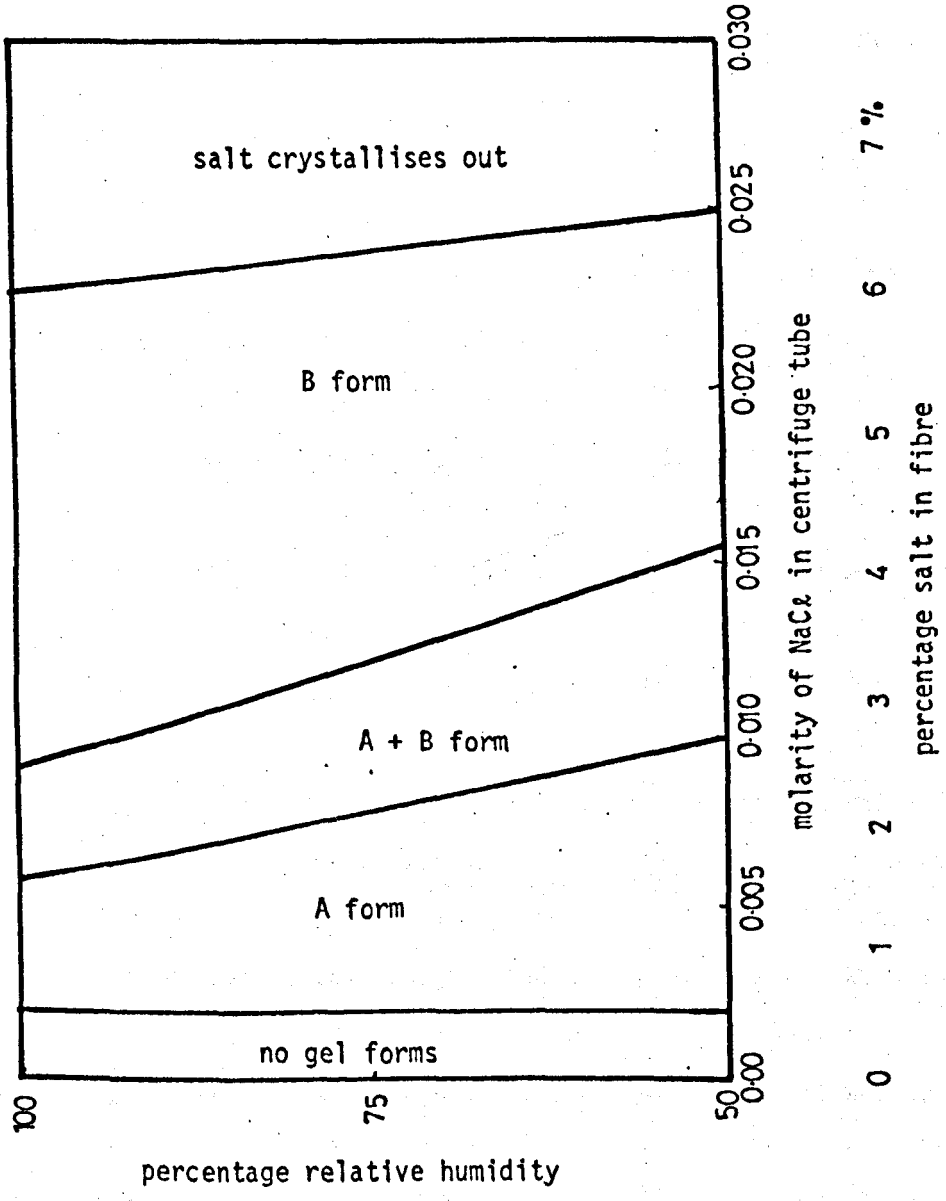


Fig. (3:12) Phase diagram showing probable conformation of D.N.A. in a fibre made from a gel prepared with known salt concentration in the centrifuge tube.

Chapter 4.

THE SPECTRAL STUDY OF D.N.A. COMPLEXES WITH DRUGS

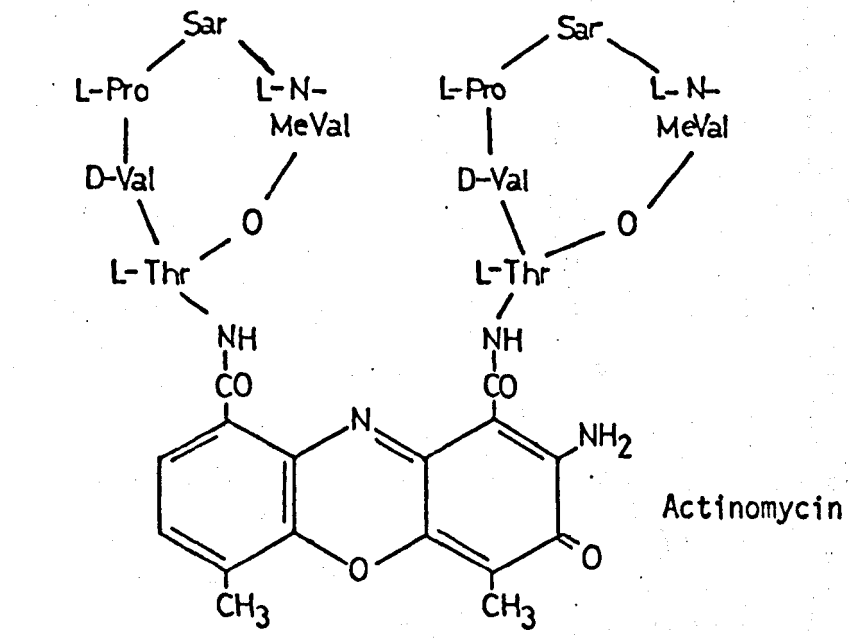
4:1 Introduction

Interactions between drugs and D.N.A. can be followed by observing changes in the spectrum of the drug chromophore over a range of P/D ratios in solution. The steffimycins are ideal drugs for this type of study since their visible absorption spectra occur where the absorption from D.N.A. is negligible; between 400 and 500 n.m. A thorough investigation of the binding of both the steffimycins to various natural D.N.A.'s was carried out. In addition, the formation of steffimycin complexes with poly d(A-T) and poly d(G-C) was examined.

A brief study was also made of the binding of daunomycin, adriamycin, actinomycin and nogalamycin to three natural D.N.A.'s. The first two were studied because of their similarity to the steffimycins. Actinomycin was used because its interaction with D.N.A. is extensively studied and it served as a check on the experimental method. Nogalamycin is relatively little studied and a spectral investigation was thought useful for future work on this drug. The chemical structure of these molecules is shown in fig. (4:1).

4:2 Steffimycin and steffimycin B spectra

Aqueous solutions of both the drugs appear yellow-orange and absorb in the U.V. and visible regions of the spectrum. These absorbtion spectra are shown in fig. (4:2) and are for solutions of



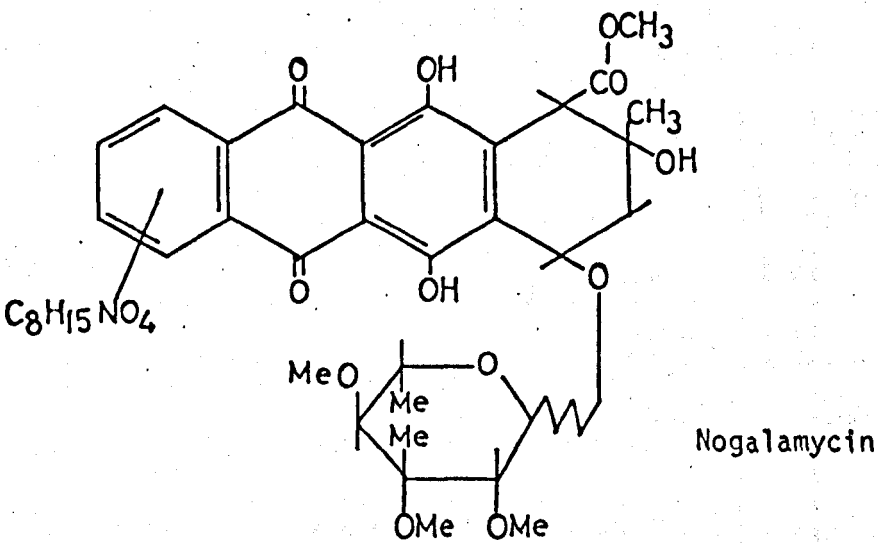


Fig. (4:1) Structural formulae of compounds used in this chapter. The others are shown in Fig. (1:5). The chromophore for nogalamycin is one of several possibilities (from ref. 31).

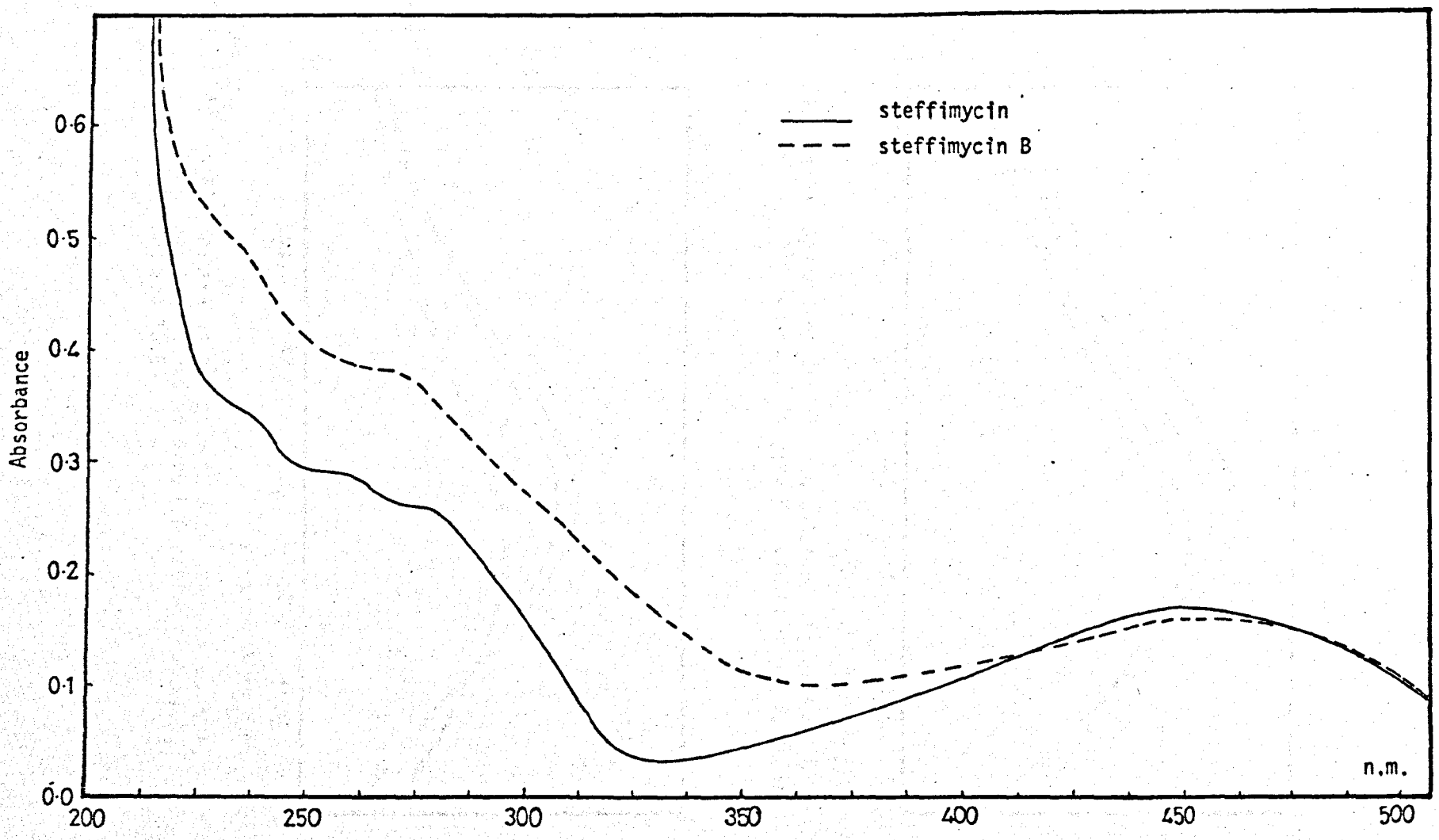


Fig. (4:2) Absorption spectra of steffimycin and steffimycin B in H_2O, pH 7.7, concentration 20μM (normalised)

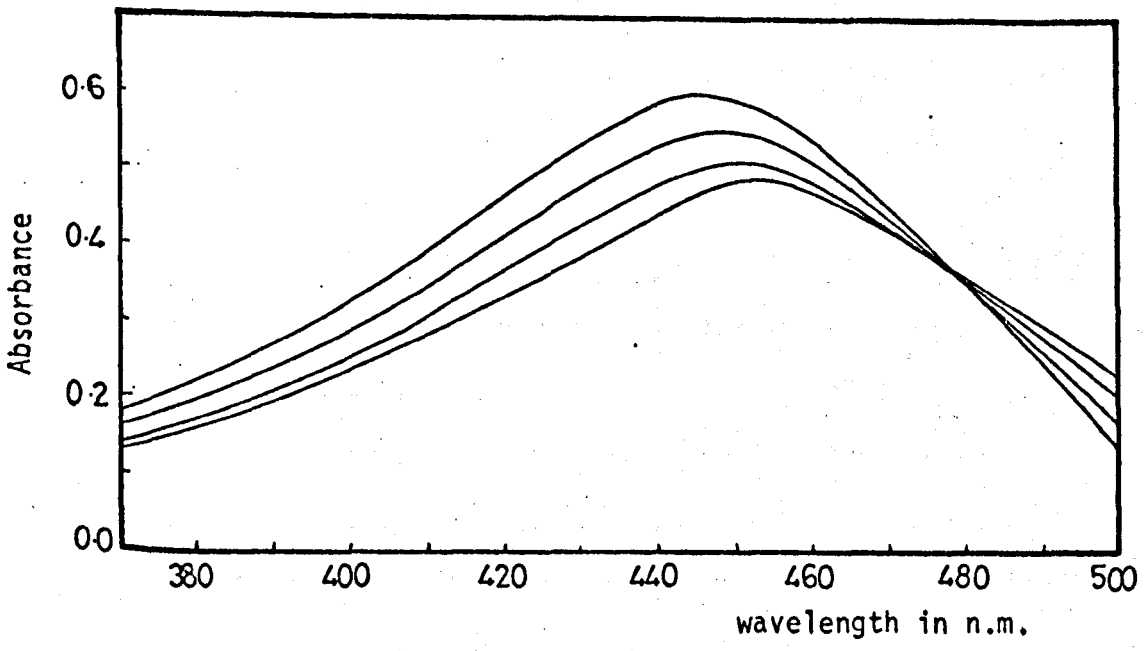


Fig. (4:3) Spectra of steffimycin solution with D.N.A. solution added to give P/D ratios of 0, 5, 10 and 20.

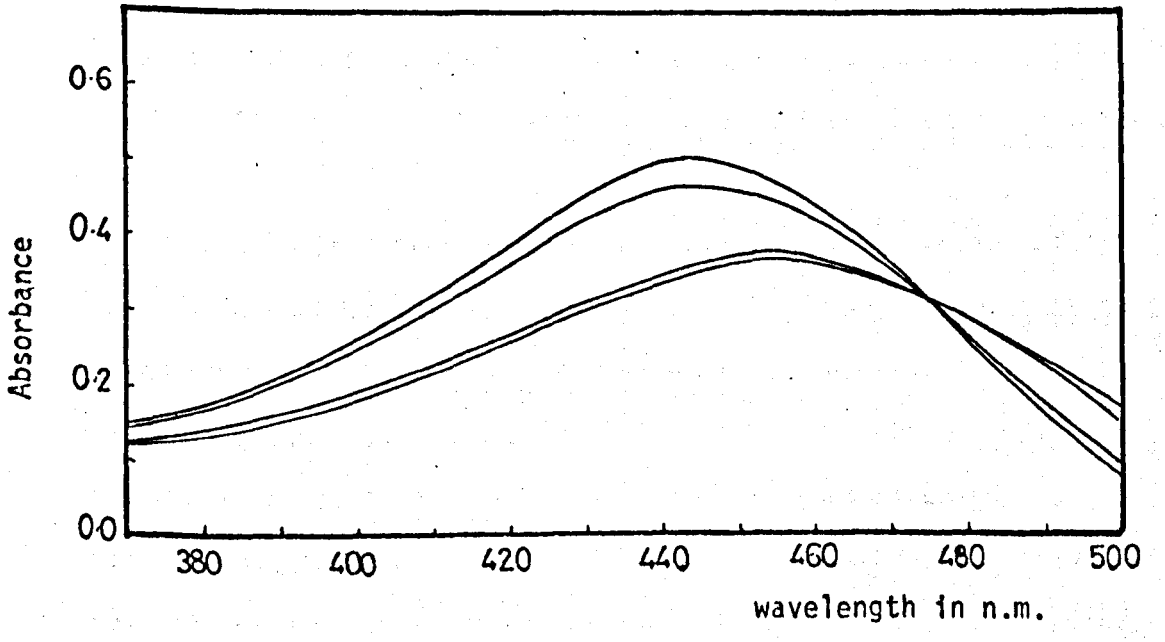


Fig. (4:4) Spectra of steffimycin B solution with D.N.A. solution added to give P/D ratios of 0, 1.5, 7.2 and 10.5

pH <8.5. Above this pH, there is an abrupt colour change from orange to purple possibly due to salification of the compound. When the drug is fully bound to D.N.A., the colour change occurs at a pH of 9-9.5. All spectral work was done on solutions with pH below 8.5.

4;3 Spectra of the steffimycins bound to Nucleic Acids

There is a shift of absorption in the visible region to longer wavelengths when D.N.A. is added to a solution of steffimycin or steffimycin B. Figs. (4:3) and (4:4) show how the absorption spectra of the drugs are dependent upon P/D ratio. The maximum change in absorption corresponds to the spectrum of the fully bound drug where $P/D \to \infty$; i.e. D.N.A. is in excess and further addition of the nucleic acid does not change the spectrum. This metachronic shift is indicative of increased conjugation of the π electrons on the chromophore due to the binding to the nucleic acid. Such a change in the absorption of the drug is typical of many which bind to D.N.A. in some way. Other samples are ethidium bromide [26] and echinomycin [75].

It can be seen from these graphs that the absorbancies of the free and bound drug are different. Both drugs exhibit an isosbestic point around 480 n.m. where absorbance is independent of P/D. The existence of an isosbestic point indicates that there are only two components in solution, viz. bound and free drug molecules. It is unlikely that there are three components in solution since they would all have to show the same absorbance at the isosbestic point.

There is a gradual change in the spectrum of the drug between the free and bound state. This variation is more pronounced at wavelengths lower than the isosbestic point. It is illustrated by

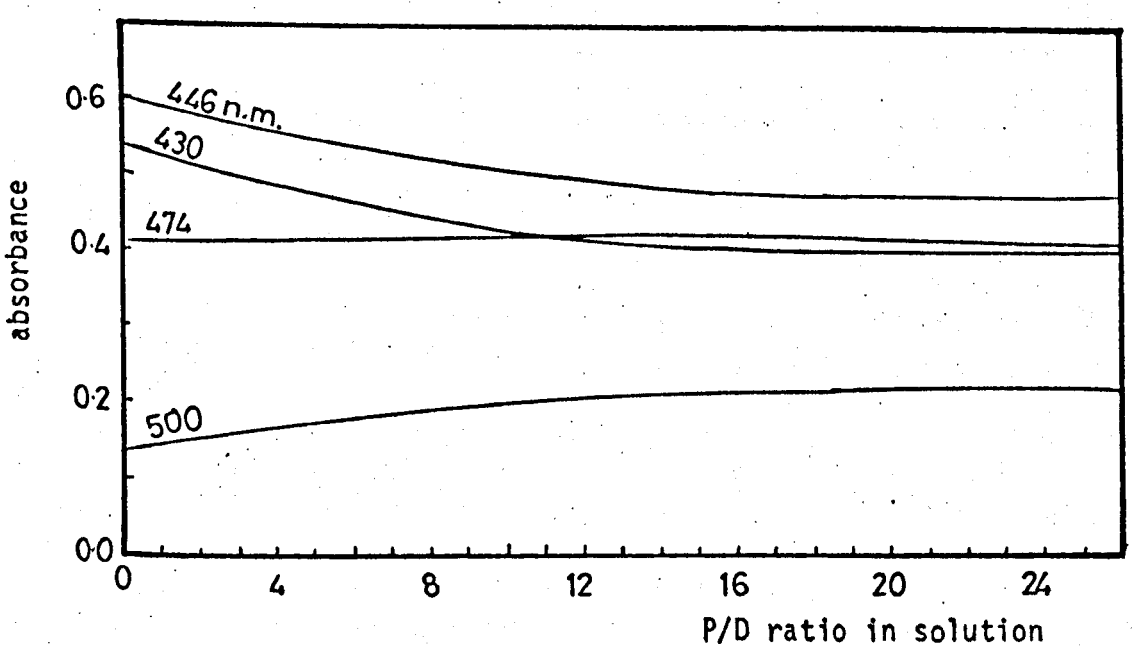


Fig. (4:5) Change in absorption of steffimycin as a function of P/D ratio at various wavelengths indicated in n.m. on each plot.

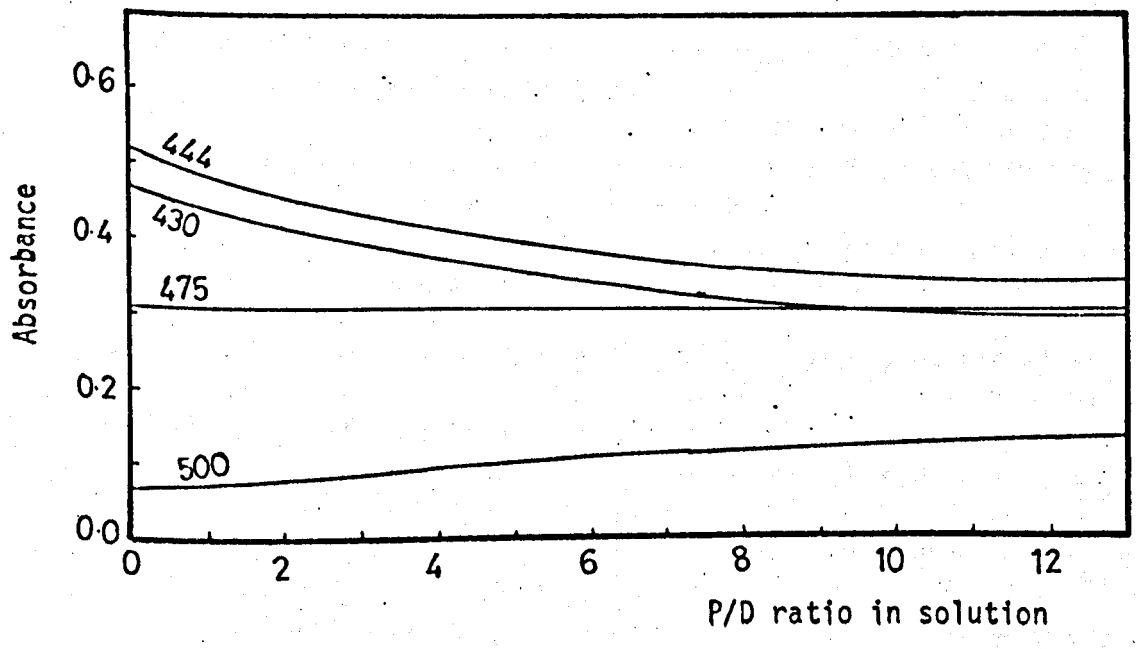


Fig. (4:6) Change in absorption of steffimycin B as a function of P/D ratio at various wavelengths indicated in n.m. on each plot.

fig. (4:5) and (4:6) where the absorbance at several wavelengths is plotted as a function of P/D and it can be seen that the absorbance reaches a minimum value. The slight increase in absorbance as P/D is increased beyond this minimum is later shown to be due to secondary binding.

Beers' law was found to hold for the steffimycin solutions and the complexes for concentrations up to 45μ M in drug and with a P/D of 4 to ∞.

4:4 Theory of the spectroscopic analysis

The effect on the visible absorption spectra of these drugs in the presence of D.N.A. is typical of others which exhibit only one mode of binding where the drug is either bound or free in solution. An analysis of this type of system was suggested by Peacocke and Skerrett [24] in their quantitative spectrophotometric study of proflavine binding to nucleic acids.

The equilibrium situation in such complex formation can be written down as:-

$$\underset{[n-r]}{\text{D.N.A.}} + \underset{[c]}{\text{Drug}} \underset{K_d}{\overset{K_a}{\rightleftharpoons}} \underset{[r]}{\text{Complex}}$$

where n = number of binding sites per nucleotide

c = concentration of drug free in solution

r = number of molecules bound per nucleotide

K_a = association constant

K_d = dissociation constant

The unit of D.N.A. for the purpose of concentration measurements was taken as one nucleotide. If there are n binding sites per nucleotide and r of them are binding drugs while c drug molecules

remain in solution then the concentration of each species in the binding reaction is shown in square brackets. The dissociation constant K_d which is smaller for a more stable complex is given by:-

$$K_d = \frac{(n-r)c}{r} \quad \text{——} \quad 1$$

The absorbancy E_λ of the solution at wavelength λ can be expressed as:-

$$E_\lambda = \frac{CE'_\lambda}{D} - \frac{rPE''_\lambda}{D}$$

where E'_λ and E''_λ are the absorbancies of the free and bound drug respectively. P is the concentration of nucleotides and D the total drug concentration. If α is the fraction of the drug bound in the mixture then:-

$$\alpha = \frac{E'_\lambda - E_\lambda}{E'_\lambda - E''_\lambda} = \frac{rP}{D} \quad \text{——} \quad 2$$

Thus for any wavelength λ where Beers' law operates, and the D.N.A. does not absorb, α can be determined for a mixture of drug and nucleotide in solution, E'_λ and E''_λ can be determined from the spectra of the free and bound drug respectively and E_λ is the absorbance of the solution for which α is to be found. The number of drug molecules bound per nucleotide, r, is $\alpha D/P$ and the concentration of free drug, c, is $(1-\alpha)D$, both of which can be calculated. A plot of r against c shows how the number of drugs bound varies with the amount of drug available in solution.

$$\text{From } 1 \quad \frac{r}{c} = \frac{n}{K_d} - \frac{r}{K_d}$$

A plot of r/c against r results in a straight line with gradient $-1/K_d$ and the intercept on the r axis gives n, the number of drugs strongly bound per nucleotide.

In practice this latter plot was found to deviate from a straight line at low r/c values where it approaches the r axis asymptotically. The straight part of the graph is indicative of strong binding with a low value of K_d and it is this slope that is extrapolated to give the value of n. The deviation from this straight line shows that more drugs are being weakly bound when there is a higher concentration of free drug. It is because of this deviation from the theoretical straight line that the values of K_d are not very reliable. The slope of the straight line is difficult to draw because it is based upon data points where α is gradually approaching the value of 1 as the drug becomes fully bound. It does, however, allow an order of magnitude comparison to be made between dissociation constants of the same drug with different D.N.A.'s and gives a qualitative indication of the variation in the strength of binding.

4:5 Complexes with the steffimycins

Section 4:3 provided a general description of the change in spectra as drug molecules bind to nucleic acids. This was followed in section 4:4 by a development of the theory used to analyse the spectra. A detailed application of this spectroscopic analysis is now applied to the steffimycins.

4:5:a Effect of solvent upon visible absorption of the steffimycins

Typical visible absorption spectra for steffimycin and steffimycin B in H_2O are shown together in Fig. (4:2) . The absorption peak is dependent upon solvent, and since it was sometimes necessary to use low concentrations of ethanol in the solvent, its

variation for both drugs at different water/ethanol mixtures is shown in fig. (4:7). There is a shift of the absorbance to longer wavelengths for an increase in the ethanol content of the solvent. The change in extinction coefficients for the drugs in different water/ethanol mixtures was given in fig. (2:3) and (2:4).

4:5:b Effect of salt on binding of steffimycin to D.N.A.

In section 4:3 the binding of steffimycin to calf thymus D.N.A. was observed by a change in the visible spectrum of the drug. In order to see the effect of sodium chloride upon the binding of steffimycin to D.N.A., two series of experiments were then performed. Aliquots of calf thymus D.N.A. were added to two separate samples of steffimycin in which the salt concentration was 0.002 and 0.050M respectively. Spectra of the complexes for various P/D's in solution were plotted.

Increased ionic strength was seen to inhibit the formation of the drug/D.N.A. complex. This is observed as a decreased metachromic effect produced by the binding of steffimycin to D.N.A. when there is a higher salt concentration. Fig. (4:8) shows the number of drug molecules bound per nucleotide as a function of the concentration of free drug in the solution. The two curves correspond to the formation of the complex at different salt molarities. It can be seen that for a given concentration of free drug, the number of drugs bound to the D.N.A. is lower for the solution with the higher ionic strength. The dissociation constants for the complexes are determined from fig. (4:9) and the value is greater for the high salt molarity indicating a less stable complex formation at this higher ionic strength. Similar observations have

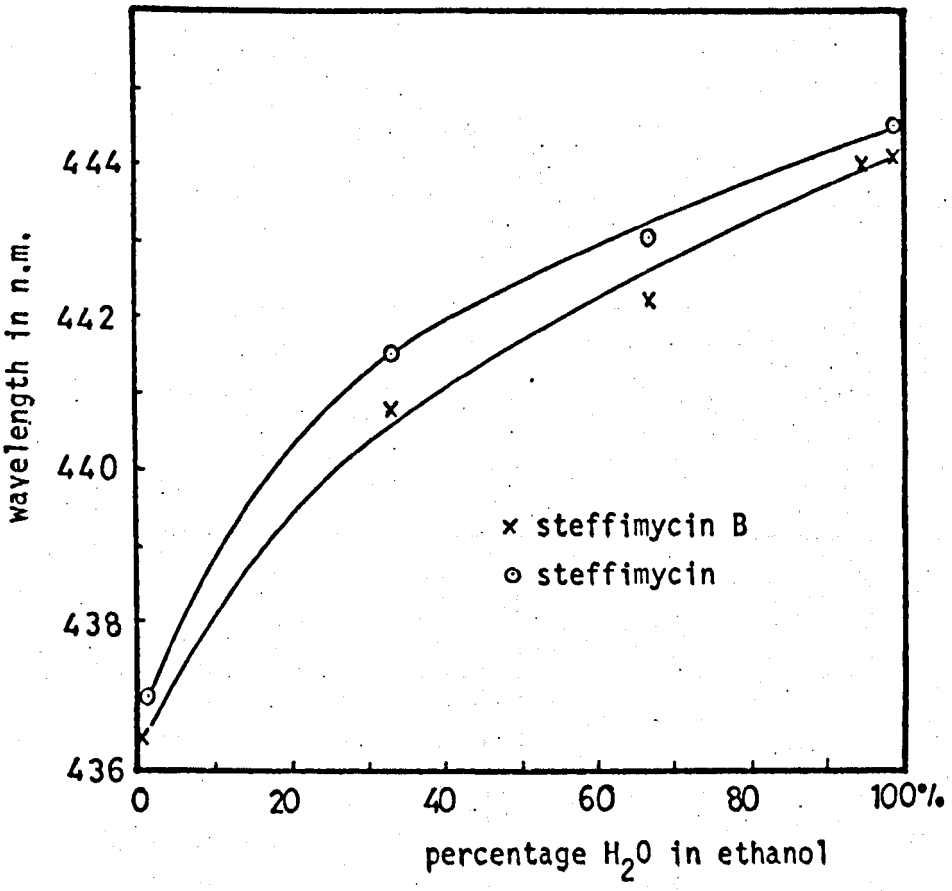
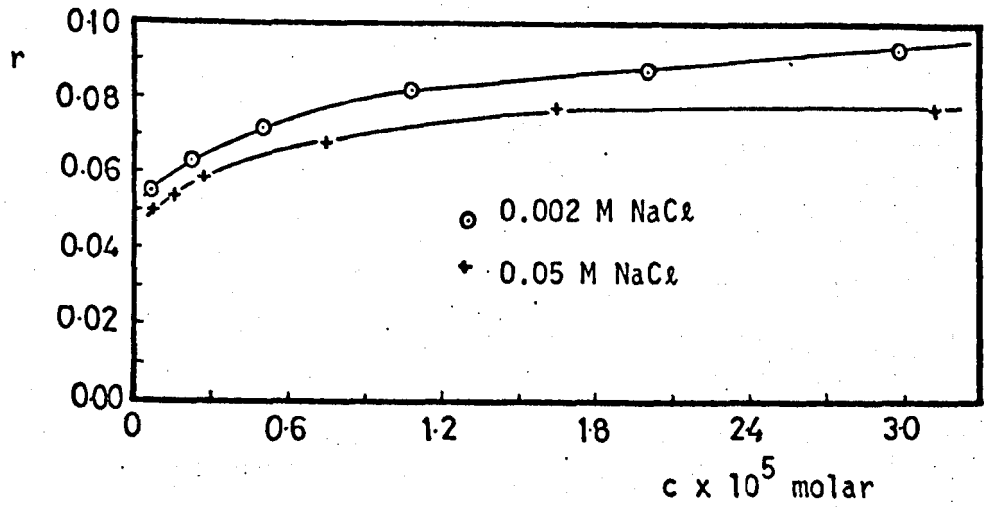


Fig. (4:7) Shift in visible absorption maximum for steffimycin and steffimycin B as a function of the H_2O/ethanol ratio of the solvent.



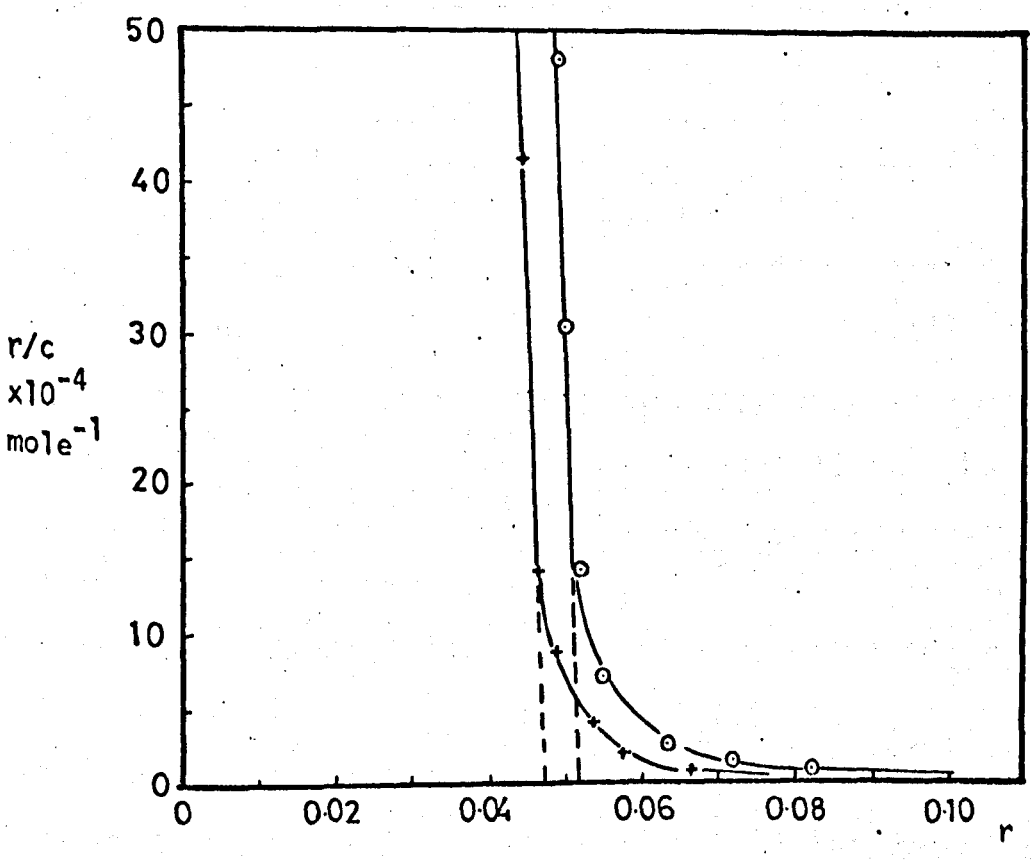


Fig. (4:8) and (4:9) Comparison of the number of binding sites on calf thymus D.N.A. for steffimycin, and the different rate constants at two ionic strengths.

been made for proflavine - D.N.A. interactions [24]. The nucleic acid double helix is negatively charged at each phosphate group so that if the preliminary attraction of steffimycin to D.N.A. was electrostatic, then the sodium cations would compete for, and tend to mask the phosphate anions. Fig. (4:10) summarises this data comparing the complex formation at two salt concentrations. The number of drugs strongly bound, n, shows an inverse relationship to the dissociation constant.

calf thymus	molarity of NaCℓ	n	K_d
D.N.A. + steffimycin	.002 .050	.052 .047	0.62×10^{-8} 1.88×10^{-8}

Fig. 4:10

All the solutions used in the spectroscopic work had a sodium chloride concentration of 0.004 N except where mentioned. The pH was around 7 and tris buffer was used to prevent this rising above 8.5 where steffimycin becomes deep purple. Aqueous solvent was used unless otherwise stated.

4:5:c Steffimycin binding to synthetic polynucleotides

The base pair specificity of steffimycin binding to synthetic polynucleotides was examined by forming complexes of poly d(A-T) and poly d(G-C) with the drug. The spectroscopic analysis outlined above leads to the determination of the number of drugs bound per binding site and thereby provides a means of discovering any binding preference. The experiments were performed by adding each

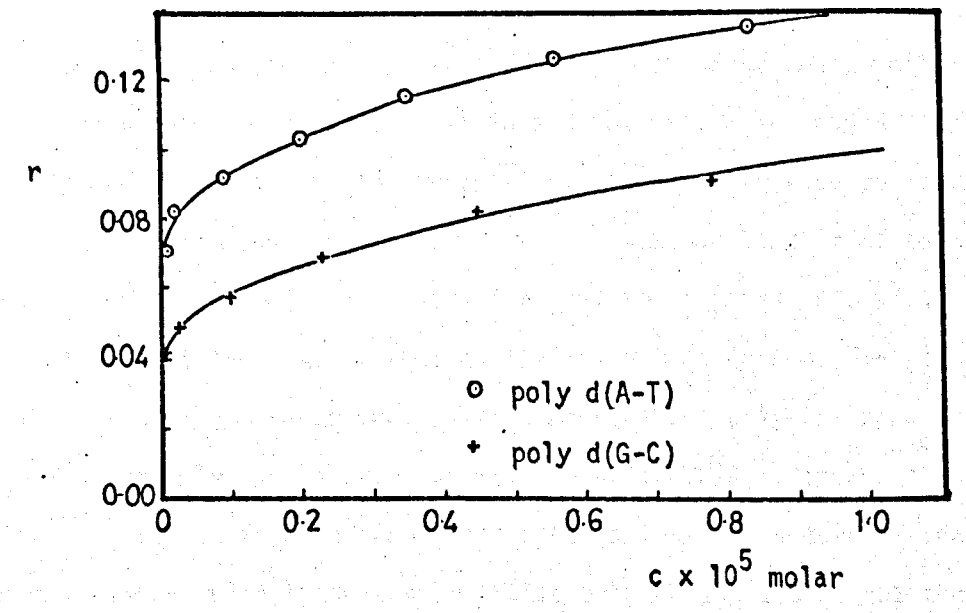
polynucleotide to the drug solution as before but two separate runs were carried out with the drug at 11μM and then at 45μM. Drug binding was examined at the lower range to enable a comparison to be made with the experiments of Reusser [23] who used concentrations of <1μM. The saturated 45μM solution was used to determine the interaction with synthetic D.N.A. at the highest drug concentration possible.

Figs. (4:11) and (4:12) show the binding of the drug to poly d(A-T) and poly d(G-C). As Reusser observed, the antibiotic does show preference for adenine-thymine base pairs; the number of drugs bound per nucleotide, n, being greater for poly d(A-T) than for poly d(G-C). The dissociation constant also indicates a more stable complex of the drug with poly d(A-T).

Reusser sought to determine the base specificity of steffimycin by observing its inhibition of D.N.A. directed R.N.A. synthesis when added to the poly d(A-T) or poly dG.dC template. He chose to use one polynucleotide with a self complementary alternating A-T base sequence and the other with a duplex of the homo polymers poly dG and poly dC. At the low drug concentrations he observed inhibition of the template activity for poly d(A-T) but not for poly dG.dC.

The experiments here used two synthetic D.N.A.'s with alternating base sequences and the binding of steffimycin was seen to be considerably higher for poly d(A-T) than for poly d(G-C). With a free drug concentration less than 1μM, as used by Reusser, there are approximately twice as many steffimycin molecules bound to the adenine-thymine base pairs than to the guanine-cytosine.

Poly d(A-T) and poly d(G-C) were then added to steffimycin at 45μM and the corresponding changes in visible spectrum are shown in



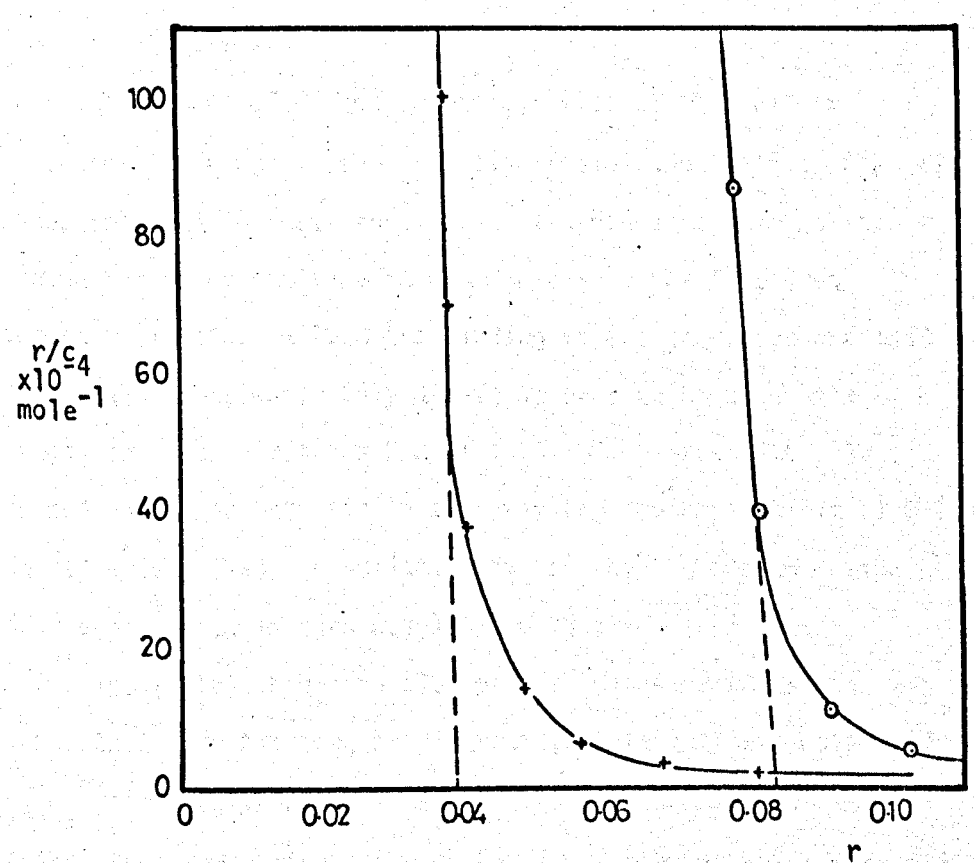


Fig. (4:11) and (4:12). Binding of steffimycin to poly d(A-T) and poly d(G-C).

poly d(A-T) $n = 0.084 \pm 0.15$ $K_d = 0.577 \times 10^{-8}$

poly d(G-C) $n = 0.038 \pm 0.15$ $K_d = 0.067 \times 10^{-8}$

fig. (4:13) and (4:14) respectively. The usual metachromic shift is observed together with a decrease in absorbancy for the bound drug. One important feature of the graphs is that the absorption spectrum for steffimycin bound to poly d(G-C) is dissimilar to that of the drug bound to poly d(A-T). The latter complex appears to absorb with a peak at 480 n.m. as well as the maximum at 455n.m. Complexes with poly d(A-T) were unusual in this respect not only compared with those using poly d(G-C) but also with natural D.N.A.'s as shall be shown later. It appears from these spectra that the nature of the complex formed with these two synthetic polynucleotides is not the same in both cases.

The numbers of drugs per binding site at this higher concentration range is shown in fig. (4:15) and (4:16). Fig. (4:15) shows that the A-T base specificity was not sustained as the concentration of steffimycin was increased. At free drug concentrations above 10μM the binding to G-C increases and that to A-T remains constant. Poly d(A-T) appears to be saturated with 0.095 drugs per binding site; a P/D of 10.5. Complexes with poly d(G-C) do not appear to saturate in this way and the upper limit of 0.115 drugs per site was a restriction imposed by the concentration of drug used, which in turn was limited by its solubility.

Fig. (4:16) shows the plot of r/c against r which was demonstrated by Peacocke and Skerret [24] to indicate n, the number of drugs strongly bound per site. The straight part of the curves result from the dominant strong binding which has a low dissociation constant and gives the value n when extrapolated to the r axis. Strong binding which is dominant in the region where the concentration of steffimycin is less than 10μM is seen to be preferential to A-T

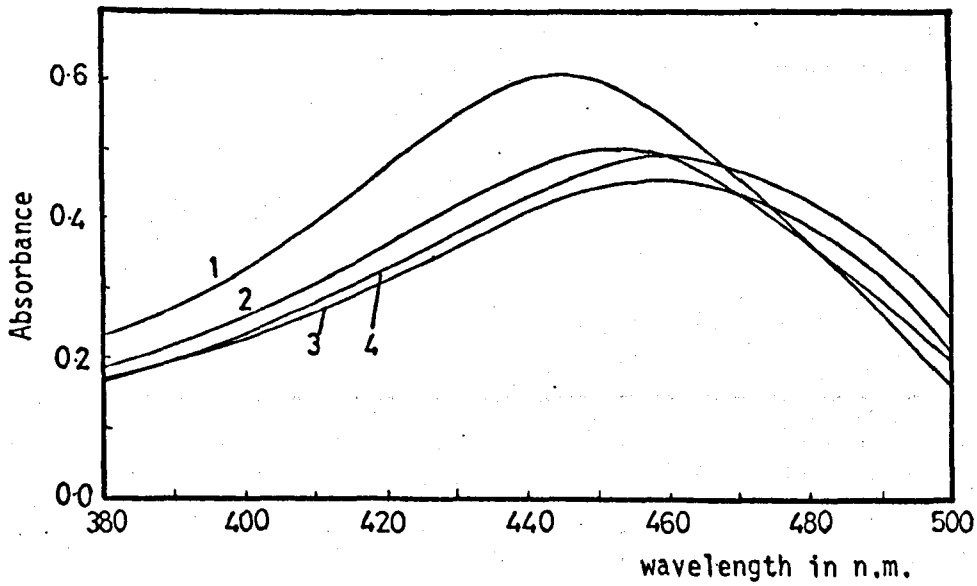


Fig. (4:13) Spectra of steffimycin with poly d(A-T) at P/D ratios of 0, 6.7, 11.3 and 19.4 in the order of 1 to 4.

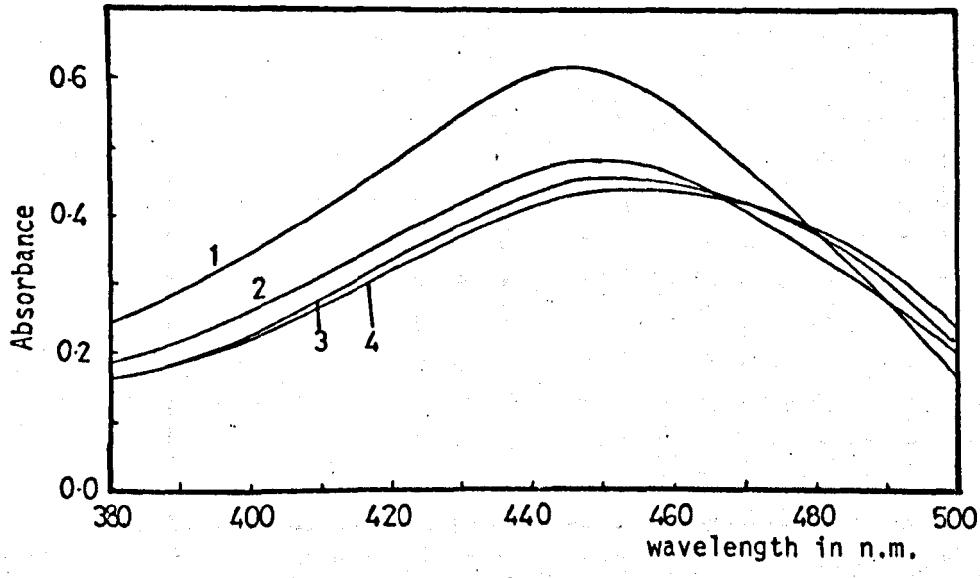


Fig. (4:14) Spectra of steffimycin with poly d(G-C) at P/D ratios of 0, 12.8, and 20.6 in the order 1 to 4.

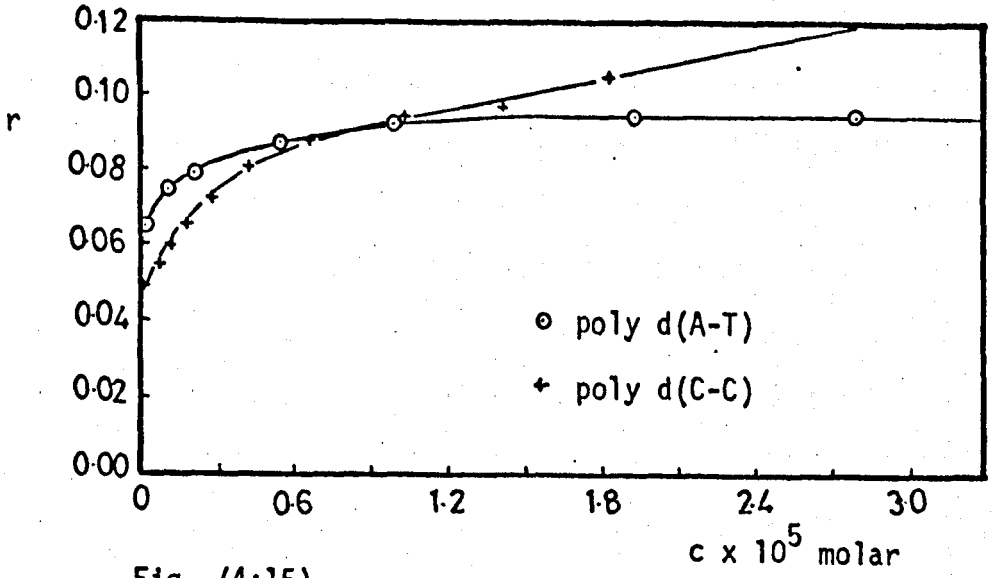


Fig. (4:15)

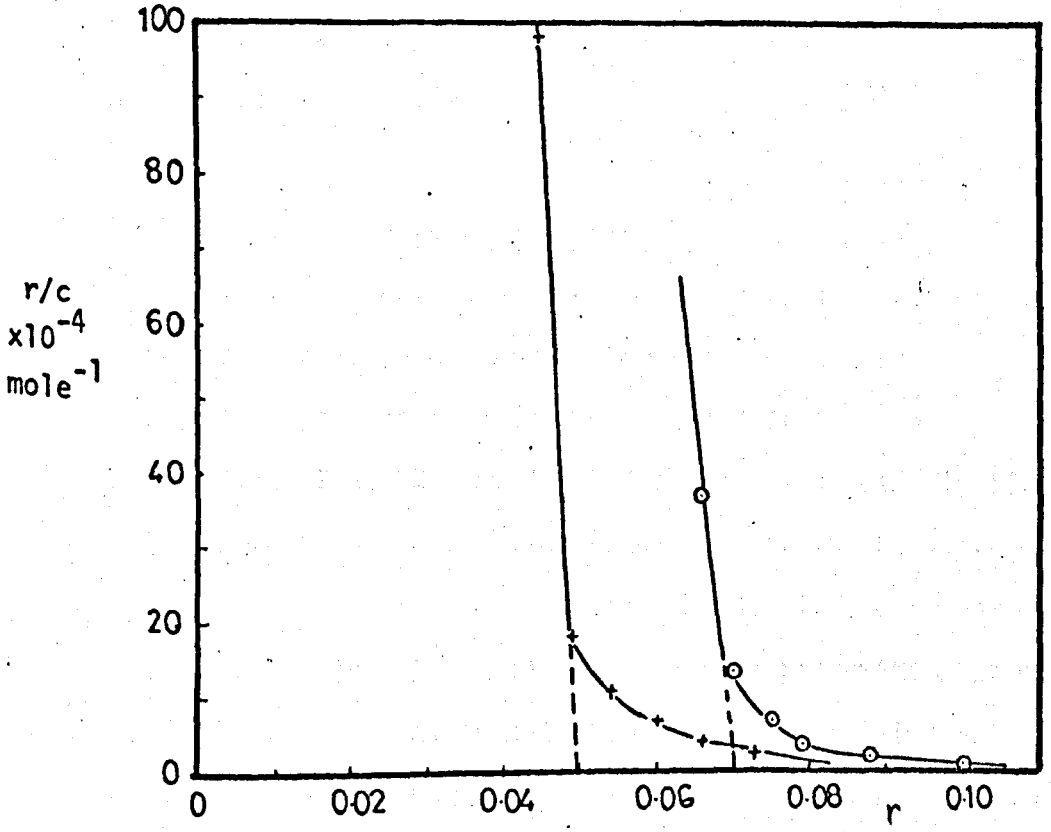


Fig. (4:16) Binding of steffimycin to the synthetic polynucleotides at the higher concentration range of the drug.

poly d(A-T) n = 0.07 ± 0.15 K_d = 1.176×10^{-8}

poly d(G-C) n = 0.05 0.15 K_d = 1.25×10^{-8}

base pairs with 0.07 drugs bound compared with 0.05 for G-C.

Thus the base specificity observed for steffimycin when using poly d(A-T) and poly d(G-C) is for A-T when the free drug is at concentrations up to 10μM. Any further increase in drug concentration results in an increase of r only for poly d(G-C) making the overall preferential binding of the drug be for G-C pairs with free drug concentrations in excess of 10μM.

10μM is a high concentration when one considers the corrections which could reasonably be used in an in vivo situation. Therefore, the behaviour of the drug in biological systems will most likely compare with the experimental results where the total drug concentration is below 10μM.

4:5:d Steffimycin binding to natural D.N.A.'s

Spectral studies on the binding to the natural D.N.A.'s from M Lysodeikticus, calf thymus and Cℓ Perfringens were carried out. These D.N.A.'s were chosen because of their different base composition, viz. 72%, 42% and 31% G-C content respectively [76] [77]. This permitted a comparison to be made of the relative number of drug molecules that each D.N.A. would bind. If the number of chromophores bound is related to the base pair composition of the nucleic acid then drug can be said to show preferential binding and is base pair specific. Once again, experiments were performed over two concentration ranges of the free drug.

The spectra of steffimycin in solution with the three different D.N.A.'s at various P/D ratios are shown in figs. (4:17-19). The curves show the gradual change in absorbance as more and more drug binds to D.N.A. Spectra of the fully bound drug with each of the

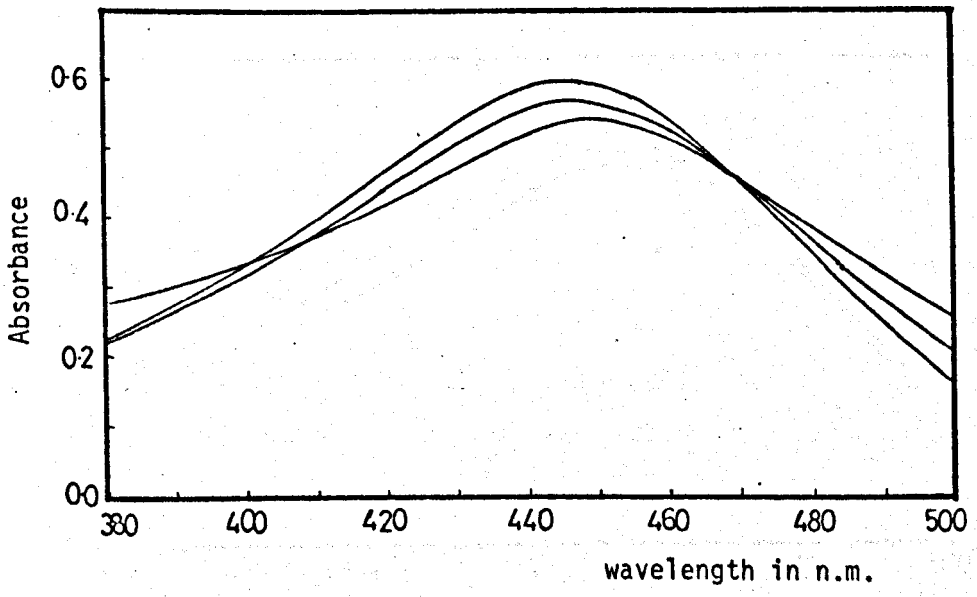


Fig. (4:17) Spectra of steffimycin with M Lysodeikticus D.N.A. at P/D 0, 4 and 11.4

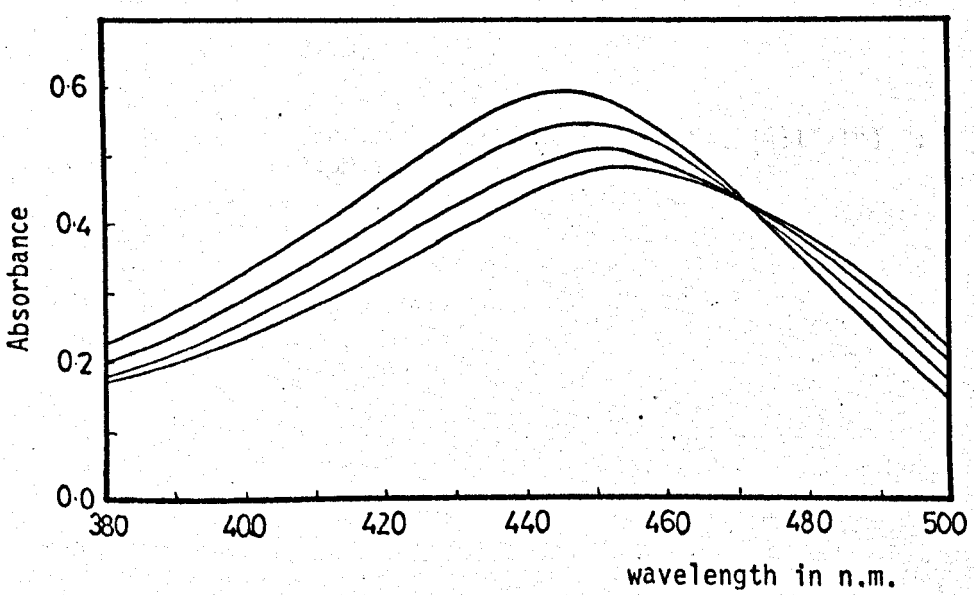


Fig. (4:18) Spectra of steffimycin with calf thymus D.N.A. in solution at P/D 0, 5, 10 and 20.5

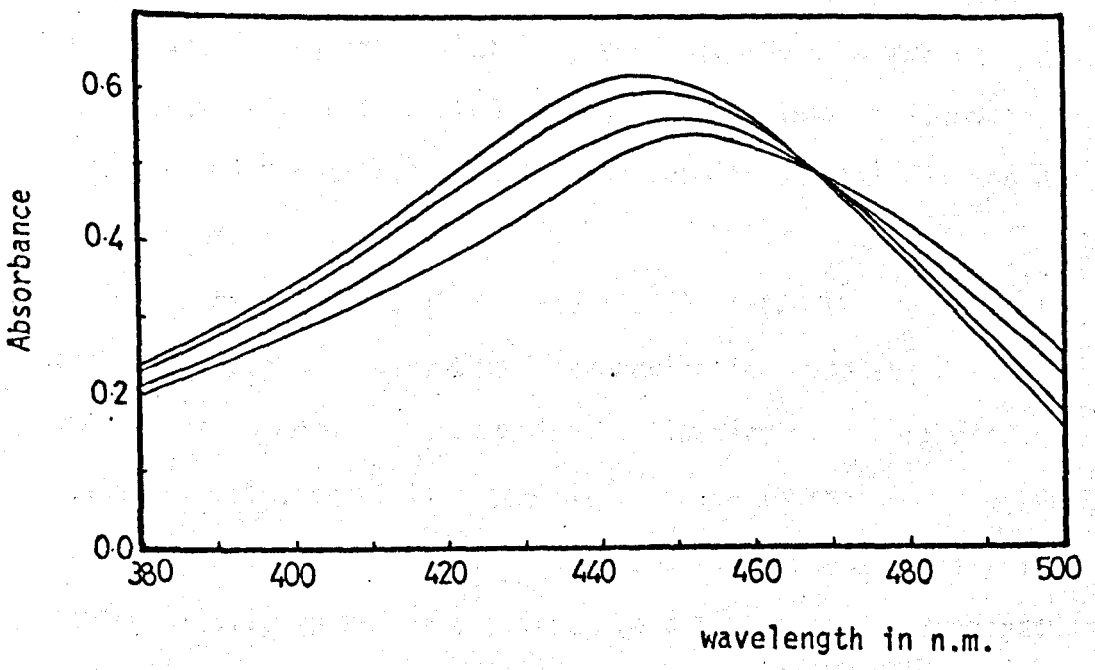


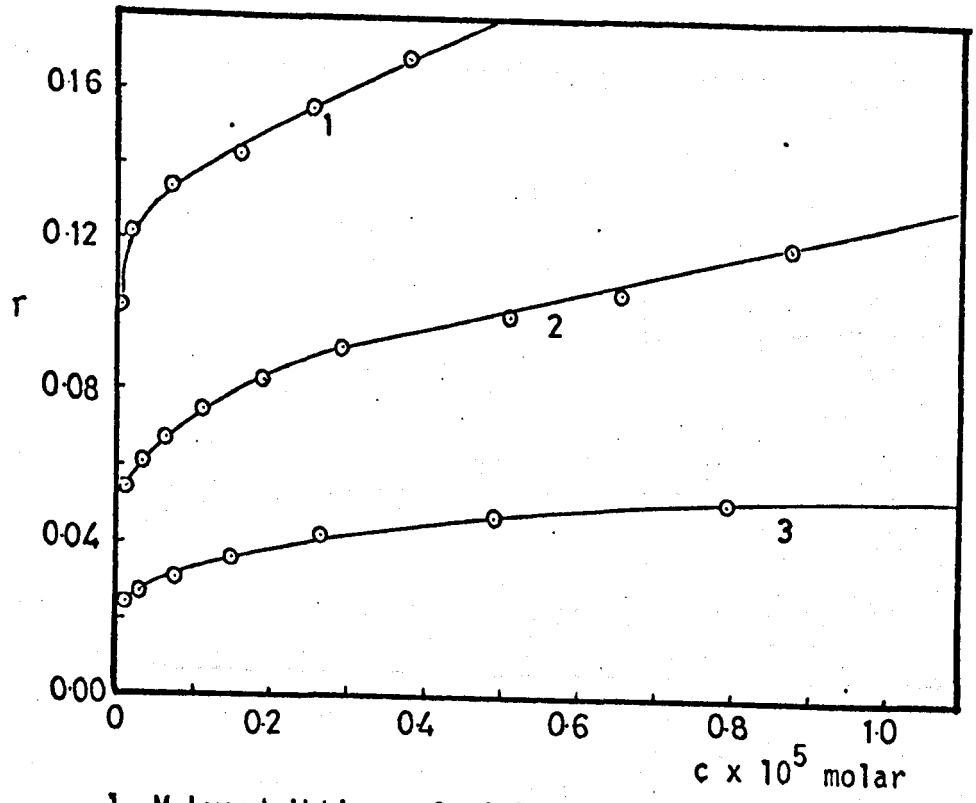
Fig. (4:19) Spectra of steffimycin with Cℓ Perfringens D.N.A. in solution at P/D 0, 4.7, 11.7 and 21.3.

Conc. of steffimycin in Figs. (4:17-19) is 45μM.

natural nucleic acids are very similar and a comparison with fig. (4:13) and (4:14) shows that they resemble the spectrum of steffimycin with poly d(G-C). This suggests that steffimycin forms a complex with poly d(A-T) different from those with all the other D.N.A.'s examined.

Referring to fig. (4:20) and (4:21) it can be seen that the number of drug molecules bound per nucleotide once again rises steeply at low drug concentrations indicative of strong binding and following this, there is a gradual increase in the number of drugs bound per site. The graphs show very clearly that steffimycin binds preferentially to G-C base pairs. This base specificity continues as the free drug concentration was increased up to the maximum possible using a saturated solution. Figs. (4:22) and (4:23) show the number of drugs bound per nucleotide over this range of higher drug concentration. Since the essence of these results is in conflict with the conclusion drawn by Reusser from more limited studies, particular attention was made to verify that these effects were fully reproducible and were also maintained when 20% ethanol was used as solvent.

It can be seen from the plots of r against c, that the number of occupied drug binding sites rises rapidly as the drug concentration increases from zero. This represents strong binding and gives the dissociation constant K_d for the complexes. The slope of the graphs then level off and for Cℓ Perfringens with lower G-C content, r increases very little with the increase of drug concentration. With M Lysodeikticus, high G-C, the number of occupied sites on the D.N.A. continues to increase giving evidence for a second weaker mode of binding. Although the base specificity



1. M Lysodeikticus 2. Calf thymus 3. Cℓ Perfringens

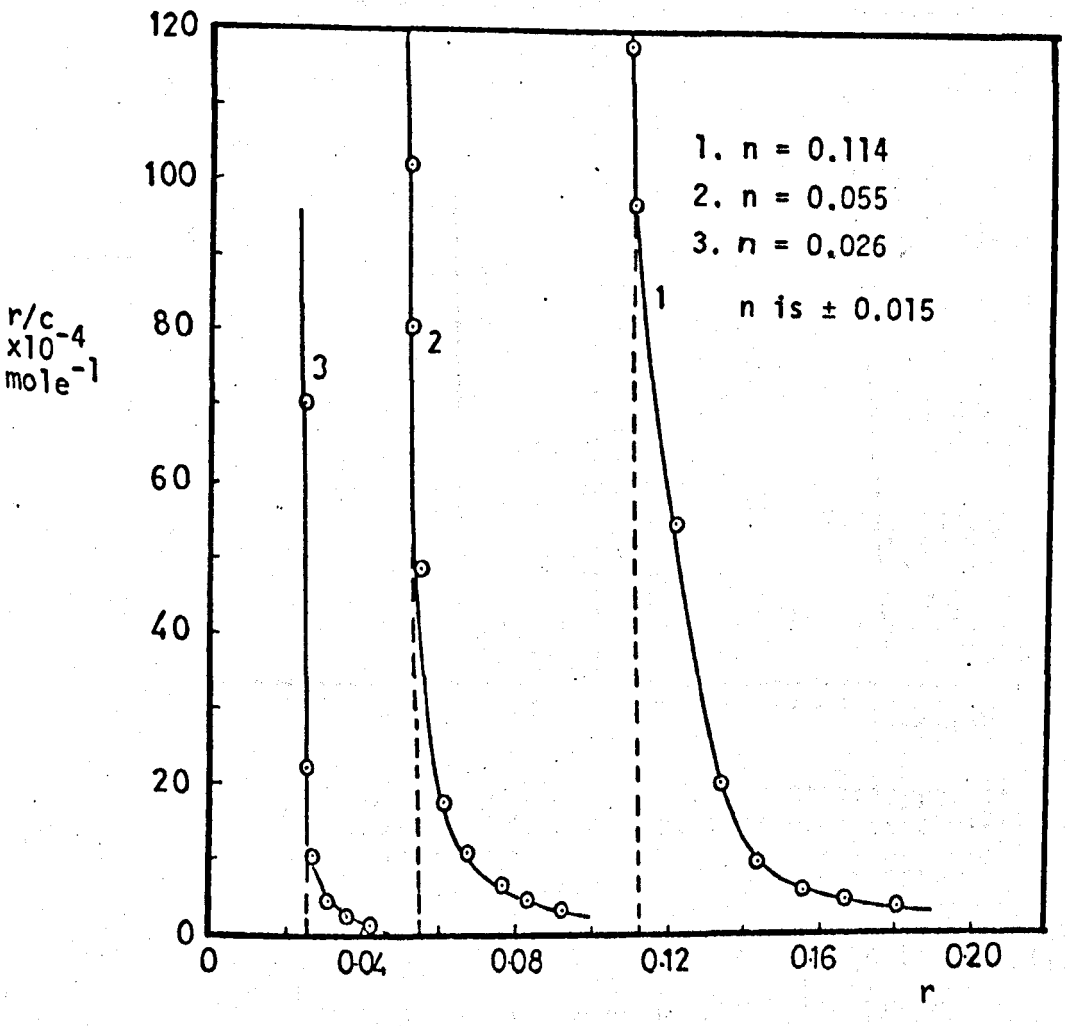
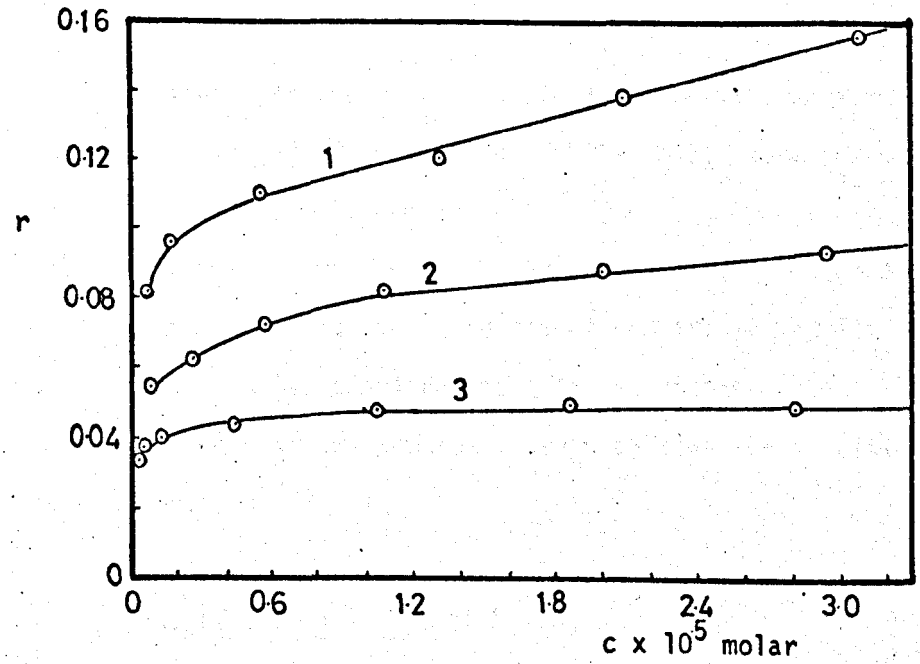


Fig. (4:20) and (4:21) Binding curves for steffimycin to natural D.N.A.'s at low drug concentrations



1. M Lysodeikticus 2. Calf thumus 3. Cℓ Perfringens

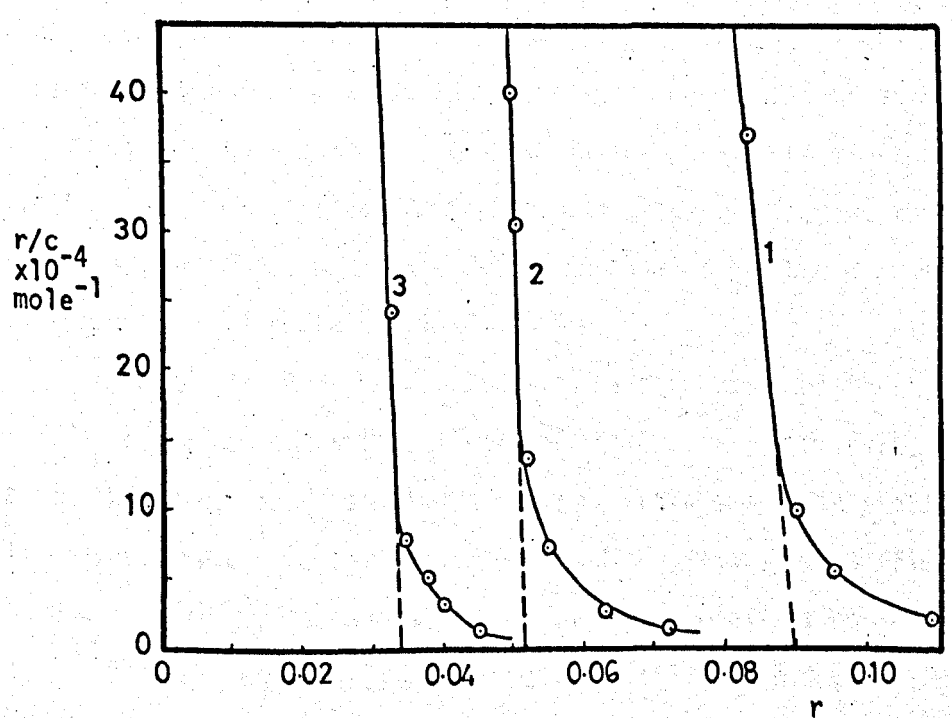


Fig. (4:22) and (4:23). Binding of steffimycin to natural D.N.A.'s at high drug concentration range.

1. n = 0.090 $K = 1.75 \times 10^{-8}$
2. n = 0.052 $K = 0.90 \times 10^{-8}$ n is ± 0.015
3. n = 0.035 $K = 0.75 \times 10^{-8}$

is more complicated in the synthetic D.N.A.'s, results show that secondary weak binding is associated with G-C base pairs for both natural and synthetic poly nucleotides.

The dissociation constants for the strong binding between the drug and all three natural D,N.A.'s are of comparable magnitude indicating that similar complexes have formed in each case. The low value of K_d shows that the complexes are stable in the conditions of the experiment.

4:5:e Binding of steffimycin B to D.N.A.

A spectroscopic study of steffimycin B binding to M Lysodeikticus, calf thymus and Cℓ Perfringens D.N.A.'s was carried out. Owing to the lower solubility of steffimycin B, the binding experiments were performed with a total drug concentration of 11μM. The change in the visible spectra due to the binding of the three D.N.A.'s is shown in figs. (4:24-26). As with steffimycin, there is no significant difference between the spectra of the bound drug with different natural nucleic acids.

The number of drug molecules bound as a function of the steffimycin B concentration is shown in fig. (4:27) and (4:28) and again this drug showed similar binding characteristics to steffimycin. In general there were fewer occupied binding sites with steffimycin B than with steffimycin for the same free drug concentration and D.N.A. type. This can be seen by comparing fig. (4:27) with fig. (4:20).

Steffimycin B exhibits both weak and strong types of binding. As with steffimycin, the strong binding saturates when the free drug concentration is ∿0.5μM. The slow increase of drugs bound as c increases beyond this concentration is an indication of a secondary

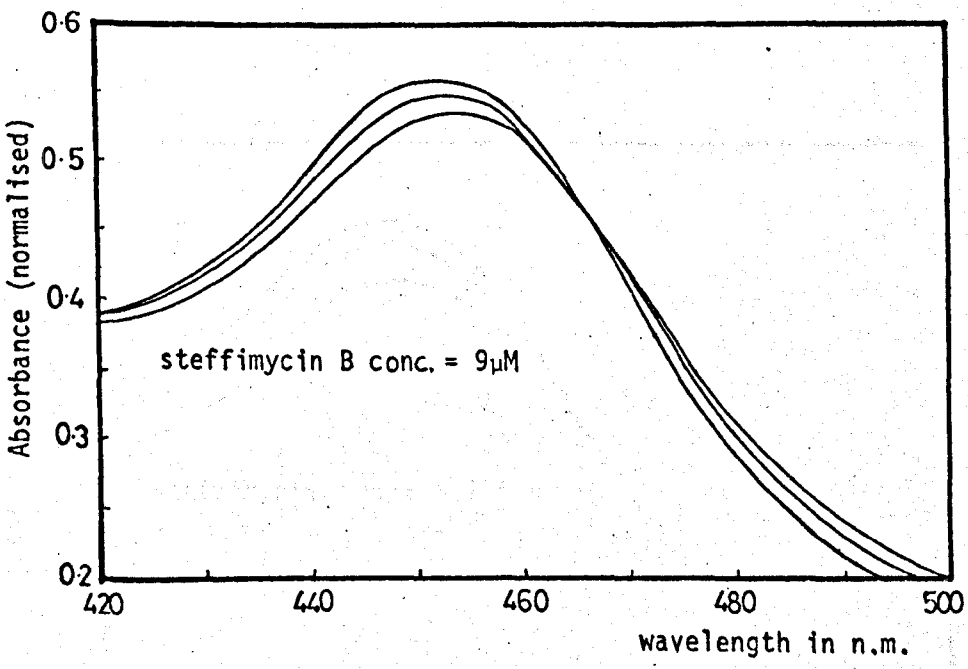


Fig. (4:24) Spectra of steffimycin B with M Lysodeikticus D.N.A. in solution with P/D of 0, 4.5 and 6.7.

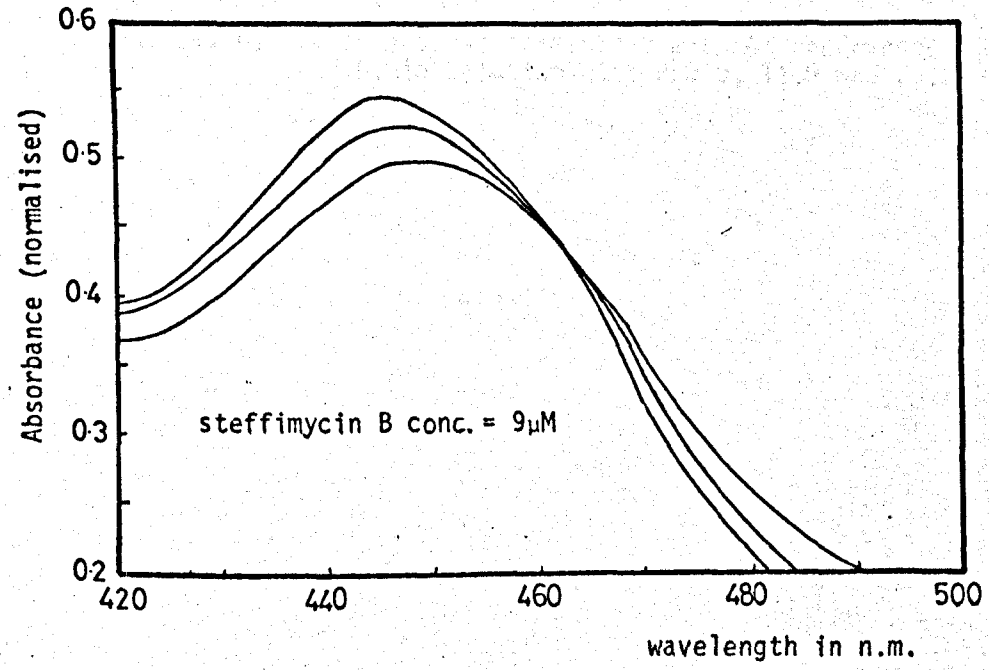


Fig. (4:25) Spectra of steffimycin B with calf thymus D.N.A. in solution with P/D of 0, 4.0 and 9.7.

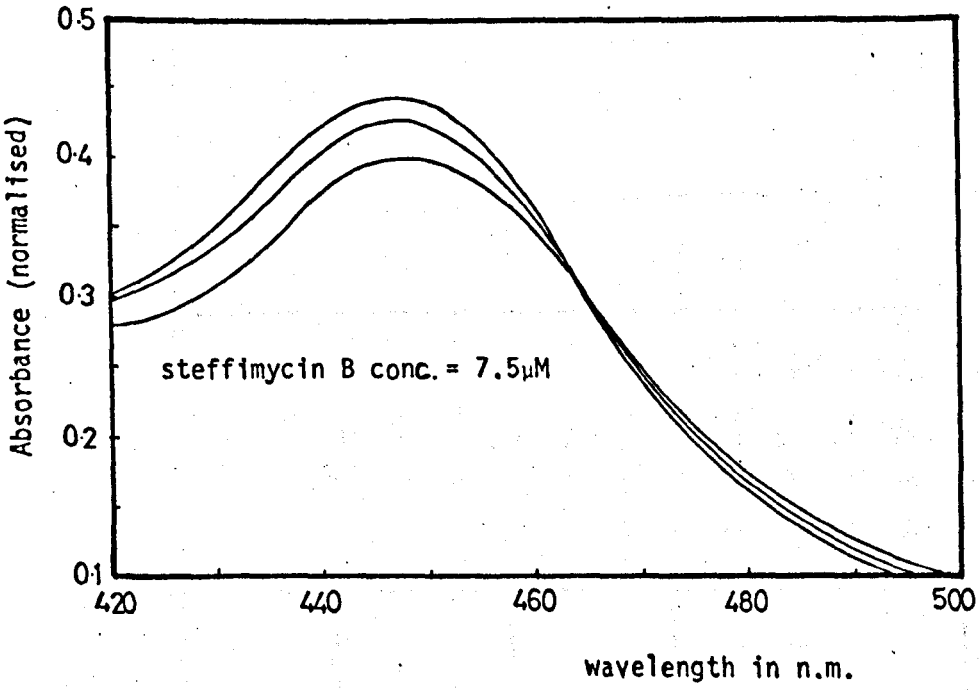
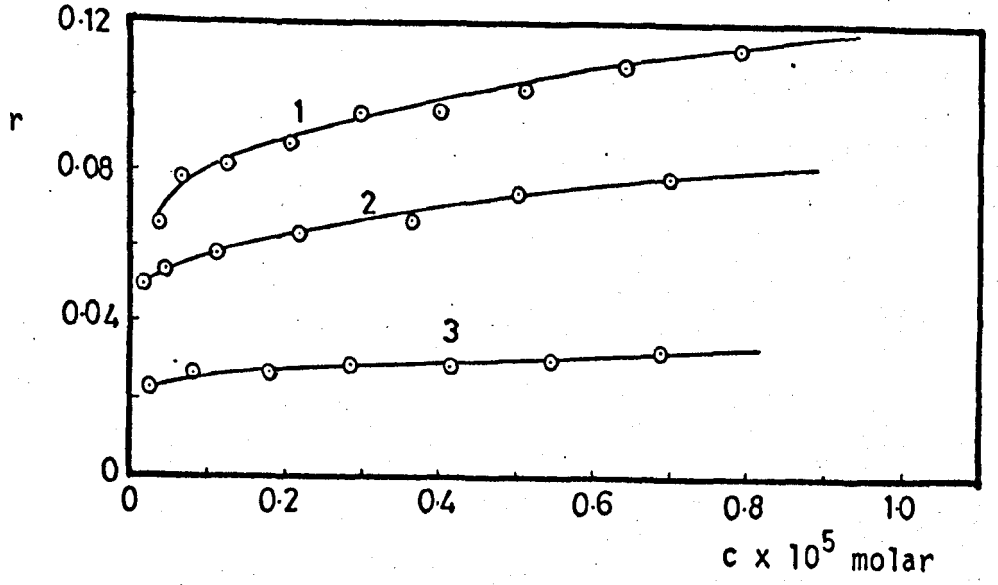


Fig. (4:26) Spectra of steffimycin B with Cℓ Perfringens D.N.A. in solution with P/D 0, 11.9 and 26.2



1 M Lysodeikticus 2. Calf thymus 3. Cℓ Perfringens

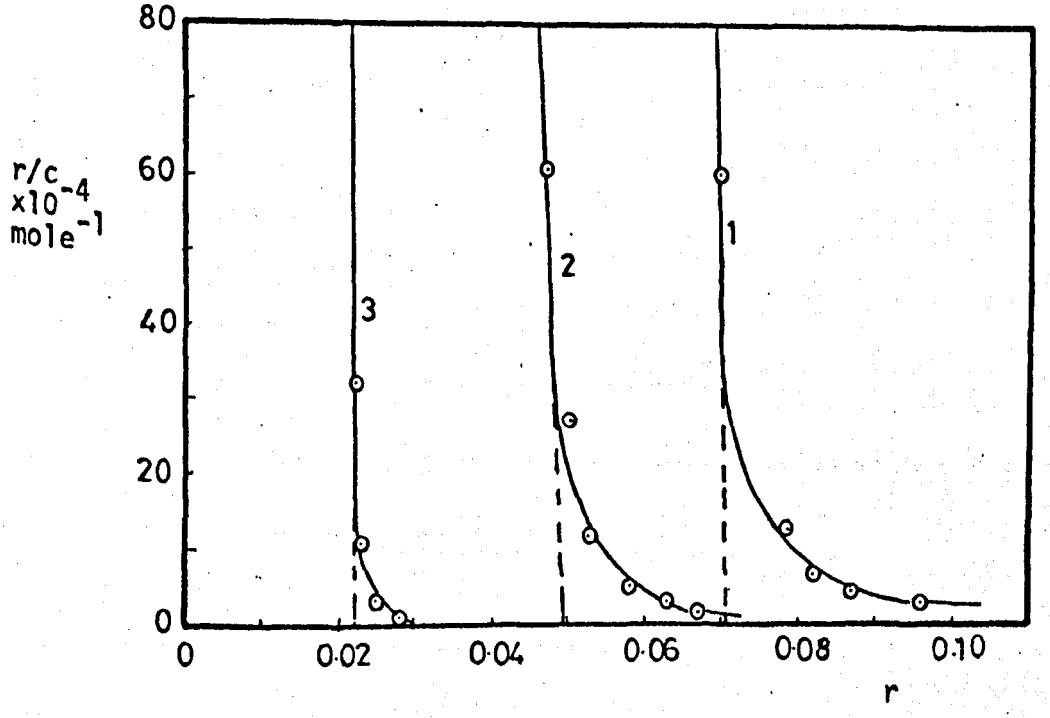


Fig. (4:27) and (4:28) Binding curves for steffimycin B to natural D.N.A.'s with max free drug concentration of 9μM.

1. n = 0.071 ± 0.15 K = 0.12×10^{-8}
2. n = 0.050 ± 0.15 K = 0.40×10^{-8}
3. n = 0.022 ± 0.15 K = 0.35×10^{-8}

weaker binding process. Complex formation with natural D.N.A.'s indicates a preference to bind to G-C base pairs with a secondary binding occurring at the higher drug concentrations associated also with G-C base pairs.

Studies of the binding of steffimycin B to synthetic D.N.A.'s were not carried out due to the expense involved and because of the difficulty of measuring the absorbance at such low concentrations. Reusser reports, however, that steffimycin B shows a preference to bind to A-T base pairs using synthetic polynucleotides [23]. He used drug concentrations of less than 1μM and based his suggestion on experiments involving the inhibition of the template activity of poly d(A-T) and poly dG poly dC. Again this is contrary to the base specificity observed in natural D.N.A.'s in the experiments reported here. Reusser remarks that reactions primed with natural D.N.A. remained insensitive to steffimycin B inhibition.

4:6 Complexes of D.N.A. with other drugs

Spectral studies were carried out on the interaction of daunomycin, adriamycin, actinomycin and nogalamycin with the three D.N.A.'s used above. Daunomycin is well known for its binding to D.N.A. [25] and to be specific in its interaction [35]. A comparison of its complex formation in solution with steffimycin is of interest because of the similarity between the two drugs. Adriamycin is less studied than daunomycin but the drugs are very similar in composition; the only difference being that the former has an OH group replacing H in the latter on the saturated ring of the chromophore. Adriamycin is reported to be non-specific in its binding to D.N.A. [78].

Actinomycin was studied because of its well characterized binding to D.N.A. and its G-C base pair specificity. The spectral work on actinomycin can be used as a control when studying other drugs whose mode of binding is uncertain or unknown. Finally, the binding of nogalamycin was examined. Most of the understanding of the interaction of this drug with D.N.A. rests on studies of its ability to inhibit the synthesis of D.N.A. and R.N.A. when added to a D.N.A. or poly dG.dC template [35,79,80]. It is evident that complexing with the template takes place but workers disagree about its specificity. It was hoped to use the spectroscopic method of this work to clarify the mode of interaction of this drug with D.N.A. of different base composition.

4:6:a Daunomycin/D.N.A. complexes

Spectroscopic investigations of the binding of Daunomycin to D.N.A.'s show that there is no detectable base specificity influencing complex formation. For the three natural D.N.A.'s used, the results show that there are 0.28 binding sites per nucleotide and that K_d is 2.7×10^{-8} in each case. See fig. (4:29) and (4:30). This strong binding saturates rapidly after which the number of drugs bound per site continues to increase linearly and at the same rate for the different nucleic acids. This secondary binding may be the result of the cooperative stacking of chromophores on the outside of the double helix. The latter explanation is likely because the complex precipitates out at higher D.N.A. concentrations. Pigram [45] concludes that daunomycin intercalates with a maximum of 4 or 5 bases per bound drug as evidenced by X-ray diffraction studies. Further binding of daunomycin then occurs until one drug was bound

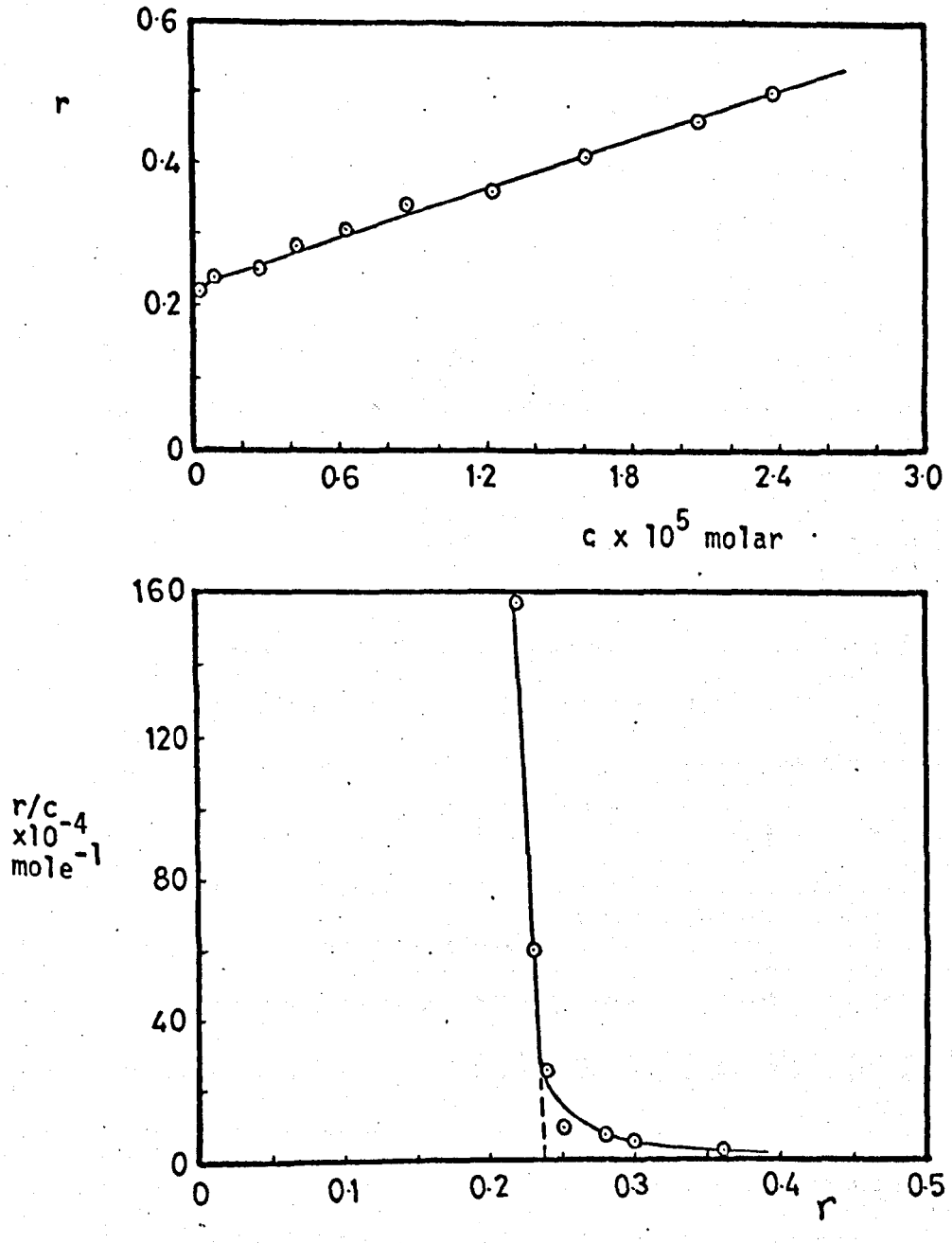


Fig. (4:29) and (4:30) Binding of Daunomycin to M Lysodeikticus, calf thymus and Cℓ Perfringens D.N.A.'s. All three curves coincide.

$n = 0.24 \pm 0.02$ $K = 2.7 \times 10^{-8}$

per nucleotide. Thus the two types of binding observed in the spectroscopic work of this thesis agree with the two types of complex observed by Pigram [45] and other workers such as Kersten et al [81].

4:6:b Adriamycin/D.N.A. complexes

Results of spectroscopic studies are shown in fig. (4:31) and (4:32). Adriamycin gave very similar binding characteristics to those of daunomycin. Once again both primary and secondary binding were observed. Similarly, no base specificity was observed for the strong binding which occurred to a slightly less extent than in daunomycin. For adriamycin, $n = 0.23$ and $K_d = 1.21 \times 10^{-8}$. Secondary binding was more prominent for adriamycin and there was some evidence to show that this binding occurred to a greater extent for higher G-C content D.N.A. The number of occupied sites per nucleotide rises to 1 for M Lysodeikticus and probably continues rising for greater drug concentrations. Possibly, adriamycin molecules which are bound to D.N.A. become binding sites for further drug molecules. This would not explain the specific nature of the secondary binding.

4:6:c Actinomycin/D.N.A. complexes

Results are shown in fig. (4:33) and (4:34). Actinomycin forms complexes which exhibit base specificity with a preference for G-C base pairs. The results coincide with those of Müller and Crothers [82] who carried out an extensive study actinomycin binding to D.N.A. using several techniques including spectrophotometry. These

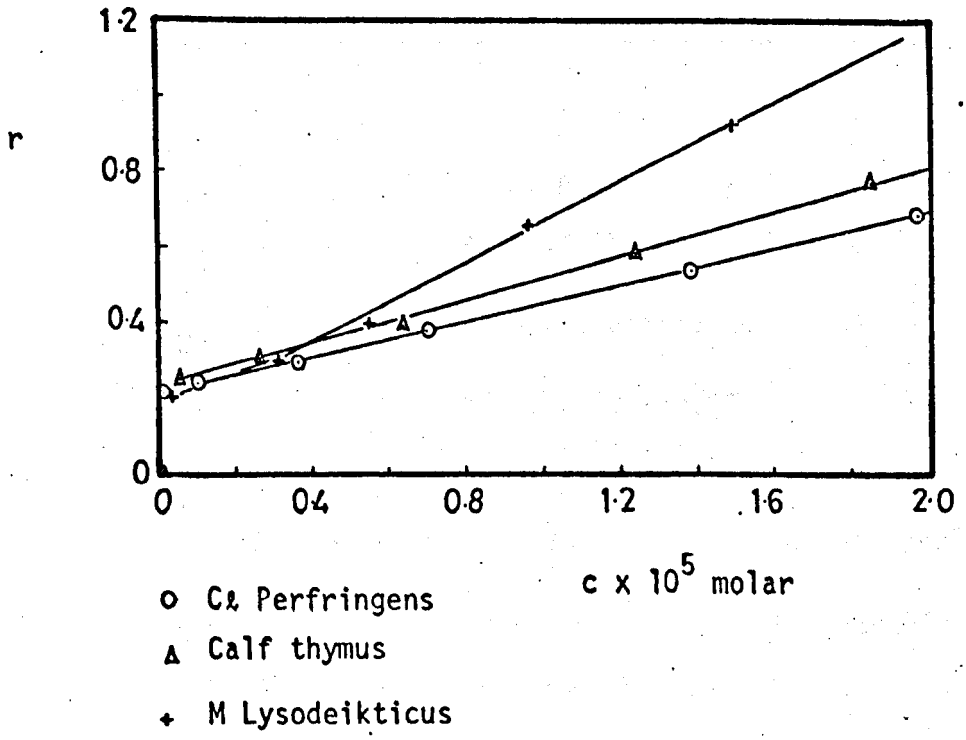


Fig. (4:31) Adriamycin binding to natural D.N.A.'s. Concentration of drug is 29.9μM for Cℓ P. and C.T., 27.8μM for M Lyso.

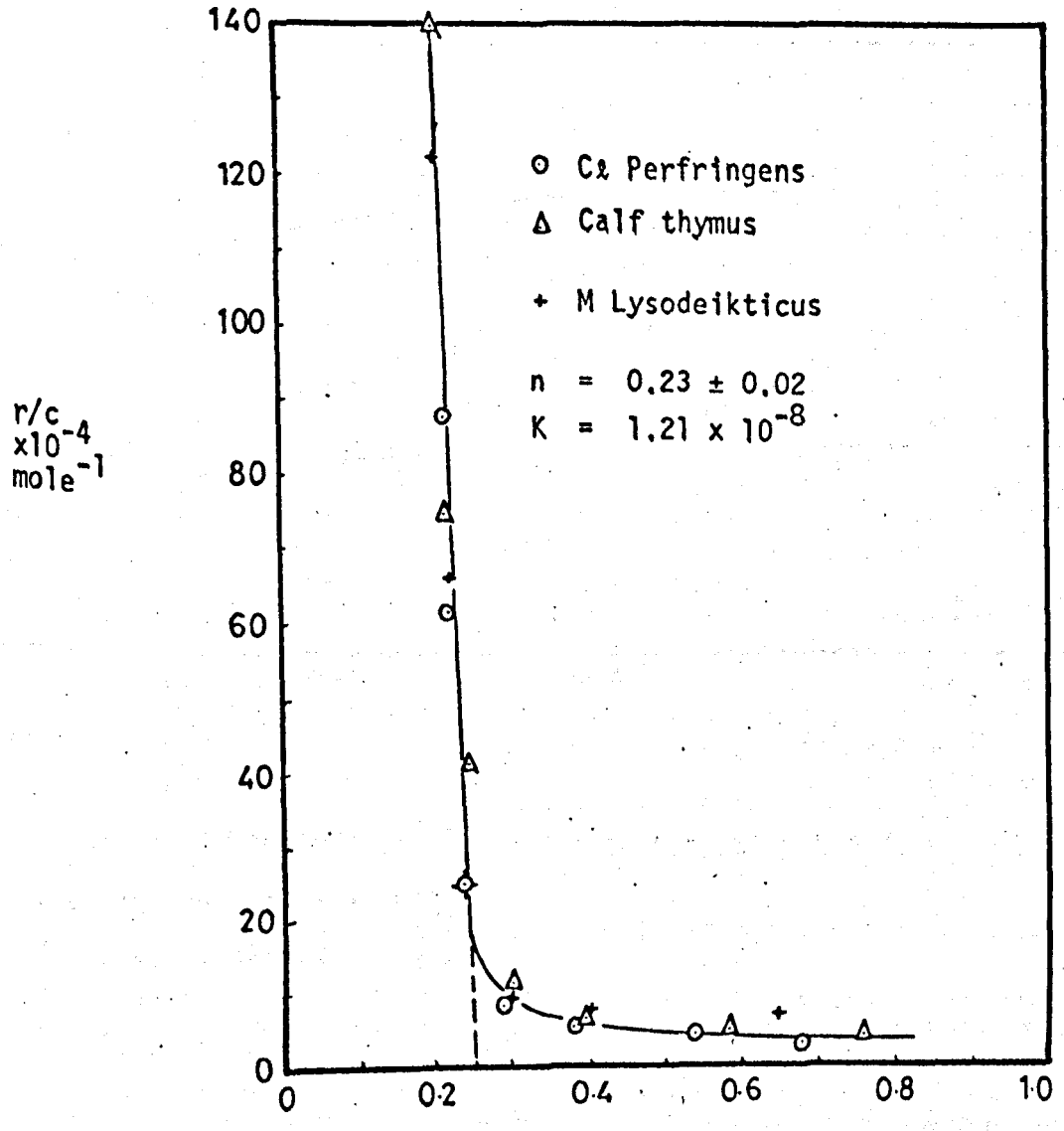


fig. (4:32) Binding of Adriamycin to natural D.N.A.'s.

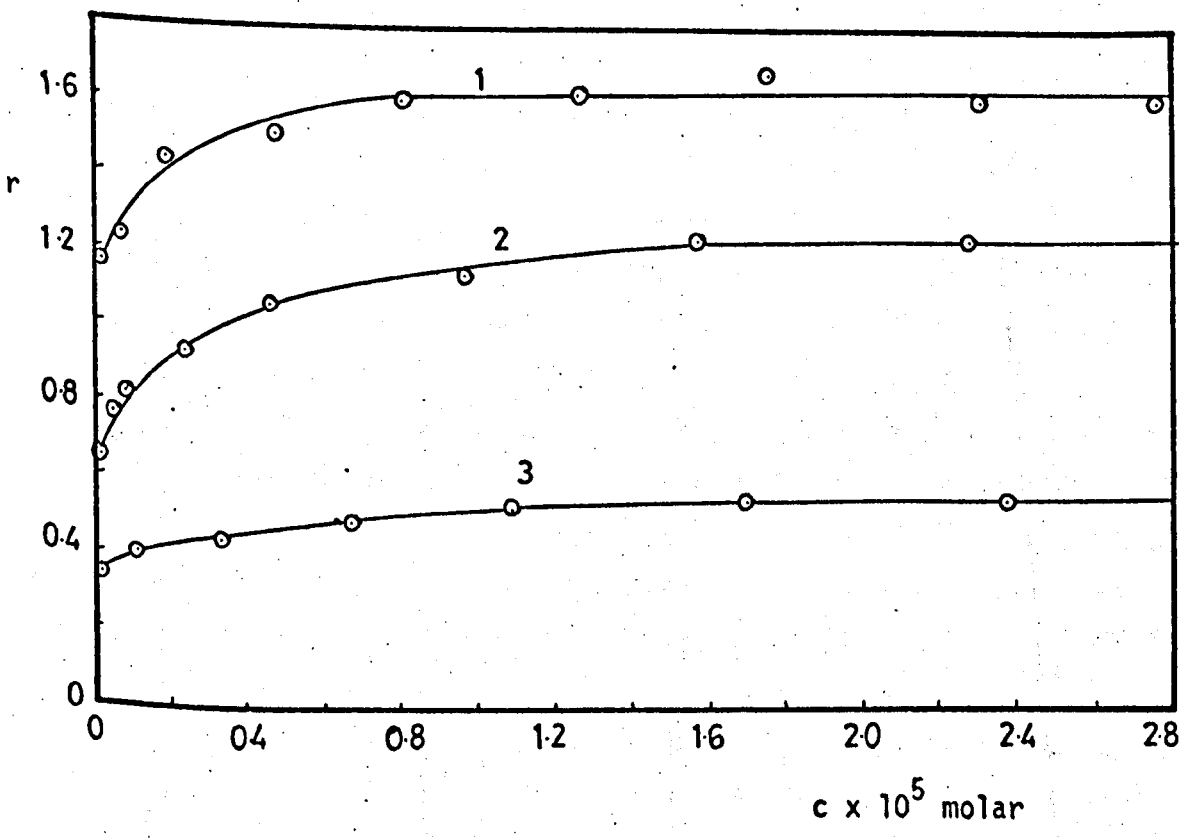


Fig. (4:33) Actinomycin D binding to natural D.N.A.'s.

Drug concentration 30.5μM.

1. M Lysodeikticus
2. Calf thymus
3. Cℓ Perfringens

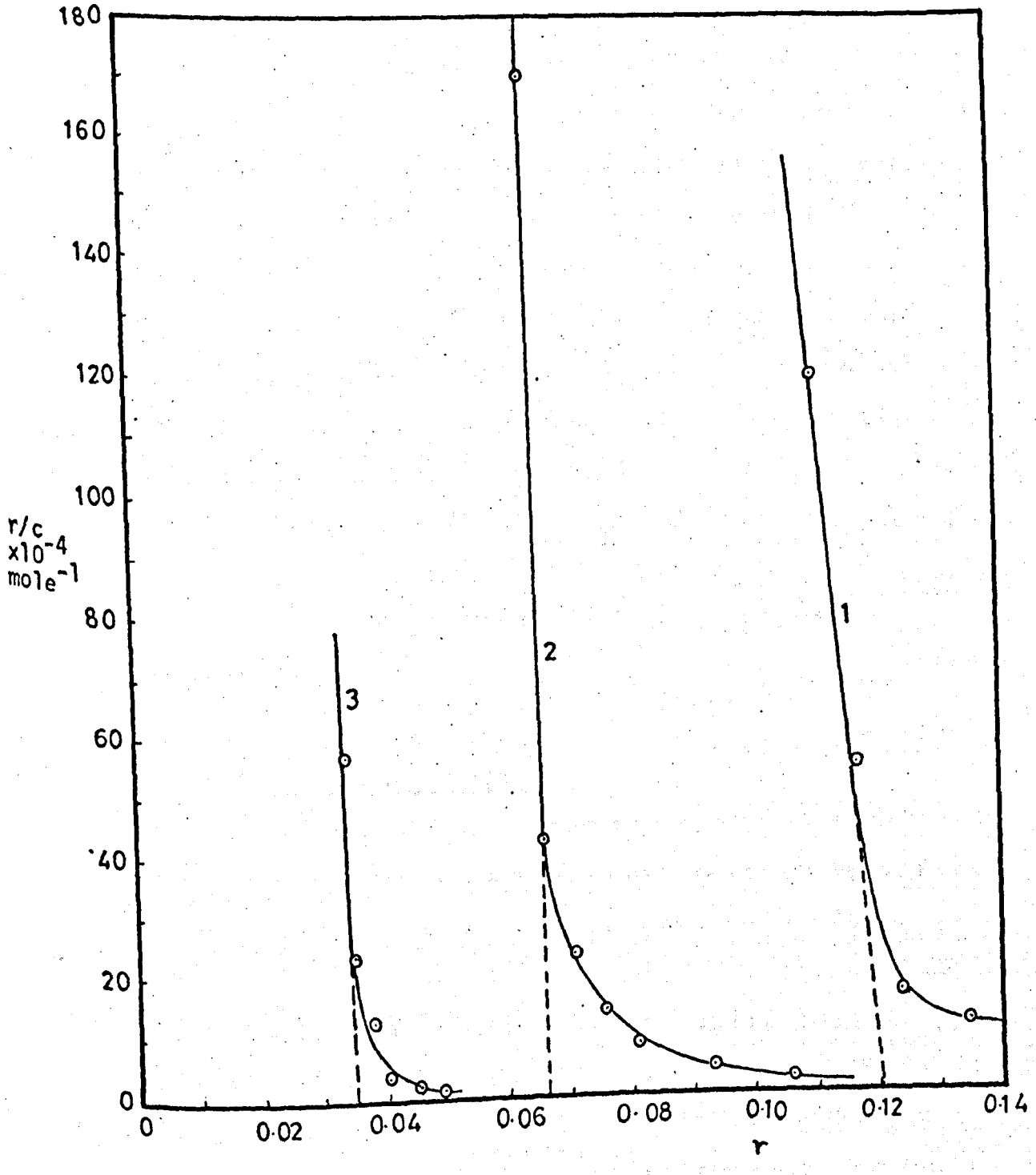


Fig. (4:34) Actinomycin binding to

1. M Lysodeikticus, 2. Calf thymus and 3. Cℓ Perfringens D.N.A.

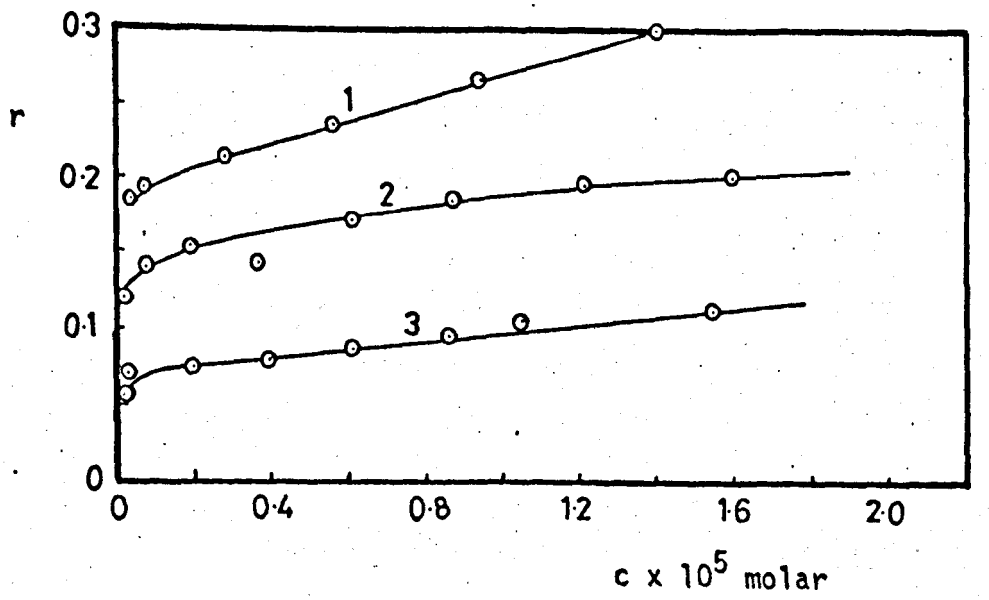
1. n = 0.120 ± 0.01 K = 0.85×10^{-8}
2. n = 0.067 ± 0.01 K = 0.10×10^{-8}
3. n = 0.035 ± 0.01 K = 0.18×10^{-8}

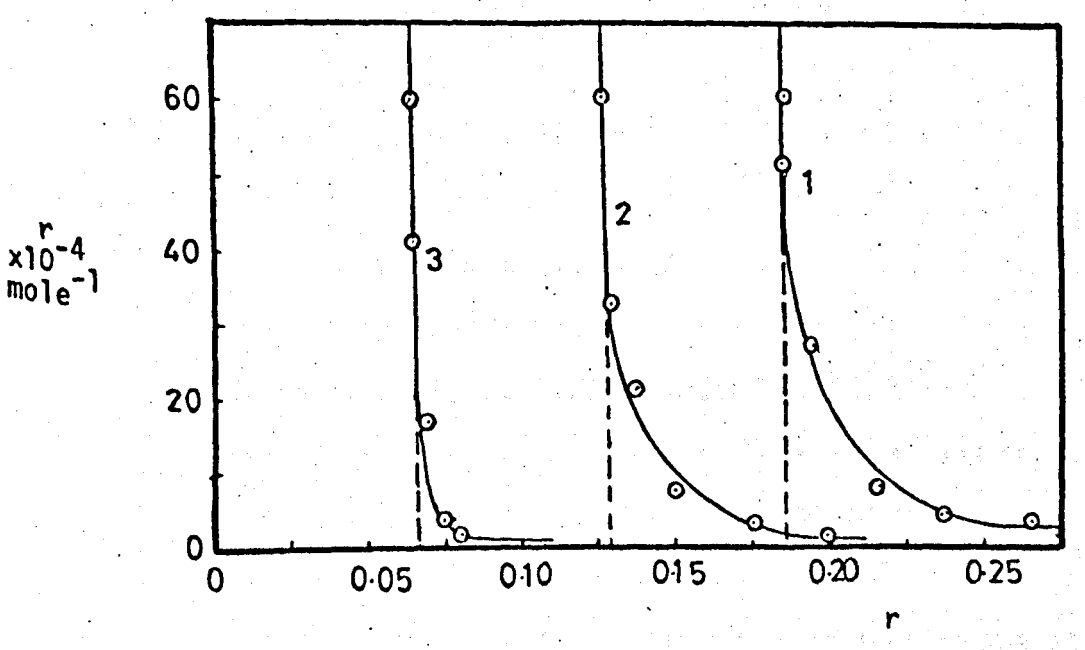
two workers give n for binding to calf thymus D.N.A. as 0.075 (0.01 M Na^+) compared with a value for n of 0.067 (0.004 M Na^+) obtained in this work. Müller and Crothers plotted r/c against r with values of r/c up to 60 x 10^4 but when r/c is plotted to 200 x 10^4 the slope then gives the lower value of n. In their discussion of the base specificity, Müller and Crothers suggest that guanine is recognised by π complex formation which takes place during intercalation. This is dependent upon the existence of specific resonance forms of the actinomycin chromophore.

Fig. (4:33) also shows how the binding of actinomycin rises to a maximum for each D.N.A. and remains constant for increased concentration of the drug. This implies that there is little or no secondary binding.

4:6:d Nogalamycin/D.N.A. complexes

The experiments on the interaction of nogalamycin with different D.N.A.'s are only qualitative due to the uncertainty of the drug concentration which had to be estimated. Because of its low solubility in water, nogalamycin was dissolved in a solution of 20% ethanol. No extinction coefficient for the drug has been quoted but this was estimated to be 4000-5000 by using a M.W. of 787 and measuring the absorption of a solution in 20% ethanol with known concentration. It can be seen, however, from fig. (4:35) and (4:36) that the drug binds to D.N.A. with a preference for G-C base pairs. This specificity is clear even though the absolute number of drugs bound per nucleotide is not known. Nogalamycin shows a small degree of secondary binding and this is more evident for higher G-C content D.N.A.





1. M Lysodeikticus 2. Calf thymus 3. Cℓ Perfringens

Fig. (4:35) and (4:36) Binding of nogalamycia to natural D.N.A.'s. Concentration of drug was estimated to be 20μM but may be as low as 10μM. Results are qualitative.

1. n = 0.187
2. n = 0.130
3. n = 0.068

4:7 Summary of the spectroscopic work

The information on the binding of the steffimycins to D.N.A. and a comparison with the other antibiotics can be summarised as follows.

1. The steffimycins are hydrophobic and are more soluble in apolar solvents. Steffimycin is more soluble than steffimycin B.
2. The steffimycins do bind to D.N.A. with the number of steffimycin molecules bound ~1.5 x the number of steffimycin B molecules for the same total drug concentration.
3. Both "strong" and "weak" types of binding were observed.
4. The number of primary binding sites on natural D.N.A.'s increases with increasing G-C content.
5. The binding to the synthetic D.N.A.'s seems anomalous in that the number of prime sites on poly d(G-C) is much lower than expected and also poly d(A-T) has quite a high number of sites.
6. Secondary binding does not occur with poly d(A-T) and for the other D.N.A.'s increases with the G-C content.
7. The extrapolation of the M Lysodeikticus data (which has the highest G-C content of the natural D.N.A.'s) indicates that there would be 1 prime site for every 12 phosphates.
8. The spectrum of the steffimycins with poly d(A-T) is different from the rest and coupled with points 5 and 6 suggests that a quite different molecular complex occurs.
9. Of the other drugs examined, only actinomycin did not exhibit both weak and strong binding.
10. Base pair specificity of daunomycin, adriamycin, actinomycin and nogalamycin are compared with results of other workers in

fig. (4:37).

Disparate results for the base specificity of daunomycin and nogalamycin are reported by different workers. It is important to note the experimental methods used to investigate the interaction of a drug with nucleic acids since methods involving the inhibition of the enzymatic synthesis of R.N.A. by addition of the drug to the D.N.A. template sometimes disagree with the more direct spectroscopic and melting point studies. It may be possible for a drug to bind to D.N.A. and not inhibit its template function. Such binding would not be observed by R.N.A. synthesis methods.

Adriamycin is very similar to daunomycin in its molecular structure and effects on D.N.A. The findings on the interactions of the drug here are in agreement with Kersten [78]. The characteristics of actinomycin fixation both in extent and specificity of binding agree with the results of Waring [26] and thereby provide a useful set of control experiments.

4:8 Extent of complex formation in sedimented gels

Gels of the steffimycin/D.N.A. and steffimycin B/D.N.A. complexes were prepared from solution by centrifugation as described in chapter 2 section 3:a. Equation 2 of this chapter shows that the number of drug molecules bound per nucleotide, $r = \alpha D/P$. Thus the P/D of the complex in the gel after centrifugation is not the same as the P/D ratio of the starting solution since α is less than one except where there is a large excess of D.N.A. when P/D>30.

In order to determine the P/D of each gel and hence of the resulting oriented fibres, the supernatant liquor was analysed spectroscopically for drug and D.N.A. content since there was always

chromophore	*base specificity*	*worker*	*details and methods*	*results from this work*
Daunomycin	not specific	Ward, Reich and Goldberg [35]	various methods	no base pair specificity
	A-T specific	Di Marco et al [36]	melting point experiments	
	G-C specific	Kersten [78]	only slightly specific, circular dichroism	
Adriomycin	not specific	Kersten [78]	concludes that anthracyclines do not show base specificity	only base specific in secondary binding
Nogalamycin	A-T specific	Ward et al [35]	R.N.A. synthesis	G-C specific
	A-T in isopropanol	Bhuyan et al [79]	D.N.A. dependent R.N.A. synthesis. Sequence important	
	G-C dependent	Honikel [80]	poly dG.dC directed D.N.A. synthesis inhibited	
Actinomycin	G-C dependent	Muller & Crothers [82]	spectral, equilibrium dialysis, viscosity, sedimentation and kinetic measurements	G-C specific
	G-C specific	Waring [26]	Spectroscopic	
	Base sequence dependent	Wells & Larson [83]	used various short strands of known base sequence	

Fig. (4:37) Comparison of the base pair specificity by other workers

some drug remaining in solution. Fig. (4:38) shows the resulting P/D of a sedimented gel as a function of P/D of the solution originally placed in the centrifuge tube for the three natural D.N.A.'s used. Similar results were obtained for the P/D of gels of steffimycin B complexes with D.N.A. Since the complex could be sedimented as a gel, it appears that the interaction between drug chromophore and D.N.A. did not depend upon being in solution.

Knowing the drug and D.N.A. concentrations in solution before and after centrifugation, it was found that D.N.A. with higher G-C content was capable of binding more drug from solution than low G-C. This gives further evidence for the G-C specificity of the steffimycins when binding to natural D.N.A.'s.

The solubility of the drugs prevented solutions (and hence gels) being prepared with P/D<6. Even at the highest drug levels used, no precipitation of the complexes was observed.

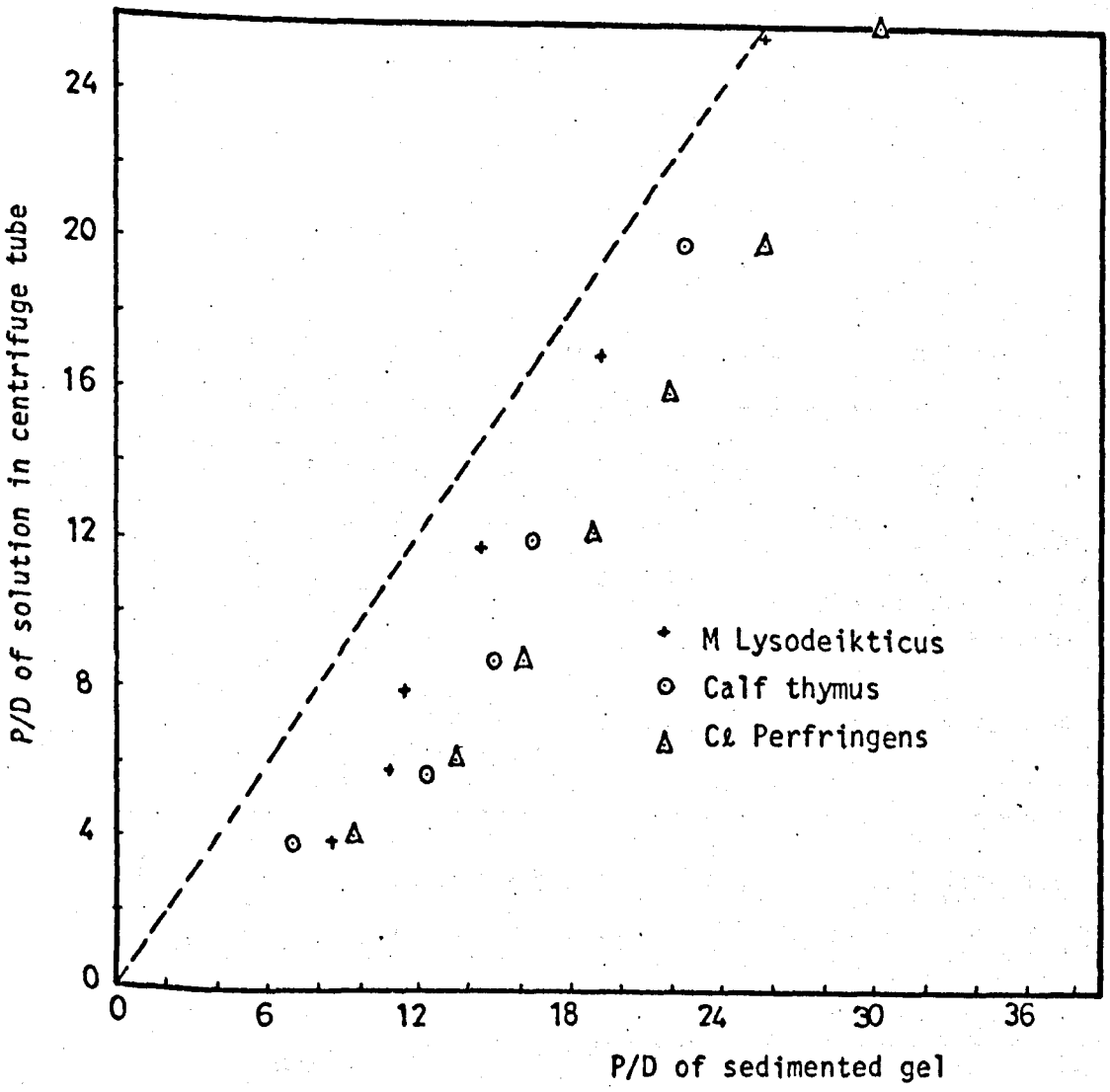


Fig. (4:38) Graph showing the resulting P/D in a gel of a steffimycin complex formed with each of the three D.N.A.'s. The dashed line represents 100% of drug bound and points farthest to the right of this indicate more of the steffimycin left in solution.

Chapter 5

X-RAY DIFFRACTION STUDIES OF D.N.A.-STEFFIMYCIN FIBRES

5:1 Introduction

The atoms which form a molecule of D.N.A. lie on a series of helices with screw symmetry relating any particular atom in a nucleotide with the equivalent atom in the neighbouring nucleotide. This collection of discontinuous helices is used as a basis for the mathematical formulation of the Fourier transform of D.N.A. This transform is found to be:-

$$G(R,\psi,\ell/c)=\Sigma\Sigma J_n\, f_j(2\pi R r_j)\, \exp\, i[n\psi - \phi_j + \pi/2) + 2\pi\ell Z_j/c]$$
$$nj$$

where R, ψ, ℓ/c are reciprocal space coordinates of the transform, ℓ is the layer line number in the transform, c is the helix pitch, r_j, ϕ_j, Z_j, are the real space coordinates of j atoms in the helix and J_n is an nth order Bessel function.

This describes a three dimensional transform which is observed on a two dimensional plane. It is zero everywhere except on a series of planes observed as layer lines. On any particular layer line the orders of Bessel functions contributing to the resulting transform are given by the integral solutions of:-

$$n = \frac{\ell - mN}{K}$$ where ℓ is the layer line,

$m=0,\pm1,\pm2$ etc., K is the minimum number of complete turns of the helix necessary to incorporate an integral number of residues, and N is the number of residues in this repeat distance. The resulting transform is therefore the sum of all values of n obtained from this

relationship.

Thus it is possible to interpret the features of the diffraction patterns of aligned D.N.A. molecules in terms of helical parameters. The following information was obtained.

1. The helix pitch. This is given by the reciprocal of the layer line separation and corresponds to c.
2. The base pair separation in the molecules. This can be determined since the Fourier transform of the D.N.A. molecule is formed from a convolution of the transform of a continuous helix with that of a function which is zero except on planes of spacing p, the axial rise per residue. The transform then has peaks with spacing 1/p and is equivalent to the transform of a continuous helix with the origin at $\zeta = 0, \pm 1/p \pm 2/p$ etc. This results in a repeat of the transform every 1/p along the meridional. Thus the base pair separation is given by the reciprocal of the meridional reflexion spacing which involves a zero order Bessel function with a maximum at $R = 0$.
3. Intermolecular separation. This distance is a feature of the crystal packing of the helices. The transform of the D.N.A. helix is sampled at the reciprocal lattice points and the resulting crystalline reflexions on the equator were indexed enabling the unit cell dimensions to be calculated. The distance between helix axes could then be calculated by simple geometry.

The sodium salt of D.N.A. can form different crystalline or semicrystalline structures. The parameters of the two main forms, A and B are given in fig. (5:1).

configuration	no. of nucleotide pairs per helix pitch	Inclination of base pairs to helix axis	crystal class	crystallinity	molecular positions	Unit cell dimensions a(A)	b(A)	c(A)	β(°)
A	11.0	70°	monoclinic	crystalline	0, 0, 0 $\frac{1}{2}$, $\frac{1}{2}$, 0	22.24	40.62	28.15	97.0
B	10	~90°	hexagonal	semi-crystalline	0, 0, 0 $\frac{1}{3}$, $\frac{2}{3}$, $\frac{1}{6}$ $\frac{2}{3}$, $\frac{1}{3}$, $\frac{-1}{6}$	46	-	34.6	-

fig. (5:1) after Langridge et al [60]

Typical X-ray photographs of the A and B forms of D.N.A. are shown in fig. (5:2) and (5:3). The pattern from the crystalline A form was obtained at low salt content ∿ <0.005 M NaCℓ in the solution used to form the original gel. With higher salt content, ∿0.01M NaCℓ, the X-ray diffraction pattern was of the B configuration. These photographs are typical of those obtained from D.N.A. gels used as a control when producing fibres with drug bound to the D.N.A. molecules.

The changes in these helical parameters and crystalline packing of D.N.A., caused by the binding of the steffimycins, were examined by taking several series of X-ray diffraction patterns of fibres containing drug bound to the nucleic acid. The X-ray studies carried out were as follows.

1. An examination of D.N.A. with steffimycin bound at a range of P/D ratios. The photographs were taken at 92% humidity. The natural D.N.A.'s used were M Lysodeikticus, Cℓ Perfringens and calf thymus.
2. Steffimycin bound to poly d(A-T) and poly d(G-C).
3. Selected steffimycin-D.N.A. fibres were photographed at a range of humidities to observe the effects of varying the proportion of water molecules in the D.N.A./drug lattice.
4. A limited study of steffimycin B/D.N.A. fibres was also carried out. This allowed the effects on D.N.A. of steffimycin B and steffimycin to be compared.

5:2 X-ray diffraction of complexes of steffimycin with natural D.N.A.

Fig. (5:4) shows an X-ray diffraction photograph obtained from a fibre of M Lysodeikticus D.N.A. with steffimycin and taken at 92%

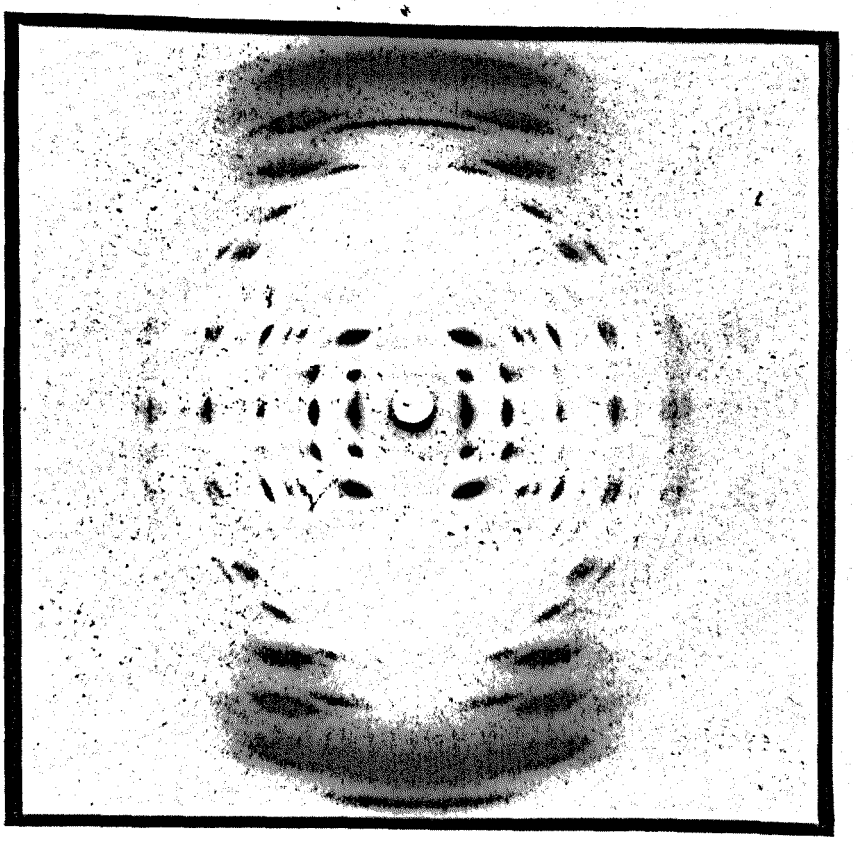


Fig. (5:2) D.N.A., A conformation

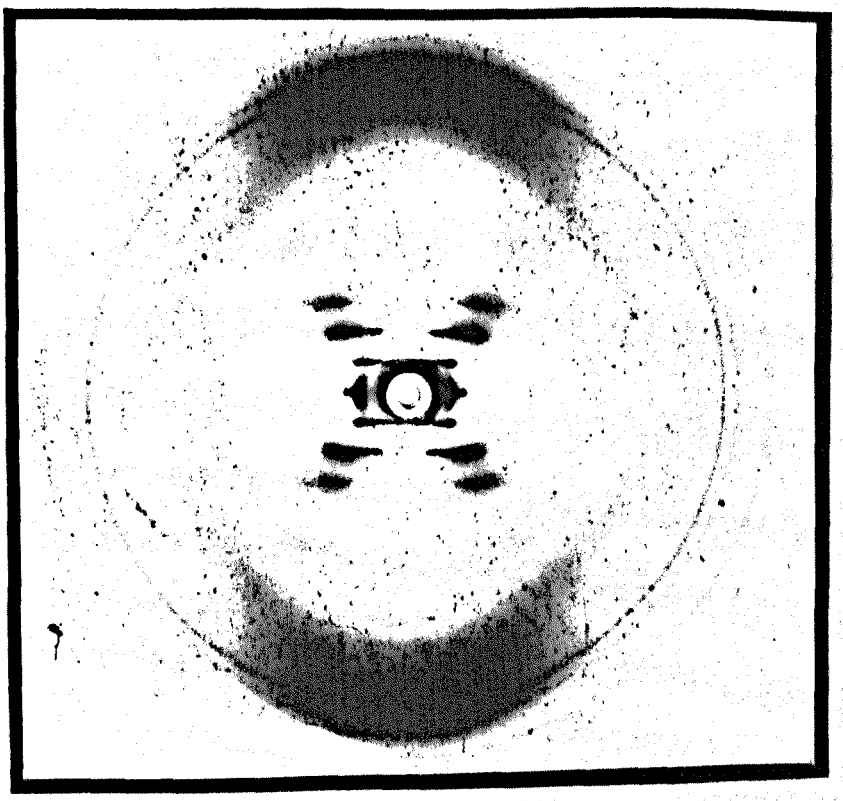


Fig. (5:3) D.N.A., B conformation

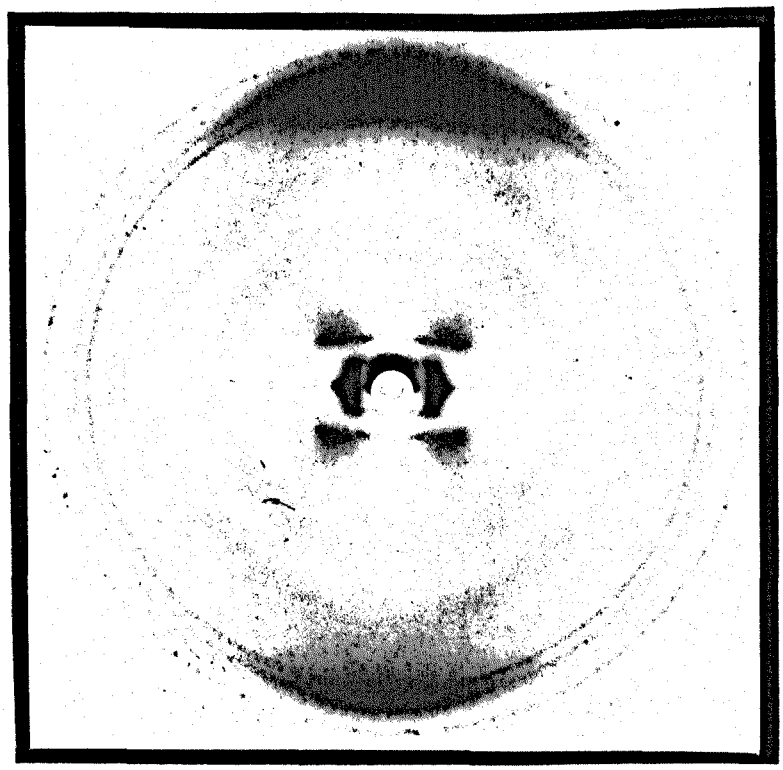


Fig. (5:4) D.N.A. - Steffimycin P/D = 8.6 R.H. = 92%

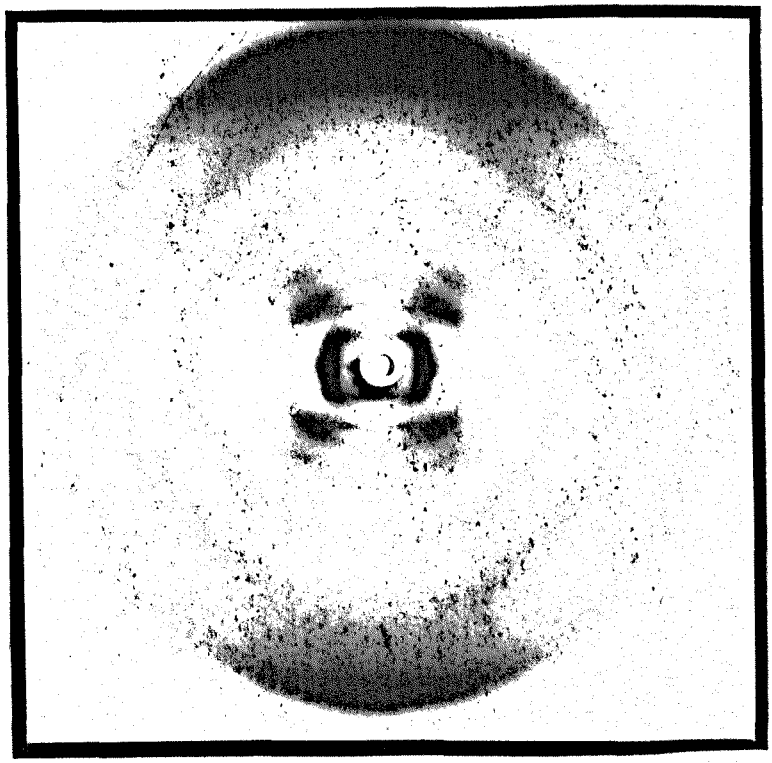


Fig. (5:5) D.N.A. - Steffimycin P/D = 14.9 R.H. = 92%

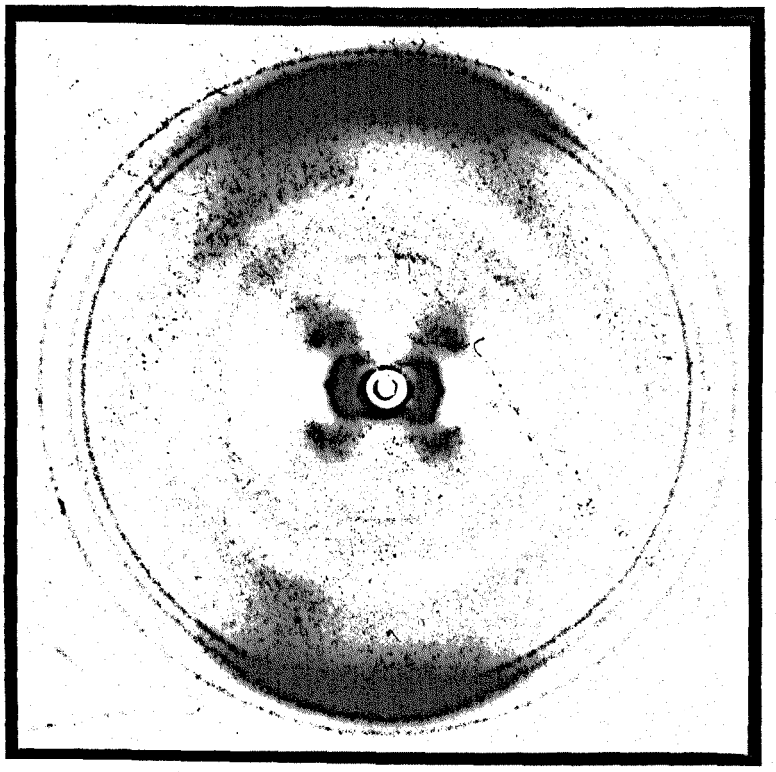


Fig. (5:6) D.N.A. - Steffimycin P/D = 18.4 R.H. = 92%

relative humidity. The P/D of the fibre was 8.6 which corresponds to the maximum number of occupied binding sites suggested by the spectroscopic studies. The photograph retains features of diffraction patterns from a double helical polymer. There are five crystalline equatorial spots which can be measured and these were indexed as follows.

$1/d^2$ observed	ratio	$1/d^2$ calculated	h k ℓ
0.00212 ± 16	3	0.00217	1 1 0
.00491 ± 16	7	.00505	2 1 0
.00669 ± 1	9	.00650	3 0 0
.00869 ± 25	12	.00866	2 2 0
.01513 ± 46	21	.01516	4 1 0

This corresponds to a hexagonal lattice with a = 42.9 ±0.1A. Thus the D.N.A. is still in the hexagonal form found for the B conformation where a = 46A. The effect of the binding of steffimycin has served to decrease the intermolecular separation of the helices but not to alter the molecular packing. It was unusual to be able to measure five equatorial diffraction spots but the intense 110 reflexion was always present and could be used to obtain the separation of the D.N.A. molecules on all patterns.

The presence of a helical polymer is indicated by the presence of layer lines on the pattern of which the first and second were almost always present. Reflexions tended to be spread out along the layer line which could be the result of irregularities of the molecular packing. The layer line spacing corresponding to the c axis of the hexagonal cell was used to determine the helix pitch. Since the 1st layer line was very close to the equator and tended to be obscured by the 110 reflexion, four positions on the +2 and also on the -2 layer lines were measured for this purpose.

There is a prominent meridional reflexion produced by the

repeat distance of the residues in the polymer. Measurements of this reflexion were used to calculate the axial separation between base pairs in the D.N.A. helix. This spacing in the photograph shown in 3.40A which was the value found, with very little deviation, in all patterns taken of complexes of the steffimycins with D.N.A. These X-ray photographs resemble the B form of D.N.A. with no clear higher layer lines, except the 10th, or crystalline reflexions as found in the A form.

The innermost of the three diffraction rings produced by veterite powder and visible in fig. (5:4) is at a spacing of 3.40A and was used to calibrate this and subsequent patterns.

5:2:a The effect of P/D on helix parameters

Data taken from the diffraction patterns show that steffimycin causes an increase of helix pitch and a decrease of helix separation. Figs. (5:4 - 6) are examples of patterns taken at different P/D ratios which illustrate these effects. The variation in pitch was noted to be a function of the P/D ratio at constant humidity and this is shown in fig. (5:7) for many X-ray patterns that were analysed. In general the pitch increases with increasing drug content but there is considerable spread of the data points. This graph is a collection of data taken from a large selection of photographs using different D.N.A.'s and the points fall into a wide band of values. If the results are plotted from any one particular experimental run, as shown in fig. (5:8), then the result is a much smoother variation in pitch change with increase of P/D. This indicates that slight differences in the experimental conditions of each run bring about the spread of data rather than the drug itself

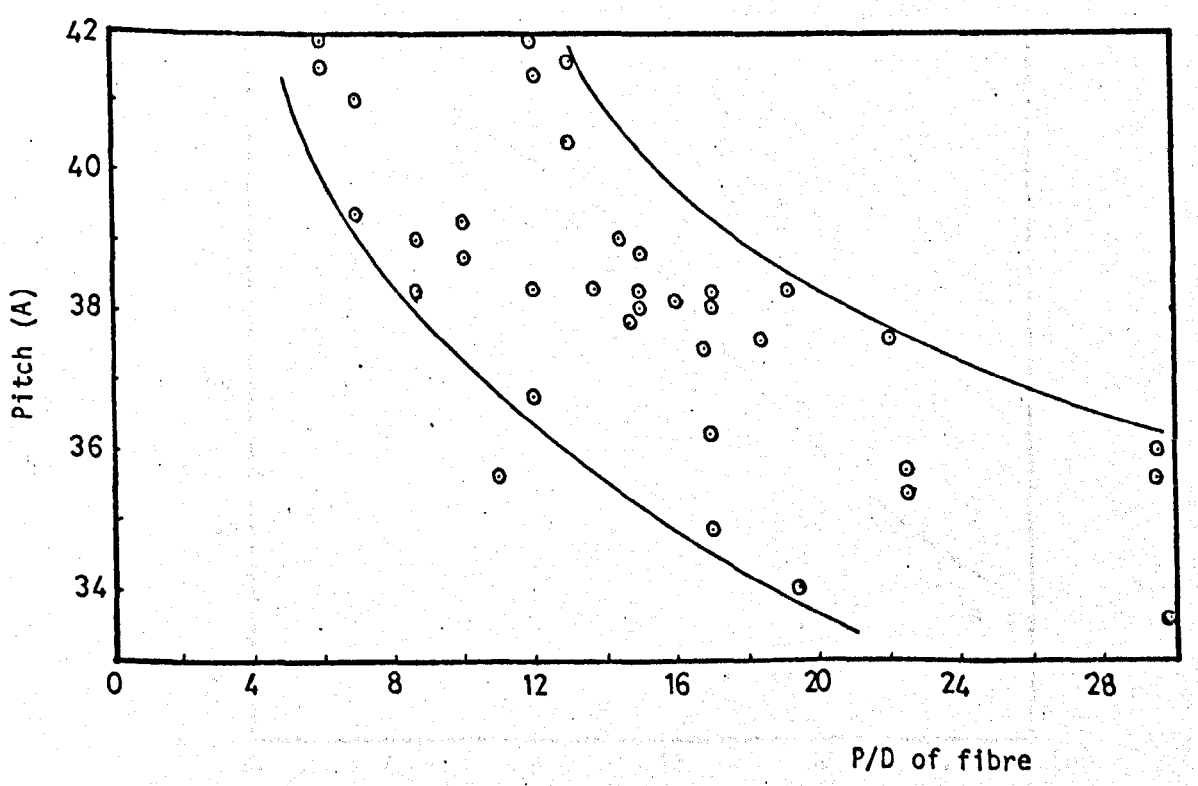


Fig. (5:7) D.N.A. pitch as a function of P/D of the complex with steffimycin. The points represent six series of gel preparations using the three natural nucleic acids mentioned in the text.

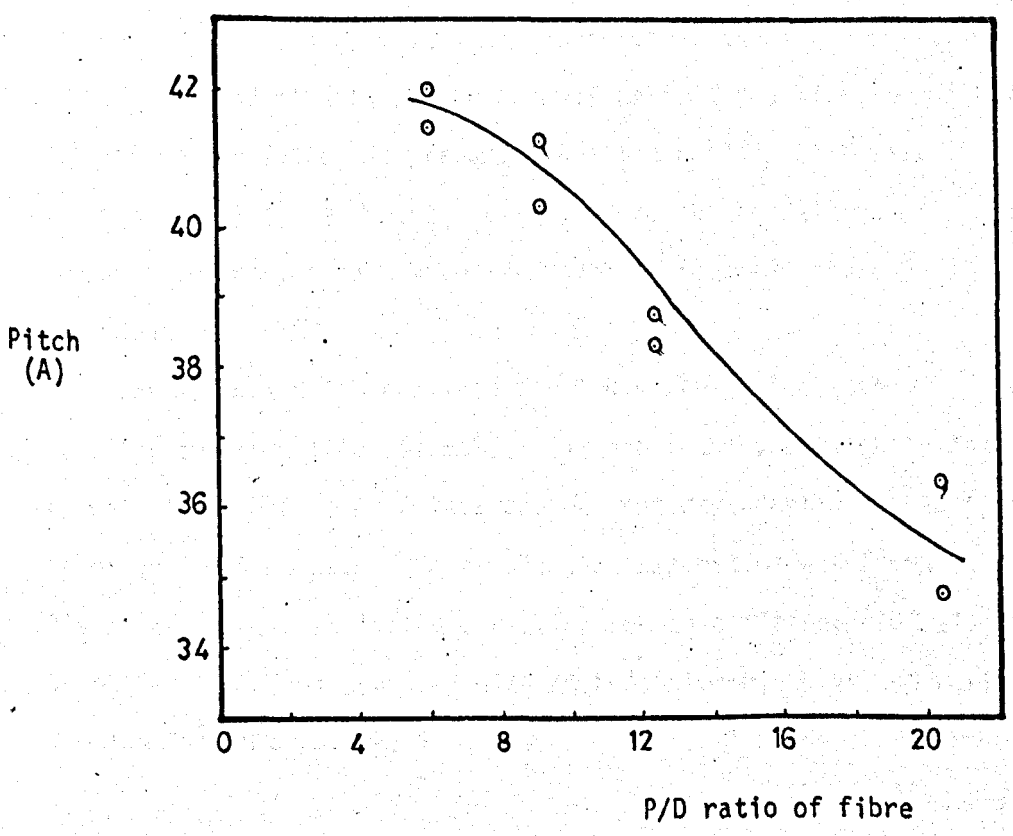


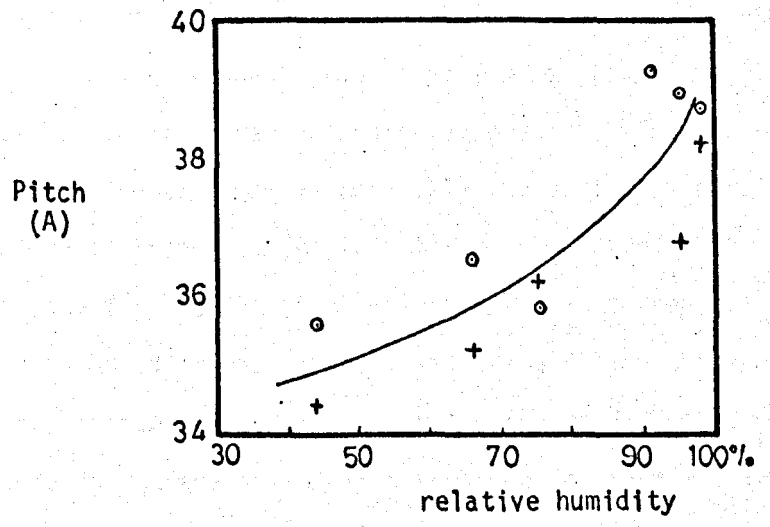
Fig. (5:8) Pitch of calf thymus D.N.A. as a function of P/D of a complex with steffimycin.

causing a variable increase of the D.N.A. pitch. Conditions which could influence the effect of drug on the nucleic acid, and hence the spread of pitch values, are:- ionic strength of the solution, temperature and time taken for centrifugation, the lifetime of the gel before the fibre was prepared, and the humidity and salt content of the fibre. Nevertheless, where the conditions of preparation are similar, then the pitch increase is closely dependent upon P/D.

The maximum pitch obtained for D.N.A. molecules with steffimycin present was 41 ±1A. This was found consistently for fibres with a P/D of 12 or less and was not dependent upon the type of natural D.N.A. used. Intermolecular separation was found to be 24A on average, with little variation due to different P/D values less than 20. This compares with 26.56A intermolecular separation for the B form of D.N.A.

5:2:b The effect of humidity on helix parameters

Helix pitch and intermolecular separation were found to depend not only on the P/D ratio but also on the humidity of the fibre at which the X-ray pattern was taken. Several specimens were photographed at 44, 66, 75, 92, 95 and 98% relative humidity in order to determine the changes in these molecular parameters. Fig. (5:9) shows the result of these measurements. Not only does the intermolecular spacing increase with the presence of water molecules but the helix is seen to increase in pitch. It was interesting to note, however, that the maximum pitch of 41A was not exceeded at increased humidity.



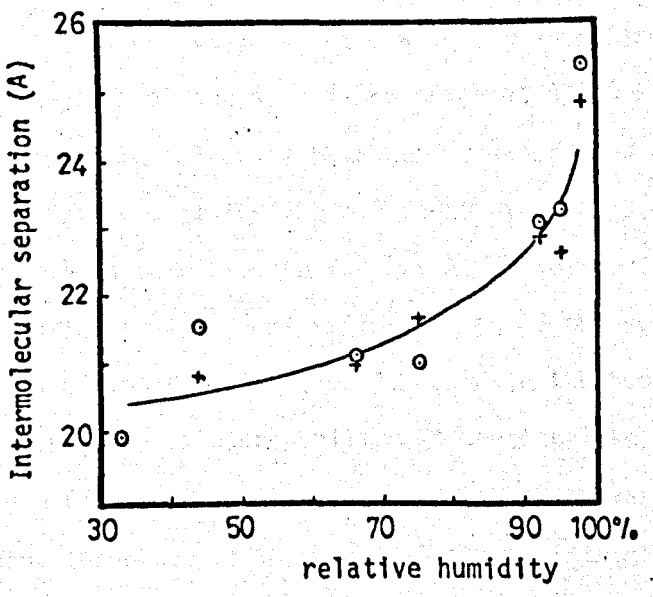


Fig. (5:9) Effect of humidity on intermolecular separation and pitch on two specimens

These effects were not usually dependent upon whether the specimen was first photographed at low relative humidity and subsequently at higher values or vice versa. There were exceptions to this and occasionally the variation of pitch was found to be irreversible after taking the fibre to low humidities. With such fibres, the pitch was observed to decrease to about 34A when the humidity was lowered, and the effect was accompanied by the appearance of a meridional reflexion at 7.2 ±.2A. Upon rehumidifying, the helix pitch remained constant and the 7.2A reflexion became sharper.

Fig. (5:10) shows an X-ray diffraction pattern of a fibre of P/D 12 taken at 92% relative humidity with the usual increase in pitch. A photograph of the same fibre taken at 44% R.H., fig. (5:11) shows that the molecules have become disordered and the pattern is diffuse except for the 110 equatorial and 3.4A meridional which is not uncommon for D.N.A. at low humidities. However, a prominent meridional reflexion can be observed which corresponds to a repeat distance of 7.2A. With the humidity raised to 95% in fig. (5:12), a poor quality but nevertheless typical B D.N.A. X-ray diffraction pattern was seen with the extra meridional reflexion superimposed. It appears that the effects of drug binding to D.N.A. were destroyed at low humidities and that once this had occurred, it was permanent. Fig. (5:13) shows the irreversible change of pitch in a fibre which is taken from high to low humidity.

It was not always necessary to reduce the humidity of a fibre to produce the normal B pattern with the 7.2A meridional reflexion superimposed. A pattern showing normal D.N.A. with a highly

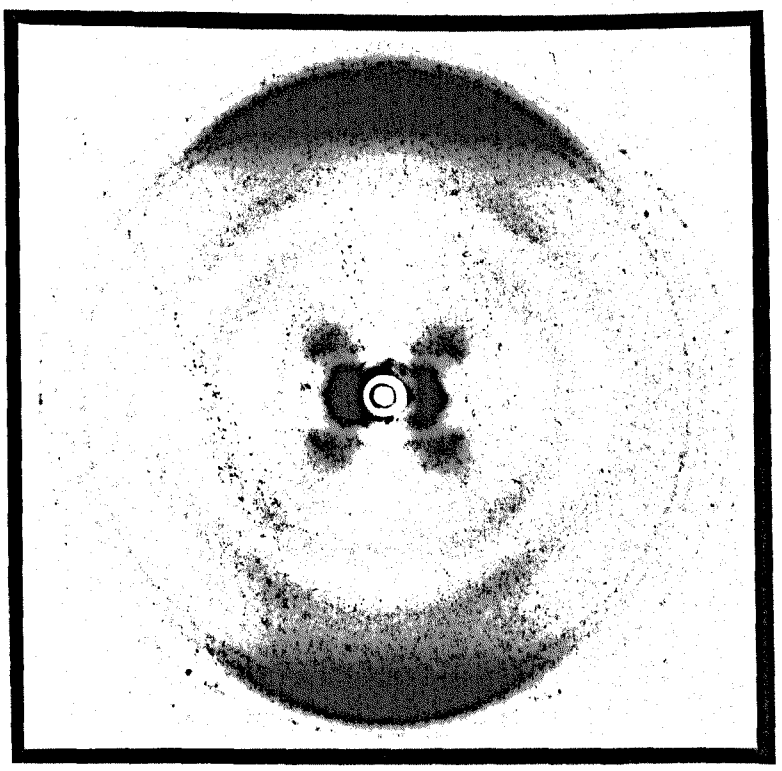


Fig. (5:10) D.N.A. - Steffimycin P/D = 12 R.H. = 92%

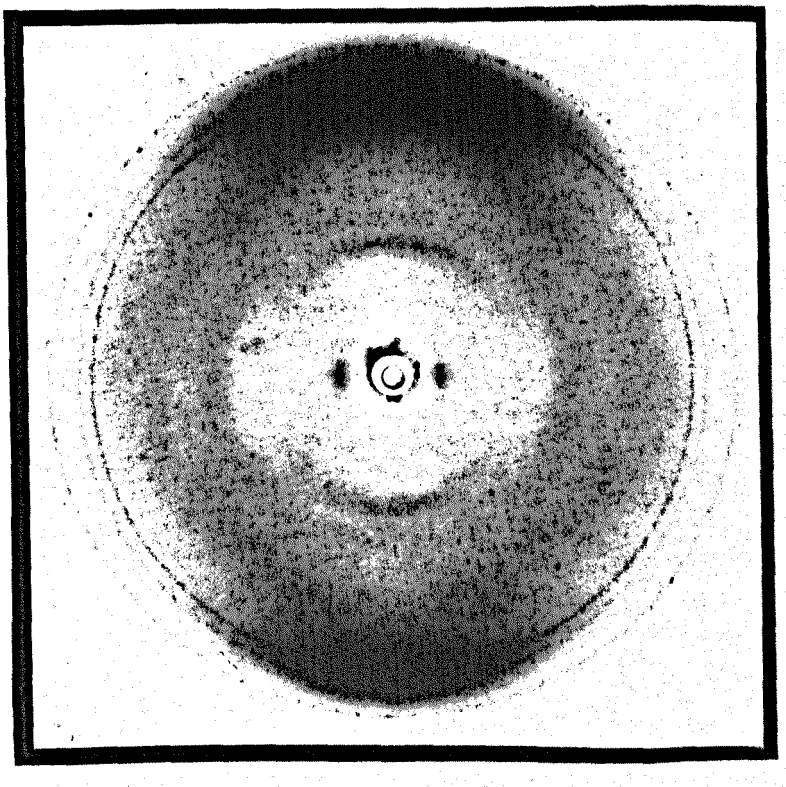


Fig. (5:11) D.N.A. - Steffimycin P/D = 12 R.H. = 44%

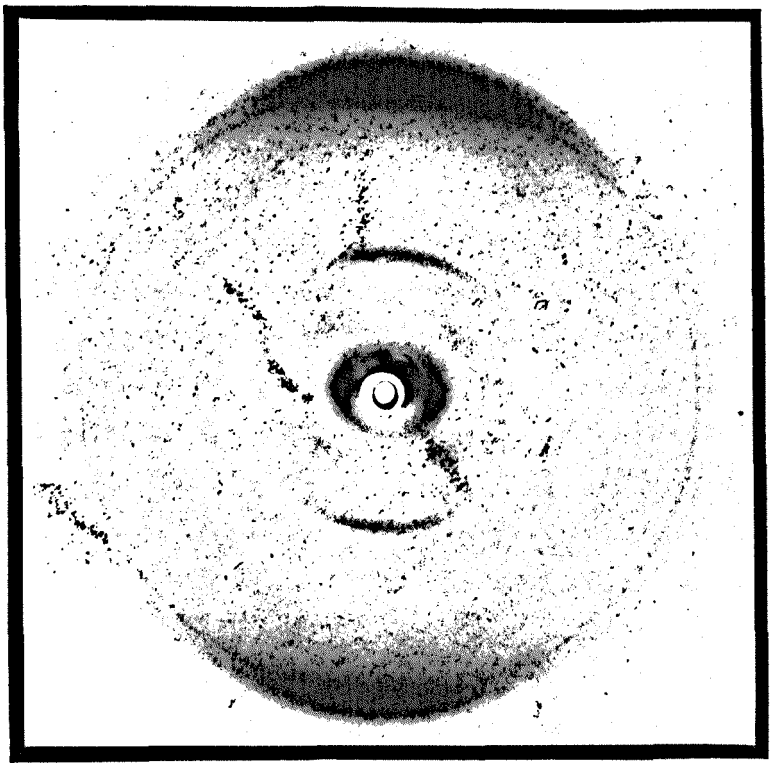


Fig (5:12) D.N.A. - Steffimycin P/D = 12 R.H. = 95%
The R.H. had previously been lowered to 44%.

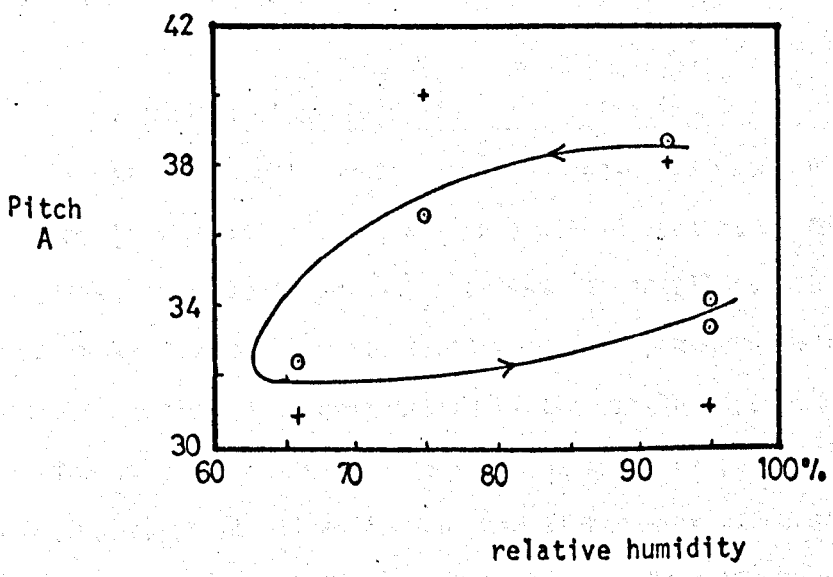


Fig. (5:13) Irreversible change in pitch resulting from lowering the humidity on two specimens.

crystalline 7.2A spacing was obtained for a fibre with P/D 10.7 which had not been placed in a low humidity environment. In all cases where the crystalline drug reflexions were observed, then the D.N.A. had a pitch of 34A.

5:3 X-ray studies of steffimycin with synthetic D.N.A.

It was possible to prepare only a single gel each of steffimycin with poly d(G-C) and poly d(A-T) because of the quantities required to prepare a pellet by centrifugation. Gels of the pure synthetic polynucleotides were also prepared to act as controls and permit an examination of the samples used.

The salt content needed to sediment a gel of poly d(G-C) was so high that crystallites formed when fibres were prepared. This difficulty of sedimentation was probably due to a low average molecular weight. The resulting X-ray diffraction patterns of poly d(G-C) fibres were diffuse due to the disorientation of molecules caused by the crystallisation of salt. With poly d(G-C)/ steffimycin fibres, the patterns obtained were also diffuse and yielded little information. A 5.4A spacing was observed which could be attributed to the presence of unwanted protein in the sample.

Poly d(A-T) gave very good crystalline A patterns and an example is shown in fig. (5:14). A fibre prepared of this alternating base pair D.N.A. with steffimycin gave a good quality X-ray photograph shown in fig. (5:15). The fibre had a P/D of 10.5 corresponding to the maximum number of bound sites for poly d(A-T) as shown in the spectroscopic studies. The pitch of the helix is 48A compared with the 41A maximum obtained with natural D.N.A. This was taken as further evidence that the complex formed between

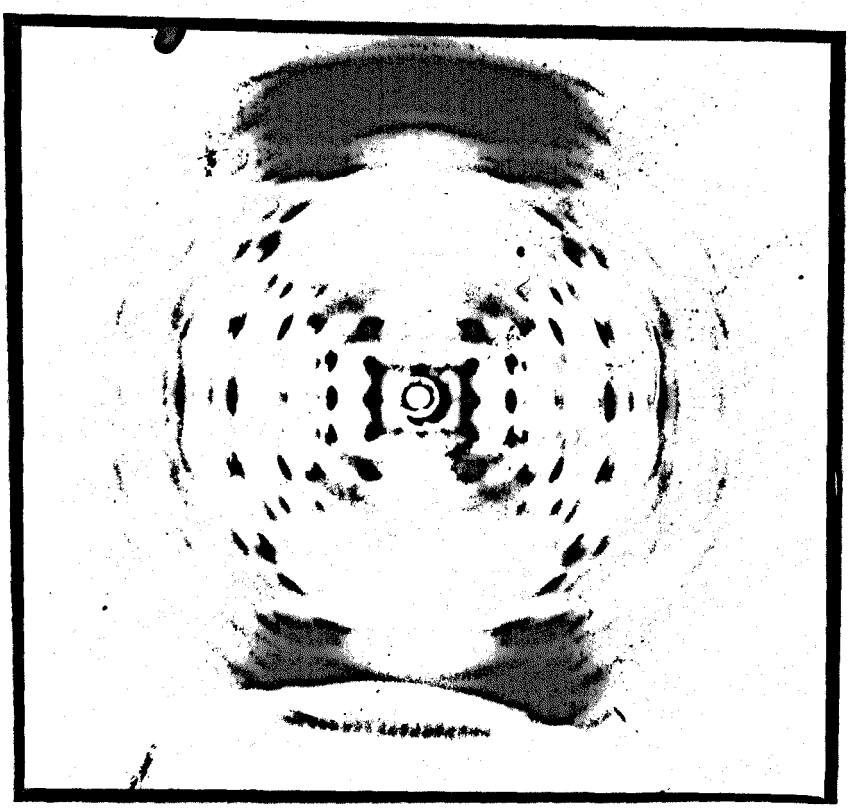


Fig. (5:14) Poly d(A-T) R.H. = 92%

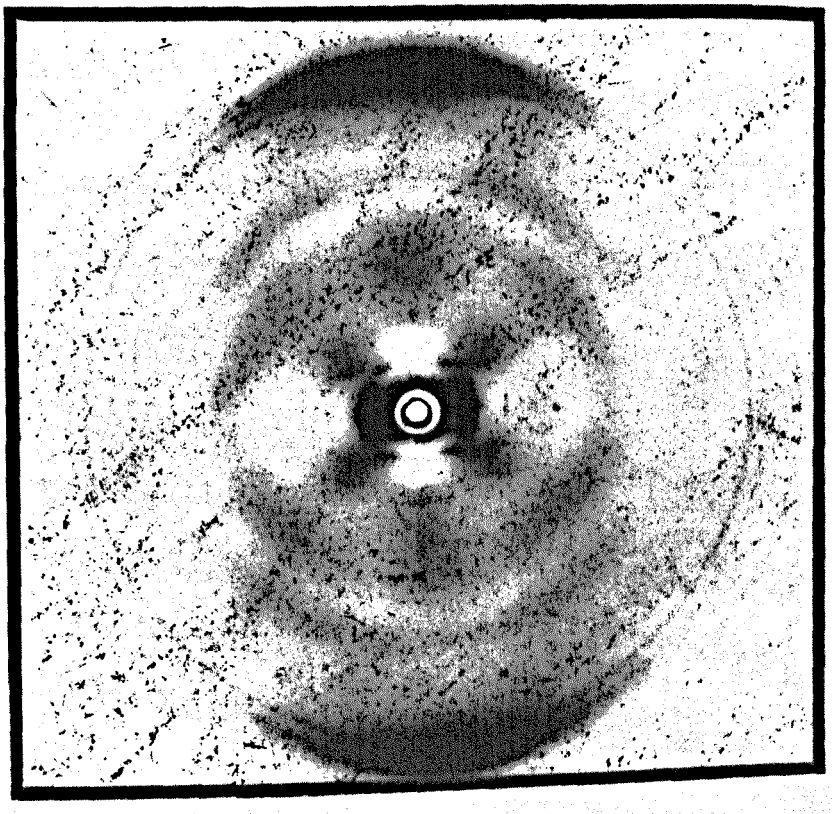


Fig. (5:15) Poly d(A-T) - Steffimycin P/D = 10.5 R.H. = 92%

steffimycin and poly d(A-T) is not the same as a complex formed with the natural D.N.A.'s.

5:4 X-ray studies of steffimycin B with natural D.N.A.'s

A series of X-ray photographs was taken of steffimycin B bound to D.N.A. and gave diffraction patterns as shown in fig. (5:16), which was produced by a fibre with a P/D of 6.6. Fig. (5:17) shows the diffraction from a fibre of a steffimycin B/D.N.A. complex with P/D 48.2. The diffraction patterns for these complexes resemble those taken with steffimycin. Analysis of the diffraction data again showed that there was an increased pitch and decreased intermolecular separation resulting from the binding of the drug. Graphs of these parameters and their variation as a function of P/D ratio are shown in fig. (5:18). As with steffimycin, the maximum pitch observed was 41A obtained with fibres of P/D 12 or less. Steffimycin B had essentially the same effect on D.N.A. pitch and molecular packing as steffimycin.

Observation of the behaviour of D.N.A. fibres with steffimycin B at humidities below 75% was difficult since the molecules tended to become disoriented. The results obtained do show that there is a decrease in pitch as the humidity is lowered. This drug, however, showed a greater tendency than steffimycin to leave the D.N.A. at low humidities with the appearance of a meridional reflexion at around 7A. Approximately 30% of the fibres with a P/D < 12 gave a reflexion at this spacing without the need of a low humidity's environment.

Since the intensity at 7A is not known to be a feature of D.N.A. molecules, it was therefore assumed to be due to the presence of the

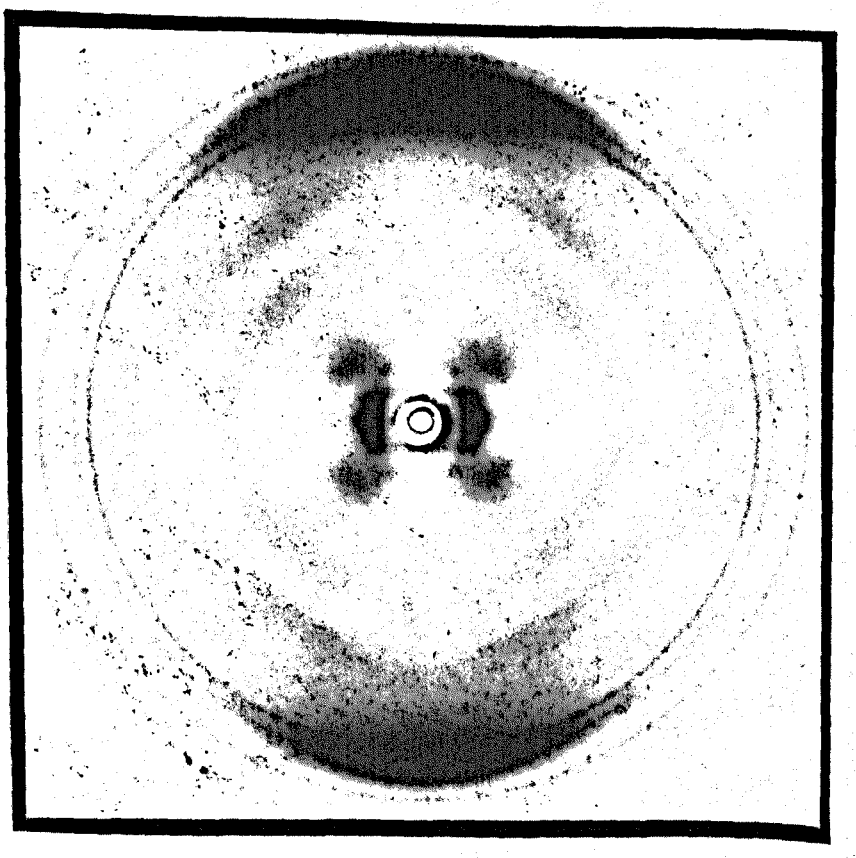


Fig. (5:16) D.N.A. - Steffimycin B P/D = 6 R.H. = 92%

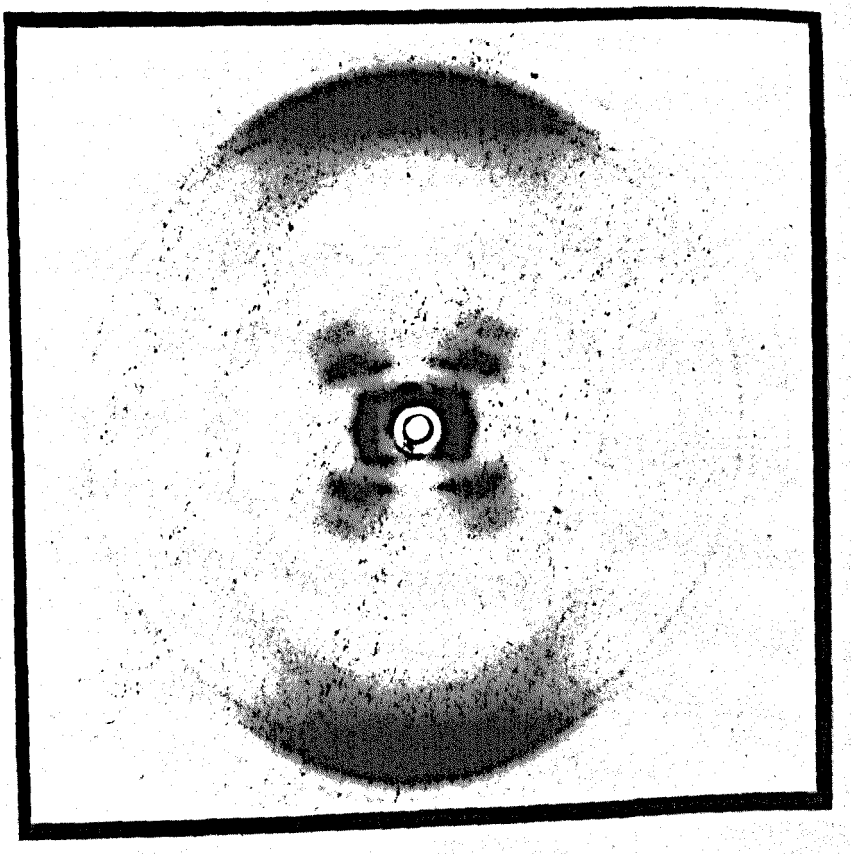


Fig. (5:17) D.N.A. - Steffimycin B P/D = 48 R.H. = 92%

the drug. It was not unusual to observe crystalline reflexions produced by steffimycin B which resembled a single crystal rotation photograph superimposed over a B D.N.A. diffraction pattern.

5:5 Steffimycin B crystallisation with D.N.A.

An example of an X-ray diffraction pattern of a steffimycin B D.N.A. fibre with crystalline reflexions produced by the drug is shown in Fig. (5:19). The P/D ratio is 6.4 and the fibre was photographed at 92% relative humidity. Several such examples were obtained with P/D in the range 6 to 12. This type of pattern resembles diffraction photographs obtained by Dr. M.E. Davies of miracil bound to D.N.A. where the drug had co-crystallised with the nucleic acid [84].

Fig. (5:19) shows the X-ray diffraction pattern of the B conformation of D.N.A. together with crystalline reflexions of the drug along layer lines. Patterns where the steffimycin B crystal structure was evident were obtained both with and without the use of ethanol in preparing the gel. Nevertheless, it does seem likely that the tendency for this type of crystal structure to arise was associated with the presence of ethanol used to prepare gels with a P/D lower than 10. The crystalline drug lattice remained when specimens were photographed at lower humidity.but became more disoriented. An example of such a fibre photographed at 66% R.H. is shown in Fig. (5:20).

The spacings of all measurable diffraction spots from the steffimycin B lattice were measured and the pattern was found to index upon the basis of a hexagonal unit cell as shown in Fig. (5:21).

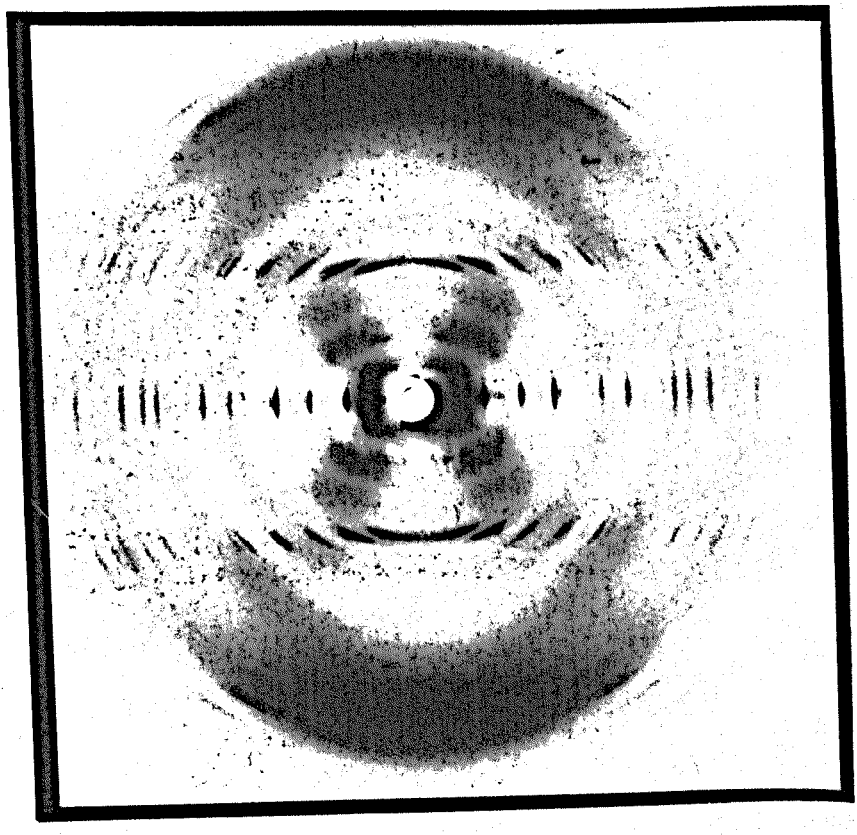


Fig. (5:19) D.N.A. - Steffimycin B P/D = 6.4 R.H. = 92%

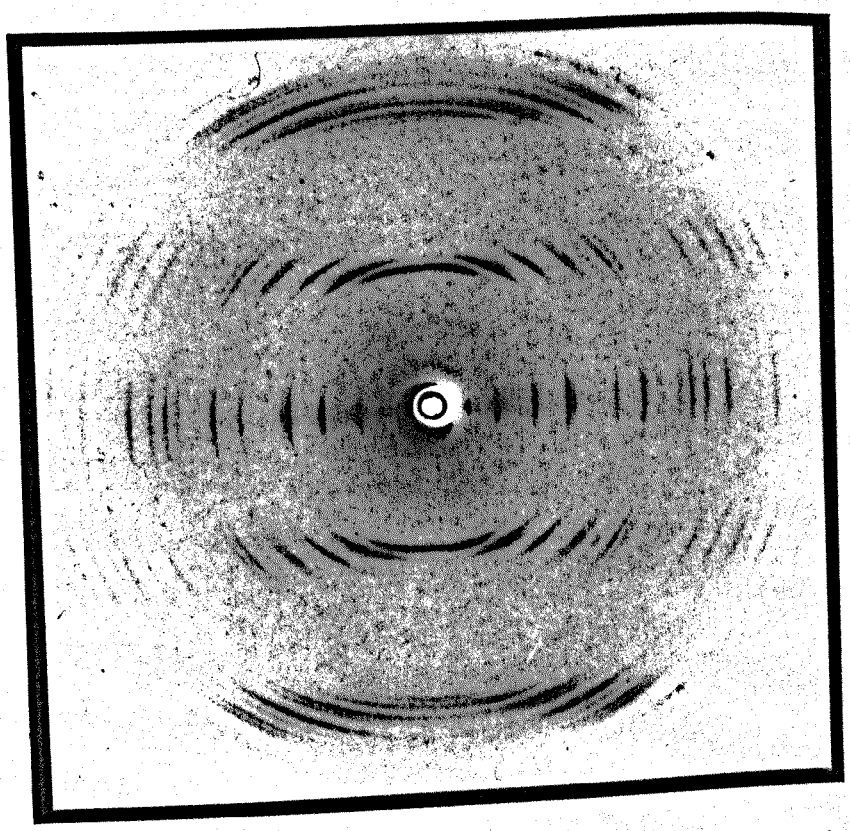
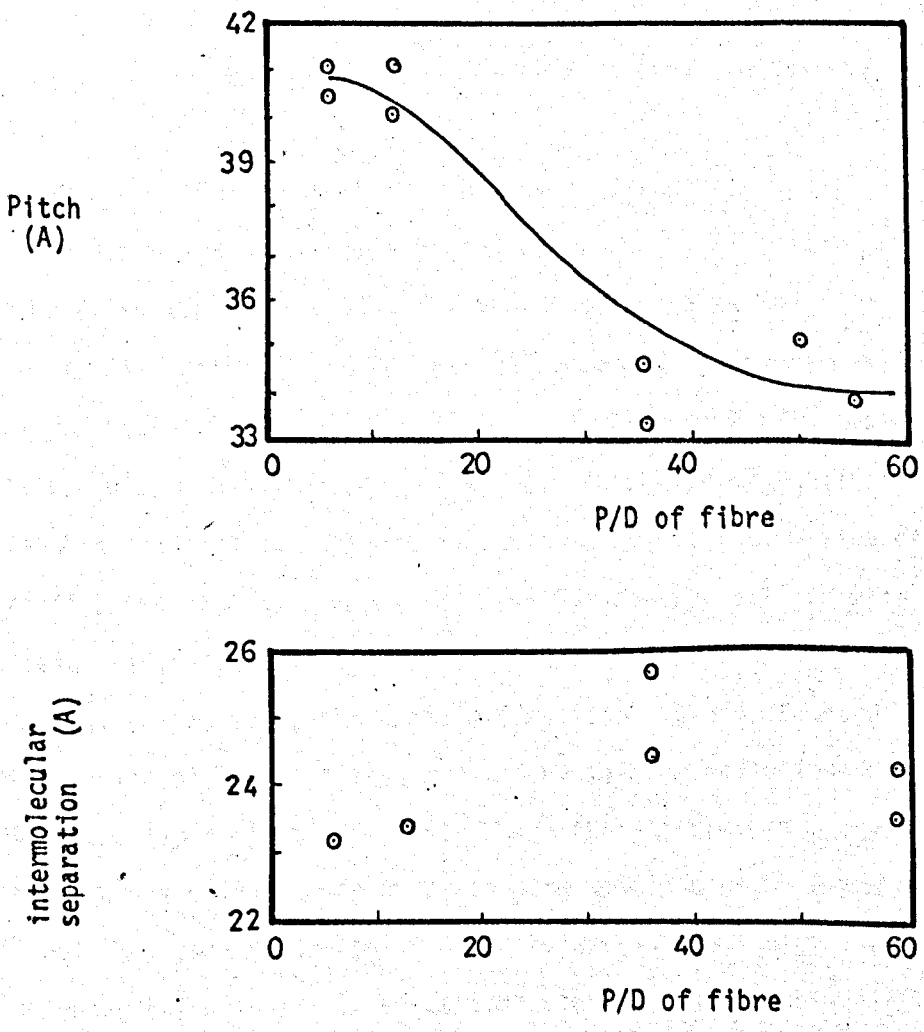


Fig. (5:20) D.N.A. - Steffimycin B P/D = 12.6 R.H. = 66%



Figs. (5:18) Pitch and intermolecular separation as a function of P/D of steffimycin B - D.N.A. fibres.

Fig. (5:21)

observed $\sin^2\theta$	calculated $\sin^2\theta$	h k ℓ
0.0027	0.0027	1 1 0
.0062	.0062	2 1 0
.0106	.0106	2 2 0
.0116	.0115	3 1 0
.0168	.0168	3 2 0
.0186	.0186	4 1 0
.0239	.0239	3 3 0
.0273	.0274	5 1 0
.0319	.0318	6 0 0
.0344	.0345	5 2 0
.0379	.0380	6 1 0
.0433	.0433	7 0 0
.0743	.0743	8 2 0
.0110	.0109	1 0 1
.0128	.0127	1 1 1
.0163	.0162	2 1 1
.0206	.0206	2 2 1
.0216	.0215	3 1 1
.0242	.0242	4 0 1
.0269	.0268	3 2 1
.0427	.0418	6 0 1
.0481	.0480	6 1 1
.0534	.0533	7 0 1
.0604	.0604	7 1 1
.0693	.0693	7 2 1
.0742	.0746	8 1 1
.0411	.0400	1 0 2
.0434	.0428	1 1 2
.0461	.0463	2 1 2
.0481	.0481	3 0 2
.0515	.0516	3 1 2
.0541	.0543	4 0 2
.0565	.0569	3 2 2
.0587	.0587	4 1 2

All observed $\sin^2\theta$ are ±0.0005 $\lambda = 1.5418$

This gives a hexagonal unit cell for steffimycin B with a = 29.93 ± 0.02A and c = 7.70 ± 0.01A. The c axis is arranged parallel to the D.N.A. helix axis. If one assumes there are six molecules in this large unit cell of volume 5973.58A^3 then the density is found to be 0.98 ± 0.01 gm/ml. This figure will be higher when the solvent molecules are taken into account.

The unit cell of the B D.N.A. present with the steffimycin B crystals was also determined. The hexagonal cell had a = 45.9 ± 2.0A compared with the 46A generally accepted, and the pitch of the helices was 33.95 ± 1.0A.

It is interesting to note that the a axis of the hexagonal cell of D.N.A. is 1.5 x that of steffimycin B, making it possible to superimpose the nucleic acid lattice over the drug lattice producing a regular arrangement of the two unit cells. This superimposing of crystal lattices would be the case if the steffimycin B and D.N.A. molecules were able to crystallise in their respective lattice sites in the same region of the fibre. An examination of this possibility reveals the difficulty of arranging the steffimycin molecules in an identical position in each unit cell without interference from the D.N.A. molecules. It appears, therefore, that the drug crystallises at sites between D.N.A. crystallites even though the fibres did not show visible crystals when viewed with a microscope at x40 magnification as can be observed when sodium chloride crystallises out.

An attempt was made to crystallise suitable crystals of the steffimycins for X-ray rotation photography. This was achieved only for steffimycin by dissolving the drug in isopropanol and slowly cooling a boiling saturated solution. Thin needles formed which were

dried and mounted on a thin glass rod to obtain X-ray diffraction patterns using a Weissenberg camera. The crystals were very thin and were twinned which resulted in poor photographs. Steffimycin B would not crystallise in this way and simply formed a colloidal suspension. A powder photograph was therefore taken of the steffimycin B crystallites and is shown in fig. (5:22).

Since good crystal data has not been obtained, the crystal forms were not solved but both appear to exhibit a different crystal structure when formed from solution from that formed in the D.N.A. fibre. This would indicate that the presence of D.N.A. molecules is sufficient to cause the formation of a new drug crystal structure. It is likely that the drug must be in close proximity to the helices in order for this to occur and it would be the orientation of the chromophore in contact with the D.N.A. molecules that would seed the crystal formation.

If the drug crystallised in regions between oriented D.N.A. helices then the effect of the presence of the polymer would be minimal. Hence, as previously mentioned, the normal B pattern for D.N.A. is always observed when crystalline drug is present and no extension of the helix was detected. It seems, therefore, from this evidence, that steffimycin and steffimycin B form microcrystallites situated between regions where the D.N.A. is crystallised but intimately associated at the crystal boundaries. Crystalline drug is aligned by D.N.A. crystal boundaries so that the disorientation of D.N.A. at high humidities also occurs in the drug crystals.

It is now possible to suggest what takes place when a fibre which originally gave a diffraction pattern showing increased pitch

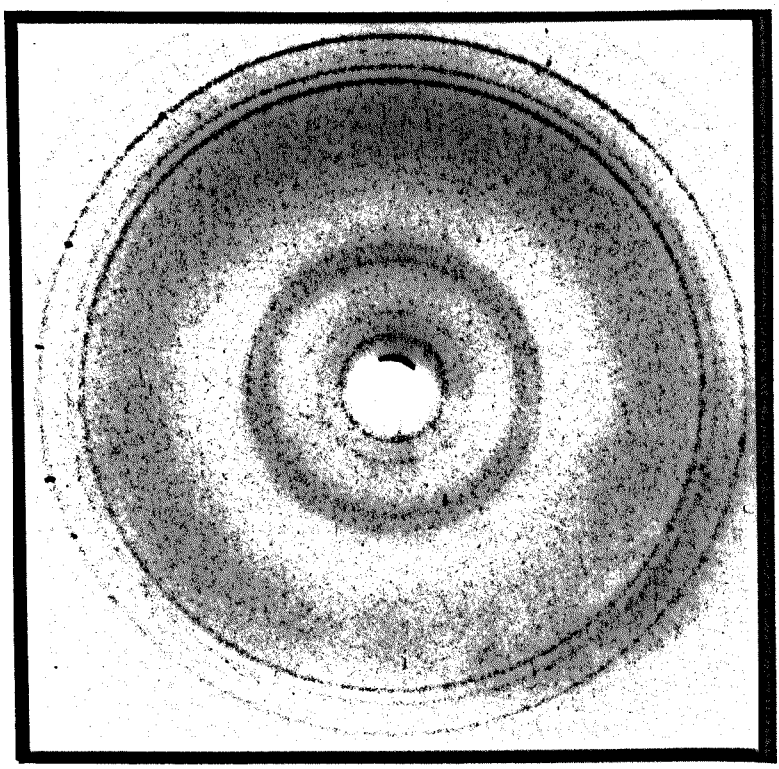


Fig. (5:22) Steffimycin B powder photograph

at 92% relative humidity and was observed at lower humidities to revert to the B form of D.N.A. with the appearance of a ∿7A meridional reflexion. It is certain that one result of the binding of drug molecules to D.N.A. is an increase in the helix pitch. If this were by intercalation then the chromophores would be positioned at least two base pairs apart according to the excluded site model where the maximum number of drugs bound is one for every two base pairs. Upon lowering the humidity the hydrophobic drug would be less tightly bound in the helix and leave the binding site. Drugs which were positioned two base pairs or 6.8A apart in the helix would be in a position to seed the formation of a crystal. This leads to the appearance of the broad reflexion around 7A and the decrease of the helix pitch to 34A. In some fibres of both steffimycin and steffimycin B, this crystallisation was complete and well oriented which led to the very clear crystalline reflexions observed. It is interesting to note that at the first appearance of crystalline drug, a broad meridional reflexion is observed at ∿7A. When the drug gives sharp crystalline reflexions, the spacing is then 7.70. This would support the hypothesis that drugs have left intercalation sites 6.8A apart and rearranged to form a unit cell with the 7.70A spacing. This would explain not only the X-ray diffraction patterns observed but suggests why the particular crystalline form found in the presence of D.N.A. is not the same as when the drug crystallises from solution.

This discussion has centred around the formation of steffimycin B crystals in D.N.A. fibres. Similar sharp crystalline diffraction patterns were also obtained for steffimycin although

they occurred more infrequently. This latter drug tended to produce an increase in the pitch of D.N.A. which was not affected by changes in humidity.

5:6 A consideration of the effects of the steffimycins bound to D.N.A.

The increase of the pitch of the D.N.A. helix could be due to:-

1. An increase of the axial separation of base pairs with corresponding changes in the conformationof the sugar-phosphate chain.
2. Induced unwinding of the helix resulting from the external binding of the drug to the D.N.A. molecule.
3. Intercalation of the drug chromophore which may also involve a rotation of base pairs.

The first possibility is discounted not only because of the drastic effect on D.N.A. stability and conformation but also because the 3.4A meridional reflexion resulting from the base pair separation is always present in the diffraction patterns. The remaining modes of interaction are considered below and are referred to as external and intercalation types of binding. Theoretical considerations lead to different predicted changes in pitch for the various P/D ratios.

When drugs bind externally to D.N.A., they may or may not change the helix pitch. If a drug molecule does so then the variation must be entirely due to an added rotation upon the base pairs either positively, winding up the helix and reducing the pitch, or negatively, unwinding the helix and leading to an increased pitch. If intercalation is involved then there would be an increase

in pitch due to the insertion of the molecule between base pairs and this could be further modified if the intercalation also caused a change in the rotation of the base pairs.

Expressions can be derived for the change in pitch produced by the two methods of binding. For external binding this is:-

$$P' = \frac{P}{1 - \emptyset\ N/360M}$$

and for binding by intercalation:-

$$P' = \frac{P + tN/M}{1 - \theta\ N/360M}$$

where P' = new pitch of D.N.A. with drug bound

P = pitch of the undistorted helix

M = number of base pairs per drug molecule

N = no. of residues in one turn of the original helix

t = thickness of the drug chromophore

θ = degree of uncoiling due to insertion of drug

$\emptyset$ = uncoiling due to external binding of drug

For any measured increased pitch with a certain P/D ratio, it is only possible to calculate the unwinding angle if the method of binding is known. This has to be determined on the basis of experimental evidence. It is probable that for the steffimycins, the binding mechanism responsible at least in part is intercalation. This is likely because:-

1. The steffimycins are hydrophobic and have a lower potential energy in a non-polar environment such as that between the base pairs of D.N.A.
2. There is a decrease in the intermolecular spacing of D.N.A. molecules associated with the drug. If the drug was externally

bound, then this would force the D.N.A. helices further apart.

3. The steffimycins are neutral molecules and have no electrostatic attraction for the charged phosphate groups on the exterior of the D.N.A. helix.

4. Decreasing the humidity has the effect of decreasing the helix pitch. This would be expected if the reduced hydrophobic interactions allowed drug molecules to leave the intercalation site.

5. The spectroscopic studies showed that strong and weak binding mechanisms were present. This strong binding has been associated with intercalation in other drugs such as daunomycin and actinomycin.

If we consider steffimycin binding to calf thymus D.N.A. then from the spectroscopic work, there can be 0.055 drug molecules strongly bound per nucleotide, corresponding to a P/D of 18.2. If this strong binding is due to intercalation then the maximum number of drug molecules bound in this way would correspond to this P/D. In this case, a fibre prepared with a P/D less than 18 would contain some drug bound to D.N.A. by another mechanism. Spectroscopic analysis showed that weak binding also takes place in the formation of steffimycin complexes with D.N.A. so that in a calf thymus D.N.A. fibre containing drug with a P/D lower than 18, the excess drug present is probably not bound by intercalation. The X-ray studies give further evidence to the existance of drug molecules bound to D.N.A. but not producing a pitch increase associated with intercalation.

We can now consider the experimental evidence to obtain an estimate for the unwinding angle associated with an intercalated

drug. Upper and lower limits for this angle will be determined due to the uncertainty in effect and extent of secondary binding.

The fibre with the lowest number of drug molecules per nucleotide which actually gave a pitch of 41.5A had a P/D of 13. If we assume that all the drugs are intercalating, this will give a lower limit to the unwinding angle per site and this is found to be 12.8°.

If we assume that drug molecules intercalate into D.N.A. with a P/D of 18 giving a pitch of 41A as predicted from spectroscopic studies to be the lower limit on the number of drugs which could produce such an increase in pitch, this will give an upper limit to the unwinding angle. This is calculated to be 25.5° per intercalated drug.

Providing, therefore, that the unwinding angle per intercalated drug is constant and does not vary with the concentration of bound drug, the spectroscopic and X-ray diffraction studies predict that this is between 12.8° and 25.5° per intercalated molecule.

These estimates are based upon experimental evidence but it may be that fewer drugs than this suggests are actually involved in intercalation. The unwinding angle would then be greater than 25.5°. Also the proportion of drugs bound to a fibre that actually intercalate may possibly vary and be sensitive to environmental conditions. This range of likely unwinding angles must therefore be taken as only approximate.

When steffimycin was bound to poly d(A-T), the maximum pitch obtained was 48A for a P/D of 10.5. If all these drug molecules are involved in intercalation and the polynucleotide is originally in the B form, the unwinding angle per site is 28°. If however,

external binding is the mechanism responsible for the increase in pitch, then the unwinding angle is 57°. The lowest possible unwinding angle per drug for poly d(A-T) is therefore 28° which falls outside the range of values predicted for the unwinding angle for the natural D.N.A.'s and adds to the mounting evidence that steffimycin complexes with poly d(A-T) are quite different from those with the natural D.N.A.'s.

Finally, we shall consider the drug-D.N.A. fibre with the lowest P/D of 7 which gave a pitch of 41A. If all the drugs in the fibre were involved in intercalation, then the unwinding angle would be -8.4°. This result is improbable because it would mean that there is a wide variation in the unwinding angle produced by the bound drug. It is much simpler to suggest that steffimycin and steffimycin B intercalate between base pairs and that this reaches a saturation point whereupon the remaining drugs are weakly bound. This weak binding may take the form of drug molecules aggregated together due to hydrophobic interactions. These molecules are then available for the formation of crystals in the fibre at low humidity.

The X-ray diffraction studies have shown that both steffimycin and steffimycin B when bound to D.N.A. cause the helix pitch to increase and the intermolecular separation to decrease; effects which were dependent upon humidity and best explained by an intercalation mechanism. The presence of crystalline drug is thought to be due not only to chromophores leaving intercalation sites due to low humidity but also the presence of agglomerations of the drugs weakly bound to the D.N.A. molecule. Although there

is a spread of data relating P/D to helix pitch it was suggested that the unwinding angle per intercalated drug lies in the range 12.8° to 25.5°. This data will be considered in the next chapter which seeks to consider a possible model for the D.N.A. complexes with the steffimycins.

Chapter 6

MODEL BUILDING STUDIES FOR THE D.N.A. COMPLEX WITH STEFFIMYCIN AND STEFFIMYCIN B

6:1 Introduction

This chapter uses the experimental results obtained in the two previous chapters to explore possible D.N.A. conformations which allow the binding of drug molecules. The various molecular parameters obtained from the X-ray studies are not sufficient on their own to define the molecular structure. This is where model building has been found to be a useful tool in the determination of molecular conformations. X-ray data are used to define the basic helix and the detailed arrangement of individual components can then be examined within these constraints. Use is made of accepted bond lengths, van der Waals radii, bond angles, and the configuration of sugar, bases and phosphate groups to construct possible conformations of the macromolecule. This can be done with the aid of the computer programme listed in chapter 2 section 7. A computer calculation of the Fourier transform of a proposed model is then compared with the observed transform from the X-ray diffraction of the oriented molecules. Further refinements can be made to the model until its transform matches as close as possible that of the actual molecule.

The aim now is to build a model which is consistent with:-

1. The experimental evidence obtained,
2. Known stereochemistry,
3. Other D.N.A. drug complexes and

4. The physical and chemical properties of the components.

6:2 Previous studies of drug bound to D.N.A.

6:2:a A consideration of the models of drug binding

It is useful to consider modes of binding which have been commonly observed for several different D.N.A.-drug complexes. Possible types of interaction are (1) Intercalation, where the flat aromatic ring system occupies a site between consecutive bases of the D.N.A. molecule [30] [41], and (2) external binding, where the drug is bonded in some way to the D.N.A. helices but where the whole of the drug molecule remains outside the nucleic acid [85]. Some drugs are able to bind both by intercalation or by external binding and in the case of daunomycin, the molecules are partly intercalated but have the sugar group of the drug bound externally to the D.N.A. phosphate anion [40]. Peacocke [24] observed that a drug can exhibit both strong and weak modes of binding and it was this strong binding that Lerman [37] [42] later suggested was due to intercalation. The spectroscopic and X-ray diffraction studies of the steffimycins have shown that they also bind to D.N.A. both by intercalation and in some other way; either externally or interstitially.

When constructing a model of the D.N.A.-drug complex, we are concerned with the effects the drug binding has on D.N.A. conformation. Pigram et al [40] consider three models for binding in reference to daunomycin. These are (1) External binding producing local distortion of the helices at the binding site. Any pitch change would be due entirely to an unwinding of the helices. (2) Intercalation between successive base pairs with a change in

pitch caused both by unwinding and the thickness of the drug itself. (3) External binding with a cooperative conformational change throughout the helical structure. An averaged unwinding angle per nucleotide would result in an increased pitch. These models were then considered in the light of the experimental evidence for daunomycin binding to D.N.A. in order to eliminate unlikely conformations and arrive at a proposed stereochemistry for the complex. In this case, the indications were that the binding mechanism was intercalation with simultaneous external binding.

The steffimycins have a similar structure to daunomycin as shown in fig. (6:1) and so it is worthwhile considering the stereochemistry of the D.N.A. complex with this other drug. Experimental results for steffimycin can then be used to modify or reject the daunomycin model for intercalation as an explanation of steffimycin binding.

6:2:b Intercalation models

Intercalation, whereby a flat aromatic ring system of a drug molecule is bound between base pairs of the D.N.A. helix was first suggested by Lerman for the proflavine molecule [42]. He proposed that the chromophore was situated directly over a base pair which necessitated a helix unwinding of 45° per bound drug. This gave a rotation per residue of -9° at the intercalation site making the overall right handed helix locally left handed. This model was later modified by Pritchard et al [41] who observed that proflavine exhibited both strong and weak binding processes. The strong binding was not reduced by denaturation or strand separation. These workers therefore postulate that the acridine interacts not with a hydrogen

bonded base pair but with two adjacent bases on the same polynucleotide chain and that the unwinding angle involved need not be as high as 45° for this to occur.

Waring [27], having studied the binding of ethidium to D.N.A. suggested that it binds strongly by intercalation between neighbouring base pairs and also weakly by a "stacking" mechanism. Fuller and Waring [30] consider the details of intercalation of ethidium. From X-ray work and model building studies, they conclude that the unwinding angle per intercalated drug is 12°. They suggest that the complex is stabilised by hydrophobic interactions between D.N.A. base pairs and the ethidium triple ring system. Hydrogen bonding between ethidium amino groups and charged oxygens of phosphate groups is also a possible additional method of binding in this case. In the intercalation of daunomycin, Pigram et al [40] again concluded that there was an unwinding angle of 12°.

This intercalation model consistently involving the 12° unwinding angle was challenged by Paoletti and Le Pecq [86] upon the basis of fluorescence depolarisation studies on D.N.A.-ethidium bromide solutions. They proposed that intercalation winds up the helix by 13°. Pigram et al [46] showed that their method of examination was insensitive to the unwinding angle over certain ranges and maintained that the most likely effect of intercalation was to unwind the helix by 12°. In studies by Waring [31] on a number of intercalative drugs, he concludes that the unwinding angle is not the same for each and usually comes in the range 5°-12°.

Thus the value for the unwinding angle is still likely to be approximate and it is not certain whether the rotation involves only the nucleotides either side of the drug or whether this angle

is averaged over more base pairs. If this is the case, the rotation per residue of the helix would be dependent upon P/D of intercalated drug. Alden and Arnott [87] considered the stereochemical requirements of an intercalation site in an attempt to generalise the effect on D.N.A. conformation of such binding. After a series of computer model building studies, they found the most favourable conformation involved the changing of two torsion angles and the change of sugar pucker, of the nucleotides either side of the drug, to C3´ endo. The model had a rotation of 90° over three polynucleotide residues at the binding site and intercalation caused the helix to unwind by 18°. Mr. D. Goodwin of Keele University has been carrying out similar studies on the conformation of nucleotide pairs either side of an intercalation site and so far considers that the sugar pucker is not the same on both sides of the site.

The extent of intercalation has also been studied. It appears that for ethidium, the strong primary binding saturates when one drug molecule is bound for every 4-5 nucleotides whereas secondary binding leads to the precipitation of a 1:1 complex [26] [88]. Daunomycin also intercalates to a maximum of one drug per 4 or 5 base pairs [40]. This would indicate that the conformational constraints of the D.N.A. molecule restrict intercalation to one drug for every two base pairs. The model of Alden and Arnott was based on one molecule for four base pairs.

Other features of D.N.A. with drug intercalated between base pairs are:-

1. A decreased diameter due to the extension of the helix. This is observed from the spacing of the equatorial X-ray diffraction

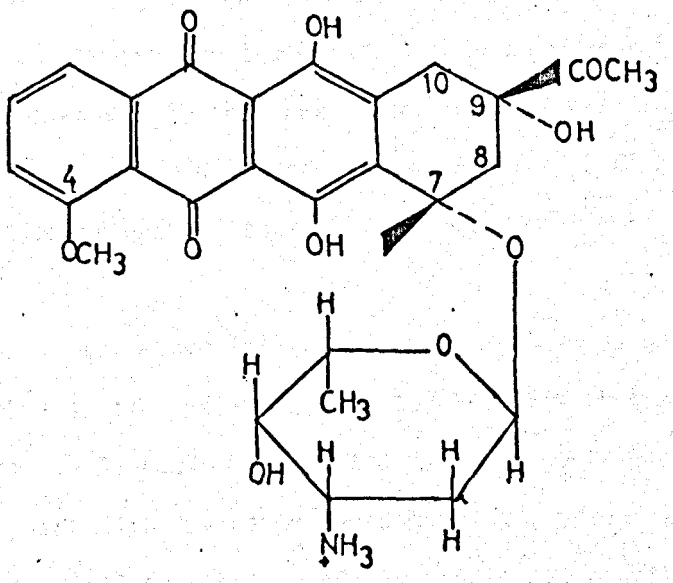
spots.

2. A pitch which increases with increasing extent of intercalation.
3. Pitch decreases with decreasing relative humidity indicating that hydrophobic interactions are involved with intercalation.
4. The translation per nucleotide pair remains at 3.4A. This is due to the aromatic ring system of the chromophore being the same thickness as the flat aromatic base pairs of the D.N.A.

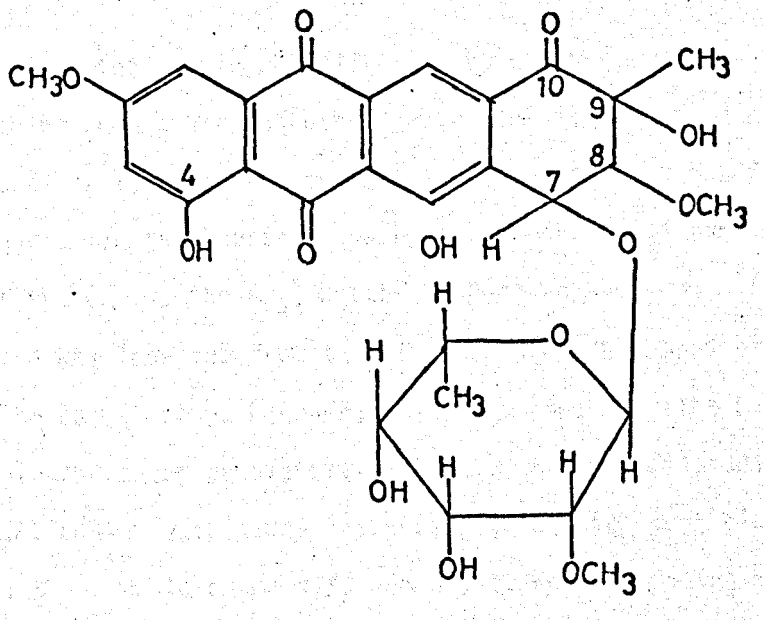
These are all characteristics of the binding of the steffimycins to D.N.A. as concluded from the X-ray studies carried out in chapter 5. Consideration is now given to the stereochemical details of steffimycin intercalation.

6:3 Stereochemistry of D.N.A. complexes with the steffimycins.

The steffimycins consist of a flat fused four membered ring system to which is attached a sugar group (fig. 6:1). The aromatic ring system is hydrophobic and the solubility in aqueous media is due mainly to the sugar group. The substitution of CH_3 in steffimycin B for the OH group in the 3′ position on the sugar explains the lower solubility of this drug in polar solvents. The stereochemistry of the steffimycins unlike that of daunomycin [89] is not known to date. When examining the possible modes of binding to D.N.A. therefore, it is necessary to consider the different isomers in which the drug could exist. Various permutations of the bonds on each of the 7, 8 and 9 carbon atoms of the saturated ring lead to the existence of eight isomers. Additional conformational freedom is provided by rotation of the oxymethyl group on C_8, the sugar ring about C_7 and also by slight puckering of the saturated



Daunomycin with protonated amine group



Steffimycin

Fig. (6:1)

ring. This leads to a wide range of possible structures for the drug. However, the binding of four isomers was surveyed. These were chosen because they involve the groups likely to be involved in stabilising the complex due to the presence of OH available to form hydrogen bonds. The four conformations are shown in fig. (6:2), and the intercalation of each of these isomers was considered in turn.

C.P.K. models of the above isomers of the steffimycins were made together with a section of the D.N.A. double helix in the B conformation. The D.N.A. could be adjusted to leave an intercalation gap between base pairs with corresponding unwinding of the helix [45]. The stereochemical feasability of the binding of steffimycin molecules was considered for each isomer.

Isomer 1

The steffimycin was difficult to build due to the steric hinderance between sugar and other groups in the saturated ring. It was possible to place the drug in the intercalation gap from the major groove. In this position, a hydrogen bond could form between the OH of the C_9 and the PO_4^- on the base above the drug. (If the chromophore was inserted from the minor groove, this bond could not form.) The sugar group, however, protrudes away from the D.N.A. molecule in the major groove giving the complex a greater diameter than D.N.A. contrary to X-ray evidence. If binding took place in this way, there would be no difference between the binding of steffimycin and steffimycin B, the C_9-OH group being common to both drugs.

Isomer 2

This was again inserted into the D.N.A. molecule from the

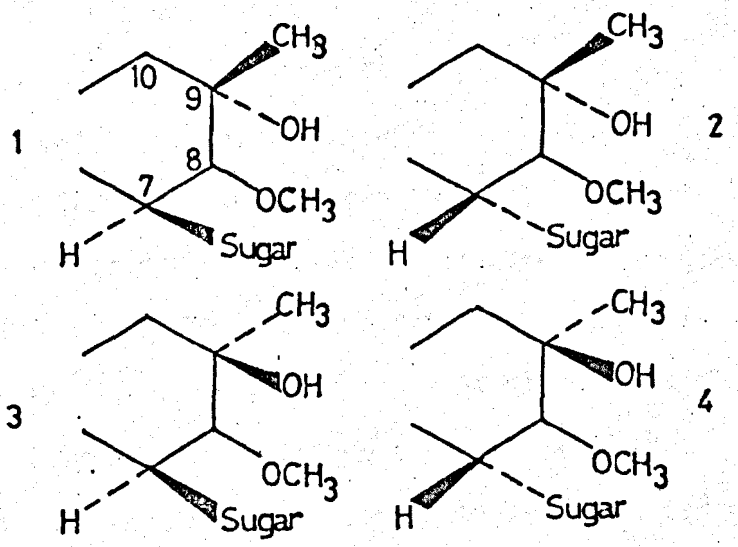


Fig. (6:2) Isomers of the steffimycin saturated ring referred to in the text.

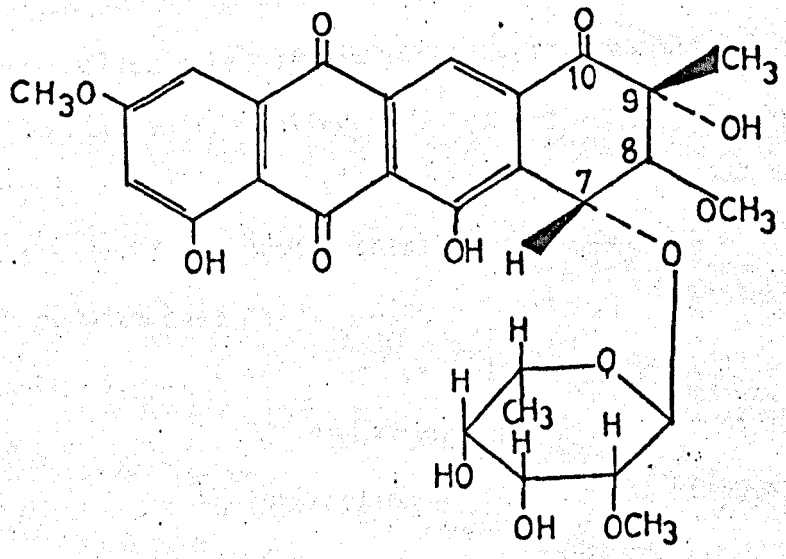


Fig. (6:3) Suggested isomer for steffimycin

major groove. In this way there was good overlap of the chromophore with the base pairs and the sugar group was tucked into the side of the major groove. There were two possibilities for H bond formation. The first was between the OH of the sugar C3' and the PO_4^- group situated two bases above. The second bond could form between the OH on C_9 of the saturated ring to the phosphate of the nucleotide above. One would expect the binding of steffimycin B to be less stable than steffimycin since there is no OH group on the C3' position of the former drug. This is in fact observed as the steffimycin B/D.N.A. complexes more reasily gave the superimposed crystalline diffraction of the drug rather than intercalation. This model would also explain the decrease in D.N.A. diameter due to the elongation of the molecule and reduced electrostatic forces because of the masking of the charged PO_4^- groups due to hydrogen bonding.

Isomer 3

This molecule has similar difficulties to model 1 since if it was intercalated from either groove, the sugar group was directed away from the double helix or, if the sugar group was placed at the side of the groove there was poor overlap of the aromatic groups with base pairs. Also, it was not possible to form any hydrogen bonds. If this isomer exists then intercalation into nucleic acid would be very difficult due to the lack of stabilisation either from dipole-dipole interaction of the aromatic ring systems or from hydrogen bonding.

Isomer 4

This steffimycin could only be made with difficulty due to steric interactions. The sugar group has little or no

rotational freedom and there are several short contacts of the van der Waals radii with groups on the saturated ring. A hydrogen bond could form between the C3'-OH on the sugar with the PO_4^- two bases above. This would not exist for steffimycin B which should therefore exhibit weaker binding. The sugar group is contained within the major groove of the double helix allowing the required decrease in helix radius.

Thus isomers 2 and 4 could intercalate into D.N.A. with good overlap of the chromophore with base pairs. Both show a decrease in molecular diameter and predict a decrease in intermolecular separation. Isomer 4, however, has some steric difficulties due to short contacts and forms only one hydrogen bond for steffimycin and none for steffimycin B. Isomer 2 forms two hydrogen bonds for steffimycin and one for steffimycin B. It does not follow that the molecule most suited to intercalation should be the one that exists. Yet because steffimycin does appear to intercalate, it seems reasonable to suggest that it exists in the conformation that is able to bind in this way.

Isomer 2 is therefore suggested as the structure of steffimycin. This has, in fact, a similar conformation to daunomycin which is known [89]. The conformation of steffimycin at the C_8 atom is left unspecified except to suggest that the stereochemically easiest molecule to build is shown in fig. (6:3). A photograph of the actual model is shown in fig. (6:4) and of this isomer intercalated into D.N.A. in fig. (6:5).

The model shows a steffimycin molecule inserted from the major groove and intercalated between base pairs with the two hydrogen

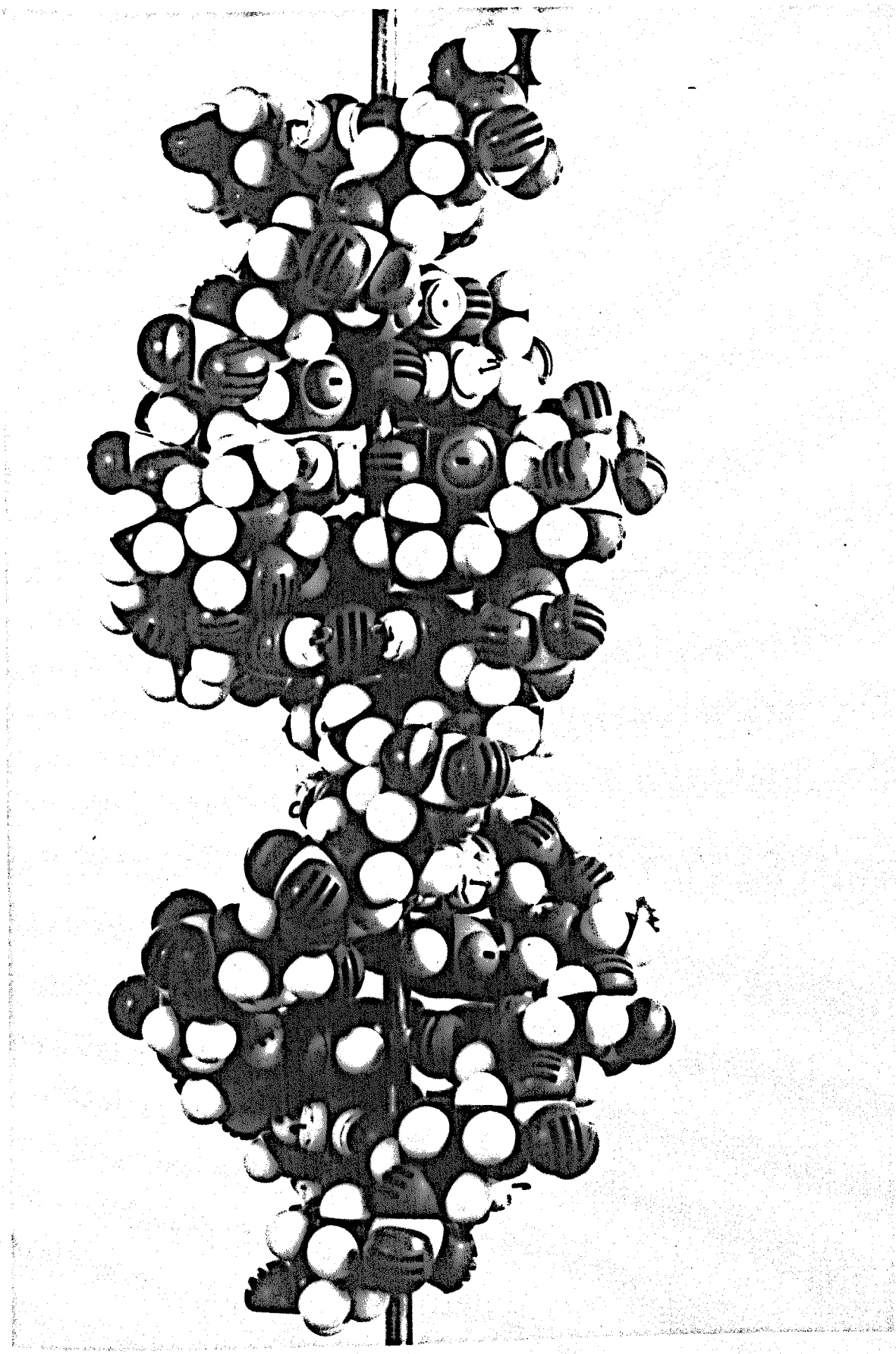
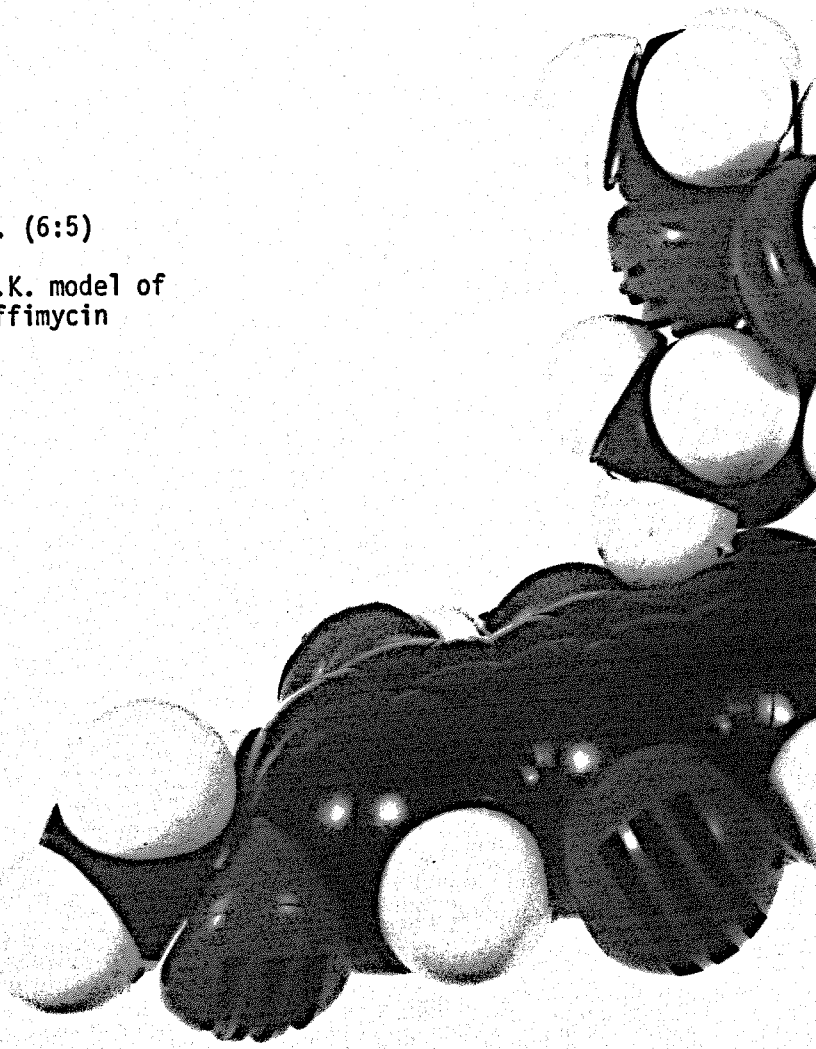
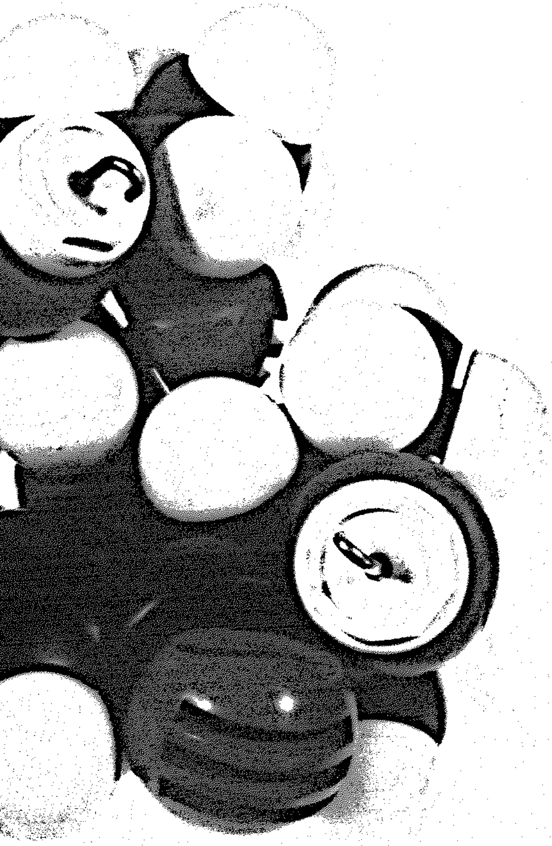


Fig. (6:4) C.P.K. model of D.N.A.-steffimycin complex

Fig. (6:5)

C.P.K. model of steffimycin





bonds linking the drug to the phosphate groups. It is inserted with the sugar group of the drug at the left side of the major groove directed up the polynucleotide away from the intercalation site. The D.N.A. molecule was originally in the B conformation and upon intercalation, the main conformational adjustments to open up the intercalation site and unwind the helix are mainly due to the rotation about the O_4-C_5' and the O_1-$C3'$ bonds of the sugar-phosphate chains. The model could be built with an unwinding angle of between 18° and 36° with the least conformational adjustment to the sugar-phosphate chain occurring nearer to the 18° value. Unwinding the helix not only opens up the intercalation site but provides for better overlap of the chromophore and D.N.A. base pairs.

6:4 Consideration of the base specificity of the steffimycins

From the experiments reported in chapter 4, we have seen that there are more primary binding sites for the steffimycin chromophore on poly d(A-T) compared with poly d(G-C). It is not until the total drug concentrations exceed 10μM that the secondary binding produces an overall preference for binding to poly d(G-C). With the natural D.N.A.'s, the drugs show specificity for binding to those with a higher guanine-cytosine content. In order to understand why a drug should be base specific, it is necessary to consider the differences between base pairs, binding sites, and between the complexes which form with the chromophores. This discussion concentrates upon the base pairs of the double helix since all glycosidic links are equivalent resulting in the sugar and phosphate groups being identical for each residue.

6:4:a Base pair - Base pair interactions

Important differences between base pairs arise from the electronic distribution in π and σ molecular orbitals. These interact between neighbouring bases leading to differences in the stabilisation energy of the helix which therefore depends upon base sequence. This stabilisation has been the object of study by several workers such as De Voe and Tinoco [90] but results tended to depend upon the model used to calculate the interactions. Gersch and Jordan [91] examined the binding of the amino acridines to nucleic acids and concluded that their free energy calculations favoured the intercalation mechanism as opposed to the external attachment of the molecule. They also found that the increase in stability of the helix due to intercalation between base pairs was greatest for A-T/A-T > A-T/G-C > G-C/G-C sites.

Polter and Sukhorukov [92] suggested that D.N.A. stability was also dependent upon the molecular environment and determined that the total energy of a G-C base pair in the D.N.A. helix was more closely dependent upon the surroundings than A-T pairs.

These theoretical studies are still too vague to do more than suggest that the stability of an intercalated drug is not the same for all sites and that the molecular environment influences this stabilisation.

An additional influence upon intercalation considered by Müller and Crothers [82] for the specificity of actinomycin was the possibility of the formation of a π complex between base and drug which could differ for each base pair and lead to different binding constants. Experimental evidence for the binding of the steffimycins suggests that a π complex between base pair and drug

is not the same for all intercalative sites since the complex formed between poly d(A-T) is different from that with poly d(G-C). Figs. (4:13) and (4:14) show the spectra of these two complexes at the same concentration and it can be observed that they are not the same. This is taken as evidence that the electronic interaction between steffimycin and the different base pairs is not the same and that the stability of a drug bound by intercalation is dependent upon the actual binding site.

We shall now discuss the likely effect on steffimycin intercalation of the different energies involved with this type of binding to particular base pairs and the resulting variations in stabilisation of the double helix.

6:4:b Forces involved with the intercalation of chromophores

The intercalation of the steffimycins is likely to be due to a combination of forces and interactions and will involve the following.

1. Hydrophobic interaction of the chromophore

Steffimycin possesses both hydrophilic and hydrophobic groups. The aromatic ring section is typical of molecules showing hydrophobic interactions whereas the substituted sugar group is hydrophilic. The molecule minimises the energy of these reactions by arranging its fused ring system in a non polar environment and the sugar group in the presence of aqueous solvent.

Hydrophobic interactions arise when a molecule, present in an aqueous environment is unable to stabilise itself in the matrix of hydrogen bonds present in the solvent. The hydrophobic molecule experiences a repulsive force as water molecules surrounding it are

pulled together by hydrogen bonds. On the other hand a hydrophilic molecular group is stabilised in the presence of water when it possesses such groups that can form hydrogen bonds with the surrounding solvent or is sufficiently polar to be stabilised by electrostatic forces.

Hydrophobic and hydrophilic forces are therefore of the same magnitude or lower than the forces involved in hydrogen bonding; very approximately, this makes them <30 kJ $mole^{-1}$.

Intercalated steffimycin is therefore stabilised by (a) the non aqueous environment of the base pairs in that any movement of the chromophore into the aqueous surroundings is met with a repulsive force and (b) the hydrophilic interaction of the sugar group projecting out from the D.N.A. molecule.

2. Hydrogen bonding

The suggested intercalation model for the steffimycins involves the presence of two hydrogen bonds for steffimycin and one for steffimycin B. Hydrogen bonds have energies between 13 and 29 KJ $mole^{-1}$ between asymmetric atoms. For the purposes of this order of magnitude comparison of forces involved in intercalation, we shall take an average value. Hydrogen bonding, therefore, stabilises steffimycin by $\sim$ 40 kJ $mole^{-1}$ and steffimycin B by $\sim$ 20 kJ $mole^{-1}$.

3. Free energy of intercalation

Gersch and Jordan [91] have shown that the amino acridines stabilise the D.N.A. helix by intercalation; the greatest stabilisation occurring with A-T base pairs. These types of theoretical calculations using monopole or dipole interactions are susceptible to the model chosen and are unreliable. However, some

stabilisation of the helix due to intercalation seems likely and the difference in electronic structure of the base pairs can lead to selectivity of intercalation. The stabilisation due to electrostatic interaction of chromophore and base pairs will be in the region of 0 to 40 kJ/2 moles of base.

Thus it can be seen that the stabilisation of the intercalated drug originates from three interactions which are around the same order of magnitude. There are several important consequences of this situation.

1. The drug would be sensitive to the differences in stabilisation resulting from the dipole-dipole interaction of chromophore and base pair. This can explain the base specificity of the steffimycins. It also shows why there is a difference in specificity between the synthetic and natural D.N.A.'s because the binding energy is dependent upon the type of base pairs on either side of the intercalation site.

   The synthetic D.N.A.'s used were poly d(A-T) and poly d(G-C) which have alternating base pairs. Since at free drug concentrations of <10μM the steffimycins are A-T specific this would indicate that an A-T/T-A intercalation site is more stabilised by the presence of a drug than a G-C/C-G site. This was reported to be the case where Gersch and Jordan [91] considered the binding of the amino acridines. Natural D.N.A.'s on the other hand possess ten distinguishable intercalation sites with different electrostatic interactions.

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

The base pair-drug-base pair complex could be stabilised differently in each of these ten possibilities showing an overall G-C specificity in natural D.N.A. even though an A-T/T-A site is more preferable than a G-C/G-C site in synthetic polynucleotides. If this were the case then G-C specificity in natural D.N.A.'s would exhibit the maximum preference of binding at some percentage G-C content less than 100%. D.N.A.'s with a G-C content higher than this percentage would show a decrease in drug binding. The range of natural D.N.A.'s used in the experiments for this thesis was not extensive enough to show if this takes place.

2. The occupation of a binding site would depend upon the total drug concentration. At increased concentration, binding to weaker binding sites could become preferential to remaining in solution. This is observed with the synthetic polynucleotides where at drug concentrations below 10μM, the A-T/T-A intercalation site is favourable. Above this concentration the strong binding sites are saturated and the secondary binding sites associated with G-C base pairs become energetically favourable.

3. Drug molecules were observed to leave the intercalation site when the humidity of a fibre was lowered. Some D.N.A./drug fibres which showed X-ray diffraction patterns of increased pitch and decreased intermolecular spacing associated with intercalation then gave a normal B pattern with crystalline

drug reflexions superimposed, when the humidity was lowered. This occurred more readily for steffimycin B than for steffimycin.

With a lowering of humidity, the hydrophobic interactions are less important and the hydrogen bonding is insufficient to stabilise the drug in the intercalation site. Steffimycin B, with only one hydrogen bond is then able to leave the intercalation site. Steffimycin, with two H bonds is slightly more stable when intercalated and in practise, it was more difficult to produce a fibre showing this drug crystallised in the presence of B D.N.A.

4. The formation of crystalline regions of drug in the presence of oriented D.N.A. is a stable arrangement. This is observed in practise since after the steffimycins have crystallised out as a result of lowering the humidity of an intercalated specimen, the drug remains in the crystalline form when the humidity is subsequently raised. The crystalline state of the drug is therefore more stable than that of intercalation.

Forces involved in the crystallisation of a non polar molecule such as steffimycin are mainly van-der-Waals and hydrogen bonding. Stabilisation energies involved in this type of crystal are around >100kJ $mole^{-1}$. They are not as stable as ionic crystals which in turn are less stable than covalent crystals. This suggests that steffimycin and steffimycin B should be somewhat more stable in the form of a molecular crystal than as intercalated molecules but the difference in stabilisation energy between the two conditions is not great. Thus in a gel with excess water present, the drug stability is

favoured by intercalation. As the water content is lowered, the crystalline state becomes more stable.

6:4:c Application to the complexes of D.N.A. with the other drugs examined in the work

It is useful to note that daunomycin shows no sign of base specificity or co-crystallisation with D.N.A., even though the molecule is very similar to steffimycin. The model of the D.N.A.-daunomycin complex suggested by Pigram et al [40] also resembles the steffimycin complex to some extent. The daunomycin model has a H bond from the OH group of C_9 on the saturated ring as does Steffimycin. However, the C3′ of the sugar possesses an amine group which is protonated as NH_3^+. This forms a strong electrostatic attraction with the neighbouring PO_4^- group verging upon covalent bond formation which becomes the dominant force in binding the intercalated daunomycin molecule in position. The strength of this bond is much greater than the differences caused by interaction with the various bases. Thus the intercalation mechanism is not sensitive to particular base pairs; no base specificity would be expected and none is observed. Such a drug molecule, bound in this way to D.N.A. would be more stable than as a molecular or ionic crystal lattice. Again, such crystallisation is not observed within the fibre.

Adriamycin also showed an absence of base specificity for the strong binding. Like daunomycin, the drug is favoured by intercalation due to the bond between NH_3^+ on the C3′ sugar and PO_4^- of D.N.A.. This masks any base specificity due to the

stabilisation of the different bases, and makes intercalation energetically more favourable than crystal formation.

It is well known that actinomycin binds to D.N.A. Reich [93] showed that the drug requires the presence of guanine to bind to the nucleic acids. Müller and Crothers [82] performed a comprehensive series of experiments which indicated that the drug intercalated adjacent to any G-C base pair forming two hydrogen bonds and a π complex. This intercalation model is generally accepted although Hamilton et al [94] proposed an external binding model on the basis of ill defined X-ray diffraction data. Cerami et al [95] note that the 2-amino group of the guanine base is important in binding since it can form a hydrogen bond with the quinoidal oxygen of actinomycin. Detailed model building studies based on single crystal data were performed by Sobell, Jain and others [96] [97] [98] and then conclude that the base specificity of actinomycin is due to hydrogen bonding to the 2-amino group of guanine.

The spectral studies of actinomycin binding to different D.N.A.'s reported in chapter 4 again show that the drug is base specific. There is a strong binding which shows a preference for G-C rich D.N.A., and no observed secondary binding. The base specificity of actinomycin was the same for synthetic and natural D.N.A.'s and was not dependent upon drug concentration. This is what one would expect since the drug is reported to form a stable π complex with guanine and to hydrogen bond specifically to the base. No such hydrogen bond formation between steffimycin and the D.N.A. bases is likely. Hence the base specificity of actinomycin is not as

susceptible to the experimental conditions of complex formation as is steffimycin. Actinomycin molecules being more complex than steffimycin are not stabilised with respect to intercalation by forming a molecular crystal. Thus the ability for crystal formation in the presence of D.N.A. observed for the steffimycins is not observed for actinomycin.

Wells [99] and Wells and Larson [83], in a study of the binding of actinomycin to a wide range of D.N.A.'s and synthetic polynucleotides deduce that deoxyguanylic acid is not necessary for the drug to bind to the nucleic acid polymer. Instead, there is a marked nucleotide sequence preference for binding where deoxyguanosine may induce a suitable configuration in a small region of the D.N.A. chain to permit binding. These workers confirm that it is, in fact, the purine 2-amino group which is sufficient to "confer on a D.N.A. a suitable steric and electronic environment to permit the binding of actinomycin."

So we can see that the analysis of drug binding to D.N.A. is extending from a study of base pair specificity to a consideration of the importance of base sequence. Bhuyan and Smith [79] have established that it is base sequence specificity which accounts for the binding of nogalamycin to D.N.A. The study of nogalamycin in this work gave this drug an apparent G-C specificity of binding.

6:5 External binding of the steffimycins

The spectral studies of D.N.A.-steffimycin complexes in solution indicated that in addition to the strong binding attributed to intercalation, there was a weak, secondary binding process. This has been associated with an external mode of binding in other drugs.

Features of the secondary binding of the steffimycins are:-

1. It was observed for poly d(G-C) and the three natural D.N.A.'s.
2. It appears to be more prevalent for higher G-C content D.N.A.'s.
3. Fibres with the higher drug contents at which secondary binding became important tended not to show increased pitch but crystalline steffimycin reflexions.

The steffimycins are not polar molecules and in the neutral or weakly acidic solutions used, there would be no protonisation as is the case with daunomycin. This latter drug shows more prominant secondary binding possibly due to electrostatic attraction between its NH_3^+ group and the phosphate chain. Therefore the steffimycin secondary binding to D.N.A. is due at most to weak electrostatic interactions and hydrophobic forces.

The D.N.A. could use the drug in this way to stabilise the helix and Polter and Sukhorukov [92] have shown that G-C base pairs are more susceptible to stabilisation by the surroundings than A-T base pairs. The external binding of steffimycin to G-C nucleotide pairs could stabilise the helix by a slight and local conformational change that makes external binding favourable at this site. This would mean that the drug would not have to interact directly with the bases themselves but the increase in helix stability would result in an apparent base pair specificity of secondary binding.

Hydrophobic interactions would be important if a chromophore were held by weak electrostatic binding to the double helix providing the drug with an aqueous environment. It would mean that the flat aromatic grouping of the steffimycins would be energetically

favoured if they were stacked upon each other thus reducing the contact between chromophore and water molecules. Such a mechanism is suggested for the external binding of daunomycin which occurs to the extent of causing the complex to precipitate [45].

X-ray work showed that it was not difficult for the steffimycin to form its own crystalline lattice which was observed superimposed over the D.N.A. diffraction pattern. Thus the weak complexing between drug and nucleic acid is not sufficiently strong enough to prevent drug crystallisation in oriented fibres. When this occurred, the pitch of D.N.A. decreased from its extended intercalated value down to 34A as in the B form.

6:6 A comparison of the results of the steffimycin studies with those of other workers

When binding to the synthetic polynucleotides, steffimycin shows a base pair specificity for adenine-thymine at drug concentrations below 10μM. Reusser suggests that steffimycin B [50] and to a lesser degree steffimycin [23], interact primarily with adenine-thymine base pairs on the basis of the template properties of poly d(A-T) and poly dG.poly dC antibiotic complexes. He used drug concentrations of <1μM and hence his results are comparable to those of this work at the same concentrations.

It is unfortunate that Reussers' conclusion about the A-T specificity of the steffimycins was based upon observations of the drug interacting with poly d(A-T) and poly dG.poly dC. Firstly, these are dissimilar synthetic polynucleotides and may not have similar conformations in solution. Secondly, both the spectral work

and X-ray diffraction studies of this thesis indicate that the steffimycin complex with poly d(A-T) is unusual. The spectrum of the complex with poly d(A-T) is not the same as with the natural D.N.A.'s and the maximum pitch obtained with poly d(A-T) was 48A compared with a consistent 41A for the natural D.N.A.'s.

The concentration of the steffimycins in the experiments with the synthetic polynucleotides is also important. The concentrations of the drugs in Reussers work, although relevant to the biological examination of the antibiotics, did not reach a level at which the total binding of the drug becomes preferential to G-C base pairs.

Reusser reports that he did not detect any base pair specificity when he used natural D.N.A.'s as templates. It is possible that at the low concentrations of drug in his experiments, the strong binding sites were never completely occupied and therefore the differences between the D.N.A.'s would be insignificant. It is evident from the results of this work that the percentage of G-C base pairs on natural D.N.A.'s correlate well with the number of steffimycin and steffimycin B binding sites.

Chapter 7

STUDIES ON THE STRETCHED FORM OF D.N.A.

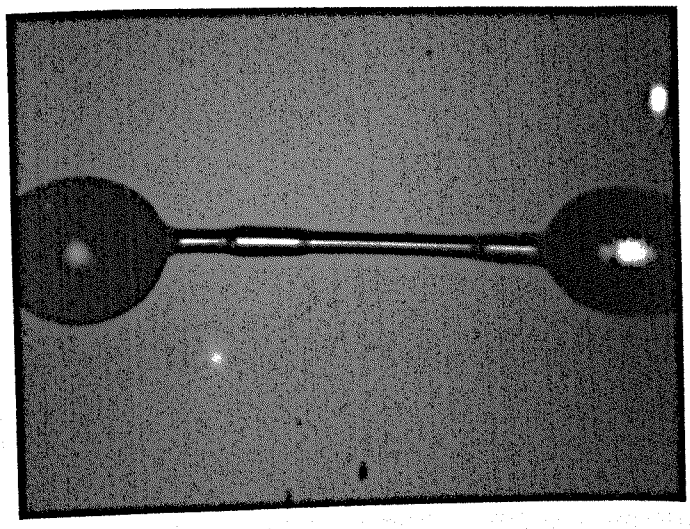
7:1 Introduction

The stretched conformation of D.N.A. can be produced when an oriented fibre held between two glass rods is made to undergo an abrupt extension. Usually the whole fibre gets longer and thinner but occasionally, a sudden narrowing is observed at one point and upon a further increase in tension of the fibre, this region extends in both directions. This is referred to as a stretched fibre and an example is shown in fig. (7:1). An outline of the method of preparing such fibres is described in section 2:4:a and the operation of the stretching frame designed to prepare and store fibres in the stretched conformation is described in 2:4:b.

Generally, stretched fibres are longer and narrower than the original specimens from which they were produced. The fibres increase in length by up to two fold on stretching. If a fully stretched fibre was further extended, it does not shear but pulls off one of the glass mounting rods, which indicates the strong binding forces that exist between molecules. There was little correlation between the change in physical dimensions upon stretching a fibre and other parameters such as birefringence and X-ray diffraction features but any relationship is probably compounded by the effects of water and salt present in the fibre.

The stretching of the fibre could be due to:-

1. A rearrangement of the packing of D.N.A. molecules,
2. A new conformation of D.N.A. helices possibly also involving a



250 mμ

Fig. (7:1) Stretched fibre viewed through crossed polarizers.

modified crystal packing, or,

3. A breakdown of the attractive forces between adjacent helices causing the long polymer molecules to slide past each other.

This latter possibility is probably not the case because of the following reasons:-

1. It is possible to cause molecules to slide past each other by stretching the fibre too vigorously causing it to shear in two. When the molecules take up the stretched conformation, a narrow neck appears which extends in both directions along the fibre as the tension is increased. This would be difficult to explain as the result of a shearing action.
2. There is an abrupt and distinct change in the value of the birefringence of a fibre between the normal and stretched portions. A more gradual change would be expected if molecules were sliding past each other.
3. The stretched process is reversible which would not be possible if molecules had moved longitudinally relative to each other.
4. X-ray diffraction reveals different conformations of the molecules in the normal and stretched regions of the fibre.

This work seeks to clarify the conditions under which the stretched fibres will form and remain stable. Optical and X-ray diffraction techniques are then used to obtain information about the conformation of the stretched molecules. The data are then used in the next chapter to carry out model building studies of the stretched form.

7:2 Notes on preparing stretched fibres

The method of preparing the stretched form of D.N.A. as outlined

above resulted in only a small percentage of all fibres pulled actually taking up this conformation. Most fibres simply elongated until they sheared and tore apart. The break was not perpendicular to the fibre axis but along a considerable portion of the specimen where the fibre would tear longitudinally. Nearly 100% of fibres were successfully stretched, however, if they had previously been briefly longitudinally compressed and then extended.

The stretched portion of the fibre could be made to extend along the specimen until one end reached the glass mounting rod whereupon a further increase of tension pulls the fibre off the glass rod.

An alternative method of stretching was to prepare a normal fibre of unstretched D.N.A. which was then sealed in a cell containing a pot of salt solution to provide a relative humidity of 95%. The fibre swelled as it took in water molecules from the atmosphere increasing the mobility of the specimen. The cell was then opened to the room humidity of around 50-60% and the fibre was immediately stretched as it was drying.

Stretched fibres were found to revert to the unstretched form again if the tension was relaxed except for those prepared from a salt free gel. The process of stretching was truly reversible as shown by X-ray diffraction photographs and fibres could be repeatedly stretched and unstretched. The stability of the stretched conformation was seen to depend upon the salt content and relative humidity. Stretched fibres made from gels prepared with Na^+ content greater than 0.003M in the pre-centrifuge solution were found to be unstable at relative humidities greater than about 50%. Such a stretched fibre not held under tension and maintained at R.H. >66%

would gradually revert to an unstretched D.N.A. conformation.

The effects of salt content and humidity upon stretched fibres will be considered in detail in section 7:5 and 7:6.

7:3 Birefringence of D.N.A. fibres

7:3:a Origin and measurement of birefringence

The refractive index of optically anisotropic materials varies according to the direction of the electric field vector of the applied radiation and upon the polarisation of the medium itself. If the polarizability of the medium, referred to a set of orthoganal axes, is identical in two directions but has a different value in the third, then the material is said to be birefringent and possesses a maximum and minimum value of the refractive index. The value of the birefringence is the difference between these two refractive indices and in an oriented specimen of D.N.A. molecules, these occur parallel and perpendicular to the helix axis.

Birefringence in a dired fibre of D.N.A. originates from two sources. One is the "intrinsic" birefringence which is present due to the polarisation of the bonds linking atoms. The maximum polarisation is found in the planes of the bases and the minimum perpendicular to these bases. The contribution by the sugar and phosphate groups is negligible since the polarization of the bonds involved averages effectively to zero.

Since the net polarizability in a D.N.A. molecule occurs in the plane of the bases, the intrinsic birefringence is seen to be dependent upon the tilt of the bases in the double helix. The maximum value of birefringence occurs when the bases are perpendicular to the helix axis and will have the value zero when

they have tilted to such an angle that the component of polarizability directed along the helix is equal to the perpendicular component. This angle of tilt is found to be 54° irrespective of any misalignment of molecules in the fibre [100].

The second term involved in birefringence is called "form" birefringence which arises from the presence of a collection of long parallel molecules in a fibre. These possess maximum polarizability along the fibre since the electronic charges along molecules are aligned in this direction. The minimum polarizability of the collection is perpendicular to the fibre axis resulting from the transverse discontinuities in the molecular matrix. The resulting form birefringence for a collection of oriented D.N.A. molecules is a constant positive term which is dependent upon the distance between helices and the presence of solvent molecules.

If the value of the form birefringence for D.N.A. is taken as 58% of the maximum intrinsic birefringence as found by Tsvetkov [101] then the maximum value of the total birefringence still occurs when the bases are perpendicular to the helix axis but the zero value results from an angle of tilt of the base pairs of 32°.

Birefringence can therefore give information about the arrangement and conformation of D.N.A. molecules in a fibre and in particular, about the tilt of the bases.

The birefringence was measured by placing the specimen between crossed polarizers of a microscope and placing a "compensator" in the light path between the fibre and the analyser. This compensator consists of a slice of crystal such as quartz or calcite cut so that the refractive index is uniform in its plane but shows birefringence when tilted. The angle of tilt can be varied until the phase

difference between the fast and slow axes of polarized light from the specimen is compensated for by an opposite phase change produced by the compensator and the fibre appears blackened. The phase changed produced by the compensator is related to the sine of the angle of tilt and can be read off tables supplied by the manufacturer. This enables the birefringence of the specimen, which is equal and opposite to that of the compensator, to be calculated.

Birefringence measurements give information about the alignment of D.N.A. helices in a fibre and about their molecular conformation. If the molecules are equally well aligned throughout the fibre then the value of birefringence is uniform along the whole specimen. Any microcrystallites present which are oriented at differing angles to the fibre axis give different values of birefringence observed as a pattern of interference colours. This reveals fibres which have disoriented molecules and would not be ideal for X-ray diffraction studies.

An indication of the conformation of D.N.A. in a fibre is provided by the value of its birefringence. In the B conformation of D.N.A. the bases are essentially perpendicular to the helix axis and defined to have a zero tilt angle. A well aligned fibre of B D.N.A. shows a value for birefringence of $\sim$ 0.09. The A form with the bases tilted by 23° has a value of $\sim$ -0.07 again in a well aligned fibre.

7:3:b Birefringence of stretched D.N.A.

When a stretched fibre is viewed between crossed polarizers as shown in fig. (7:1) then the birefringence is observed to have a value close to zero. The theory of birefringence in D.N.A. fibres

mentioned above suggests that this value occurs when the base pairs are tilted by between 54° (by considering intrinsic birefringence) and 32° (if the estimated contribution of form birefringence is taken into account). This indicates therefore that stretched D.N.A. has a different conformation from normal fibres and predicts a base tilt of between 32°-54°.

The values of birefringence measured for a selection of stretched fibres are shown in fig. (7:2) plotted against the diameter of the fibre expressed as the percentage of the diameter of the unstretched portion. Values are seen to lie between +0.015 and -0.03 and show little correlation with the decrease in diameter of the fibre. In all cases, however, the change in the value of birefringence between the unstretched and stretched conformation was abrupt and no intermediate values were observed.

This is not the case if fibres of D.N.A. are crushed between microscope slides where the value of birefringence is again made to approach zero. If the birefringence is measured as the fibre is crushed, the change in its value is seen to follow the variation in fibre thickness as shown in fig. (7:3). This may indicate that the change of molecular structure in crushed fibres is not the same as when they are stretched and is possibly due to an increasing misalignment of the helices. The result would be a gradual change in birefringence without any discontinuity and it would never become positive as is observed.

The spread of birefringence values found for the different stretched fibres has already been mentioned and possible reasons for this will be considered in section 7:4 and 7:5. Occasionally, however, a fibre showed different values for the birefringence in

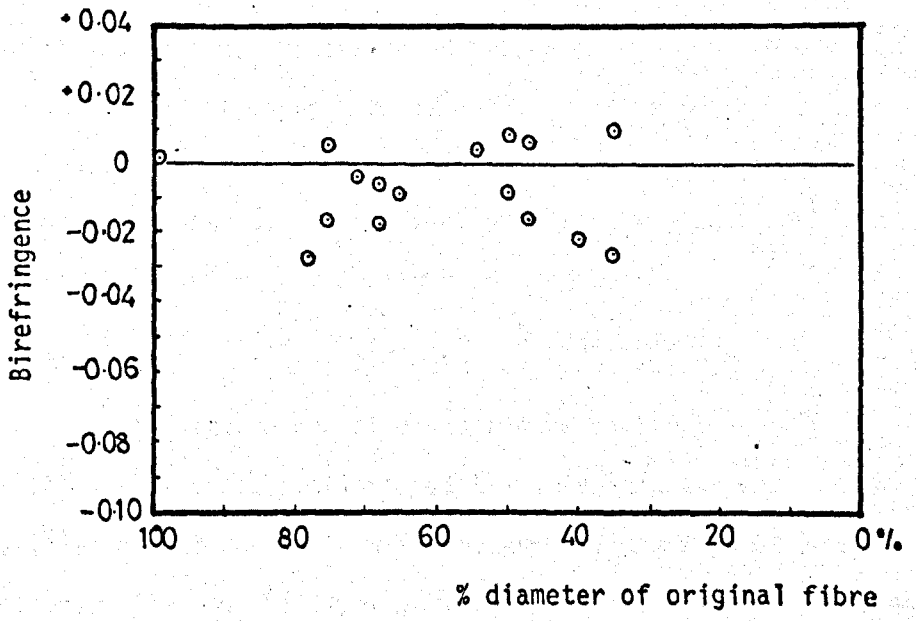


Fig. (7:2) values of birefringence for stretched fibres as a function of percentage decrease in diameter.

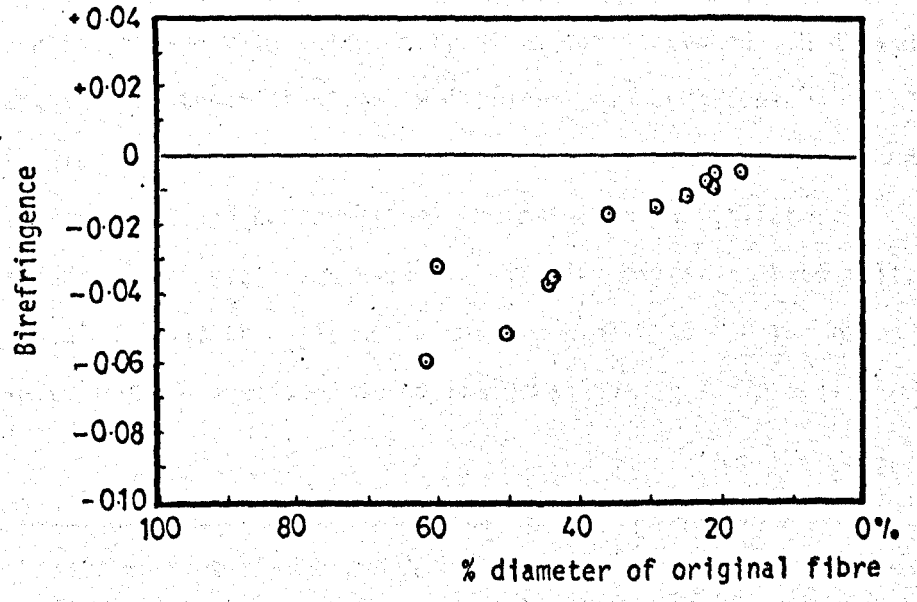


Fig. (7:3) values of birefringence for crushed fibres as a function of percentage decrease in diameter

different sections of the stretched region. When this occurred there was a distinct change in the value from one section to the next. Two fibres were prepared with four such regions and the values of birefringence in each section was as follows.

Fibre	Birefringence values			
S 72	+0.0048	-0.0005	-0.0192	-0.0668
S105	+0.0057	-0.0010	-0.0223	-0.0430

(maximum error of measurements ±0.0010)

All four separate sections had the same diameter and it was impossible to distinguish them except when viewed between crossed polarisers. The possibility that the different values were due to a non uniform salt content was discounted because this would lead to a gradual variation instead of the distinct differences observed.

The two highly negative birefringences of -0.067 and -0.043 fall within the range of values given by poorly oriented nonstretched D.N.A. fibres. However, the remaining three sections in each fibre have values typically found for stretched D.N.A. and suggest the existence of slightly different conformations. Distinctly different birefringence values would be found for example if several stretched conformations could form with similar stabilisation energies each having a slightly different base tilt. This predicts that there is no unique angle of tilt for the bases but several which are found to be conformationally acceptable.

7:4 The effect of sodium chloride on stretched D.N.A. fibres

Stretched fibres were prepared from gels of sedimented D.N.A. with a sodium chloride content that was dependent upon the salt concentration in the centrifuge tube. Higher salt contents made it

easier to induce the fibre to take up the stretched form but the result was a more negative birefringence and poorer quality X-ray patterns. Fibres made from gels with the lowest salt contents were only produced with difficulty but gave the most positive values of birefringence and the best diffraction photographs. Fig. (7:4) shows how the value of the birefringence of a specimen of stretched D.N.A. is greatly affected by the salt content. It was fibres with the most positive birefringence that gave the best diffraction patterns. In order to obtain such specimens, D.N.A. was used which, after purification, was washed in several changes of aqueous ethanol to reduce residual traces of sodium chloride. The fibre was then prepared by placing a portion of this D.N.A. onto a droplet of water between glass rods. A gel then formed with very low salt content and a fibre could be formed and stretched in the usual way.

Fibres with lower salt content were also found to be more stable at higher humidities. It is difficult to quantify this relationship but the extent of hydration of a fibre is dependent upon salt content of the specimen. Stretched fibres prepared after removing the salt from the D.N.A. were sometimes found to be stable even after the tension was removed.

Differences of birefringence measurements between fibres are therefore partly due to variation in the salt concentration in the D.N.A. lattice. This effects the crystallinity of the molecules and the contribution of the "form" term to the overall value of birefringence.

7:5 Effect of humidity on stretched D.N.A.

The birefringence of three stretched fibres was measured at

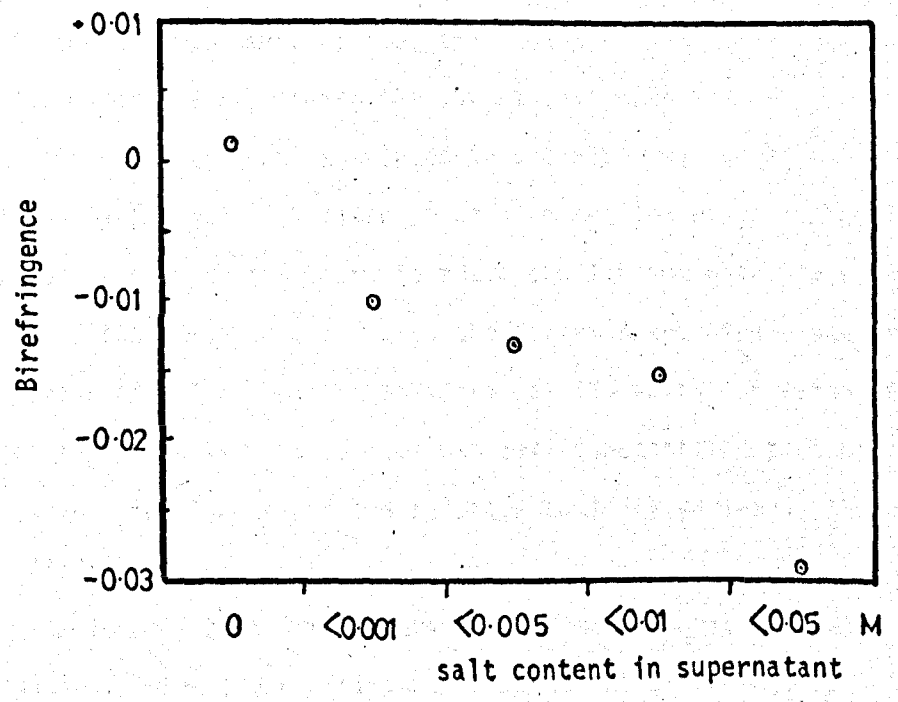


Fig. (7:4) Average values of birefringence for stretched fibres for different concentrations of NaCℓ used to prepare the gel.

different values of the relative humidity. The fibres chosen were of different salt content and the usual effect of NaCℓ upon birefringence was observed; i.e. the most positive birefringence was found for the fibre with the lowest salt content.

Each fibre in turn was placed in a sealed cell which was continually flushed with nitrogen at a controlled relative humidity. The fibre was given two hours to reach equilibrium with the water vapour of the atmosphere and then the diameter and birefringence were measured. The starting humidity was 44% which was raised in stages to 92% whilst the tension was left constant. A plot of the change in birefringence as the relative humidity varies is shown in fig. (7:5).

Stretched fibres were observed to be more stable at lower humidities. When the humidity was raised, the birefringence gradually became more negative until a value of -0.03 was reached whereupon the stretched fibre reverted to the unstretched state with an abrupt change in the value of birefringence. It can be seen from fig. (7:5) that the exact value of the relative humidity at which the fibre was found to have a birefringence of -0.03 was itself apparently dependent upon the salt content of the specimen.

The stretched conformation is stable at low relative humidities and for this reason X-ray diffraction patterns were usually taken at 66% or 44% R.H. Increased humidity provides water molecules which occupy sites between D.N.A. molecules and interfere with the crystal lattice. It then becomes energetically more favourable to revert to the unstretched form. When this occurred, it was necessary to reduce the humidity and apply tension once more to return to the stretched conformation.

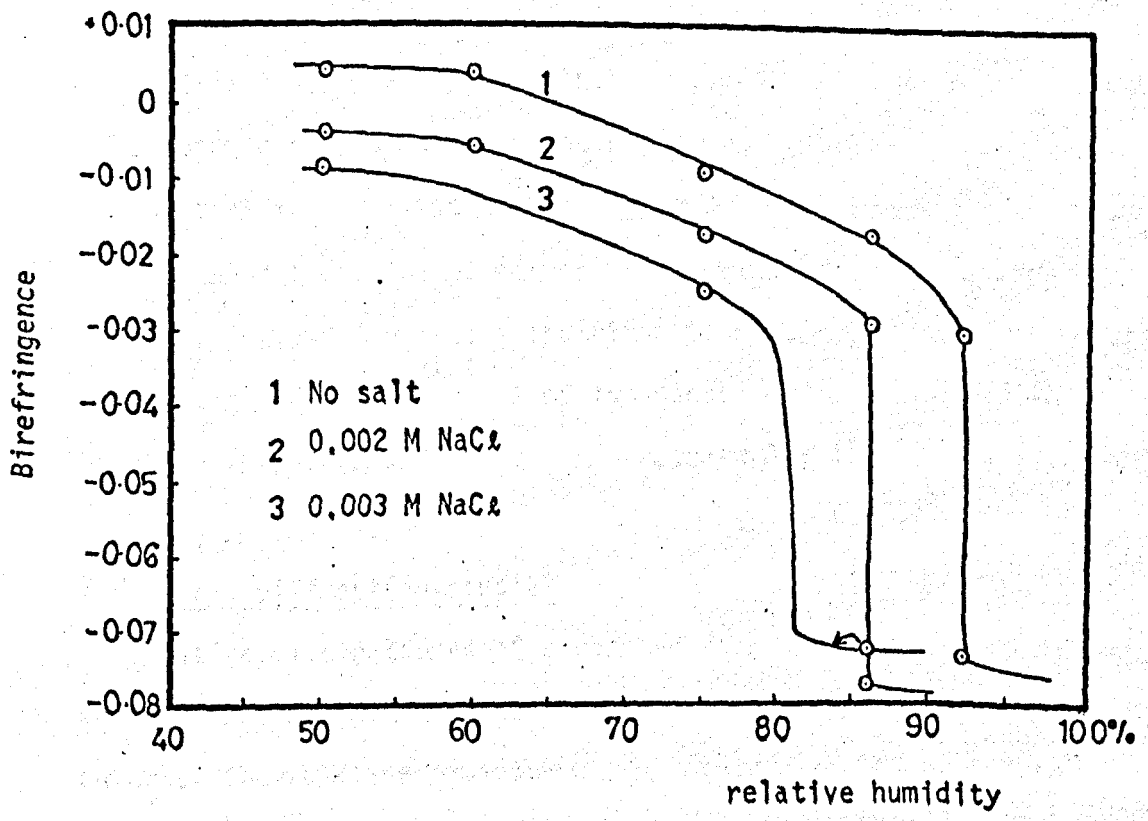


Fig. (7:5) Birefringence of three stretched fibres as a function of humidity

It can be seen from fig. (7;5) that a fibre with little or no salt content should be stable up to 90% relative humidity. It was found possible to remove such fibres from the stretching frame without them returning to the unstretched form.

It is also interesting to note that the measured birefringence of stretched fibres falls in the range +0.15 to -0.03. It is this region that is sensitive to the salt and humidity conditions of the fibre. When the value of birefringence reaches -0.03, any further increase in humidity causes the fibre to return to the unstretched state with the disappearance of the necks, and hence no value more negative than this was found for stretched D.N.A.

7:6 X-ray diffraction studies

Diffraction patterns of stretched fibres were obtained by placing the specimen mounted on a stretching frame, inside the X-ray camera. The relative humidity was maintained at 44% or 66% to prevent the fibre from reverting to the unstretched form. The fibre diameters were ∿50 microns and necessitated exposures of 24 hours using toroidal optics or 48 hours for Franks optics in a Searle camera, specimen to film distance 3 to 4 cm., mounted on a rotating anode generator using a tube current of 60mA at 35KV.

Examples of X-ray photographs of stretched D.N.A. fibres are shown in figs. (7:6) and (7:7). Resolution is not good, yet photographs of this quality were consistently obtained. Some details of the stretched D.N.A. structure were obtained from the X-ray diffraction studies and these are now listed.

1. Crystalline reflexions on the equator. Usually only two reflexions (110 and 220) were observed on the equator but one

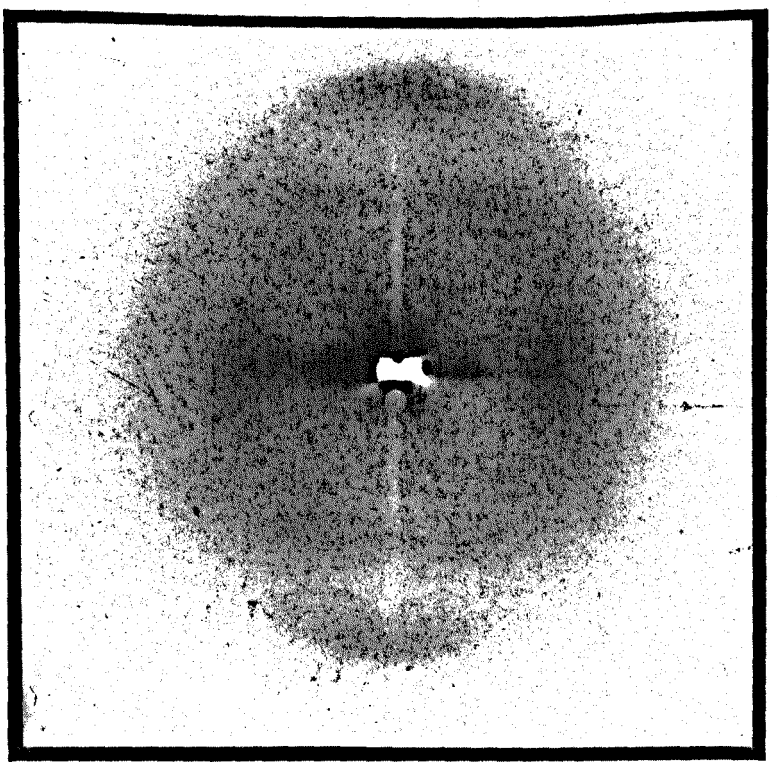


Fig. (7:6) Stretched D.N.A. R.H. = 66%

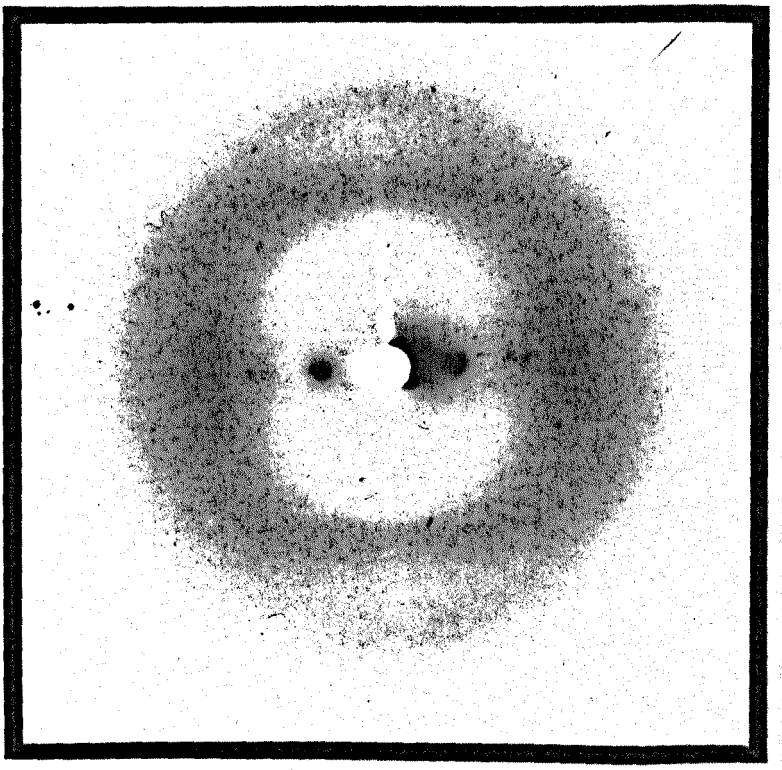


Fig. (7:7) Stretched D.N.A. R.H. = 44%

photograph led to the measurement of four such reflexions from a densitometer trace. These were indexed on a hexagonal lattice as follows.

$1/d^2$ observed	0.0064	0.0169	0.0250	0.0548
$1/d^2$ calculated	0.0061	0.0182	0.0243	0.0546
index	110	300	220	330

(errors in the observed reflexions, ±0.0005).

This gives the hexagonal dimension as a = 25.6 ± 1.0A. Measurements by Dr. C. Nave from an X-ray pattern of a stretched fibre produced by Professor W. Fuller gave a hexagonal lattice with a = 23A. This difference between X-ray data will be considered below.

2. There is a strong near-meridional reflexion along a layer line with a spacing of 5.4 ± 0.1A which was present on all photographs taken.

3. The 3.4A meridional reflexion associated with the base pair separation in the D.N.A. molecule was sometimes present and an average measurement for this spacing from photographs of stretched fibres gave the value of 3.48 ± 0.02A. This meridional reflexion was sometimes absent and was never so intense as in normal D.N.A. This could be due to the sphere of reflexion not intersecting this region of reciprocal space or it could be a feature.of the structure of the molecule which gives a weak 3.4A reflexion and is variable due to the conformational flexibility of the stretched molecules.

4. The central region of the diffraction pattern is generally devoid of intensity so that there is little evidence of layer lines which would correspond to the helical repeat of the D.N.A. It was possible to observe some intensity at 30.4 ± 0.4A

however and this was taken as a layer line spacing when considering possible models of the structure.

5. Diffuse scatter can be observed around the sides of the pattern at about 5A spacing. Although no conformational data can be obtained from this, it must be considered when possible models of the molecules are examined. The absence of details in this diffuse scatter could be due to the existence of helical molecules with variable pitch which would lead to a broadening of layer lines.

7:7 Conformational route between stretched and unstretched D.N.A.

Fibres were usually held under tension during the X-ray exposure, which was sufficient to maintain the fibres in the stretched configuration. Sometimes, however, a fibre would revert to the unstretched form and provided an opportunity to examine the structure of D.N.A. after it had been through the stretched phase.

The fibre used to produce fig. (7:7) subsequently returned to the unstretched form, probably because of the high relative humidity of the room, and the resulting diffraction pattern is shown in fig. (7:8) which is typical of photographs given by the B conformation of D.N.A. The gel used to make the fibre was prepared with 0.005M NaCℓ in the centrifuge tube and it is unusual that the B conformation is found with this low salt content at 44% humidity. This is possibly due to the tension applied to the fibre which prevented the molecules taking up the A form. One end of the fibre was then cut from the glass rod releasing the tension and the specimen then produced the photograph shown in fig. (7:9). The pattern is that of D.N.A. in a mixture of the A and B conformations. This same fibre

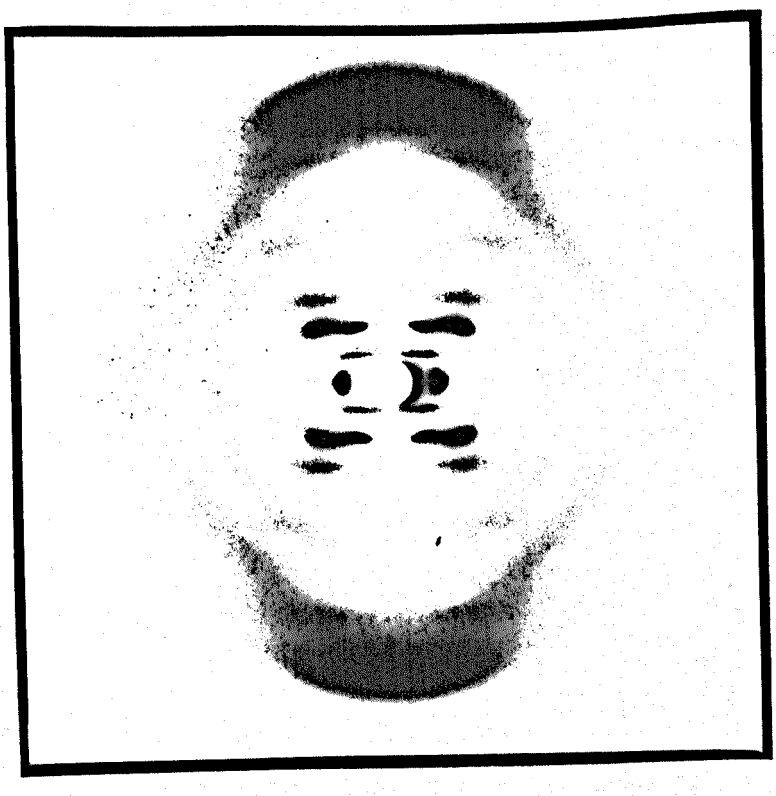


Fig. (7:8) D.N.A. previously stretched but reverted to the unstretched form whilst under tension. R.H. = 44%

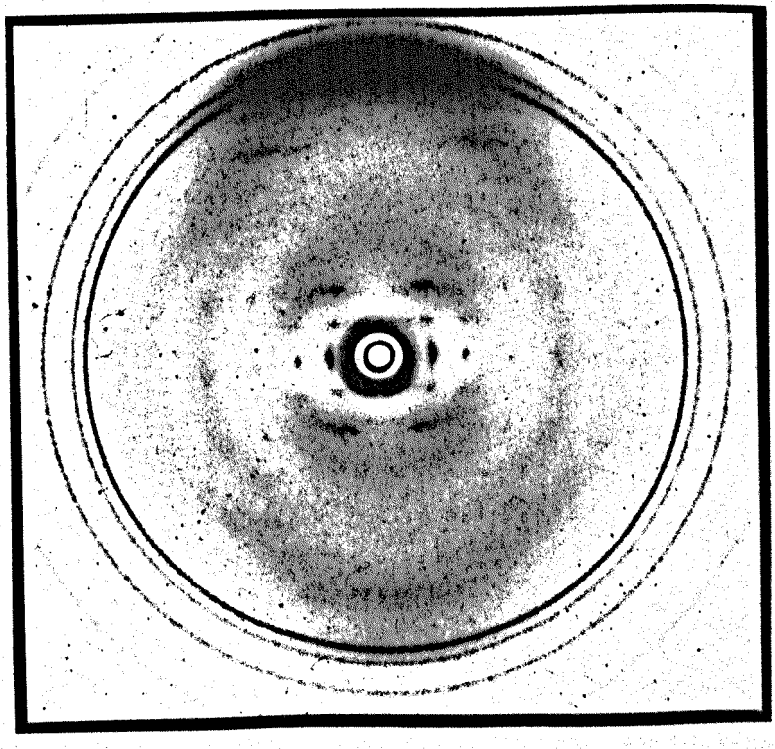


Fig. (7:9) D.N.A. previously stretched, now in the unstretched form without tension. R.H. = 66%

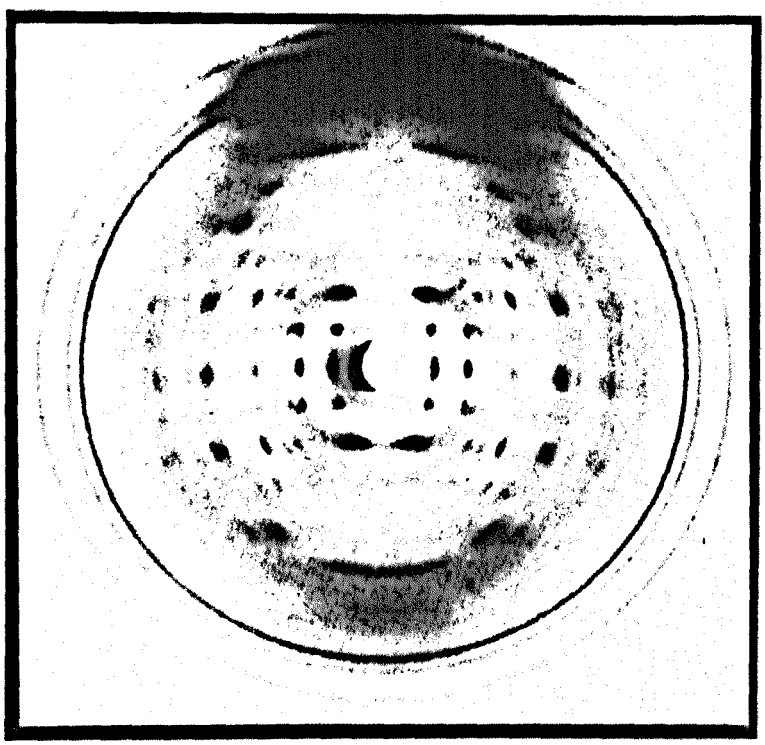


Fig. (7:10) D.N.A. previously stretched, now in the unstretched form without tension having been stored for several weeks. R.H. = 66%

was stored for several weeks and then used to produce the X-ray photograph shown in fig. (7:10) where all the D.N.A. is now in the A form.

The X-ray study of this fibre shows that when the stretched molecules revert to the unstretched form, they change to the B conformation of D.N.A. It seems that the tension still applied to the fibre had prevented the formation of the A form and constrained the molecules to the B conformation. There is only a 2.4% decrease in the length of the molecules as the conformation changes from the stretched to the B form. The B to A transition requires a 25% decrease in the length of the molecules which can only occur when the tension is no longer applied.

Therefore, the conformational route of a stretched fibre when the tension is relaxed, is to go from the stretched form to the B form and then to the A form. The process presumably takes place in the reverse direction when a stretched fibre is being prepared.

7:8 Summary of the work on stretched D.N.A.

Stretched fibres have birefringence values between +0.015 and -0.03 whilst a value of zero suggests a tilt of the base pairs of between 32° and 54°. Fibres showing distinct regions of slightly different birefringence values indicate the possibility of several related conformations of the stretched molecules.

It appears that salt stabilises unstretched B D.N.A. with respect to the stretched form since only stretched fibres with residual NaCℓ removed are stable without tension being continually applied. Fibres produced from gels with the high salt content required for the B form can easily be made to take up the stretched

form but with a high negative birefringence and resulting in poor X-ray patterns. If salt assists the transformation of the A form with its tilted base pairs, to the B form, then it could also stabilise bases at 90° to the helix axis and hinder the distortion of the D.N.A. into the stretched form.

Low salt fibres tend to become elongated without transition to the stretched form. They are difficult to stretch possibly because of the more drastic conformational change which takes place in going from the A to the stretched conformation. Such fibres give zero or positive birefringence and the sharpest diffraction patterns.

The effect of raised humidity on stretched fibres is to stimulate the return to an unstretched conformation when the birefringence reaches a value of -0.03.

The X-ray studies provided parameters which were used to direct the construction of the molecular models described in the next chapter. Stretched D.N.A. fibres are extended by up to two times the length of the unstretched fibres. This could be achieved by unwinding the double helix. The unwinding may be complete so that a step ladder type of molecule results which has a long and possibly variable pitch. This would explain the absence of layer line information and lower angle diffraction data.

Consideration must be made of the evidence of a layer line at 30.4A which suggests that the stretched molecule exists as a helix. If so the stretched conformation would need to result in the paucity of radiation being observed for the lower angle region of the pattern.

The 3.48A meridional was often present for stretched D.N.A. and must be taken into account. It could be that unstretched sections of

molecules are always present in stretched fibres or alternatively that one feature of the stretched molecules is that slight variations of the conformation greatly effect the observed diffraction intensities of this spacing.

Chapter 8

MODEL BUILDING STUDIES OF STRETCHED D.N.A.

8:1 Introduction

The purpose of this chapter is to use the data obtained from the optical and X-ray diffraction studies to construct possible models of the stretched D.N.A. conformation. Previous discussion [17] [20] [102] centred on the idea of a "step ladder" type of molecule with the double helix unwound to give an infinite or at least very large and possibly variable pitch. The second type of model examined is of a helix based upon parameters suggested by the appearance of a layer line on some diffraction patterns. The conformations were examined using rigid wire skeletal models of scale 4 cm. per A which enabled the atomic coordinates to be measured. Use was made of the computer model building programs described in chapter 2.

8:2 "Step ladder" molecules

Preliminary models were built to see if this type of model were stereochemically possible and the first models then helped to develop a satisfactory method of refining the crude structures. As a result, it was found easiest to proceed by considering the stretched D.N.A. firstly as a series of base pairs. They were fixed in position with an axial separation of 5.4A which would produce reflexions on a diffracted layer line at this spacing. The base tilt and rotation between base pairs, if any, could then be adjusted followed by the construction of the sugar-phosphate chain

between glycosidic links, arranged to minimise any steric interference. If the constructionof this chain between base pairs was feasible, then the coordinates of the model were measured and refined by the computer programs to minimise any short contacts between atoms. Not all models built would refine in this way without some unacceptably short contacts between atoms.

The base coordinates were taken from Langridge et al [60] and when models were refined, the base pairs were positioned with respect to the helix axis as defined by Arnott and Hukins [4]. Sugar coordinates were those used by Pigram [45] and the puckers were chosen as necessary in order to link the bases with the sugar-phosphate chain. The "step ladder" molecules built are summarised in fig. (8:1).

Models were built firstly with base pairs tilted at 45° as suggested by Wilkins [17] based on the change of physical dimensions and birefringence measurements. These models, which had an infinite pitch were unsatisfactory because the sugar-phosphate chain either had serious short contacts between atoms or would not link the base pairs together.

The model would refine when the bases were tilted at 54°, the angle at which intrinsic birefringence has the value zero. The stereochemically most favourable molecule of this type has an infinite pitch but there was no difficulty in building the model with a pitch of between 388A and ∞ for a right handed helix and between 200A and ∞ for a left handed model.

Built in this way, the models would give a 5.4A near-meridional reflexion with no other layer lines present for those helices with infinite pitch. Equatorial reflexions would be sampled according to

Model	Base tilt (°)	Rot per base pair (°)	Pitch (A)	Axial rise per residue (A)	Sugar pucker	Details
1A	45	0	∞	5.4	C2 Endo	P has short contacts
1B	45	0	∞	5.4	C2 Endo	Sugar rotated from 1A. Difficult to fit the phosphate group between sugars
2	45	0	∞	5.4	C3 Endo	Short contact between phosphate O3 and sugar group. Phosphate sugar chain will not reach between glycosidic bonds
3A	54	0	∞	5.4	C3 Endo	model refined
B	54	5	388.8	5.4	C3 Endo	" "
C	54	7.5	260.0	5.4	C3 Endo	slight short contacts
D	54	10	200.0	5.4	C3 Endo	" " "
E	54	-5	388.8	5.4	C3 Endo	model refined
F	54	-7.5	260.0	5.4	C3 Endo	" "
G	54	-10.0	200.0	5.4	C3 Endo	" "
4	45	0	∞	5.4	C3 Endo	Used base coordinates from Arnott et al [6]. Would not build

Fig. (8:1) Details of step ladder models of stretched D.N.A.

the molecular lattice but there would be little else on the X-ray pattern. Details of these models and the refinement procedure are not discussed because they do not give a layer line at ∿30A which was observed in the better, later X-ray photographs.

8:3 Helical molecules

8:3:a Relevant Diffraction Theory

Diffraction from a continuous helix of unit scattering power can be expressed in terms of its Fourier transform.

$$G(R, \psi, \ell/c) = \sum_n J_n(2\pi Rr) \exp[in(\psi + \pi/2)] \quad - \underline{7:1}$$

The terms of this equation are defined in equation 1 of chapter 4. J_n, the nth order Bessel function, is periodic with the higher orders contributing to the diffracted intensity increasingly farther out in reciprocal space. As described in chapter 4, the transforms contributing to any particular layer line are summed over all values of n from the relationship,

$$n = \frac{\ell - mN}{K} \quad - \underline{7:2}$$

The computer generated values of the squared transform were calculated using values of n up to 44 but an insignificant contribution was made when n >20.

If we consider j series of atoms each lying on its own helix and with atomic coordinates r_j, ϕ_j, z_j, then the Fourier transform is summed over all discontinuous helices and becomes:-

$$G(R, \psi, \ell/c) = \sum_{nj}\sum f_j J_n(2\pi Rr_j) \exp i[n(\psi - \phi_j + \pi/2) + 2\pi \ell z_j/c] \quad - \underline{7:3}$$

The function describes a series of layer lines, where the

transform is non zero, along which diffraction from the discontinuous helix is observed. With a two fold rotation axis, (dyad axis), perpendicular to the helix axis where every atom r_j, ϕ_j, z_j, has an equivalent atom at r_j, $-\phi_j$, $-z_j$, the equation is

$$G(R, \psi, \ell/c) = \Sigma\Sigma f_j J_n(2\pi R r_j) \cos(2\pi \ell z_j/c - n\phi_j) \operatorname{expin}(\psi + \pi/2) \quad - 7:4$$
$$nj$$

It can now be seen from equation 7:4 that the contribution of any Bessel function to any particular layer line is dependent upon the atomic coordinates. If all the atoms in the helix are approximately at the same radius then the transform is modulated by the term $\cos(2\pi \ell z_j/c - n\phi_j)$ depending on values of Z_j and ϕ_j, the term $(2\pi R r_j)$ being the same for all atoms.

The helical parameters will now be determined in order to maximise the contribution of Bessel functions on layer lines where diffracted intensities are observed.

8:3:b Determination of helical parameters

X-ray photographs of stretched fibres reveal diffracted intensities on the meridional at 3.48A, a near meridional at 5.40A and on a 30.4A layer line. It was found that if 30.4A was designated the 3rd layer line then 5.40A was on the 17th and 3.48A on the 26th. This corresponds to a non integral helix with a repeat distance of 90.1A and $8\frac{2}{3}$ base pairs per helical turn.

Since the maximum intensity on the diffraction pattern occurs at 5.4A on the 17th layer line, it is necessary to build a model in which the atoms contribute to the Bessel function most responsible for this value of ℓ which is found by inserting the appropriate

values in equation 7:2.

$$n = \frac{\ell - mN}{K} = \frac{17 - 26}{3} \quad \text{for } m = 1$$

This gives n = -3 which is equivalent to a left handed helix where $\ell = 3$, $n = 3$, $m = 0$.

Therefore, maximum intensity on the 17th layer line is produced when as many atoms as possible have coordinates which maximise the term $\cos(2\pi\ell z_j/c - n\phi_j)$ when $\ell = 17$ and $n = -3$ and which lie along the left handed helix with $\ell = 3$ and $n = 3$. This will occur providing the atoms have approximately the same radius in the helix.

This analysis suggests that the atoms should lie on a left handed helix and yet stretched D.N.A. is prepared by applying tension to right handed helical molecules. This can be achieved by moving the nucleotide pair away from the helix axis and tilting the bases so that the atoms of each residue lie close to a left handed helix, each with approximately the same radius, whilst the sugar-phosphate chains form the characteristic right handed double helix.

In order to construct models with values of z_j and ϕ_j which maximise the appropriate cos term, it was found useful to project the coordinates onto a radial projection showing these two dimensions [103]. A grid was superimposed upon this representing the positions of maximum and minimum values of the cos term for different values of n, which occur n times in 2π radians. This enabled the contribution of the atoms to each Bessel function to be readily estimated.

8:4 Models of stretched D.N.A.

8:4:a Construction of the models

Models were built with an axial rise per base pair of 3.48A as suggested by the meridional reflexion. The J_{-3} Bessel function, responsible for diffraction on the 17th layer line was found to be maximised when the base pairs were tilted by 24.5°. The atoms were arranged at similar radii around 7A by moving the base pairs away from the helix axis. Both tilt and displacement were in the same direction as in C D.N.A. [7]. In this arrangement the sugar-phosphate chains made up a right handed double helix with three turns in the 90.1A pitch. All the atoms of any particular nucleotide are concentraded close to a left handed helix inclined at an angle of 65.5° to the helix axis.

Three slightly different skeletal wire models were built to these parameters and can be seen in fig. (8:2) (8:3) (8:4) where z is plotted against ϕ and the contributions to the different Bessel function can be identified. The base, sugar and phosphate groups can be seen to lie about the centre of gravity of the line representing the maximum contribution of the J_{-3} Bessel function whilst the base is centred on the line indicating a zero contribution to J_2 and the sugar is positioned where J_1 is 0. There is little electron density concentrated at the position which contributes most to the J_3 Bessel function.

The sugar pucker in these models was C3 exo, as found in B D.N.A. This was thought a reasonable assumption because the conformational route of the D.N.A. helix is from B D.N.A. to the stretched form as established in chapter 7.

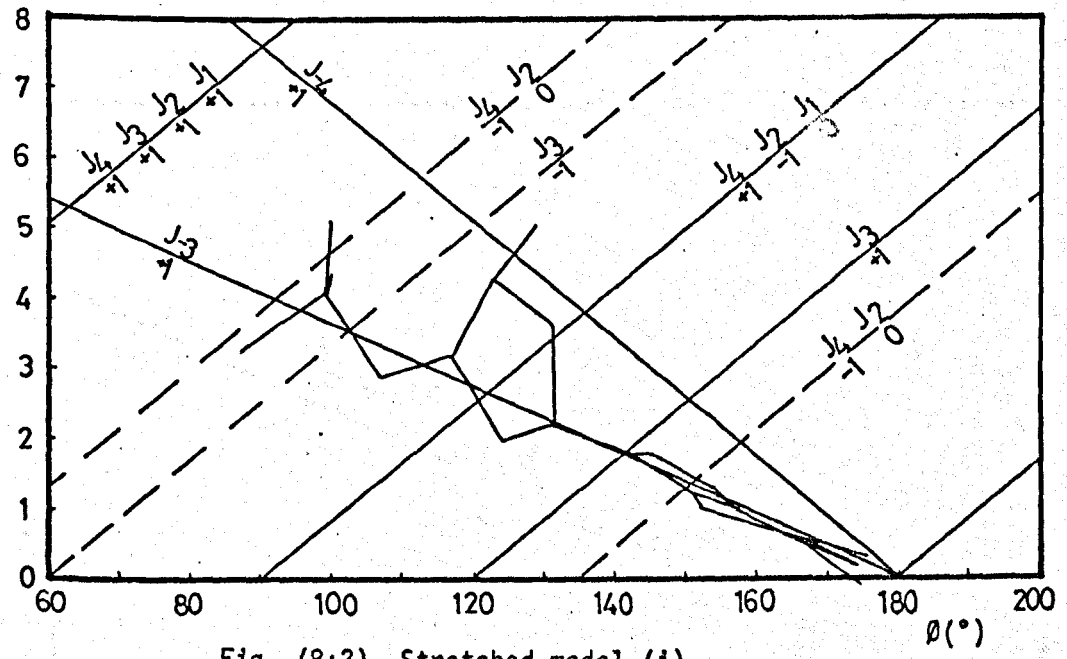


Fig. (8:2) Stretched model (i)

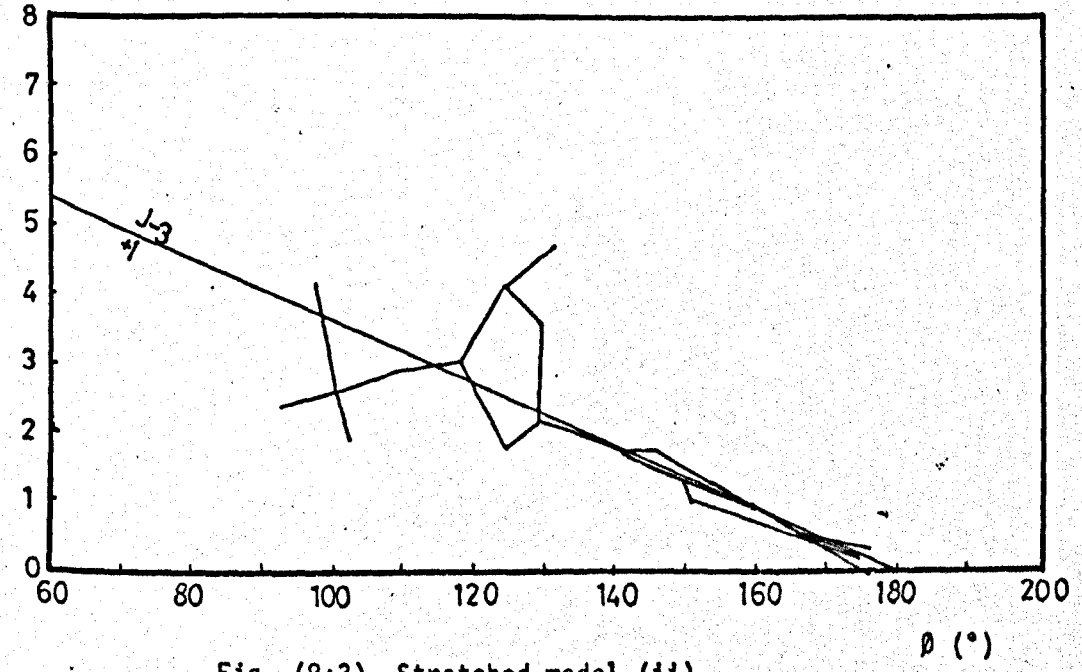


Fig. (8:3) Stretched model (ii)

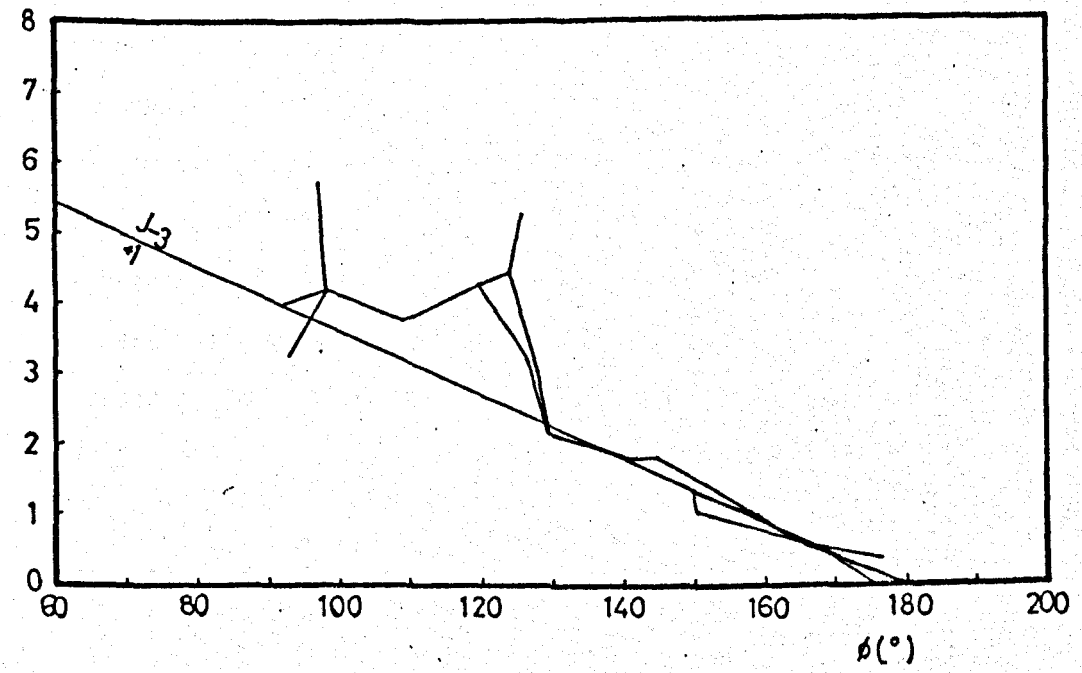


Fig. (8:4) Stretched model (iii)

8:4:b Coordinates of the models

The z coordinates were measured using a pointer attached to a vertical vernier scale and r and ϕ were read off from the calibrated base board. There were no serious short contacts between atoms and the atomic coordinates for each of the three models are listed below. The differences between the models is found in changes in the rotation of the sugar and phosphate groups. The displacement from the helix axis to the line joining the nitrogens of the glycosidic link is 5.4A for model (i) and 5.25A for models (ii) and (iii).

Model (i)

Pyrimidine bases

Atom	r(A)	ϕ(°)	z(A)
N9	6.85	-38.00	1.76
C8	8.03	-35.50	1.76
N7	8.20	-27.00	1.26
C6	6.83	-12.00	0.36
C5	6.96	-22.50	0.91
C4	6.00	-30.50	1.22
N3	4.67	-28.50	1.05
C2	4.50	-14.00	0.51
N1	5.51	- 6.50	0.19

Guanine only

NH2	3.45	- 4.50	0.25
O	7.85	- 5.50	-0.14

Adenine only

NH2	7.85	- 5.50	-0.14

Purine bases

N1	6.85	38.00	-1.76

Purine Bases

Atom	r(A)	ø(°)	z(A)
C2	6.00	30.00	-1.28
N3	6.50	19.50	-1.03
C4	7.70	19.63	-1.34
C5	8.51	26.60	-1.86
C6	8.10	35.00	-2.10
=O	4.72	30.50	-1.00

Cytosine only

NH2	8.50	10.50	-1.15

Thymine only

O	8.50	10.50	-1.15

Sugar

C1	6.50	-49.00	2.19
C2	7.81	-56.10	1.99
C3	7.80	-63.70	3.15
C4	7.13	-57.60	4.22
C5	8.22	-51.60	5.02
O5	6.32	-49.20	3.60

Phosphate

O1	7.20	-73.50	2.87
P	6.85	-81.50	4.01
O2	5.27	-80.60	4.35
O3	7.72	-80.60	5.09
O4	7.09	-93.00	3.24

Model (ii) and (iii)

Pyrimidine bases

N9	6.78	-39.10	1.77
C8	7.92	-35.10	1.77
N7	8.05	-26.60	1.27
C6	6.73	-10.60	0.37
C5	6.77	-21.60	0.92
C4	5.88	-29.90	1.30
N3	4.60	-29.10	1.03
C2	4.43	-13.10	0.50
N1	5.40	- 5.60	0.17

Guanine only

Atom	r(A)	ϕ(°)	z(A)
NH2	3.32	- 4.10	0.33
O	7.75	- 4.30	-0.06

Adenine only

NH2	7.75	- 4.30	-0.06

Purine bases

N1	6.78	39.10	-1.77
C2	5.90	30.40	-1.22
N3	6.39	19.40	-1.06
C4	7.68	19.50	-1.39
C5	8.42	27.20	-1.90
C6	8.00	35.60	-2.15
O	4.59	33.60	-0.97

Cytosine only

NH2	8.39	11.40	-1.14

Thymine only

O	8.39	11.40	-1.14

Model (ii)

Sugar

C1	6.50	-50.30	2.18
C2	7.85	-55.10	1.80
C3	8.37	-61.80	3.00
C4	7.75	-55.70	4.11
C5	8.48	-48.60	4.68
O5	6.50	-50.90	3.66

Phosphate

O1	8.33	-71.10	2.89
P	7.18	-79.70	2.60
O2	6.72	-82.30	4.14
O3	6.30	-77.60	1.96
O4	8.39	-87.40	2.36

Model (iii)

Sugar

Atom	r(A)	ø(°)	z(A)
C1	6.50	-50.10	2.18
C2	7.78	-53.80	3.15
C3	7.18	-60.60	4.23
C4	5.81	-56.20	4.44
C5	5.78	-54.20	5.26
O5	5.33	-52.10	3.08

Phosphate

O1	7.47	-71.60	3.79
P	7.16	-82.00	4.26
O2	6.62	-83.00	5.70
O3	8.39	-87.70	3.99
O4	5.94	-87.10	3.26

These coordinates describe nucleotides constructed with base pairs tilted at 24.5° with the sugar linked to the pyrimidine bases. Glycosidic links on base pairs are related by a dyad axis.

8:5 Fourier transforms of the models

Once each model had been built the cylindrically averaged Fourier transform was generated using "Helix 1" described in chapter 2. The square of the transform for each model and the diffracted intensities measured with a microdensitometer from X-ray photographs are compared in figs. (8:5) (8:6) (8:7). Calculated and observed intensities of the fibre show good agreement particularly for model (i). Observed reflexions are labelled as strong, weak or variable and the spots are indexed according to the hexagonal unit cell determined in chapter 7:6. Of the observed equatorial spots, the 110 and 220 were strong and almost always present and the 300 and 250/330 reflexions were weak and could not

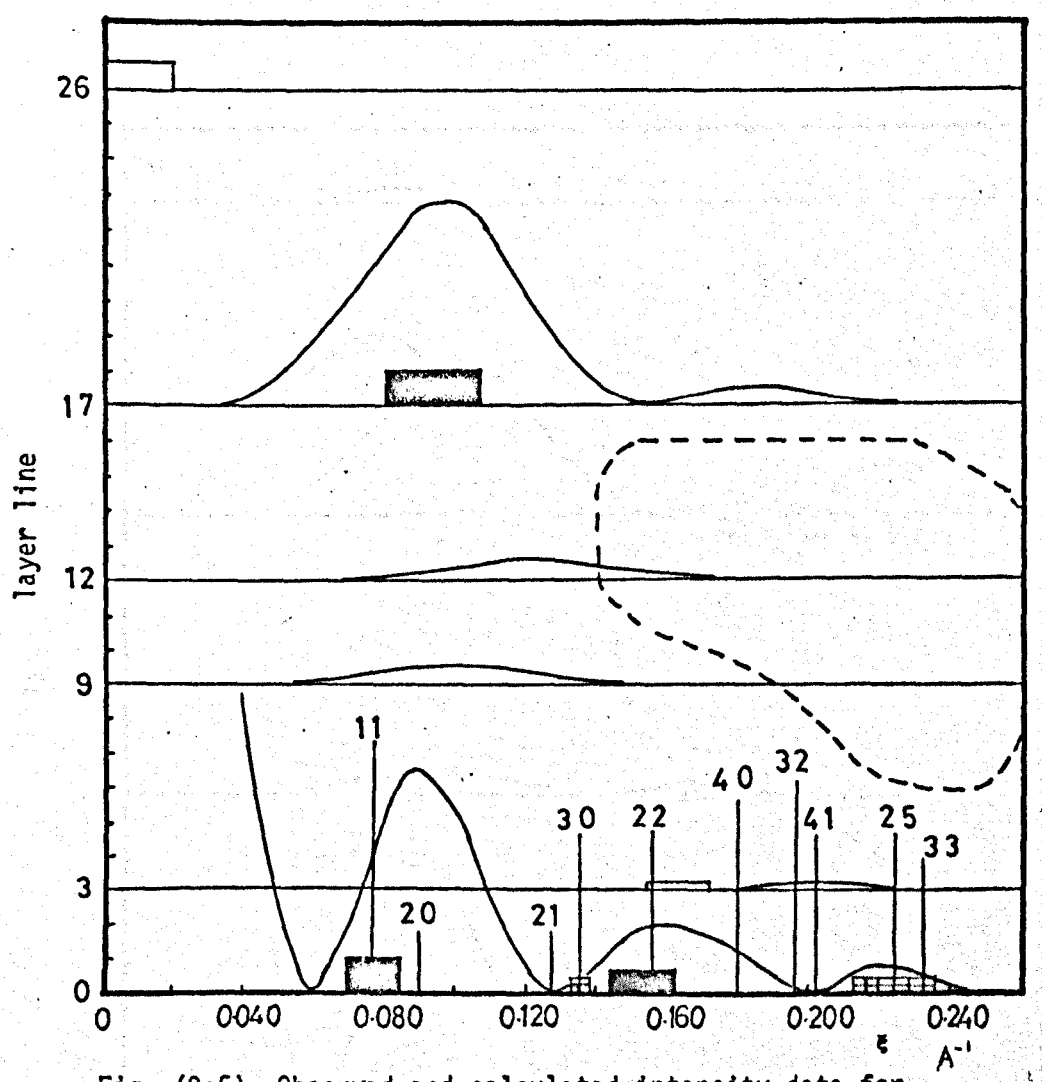


Fig. (8:5) Observed and calculated intensity data for model (i)

variable intensity

weak

strong

area of diffuse scatter

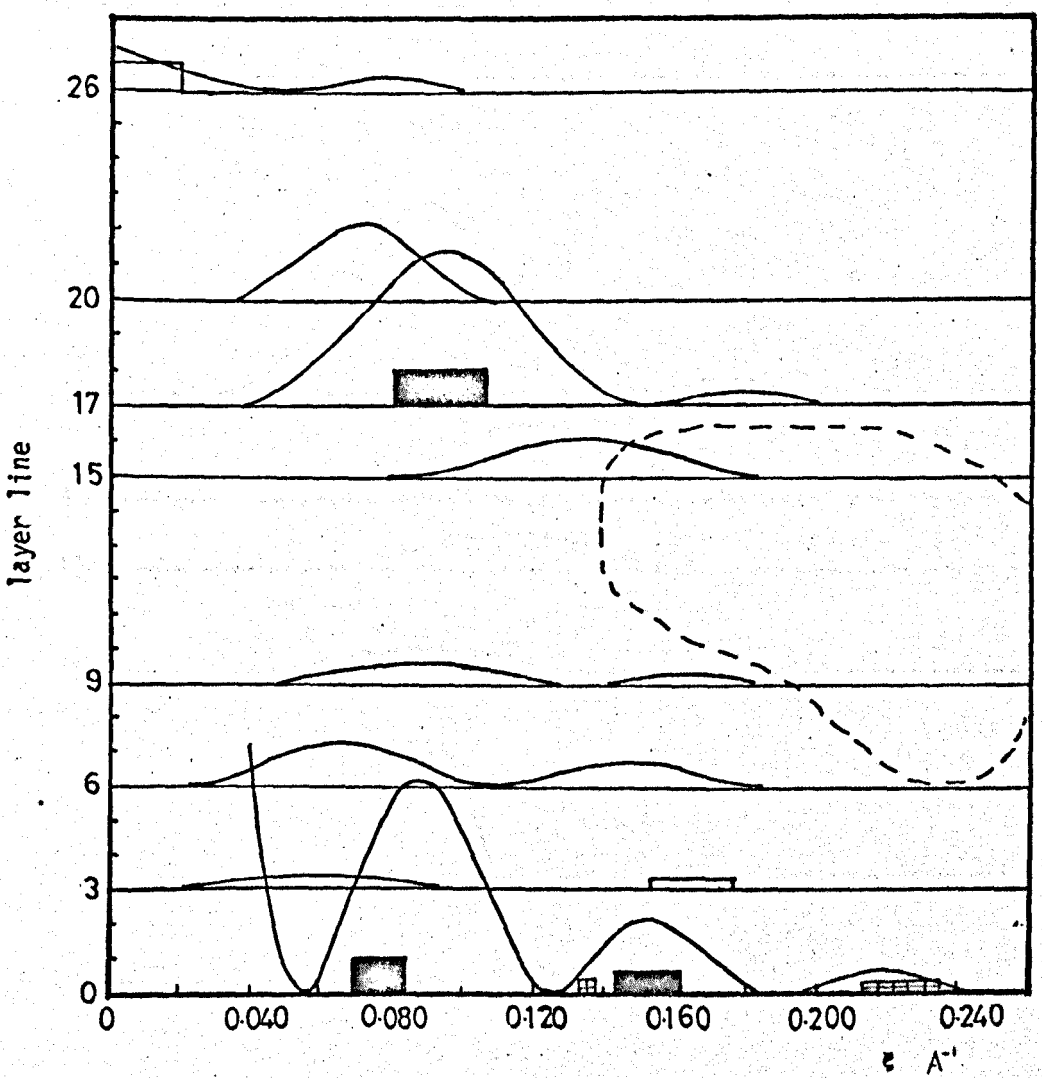


Fig. (8:6) Observed and calculated intensity data for model (ii)

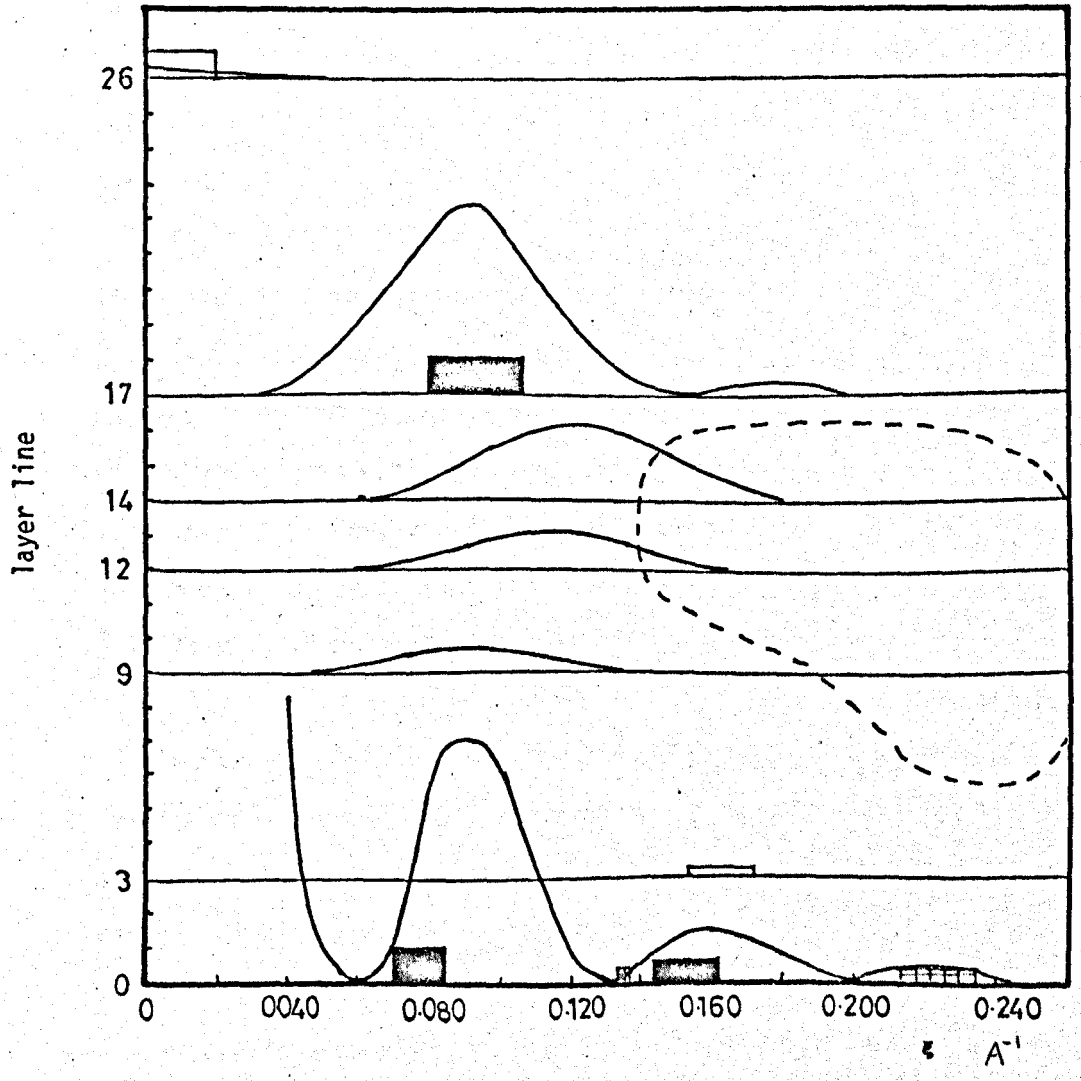


Fig. (8:7) Observed and calculated intensity data for model (ii).

always be measured. All but the 300 fall near the maxima of the theoretical squared transform which also correctly predicts the absence of the 210, 320 and 410 reflexions. The 200 and 400 reflexions also fall in maxima of the square transform but were not observed.

The models generated only a weak reflexion on the third layer line which is consistent with the difficulty found in recording this peak. The position of the maximum of the calculated squared Fourier transform on this layer line was highly dependent upon slight changes in conformation of the sugar and phosphate groups.

There is a strong 5.4A near-meridional reflexion from the models which is to be expected from their construction and this 17th layer line maximum occurs at around the measured value of z.

On the 26th layer line, the meridional reflexion varied widely in intensity between the three models. It was sensitive to slight changes in the sugar-phosphate chain conformation which was consistent with the observation that not all X-ray diffraction patterns showed a meridional reflexion on this layer line.

There is generally an absence of radiation elsewhere on the pattern. Weaker intensities are predicted however, on layer lines intermediate between 3 and 17 which are also sensitive to slight conformational differences. No layer lines were resolved in this region on the X-ray photographs but there is considerable diffuse scatter in this area which is shown on the squared transform diagrams outlined by the dashed line.

These models of stretched D.N.A. predict a diffraction pattern similar to that obtained from actual specimens. One noteable

feature is that minor changes in the conformation have a negligible effect on the strong reflexions on the equatorial and 5.4A layer lines. Simultaneously there is a considerable effect on the intensities of the 3.48A and 30.4A reflexions with large variations in the areas of more diffuse scattering.

8:6 Discussion of the helical models of stretched D.N.A.

The models built of stretched D.N.A. were built with helical parameters consistent with the X-ray diffraction patterns. The resulting calculated cylindrically averaged intensity agrees well with the observed diffraction data. The slight differences in conformation of the three models result in a variation in the predicted intensity on the 3rd and 26th layer line which was actually observed. Birefringence measurements also suggest a variable form for stretched D.N.A.

The differences between the models are not sufficient to decide the one that best fits the observed intensity data. The variation in calculated intensities for the models compares well with the range of diffraction patterns observed. This leads to the conclusion that if this is the correct type of model for stretched D.N.A. then it probably has a somewhat flexible conformation within the basic stretched form.

Dr. C. Nave of Keele University, who suggested building models with base pairs moved out from the helix so that the scattering from bases, sugar and phosphate interfere destructively, has built 9 and 10 fold helices constructed to the data obtained from a diffraction pattern of a stretched fibre prepared by Professor W. Fuller [104]. The photograph was similar to those obtained by

the author but gave slightly different helical parameters. The 10 fold helix gave the best fit to the observed intensity data from this photograph which had equatorial reflexions, a 3.4A meridional, a 5.7A layer line and a diffuse layer line between 30 and 34A. The bases were displaced from the axis by 5.4A and were tilted by 30°. Again, the bases, sugar and phosphate groups lay close to a left handed helix which gave a strong reflexion corresponding to the J_{-4} Bessel function on the sixth layer line.

All these proposed structures of stretched D.N.A. result in the base pairs being positioned on the outside of the double helix in contrast to the A and B forms where they are centrally placed. This suggests a mechanism for recognition of bases or base pairs without the need for hydrogen bonds to be broken or for slight variations in the sugar phosphate chain conformation [104]. This is because the exposed groups in positions 7 and 6 of the purines and 6 and 5 on the pyrimidines offer a "signature" which is unique for all four base pairs and characterises the base sequence. (This would not be the case if the groups exposed were those on the other side of the bases because they are not unique for each base pair.) In vivo, protein would need to induce the local conformational transition to the stretched form, unless the molecular environment already stabilises this conformation so that a site could be recognised by the groups on the base pairs. The genetic code of D.N.A. molecules is then available for such functions as the direction of points where transcription and replication should begin.

The work on the stretched form of D.N.A. has shown that it probably does not have a precisely defined conformation and is

unstable except in conditions equivalent to applying tension along the molecule. If the role of the conformation is to locally expose the information of the base sequence then all that is needed is that the stretched molecules should be temporarily stable for the purpose of base recognition. When this is no longer required, then the molecule can return to the B form which is thought to be favoured in solution. This was observed to be the conformation to which stretched molecules revert when the tension is relaxed. An increase of humidity was observed to cause the abrupt change from stretched to nonstretched molecules. It is possible that this could be involved in the formation of stretched D.N.A. in vivo since cell components can locally infleunce the hydration of the helical molecules.

Chapter 9 CONCLUSIONS

The questions in mind at the start of this work centred upon how the conformation of D.N.A. is affected by the action of tension along the molecules and also by interacting with two antibiotics, steffimycin and steffimycin B. In response to mechanical tension, D.N.A. had been observed to give unusual X-ray diffraction data which did not resemble patterns produced by the other helical forms of the nucleic acid and this work set out to examine this extended conformation of the molecule. Some antibiotics with flat aromatic groupings were known to bind to D.N.A. producing an increased helix pitch. Two recently isolated drugs, the steffimycins, provided an opportunity to examine the influence of closely related anthracyclines on the double helix and enabled a comparison to be made with similar antibiotics which have been studies for some time.

The studies on stretched D.N.A. sought to ascertain the conditions which stabilised this conformation and it seems that it requires a narrow range of environmental properties. The application of tension along the helical polymer is one way of stabilising this form for the purpose of conformational investigations. The presence of low salt contents and low relative humidity of oriented fibres was also important in maintaining the stretched form.

X-ray diffraction studies supplemented by birefringence measurements were used to obtain some basic parameters for the stretched conformation and two possible explanations for this experimental data were examined. These were step ladder molecules, with a very large helix pitch and helical molecules which have bases, sugar and phosphate groups arranged so that scattered X-rays would

interfere destructively resulting in sparse intensity data.

Step ladder models with a small or zero angle of rotation per residue were rejected on several counts. The axial rise of 5.4A which was incorporated to produce the reflexion at this spacing meant that bases were no longer stacked upon each other resulting in a lack of stability for the molecule. Model building studies found that it was difficult to produce such molecular models without short contacts in the sugar phosphate chain. Also, these models with a very long axial rise per helix turn would not account for the layer line that was observed around 30A.

Helical models with $8\frac{2}{3}$ residues per turn were built using a radial projection [103] to indicate the contribution of atoms to the various Bessel functions. A comparison between observed and caclulated diffracted intensity data was found to give reasonable agreement. Models built by Dr. C. Nave with 9 and 10 base pairs per helix turn showed that a family of closely related conformations could exist with acceptable stereochemistry.

The unusual structure of the nucleic acid in these models has various features which could be exploited in biological systems since the code carrying sequence of base pairs is positioned on the outside of the double helix. This reveals groups on the bases which give each of the four pairs a unique identification easily accessible to other molecules such as protein and thereby provides a possible means for the direction of such mechanisms as replication or transcription. For this to take place in vivo then the molecular environment needs to be capable of producing the stretched form at least along short sections of the molecule. Once the nucleic acid is again in aqueous surroundings, the polymer is free to

revert to the more stable unstretched conformation.

Thus the stretching of D.N.A. which could be said to be an artificial process may provide a clue to a mechanism which is important in the functioning of D.N.A. in the living cell.

Steffimycin and steffimycin B are two antibiotics known to interfere with the replication mechanisms. Reusser [23] [50] had claimed that the drugs specifically bound to adenine-thymine base pairs. The work for this thesis confirmed that the antibiotics do interact with the nucleic acid polymer but found that the adenine-thymine specificity was an artifact of using synthetic polynucleotides in the experiments with the drug at low concentrations of less than 1μM.

When the binding to natural D.N.A.'s from M Lysodeikticus, calf thymus and Cℓ Perfringens was examined, then both drugs were found to bind with a preference for sites associated with guanine-cytosine base pairs. This occurred for all concentrations of antibiotics used up to the limit imposed by solubility restrictions of 11μM for steffimycin B and 45μM for steffimycin.

The results of X-ray studies of D.N.A. complexes with the steffimycins indicated that a conformational change takes place in the nucleic acid polymer due to the binding of the drug molecules. The mechanism of binding which best fits the experimental data is that of intercalation of the chromophore between base pairs although some secondary binding to the outside of the helix also occurs.

One consequence of intercalation would be to interfere with the template activity of D.N.A. due to a disruption of the genetic code when a drug molecule is sandwiched between base pairs with a bulky

sugar group which protrudes into the side of the major groove of the double helix.

Crystallisation of both drugs with a hexagonal lattice was sometimes observed to occur within the D.N.A. fibre when the P/D was less than 12 but this would probably not occur in vivo where the drug concentrations are necessarily low. It could in fact be an advantage for a drug to be stable in crystalline form with low solubility thus enabling an implanted antibiotic crystal to be effective in treating a localised region such as a malignant tumour.

Steffimycin and steffimycin B produce similar changes on D.N.A. conformation. However, steffimycin B is less soluble than steffimycin and this means that the effects on D.N.A. of this drug are limited due to the lower concentrations available. Steffimycin was seen to be more persistent in intercalating into D.N.A. whilst steffimycin B more easily leaves the intercalation site at lower relative humidities. The stabilisation of the intercalated form is sensitive to changes of drug concentration and humidity and the drugs are not as tightly bound as other known intercalators such as Daunomycin or Adriamycin. This can be explained by the charged amine groups of these two drugs which bind to the negative phosphate groups of the D.N.A. helix. The steffimycins possess no such charged groups and probably rely upon two or only one (in the case of steffimycin B) hydrogen bonds to maintain the drugs in the intercalation site.

The steffimycins provided an opportunity of examining the mode of action of two non charged anthracyclines. The slight difference between the drugs of having either OH or OCH_3 on the sugar C3' position was seen to effect the stabilisation of the intercalated

molecule. The amine group of daunomycin and adriamycin is also on this C3′ atom and leads to strong binding. Nogalamycin has an oxymethyl group at C3′ and again shows relatively weak binding properties. It is likely that this position on the sugar group of similar anthracyclines is very important in the effectiveness of such drugs in the inhibition of the D.N.A. template.

Thus the conformational adjustment of D.N.A. due to the binding of the steffimycins is relevant to the understanding of the characteristics and action of other related antibiotics and their mechanism of chemotherapeutic activity.

1. Watson, J.D. and Crick, F.H.C., (1953), Nature 171, 737.

2. Franklin, R.E. and Gosling, R.G., (1953), Nature 171, 740.

3. Wilkins, M.H.F., Stokes, A.R. and Wilson, H.R. (1953), Nature 171, 738.

4. Arnott, S. and Hukins, D.W.L., (1973), J. Mol. Biol. 81, 93-105.

5. Arnott, S. and Hukins, D.W.L., (1972), Biochem. J. 130, 453-465.

6. Arnott, S. and Hukins, D.W.L., (1972), Biochem. Biophys. Res. Commun. 47, 1504-9.

7. Marvin, D.A., Spencer, M., Wilkins, M.H.F. and Hamilton, L.D., (1961), J. Mol. Biol. 3, 547.

8. Bram, S., (1973), Proc. Nat. Acad. Sci. U.S.A. 70, 7, 2167-2170.

9. Wilkins, M.H.F., Aspects of Protein Structure. Acad. press, London Ltd., p23 ff.

10. Premilat, S. and Albiser, G., (1975), J. Mol. Biol. 99, 27-36.

11. Hamilton, L.D., Barclay, R.K., Wilkins, M.H.F., Brown, G.L., Wilson, H.R., Marvin, D.A., Ephrussi-Taylor, H., Simmons, J.S., (1959), J. of Biophysical and Biochemical Cytology, vol. 5, no. 3, 397-404.

12. Milman, G., Langridge, R. and Chamberlin, M.J., (1967), Proc. Nat. Acad. Sci. 57, 6, 1804-10.

13. Crick, F.H.C. and Klug, A., (1975), Nature, 255, June 12.

14. Casazza, A.M., Di Marco, A. and Di Cuonzo, G., (1971), Cancer Research, 31, 1971-1976.

15. Blum, R.H., Friedman, M.A. and Carter, S.K., (1972), Nat. Cancer Inst. Chemotherapy, Adriamycin Clinical Brochure.

16. Trouet, A., de Campeneere, D.D. and de Duve, C., (1972), Nature New Biology, 239, 91, 110-112.

17. Wilkins, M.H.F., Gosling, R.G. and Seeds, W.E., (1951), Nature, May 12, p759-60.

18. Seeds, W.E., Ph.D. Thesis, (1951), Univ. of London.

19. Wilson, H.R., unpublished work.

20. Wilkins, M.H.F., (1961), Journal O de Chemie Physique et de Physico Chemie Biologique, 58, 891-8.

21. see example in Arnott, S., (1973), Trans. of the Am. Cryst. Assn. *9*, *38*.

22. Albert, (1951), The Acridines, London.

23. Reusser, F., (1975), Biochimica et Biophysica Acta, 383, 266-273.

24. Peacocke, A.R. and Skerret, J.N.H., (1956), Trans. Farad. Soc. 52, p2ôl ff.

25. Calandi, E., Di Marco, A., Reggiani, M., Scarpinato, B. and Valentini, L. (1965), Biochim. Biophys. Acta. 103, 25-49.

26. Waring, M.J., (1965), J. Mol. Biol. 13, 269-282.

27. Waring, M.J., (1966), Biochim. Biophys. Acta, 114, 234-244.

28. Neville, D.M. Jr. and Davies, D.R. (1966), J. Mol. Biol. 17, 57.

29. Peacocke, A.R. (1969), Chemistry and Industry, 17 May.

30. Fuller, W. and Waring, M.J., (1964), Ber. Bunsen. Phys. Chem. 68, 805.

31. Waring, M.J., (1970), J. Mol. Biol. 54, 247-279.

32. Luzzati, V., Masson, F. and Lerman, L.S. (1961), J. Mol. Biol. 3, 634-9.

33. Zunino, F., (1971), FEBS Letters 18, 249.

34. Zunino, F., Gam betta, R.A., Di Marco, A. and Zaccara, A., (1972), Biochim. Biophys. Acta, 277, 489.

35. Ward, D.C., Reich, E. and Goldberg, I.H., (1965), Science, 149, 1259-1263.

36. Di Marco, A., Arcamone, F. and Zunino, F., (1974), Antibiotics, vol. III, Springer-Verlag, Berlin, Heidelberg, New York.

37. Lerman, L.S., (1963), Proc. Nat. Acad. Sci. U.S. 49, 94.

38. Nagata, C., Kodama, M., Tagashira, Y. and Iamamura, A., (1966), Biopolymers, 4, 409.

39. Li, H.J. and Crothers, D.M., (1969), J. Mol. Biol. 39, 461-477.

40. Pigram, W.J., Fuller, W. and Hamilton, L., (1972), Nature New Biol. 235, 17-19.

41. Pritchard, N.J., Blake, A. and Peacocke, A.R., (1966), Nature, 212, 1360-1361.

42. Lerman, L.S., (1961), J. Mol. Biol. 3, 18.

43. Drummond, D.S., Pritchard, N.J., Simpson-Gildemeister, V.F.W. and Peacocke, A.R., (1966), Biopolymers, 4, 971.

44. Mauss, Y., Chambron, J., Daune, M. and Benoit, H., (1967), J. Mol. Biol., 27, 579.

45. Pigram, W.J., Ph.D. Thesis, (1968), Univ. of London.

46. Pigram, W.J., Fuller, W. and Davies, M.E., (1973), J. Mol. Biol. 80, 361-365.

47. Crothers, D.M., (1968), Biopolymers, 6, 575-584.

48. Bergy, M.E. and Reusser, F., (1967), Seperatum Experientia, 23, 254.

49. Brodasky, T.F. and Reusser, F., (1974), J. of Antibiotics, Nov.

50. Reusser, F., (1967), J. Bacteriol., 65-70.

51. Jain, S.C. and Sobell, H.M., (1971), Nature New Biol., 231, June 16.

52. Peacocke, A.R. and Walker, I.O., (1962), J. Mol. Biol., 5, 550.

53. Rusconi, A., (1966), Biochem. Biophys. Acta, 123, 627.

54. Gellert, M., Smith, C.E., Neville, D. and Felsenfield, G. (1965), J. Mol. Biol. 11, 445.

55. Fuller, W., Hutchinson, F., Spencer, M. and Wilkins, M.H.F., (1967), J. Mol. Biol., 27, 507.

56. Chesley, G.F., (1947), Rev. Scient. Instrum., 18, 422.

57. Koltun, W.L., (1965), Biopolymers, 3, 665.

58. Goodwin, D.C., (1976), Ph.D. Thesis, Univ. of Keele.

59. Fuller, W., (1961), Ph.D. Thesis, Univ. of London.

60. Langridge, R., Wilson, H.R., Hooper, C.W., Wilkins, M.H.F. and Hamilton, L.D., (1960a), J. Mol. Biol. 2, 19.

61. Kirby, K.S., (1957), J. of Biochem., 66, 495.

62. Marmur, J., (1961), J. Mol. Biol. 3, 208.

63. Eigner, J., Methods in Enzymology, Colowick and Kaplan, Vol. XII, Nucleic Acids, Part B, Ed. Grossman and Moldare.

64. Klotz, I.M. Role of Water Structure in Macromolecules, Cryobiology, 5-24.

65. Lewin, S., (1967), J. Theoret. Biol. 17, 181-212.

66. Falk, M., Hartman, K.A., and Lord, R.C., (1963), J. Amer. Chem. Soc. 85, 391.

67. Falk, M., Hartman Jr., K.A. and Lord, R.C., (1963), J. Amer. Chem. Soc. 85, 387.

68. Bloomfield, V.A., Crothers, D.M., Tinoco, Jr. I, (1974), Physical Chemistry of Nucleic Acids. Harper and Row.

69. Langridge, R., Seeds, W.E., Wilson, H.R., Hooper, C.W., Wilkins, M.H.F., and Hamilton, L.D., (1957). J. of Biophys. Biochem. Cytology, 3, 5, 767-778.

70. Cooper, P.J., and Hamilton, L.D., (1966), J. Mol. Biol. 16, 562.

71. Bram, S., and Tougard, P., (1972), Nature New Biol., 239, 094.

72. Bram, S., and Baudy, P. , (1974), Nature, 250, 414-6.

73. Thomas, R., (1953), Bul Societe der Chemie & Biol. 35, 609.

74. Ruppreckt, A., and Forslind, B., (1970), Biochimica and Biophysica Acta. 204, 304-316.

75. Waring, M.J., Wakelin, L.P.G., and Lee, J.S., (1975), Biochimica et Biophysica Acta, 407, 200-212.

76. Schildkraut, C.L., Marmur, J., Doty, P., (1962), J. Mol. Biol. 4, 430.

77. Belozersky, A.N., and Spirin, A.S., (1960). In "The Nucleic Acids", ed by E. Chargaff & J.N. Davidson, Vol. 3, p147, New York: Academic Press.

78. Kersten, W., (1971), Prog. in mol. and subcellular biol., Springer, 2, 48.

79. Bhuyan, B.K. and Smith, C.G., (1965), Proc. Nat. Acad. Sci., Wash., 54, 566.

80. Honikel , (1968), Ph.D. Thesis, Univ. of Würtzberg.

81. Kersten, W., Kersten, H., and Szybalski, E., (1966), Biochemistry, 5, 236.

82. Müller, W., and Crothers, D.M., (1968), J. Mol. Biol., 35, 251-299.

83. Wells, R.D., Larson, J.E., (1970), J. Mol. Biol., 49, 319.

84. Davies, M., (1973), Private communication.

85. Porumb, T., and Slade, E.F., (1976), Eur. J. Biochem., 65, 21.

86. Paoletti, J. and Le Pecq, J.B., (1971), J. Mol. Biol., 59, 43.

87. Alden, C.J., and Arnott, S., (1975), Nucleic Aid Res. 2, 10, 1701.

88. Le Pecq, J.B., Yot, P., and Paoletti, C., (1964), Compt. Rend. 259, 1786.

89. Angiuli, R., Foresti, E., Riva di Sanseverino, L., Isaacs, N.W., Kennard, O., Motherwell, W.D.S., Wampler, D.L., and Arcamone, F., (1971), Nature New Biol., 234, 78-80.

90. DeVoe, H., and Tinoco, J., (1962), J. Mol. Biol., 4, 500.

91. Gersch, N.F., and Jordan, D.O., (1965), J. Mol. Biol., 13, 138.

92. Polter, V.I., and Sukhorukov, B.J., (1968), Biofizika, 13, 941.

93. Reich, E., (1963), Cancer Research, 23, 1428.

94. Hamilton, L., Fuller, W., and Reich, E., (1963), Nature, 198, 538.

95. Cerami, A., Reich, E., Ward, D.C, and Goldberg, I.H., (1967), Biochemistry, 57, 1036.

96. Sobell, H., Jain, S.C., Sakore, T., and Nordman, C.E., (1971), Nature, 231, 200.

97. Jain, S.C., and Sobell, H., (1972), J. Mol. Biol., 68, 1.

98. Sobell, H., and Jain, S.C., (1972), J. Mol. Biol., 68, 21.

99. Wells, R.D., (1969), Science, 165, 75.

100. Porumb, H., (1976), Univ. of Keele, Ph.D. Thesis.

101. Tsvetkov, V.N., (1963), Polymer Sci, U.S.S.R., 4, 1456.

102. Fuller, W., (1973), private communication.

103. Klug, A., Crick, F.H.C., and Wyckoff, H.W. (1958), Acta. Cryst., 11, 199-213.

104. Nave, C., (1976), Report in course of preparation.