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INTERACTION OF Cu^{++} IONS WITH DNA, ITS CONSTITUENTS AND RELATED COMPOUNDS

by

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ABBREVIATIONS

Abbreviations of the bases, mononucleosides, mononucleotides and polynucleotides are tabulated at the end of the following abbreviations which have been used in this thesis:

ApAp! and $(Ap)_5Ap!$	Cyclic dimer and hexamer oligoribonucleotides of adenine.
CD	Circular dichroism
DNA	Deoxyribonucleic acid
(Denatured DNA)-Cu^{++}	The complex formed by adding Cu^{++} ions to denatured DNA.
Denatured (DNA-Cu^{++})	The complex formed by denaturing DNA in the presence of Cu^{++} ions.
ESR	Electron spin resonance
(G + C)	The total guanine and cytosine residue content of DNA; usually expressed as a percentage of the total number of base residues.
G - C	The guanine and cytosine base pairs in DNA.
I	Ionic strength
IR	Infra-red
Me-	Methyl
M_P	Molar concentration of phosphate or base residues.
Native DNA-Cu^{++}	The complex formed by adding Cu^{++} ions to native DNA.
NMR	Nuclear magnetic resonance
ORD	Optical rotatory dispersion
Poly (A + U)	Double strand complex containing one strand of poly A and one strand of poly U.

Poly dAT	Copolymer containing adenine and thymine deoxyribomononucleotides in an alternating sequence.
Poly dG:dC	Double strand complex containing one strand of poly dG and one strand of poly dC.
poly (I + C)	Double strand complex containing one strand of poly I and one strand of poly C.
PTFE	Polytetrafluoroethylene
r	Moles of Cu^{++} bound to DNA and related compounds per residue.
R	Total number of moles of Cu^{++} present per residue of DNA or related compounds.
RNA	Ribonucleic acid
UV	Ultra-violet

NOMENCLATURE AND ABBREVIATIONS[1] OF THE BASE RESIDUES

Base		Nucleoside		Nucleotide[2]	
Adenine	A'	Adenosine	A	Adenosine-5'-phosphate	AMP(5')
		Deoxyadenosine	dA	Deoxyadenosine-5'-phosphate	dAMP(5')
Cytosine	C'	Cytidine	C	Cytidine-5'-phosphate	CMP(5')
		Deoxycytidine	dC	Deoxycytidine-5'-phosphate	dCMP(5')
Guanine	G'	Guanosine	G	Guanosine-5'-phosphate	GMP(5')
		Deoxyguanosine	dG	Deoxyguanosine-5'-phosphate	dGMP(5')
Hypoxanthine	Hy	Inosine	I	Inosine-5'-phosphate	IMP(5')
Thymine	T'	Ribosylthymidine	rT	Thymidine-5'-phosphate	rTMP(5')
		(deoxy)Thymidine[3]	T	(deoxy)Thymidine-5'-phosphate[3]	TMP(5')
Theophylline	Th	-		-	-
Uracil	U'	Uridine	U	Uridine-5'-phosphate	UMP(5')
Xanthine	X'	Xanthosine	X	Xanthosine-5'-phosphate	XMP(5')

1. These abbreviations also apply to the polynucleotides poly A, poly G, poly I, poly C, poly U, poly T and poly rT, e.g. poly A represents polyadenylic acid (5').
2. The 2', 3' and 5' nucleoside phosphates are commercially available. Unless otherwise specified, the 5' isomer may be assumed.
3. The deoxy- term is generally omitted.

SUMMARY

Three distinct types of DNA-Cu^{++} complexes have previously been distinguished as native DNA-Cu^{++}, single strand DNA-Cu^{++} and DNA denatured in the presence of Cu^{++} ions, and have been studied independently in the work presented in this thesis. The native DNA-Cu^{++} interaction was investigated extensively by determining the binding parameters using the techniques of gel exclusion chromatography and Cu^{++} ion potentiometry, and by following the accompanying conformational changes (by viscometry and ultracentrifugation) and spectrophotometric changes. The dependence of the binding parameters of the native DNA-Cu^{++} interaction with ionic strength was confirmed, and a dependence of two types of interactions with the (G + C) content of DNA was established. The binding parameters for the other two types of DNA-Cu^{++} complexes were also determined, those for the single strand DNA-Cu^{++} interaction being the same as for the native DNA-Cu^{++} interaction.

All of the binding parameters were determined from Scatchard plots by means of an objective geometrical analysis. The presence of more than one type of site was indicated by curvature of an electrostatically corrected Scatchard plot, from which an apparent intrinsic constant, K_o, was obtained and compared to K_c determined by an independent procedure, thereby confirming the accuracy of the electrostatic correction function.

The interpretation of the nature of the individual complexes involved in the various DNA-Cu^{++} complexes was clarified from hyperchromicity and binding studies of more simple structures resembling DNA (the homo-polynucleotides, poly G, poly I, poly C and poly A) and from determinations of the stability constants for the oligo- and mono-nucleotide constituents of DNA and similar compounds.

This thesis contains no material previously submitted for a degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

D.R. Phillips

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CHAPTER I

INTRODUCTION

References

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The role of DNA in the physiology and biochemistry of cells has received great attention during the last thirty years, and it is now well established that DNA stores the genetic information of living cells.[1] The mechanism of the transfer of this information is still the subject of intensive study, although the basis of the translation and transcription processes has been clarified.[1,2] Because of the importance of the biological role of DNA and the many related questions still unanswered, the initial impetus for fundamental research on DNA has not diminished.

The physico-chemical properties of DNA have been extensively documented,[3,4] and confirm the Watson-Crick double helical structure (as determined from X-ray diffraction studies of DNA fibres) with slight modifications.[5] Many studies have indicated that this structure is maintained in solution,[6] and the "B" form[7] is now commonly used for molecular models[8] of DNA when considering the structure and reactions of DNA in solution.

A lower limit exists to the concentration of added electrolyte required to maintain DNA in the ordered double helical form[9,10] (native DNA). If the DNA concentration is sufficiently high, its secondary structure can be maintained even in the absence of added electrolyte.[11,12] These effects indicate that the repulsion between adjacent charged phosphate groups (pK_{a1}), is diminished by the presence of shielding cations, and allow the helix stabilising forces[13,16-19] to dominate.

From a knowledge of DNA nucleotide composition the ultra-violet absorption of various DNA's has been predicted assuming that the monomer constituents of DNA do not interact in any way to affect their optical properties. The predicted values are typically 30% smaller than those observed at the DNA absorbance maxima near 260 nm. The decrease of the predicted absorption, termed hypochromicity, has been attributed[13,16-20] primarily to base stacking forces and partially to hydrogen bonding between base pairs, both of which tend to orientate the base residues. Any effect which perturbs the orientation of the base residues causes an increase of DNA absorbance and is known as hyperchromicity.

Collapse of the hydrogen bonded structure of DNA is accompanied by a large hyperchromic effect although a residual hypochromicity of the order of 10% remains even when all of the hydrogen bonds have been severed. This hypochromicity is due to base stacking of adjacent residues,[16-19] and increases with chain length (to a limiting value at a degree of polymerisation greater than 10) for single strand polynucleotides.[21,22]

Hyperchromicity is readily induced in DNA by raising the temperature, the change occurring over a narrow temperature range. The point at which the DNA secondary structure is lost (indicated by a rapid increase in hyperchromicity) is known as the melting temperature (T_m), and is defined as the temperature at which the hyperchromicity is half that of the total absorbance increase. At T_m, thermal energy is just

sufficient to overcome the hydrogen bonding and base stacking forces. Consequently, the T_m is a measure of the stability of the DNA secondary structure.

DNA exhibiting maximum hyperchromicity (about 40% at the absorbance maxima for DNA with 50% (G + C) content), is termed denatured DNA. However, it is important to distinguish between two types of denatured DNA. DNA heated at $(T_m + 10)^{\circ}C$ for ten minutes, results in maximum hyperchromicity, and is accompanied by complete strand separation.[23] Such DNA is referred to in all subsequent discussions as single strand denatured DNA, and is generally characterised by the lack of a normal T_m curve. However, by heating DNA at T_m for short periods of time (less than ten minutes), hydrogen bonding may be disrupted, causing large hyperchromic changes without causing strand separation.[24] This is referred to simply as denatured DNA.

Metal ion interaction with nucleic acids has been indicated by the detection of metal ions (Ca^{++}, Mg^{++}, Ni^{++}, Cu^{++}, Zn^{++}, Fe^{+++}, Mn^{++}) in DNA and RNA isolated from biological sources.[25-33] In all such studies precautions were taken to prevent metal ion contamination of the nucleic acids during the isolation procedure. However, an ESR study has indicated that the presence of Fe^{+++} ions in DNA may be due to contamination.[34] These and other studies indicate the probable biological role of metal ions in biological systems. A summary of

the main conclusions follows.

a. The biological activity of nucleic acids depends largely on the trace element content.[28,35,36]

b. DNA containing metal ions possesses a large degree of resistance to cell radiation.[32]

c. The enzymatic synthesis of DNA requires the presence of Mg^{++}.[37]

d. The ability of nucleic acids to be infected is influenced by traces of metal ions.[28,36]

e. Particular ions are required for the growth and multiplication of organisms.[38,39]

f. Experiments by Altman[40,41] indicate that particular metal ions are incorporated into DNA during its synthesis.

g. The biological role between the nucleic acids and proteins is affected by metal ions.[42]

The many studies mentioned above indicated a link between metal ions and nucleic acids, and prompted further specific studies on the effect of metal ions on DNA. The native DNA stability, as measured by T_m,

was found to depend on the logarithm of the ionic strength,[43-46] while divalent cations were found to be significantly more effective than monovalent ions in their ability to stabilise the native DNA structure.[9]

Systematic studies of the effect of divalent cations on DNA first indicated that three groups could be distinguished. They were classified with respect to their effect on the stability, the renaturability of DNA on cooling[47] and on increasing the ionic strength.[48] One group consists of the ions Mg^{++}, Ca^{++}, Ba^{++}, Mn^{++}, Co^{++}, Ni^{++} and Zn^{++} which stabilise the DNA secondary structure. The effect has been attributed to screening of the phosphate charges.[47,48] The hypochromicity of DNA denatured in the presence of these metal ions is partially reduced on cooling. A second group contains Cd^{++}, Pb^{++} and Cu^{++} ions which destabilise DNA, most markedly in the case of Cu^{++} ions. It has been suggested that this is due to interaction of the ions with the DNA base residues, causing a weakening of the hydrogen bonds.[47,48] As Cu^{++} ions cause the largest decrease of DNA stability, it has been the most extensively studied. Renaturation of DNA denatured in the presence of Cu^{++} ions does not occur on cooling unless the ionic strength of the solution is substantially increased, suggesting that the interaction of Cu^{++} ions with the base residues is capable of holding the two strands together in some way. The third group contains other ions such as Hg^{++}, Ag^{+}, Fe^{+++} and Fe^{++} which

cause substantial changes to the properties of DNA. Hg^{++} and Ag^{+} interact with the bases of native DNA and cause large spectral shifts.[49-52] The thermal denaturation of DNA in the presence of Fe^{++} ions is anomalous as the hyperchromicity increases on cooling,[47] in contrast with the effect of all other ions.

A unifying explanation of these facts has been presented by Eichhorn and Shin.[53] They suggest that the properties of all the metal ion-DNA complexes result from the relative ability of the metal ions to interact with the phosphate groups, compared with their ability to interact with the base residues. This proposal has been supported by thermal stability and spectrophotometric studies for a range of cations.[53]

The unique properties of the DNA-Cu^{++} complex is the main reason for the many studies on the effect of Cu^{++} ions on the primary, secondary and tertiary structure of DNA. Interest in these studies has also stemmed from the increasing number of indications that Cu^{++} ions play some part in the biological role of DNA. An outline of this evidence is presented below.

Many studies have indicated that Cu^{++} ions are present in DNA obtained from biological sources. Tracer studies by Altman[40] have shown that this is not due to contamination during the recovery process of DNA. Nucleoproteins were incubated in an excess of labelled Cu^{++} ions, and on isolation of the DNA, all of the labelled Cu^{++} ions were detected in the protein fraction.[40] This indicates that contamination could not

account for the Cu^{++} ions detected in DNA, and that their presence is controlled by biological processes. Other studies have shown that the presence of Cu^{++} ions renders the DNA less easily attacked by nucleases[54,55] and also decreases the effects of radiation damage to the DNA.[32,56,57] Furthermore, two independent studies[58,59] have confirmed that DNA synthesised in the presence of Cu^{++} ions produces an increase of (G + C) content of up to 23%. That the formation of such mutants is accompanied by an increase of the (G + C) content of DNA has also been detected by Bollum,[60] who found that the re-duplication of ultra-violet irradiated DNA was accompanied by an increase of guanine residues.

The above evidence for the interaction of Cu^{++} ions with DNA has led some workers to propose a scheme to explain not only the physico-chemical facts, but also the biological studies. A redox role has been suggested for Cu^{++} ions acting as a switching mechanism for strand duplication.[61,62] The presence of Cu^{+} ions in DNA stabilises the helix,[62,63] but when oxidised to Cu^{++} ions de-stabilises the helix by weakening the hydrogen bonds, thus converting the DNA into a state in which it can be duplicated if in the right conditions.[64] After duplication, the cyclic process is completed by reduction of Cu^{++} ions to Cu^{+} ions, thereby re-stabilising the helix.

The present study of the DNA-Cu^{++} interactions has been undertaken primarily for three reasons. The interaction itself is a fundamental area of study in its own right. Secondly, any knowledge

of the interaction furthers the understanding of the nature and properties of DNA. Finally, any physico-chemical studies of the interaction may assist in clarifying its biological significance.

Interaction studies of this nature require answers to the questions posed by Scatchard:[65] "How many? How tightly? Where? Why? What of it?" As re-stated by others,[66] in order to have a complete understanding of the interaction, we must determine the number of sites available, the stability constants for the various sites or groups of sites, the nature and position of the sites, and the significance of the interaction. Attempts have been made in the present study to answer the first four of these questions. Biological aspects of the DNA-Cu^{++} interactions will also be discussed briefly.

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CHAPTER II

STUDIES OF THE INTERACTION OF Cu^{++} AND OTHER IONS WITH DNA AND RELATED COMPOUNDS

1. INTRODUCTION

DNA-Cu^{++} complexes may be formed by three methods, each producing a compound with characteristic properties. The methods of preparation are:

a. Cu^{++} ions added to native DNA at room temperatures (i.e. $T << T_m$).

b. DNA denatured in the presence of Cu^{++} ions (i.e. $T > T_m$).

c. Cu^{++} ions added to single strand DNA.

As the properties of the three types of DNA-Cu^{++} complex vary with method of formation, it is probable that some of the sites of interaction may also differ. For this reason, these three types of compounds have been discussed separately in this thesis.

The interaction of Cu^{++} and other ions with DNA involves essentially two types of reactive sites on the DNA macromolecule: the external charged phosphate groups, and the internal basic nitrogen sites of the base residues. It is necessary to clarify here that the basicity of the nitrogen sites, and not the charge, is important in determining the metal ion interacting sites (see Sect. II.3).

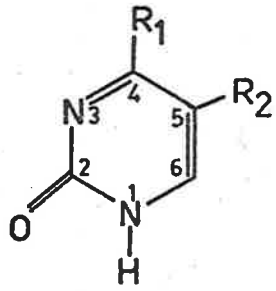
Many studies with Cu^{++} ion have attempted to define the relative importance of the base and phosphate sites of DNA, and the nature of the interaction. Evidence has been presented to indicate that the interaction of Cu^{++} ions with native DNA occurs with the phosphate groups,[1-20] or with the base residues.[4,5,10,12,14,16,17-28] Some studies indicate that both sites are involved.[4,5,10,12,14,16,17-20] A wide range of techniques has been used in these studies: spectral

techniques (UV and visible absorbance, IR, NMR, ESR, ORD, CD) conformation studies (viscosity, light scattering, density gradient and ultra-centrifugation), direct binding studies (gel chromatography, equilibrium dialysis, polarography, conductance, labelled compounds), pH titration and DNA melting temperature.

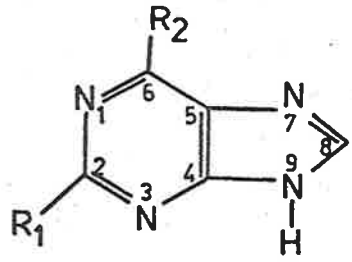
Data for the interaction of Cu^{++} ions with mono-nucleotides is discussed independent of the DNA-Cu^{++} interaction, as such interactions can only indicate the possibility of a similar interaction with DNA. This is due to the completely different environment (electronically and stereochemically) in which the nucleotides occur in DNA, as compared with the mono-nucleotides themselves.

The synthetic polynucleotides, poly dG:dC and poly dAT, are included in discussions of DNA, as they possess double helical structures and are therefore directly comparable with native DNA. However, the polyribonucleotides often occur as single strand structures,[29,30] and are therefore comparable with single strand denatured DNA. As detailed reference is made throughout this thesis to a range of bases, base residues, nucleosides and nucleotides, the structure and numbering system of these compounds is presented here. The numbering system used is that adopted by "Chemical Abstracts" and most current biochemical journals, and is indicated in Fig. II-1. However, it differs from that used in some earlier texts on pyrimidines.[31]

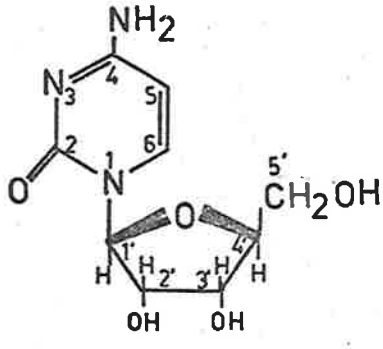
The structures of the base residues referred to in this thesis



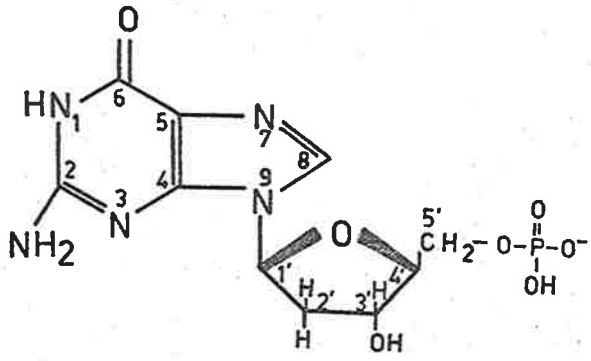
A Pyrimidine



A Purine



Ribo - nucleoside
(Cytidine)



2'deoxy-ribo-mono-5'-nucleotide
(d GMP)

Fig. II-1. The numbering system used.

The names "deoxy-ribonucleoside" and "deoxy-ribonucleotide" are frequently abbreviated to "deoxy-nucleoside" and "deoxy-nucleotide".

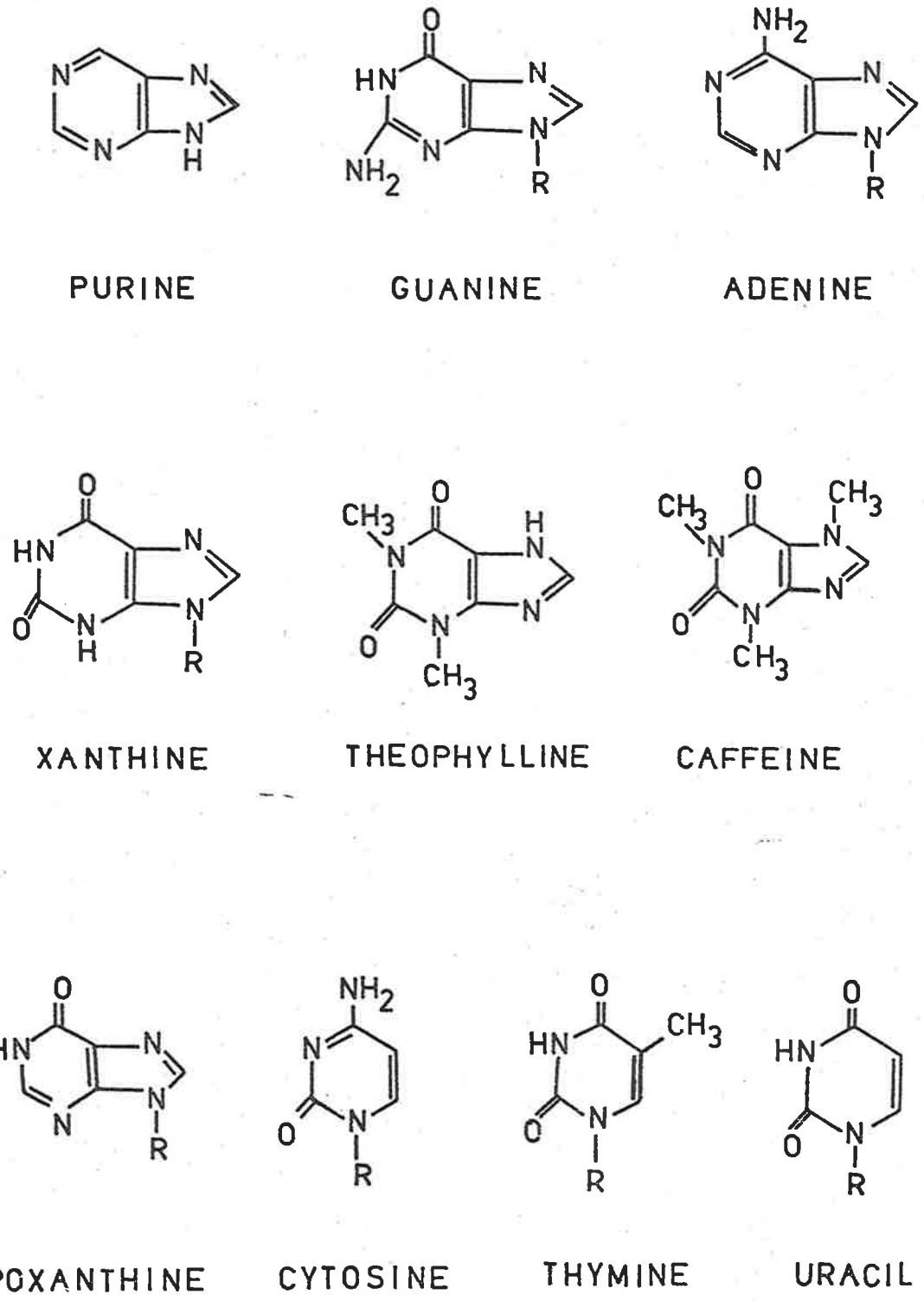


Fig. II-2. Structure of the base residues at pH ≃ 7.

R represents H, ribose, deoxyribose, ribose-phosphate or deoxyribose-phosphate.

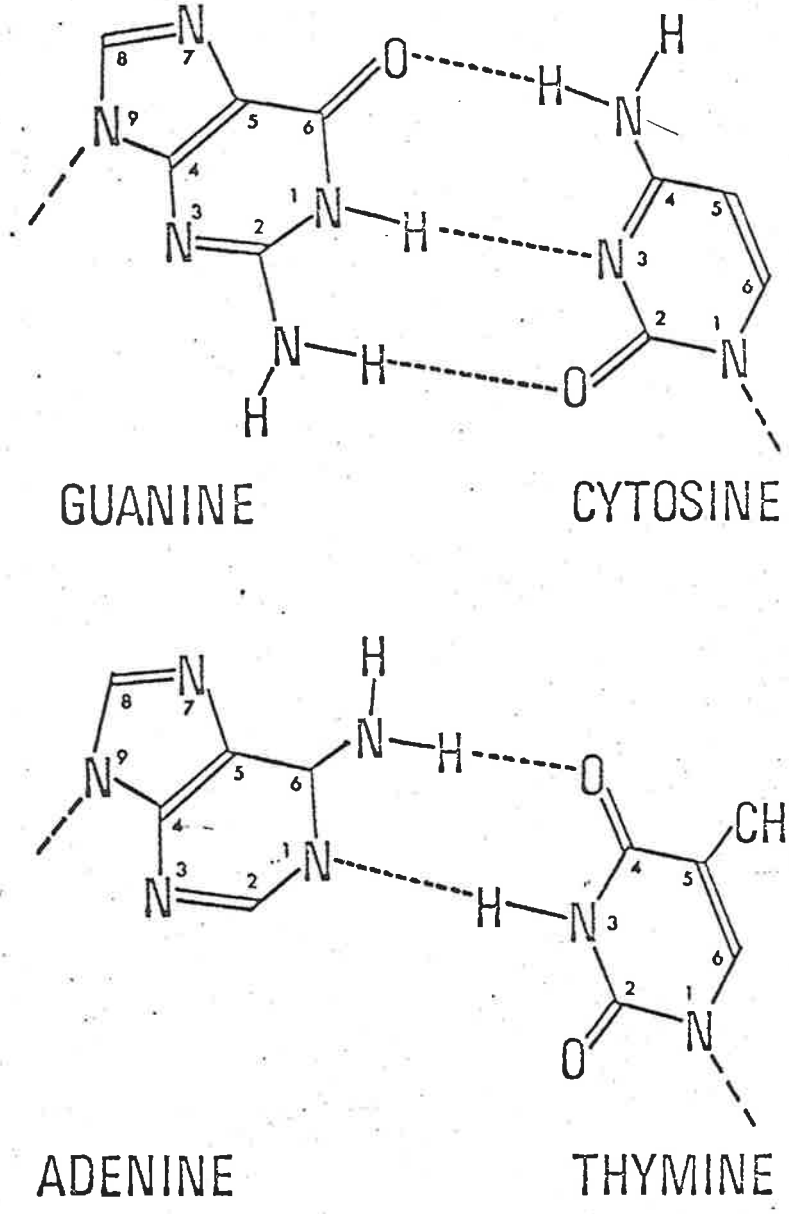


Fig. II-3. The hydrogen bonding sites of the base pairs in DNA.

are indicated in Fig. II-2. The hydrogen bonding sites of the base residues in DNA are referred to frequently and are shown in Fig. II-3.

2. PROPERTIES OF THE DNA-Cu^{++} COMPLEXES

a. The Native DNA-Cu^{++} Complex

(1) Ultra-violet and Visible Spectrophotometry

Interaction of Cu^{++} ions with the base residues of DNA is expected to perturb the energy levels of the base residues, producing changes in the ultra-violet spectrum of DNA. The extent of the wavelength shift of the absorbance maxima, as well as hypo- or hyperchromic changes, may be used as a measure of the degree of interaction. The lack of any ultra-violet spectral changes of native DNA in the presence of Cu^{++} ions has been interpreted as indicating that Cu^{++} ions are not bound to the base residues of native DNA.[7-9,13] However, other more recent studies have shown that a spectral shift to longer wavelengths does occur.[12,14,16,19,23,32,33] Based solely on ultra-violet difference spectral measurements, Yatsimirskii and Kriss,[12] and Bryan and Frieden,[16] have determined association constants for the native DNA-Cu^{++} complexes (at low ionic strengths, $I \leq 0.01M$), which agree well with values determined by techniques measuring the total Cu^{++} ions bound to native DNA.[15-17] This indicates that essentially all of the Cu^{++} ions bound to DNA perturb the energy levels of the base residues. However, these spectral changes may be due to direct Cu^{++} ion interaction with the base residues, or to re-orientation of the

base residues due to Cu^{++} ion interaction with the phosphate groups, or both. At high ionic strength ($I \geq 0.1M$) where the electrostatic effect of the phosphate groups is diminished, the ultra-violet difference spectra studies of Schrieber and Daune[19] indicate that Cu^{++} ion interactions with the base residues account for all of the spectral change at high ionic strength, and may account for at least some of the change at low ionic strength.

Zimmer and Venner[27] have reported hyperchromicity studies (at low ionic strength), with DNA containing a range of (G + C) content. They concluded that the interaction of Cu^{++} ions with native DNA occurs mainly with the guanine and cytosine residues, and is probably more specific for the guanine residues.

Bryan and Frieden[16] observed that the maximum spectral changes induced by Cu^{++} ions in native DNA were the same as those from DNA denatured in the presence of Cu^{++} ions, although different reaction times and Cu^{++} ion concentrations were required. Other studies[12,14,27,33] do not support such a large hyperchromicity for the native DNA-Cu^{++} interaction, and suggest that the DNA used by Bryan and Frieden[16] may be partially denatured.

The fact that earlier workers did not observe spectral changes of native DNA in the presence of Cu^{++} ions, is undoubtedly due to three factors. An isosbestic point has been determined at 260 nm,[19] the native DNA absorbance maxima and the wavelength used for some

earlier hyperchromicity studies. Secondly, the more sensitive technique of difference spectroscopy was not used in earlier studies. Finally, high Cu^{++} ion and DNA concentrations are required to produce small hyperchromic changes.[19] However, under these conditions, DNA-Cu^{++} aggregates are formed,[7,16,19] and must be eliminated, or allowed for, to avoid light scattering errors in absorbance studies.[19]

Visible absorbance studies of Cu^{++} ions in the presence of native DNA are contradictory. Coates et al.[8] deduced that there was no effective interaction between Cu^{++} ions and native DNA, although a small spectral shift was evident. Zimmer and Venner[27] detected a spectral shift from 818 nm to 775 nm for Cu^{++} ions in the presence of 72% (G + C) content native DNA. This shift to lower wavelengths has previously been detected for Cu^{++} ions bound to nitrogen ligands,[34] and indicates Cu^{++} ion interaction with the base residues of native DNA. The difference between the two studies has been attributed[27] to the different (G + C) content of the DNA used, implicating the guanine and cytosine residues as sites for Cu^{++} ion interaction in native DNA.

(ii) Thermal Stability

Since Eichhorn[35] first recognised that the presence of Cu^{++} ions lowers the melting point of DNA, numerous other similar studies have been reported,[6-9,11,18,19,23,25,36,37] all confirming the destabilisation of DNA in the presence of Cu^{++} ions. A small, initial increase of

T_m,[6,11] or a constant T_m [7,9,37] at low Cu^{++} ion to base residue concentrations (low R values), has been interpreted[35] as stabilisation of the helix due to Cu^{++} ion interaction with the phosphate groups, thereby screening the phosphate charges and reducing the repulsion forces between these groups. At higher Cu^{++} ion concentrations, interaction with the base residues is assumed to predominate,[35] destabilising the helix. This interpretation has received added support from Eichhorn and Shin,[18] who established that the interaction of metal ions with the phosphate and base groups of DNA is a relative effect, and explains the balancing of destabilising and stabilising effects of the ionic and covalent interactions of Cu^{++} ions. The stabilisation of DNA at low total Cu^{++} ion to base residue ratios (R) at low ionic strength does not occur at high ionic strengths,[28] supporting the proposed electrostatic phosphate-Cu^{++} ion interaction.

Ivanov and Minchenkova[37] have offered an alternative explanation for the initial stabilisation. They have shown that the T_m of DNA increases in the presence of Cu^{+} ions, and suggest that oxidising contaminants in DNA may reduce some Cu^{++} ions to Cu^{+}, thus stabilising the DNA. However, this suggestion has not been supported by any other experimental studies.

The alternating copolymer polydAT is stabilised in the presence of Cu^{++} ions,[7,11] whereas poly(A + U) is destabilised by Cu^{++} ions,[38] suggesting that the primary structure of polynucleotides and DNA may be vitally important in determining the effect of these Cu^{++} ion

interactions.

(iii) Tertiary Structure

Many studies have indicated that native DNA in solution can be adequately described as possessing a flexible, rod-like structure.[39,40] The presence of Cu^{++} ions causes the tertiary structure of native DNA to collapse slightly. This has been indicated by a decrease of viscosity,[1,16,19] an increase of the sedimentation coefficient,[16,19,28] and by light scattering studies,[19] at both high[1,19] and low[16,28] ionic strengths. The light scattering study[19] clearly indicates a decrease of the radius of gyration of native DNA, from 3100 Å to 2960 Å.

These conformational changes have been demonstrated at high ionic strengths,[1,19] where electrostatic Cu^{++}-phosphate interactions are suppressed, indicating that the changes are due to Cu^{++} ion interaction with the base residues. This has been confirmed by difference spectra studies at high ionic strengths,[19] and has led to the conclusion[19] that the conformational changes are caused by local flexibility of the DNA chains induced by Cu^{++} ion interaction with the base residues. Aggregation at high Cu^{++} ion to base residue ratios has been attributed[1,1] to interstrand bridging by Cu^{++} ions between phosphate groups of different DNA molecules.

From hydrodynamic studies, Bryan and Frieden[16] have concluded that two sites of Cu^{++} ion interaction exist in native DNA, as viscosity and sedimentation changes occur at different R ratios.

(iv) Other Properties

Cheng[22] has shown that the ultra-violet rotary dispersion is much more sensitive to the interaction of Cu^{++} ions with native DNA than ultra-violet absorbance studies. As the ORD spectrum of DNA has been explained in terms of delocalisation of the excited states of the base residues,[41] it appears that the technique of ultra-violet ORD is extremely sensitive to the interaction of Cu^{++} ions with the base residues of native DNA. This has been confirmed by Zimmer et al.[28] at extremely low Cu^{++} ion concentrations (R = 0.02), and contrasts with the inability of Mg^{++} ions, which interact essentially with the phosphate sites,[11,18,35,42-44] to alter the ORD spectrum of native DNA at much higher concentrations (R = 0.2). The maximum change of the native DNA-Cu^{++} ORD spectrum occurs at R = 0.2. Under the same conditions (I = 0.006M) maximum thermal stabilisation at R ~ 0.2 has been established,[11] indicating that Cu^{++} ion interaction with the phosphate groups (Sect. II.2a(iii)) and possibly also with the base residues, may be involved in the stabilisation of the helix at low R ratios.[6,11]

Circular dichroic studies with a range of (G + C) content DNA indicate that Cu^{++} ions interact with the G - C base pairs of native DNA.[28]

Infra-red spectral changes[24,28,45] infer a Cu^{++} ion interaction with the base residues of native DNA, and establish that the guanine and cytosine residues are predominantly involved.

Other properties of either Cu^{++} ions or native DNA, which have

been found to change after the formation of a native DNA-Cu^{++} complex, include NMR studies of the shortening of relaxation times of water protons bound to Cu^{++} ions,[2,3] ESR studies of the Landé splitting factor,[4,5,10,20] pH titrations[13] and conductance studies.[1] These studies are examined in detail in Section II.3e where they are more pertinent to discussions of the site of Cu^{++} ion interaction with native DNA.

b. DNA Denatured in the Presence of Cu^{++} Ions

Many studies[6-9,11,18,35,37] have indicated that there is no decrease of hyperchromicity on cooling DNA which has been denatured in the presence of Cu^{++} ions. Ivanov and Minchenkova[37] found that this occurs only if $R \geq 1.3$, the condition fortuitously employed in earlier studies. By comparison with the effect of formaldehyde on DNA,[46] cleavage of the hydrogen bonds has been shown to accompany this temperature independent hyperchromicity.[7] However, complete reversal of the hyperchromicity occurs if the ionic strength of the solution is raised to greater than 0.1M by the addition of 1:1 electrolytes. Using a wide variety of techniques (viscosity,[7] sedimentation coefficient,[6] density gradient,[6] optical rotation,[6] ORD,[28] UV absorbance,[6,7,9] transforming activity,[6] chromatographic pattern[6] and melting temperature[6]), it has been demonstrated that the ionic strength induced renaturation process forms DNA possessing all of the properties of native DNA. Hiai[6] has indicated that the removal of Cu^{++} ions (by

dialysis or by complexing with EDTA) is the fundamental step for the renaturation process.

It has been shown that DNA denatured in the presence of Cu^{++} ions, is not completely reversed by raising the ionic strength of the solution if the denaturing temperature was substantially greater than T_m,[7,28] suggesting that some regions of strand separation occur. If the Cu^{++} ion concentration is sufficiently low, complete strand separation may occur at high temperatures.[28]

The UV absorbance maxima of DNA in the presence of Cu^{++} ions shifts to longer wavelengths on raising the solution temperature,[8,13] and is accompanied by up to 55% hyperchromicity. These effects have been attributed to perturbation of the allowed energy levels of the base residues by interaction with Cu^{++} ions.[8,13] This interpretation has been confirmed by visible absorbance studies in which the Cu^{++} ion absorbance maxima shifts from 800 nm to 740 nm after heating the DNA-Cu^{++} system.[8,32] Such a shift is identical to that observed for the formation of $[Cu(NH_3)(H_2O)_5]^{++}$, which has an absorbance maxima at 740 nm, and is similar to other nitrogen ligand-Cu^{++} ion complexes.[34]

The enhanced hyperchromicity of the denatured DNA-Cu^{++} system has been attributed[11] primarily to the disorientation of the stacked base pairs (as observed for denatured DNA alone)[47] and partly to perturbation of the electronic structure of the base residues. This has been supported by small hyperchromic changes observed by Zimmer and Venner[27] for the interaction of Cu^{++} ions with deoxymononucleotides. Another

explanation for this enhanced hyperchromicity has been proposed by Sutherland and Sutherland.[26] They suggest that the extra hyperchromicity (10-15%) relative to denatured DNA indicates that Cu^{++} ions hold the base residues further apart and allow less stacking of bases than in DNA denatured in the absence of Cu^{++} ions. However, direct support for this proposal is still lacking.

The guanine and cytosine residues have been implicated by several thermal studies as the site of Cu^{++} ion interaction. The decrease of T_m in the presence of Cu^{++} ions is proportional to the (G + C) content of DNA.[7,11] Dispersion analyses of the hyperchromic effect[19,25] have indicated that Cu^{++} ions are bound essentially to the G - C base pairs. The N_7 position of guanine residues has been indicated as a specific site of Cu^{++} ion interaction by thermal studies using DNA containing N_7 methylated guanine residues.[28]

The tertiary structure of DNA denatured in the presence of Cu^{++} ions (at low ionic strength) is significantly altered to an extremely collapsed and compact configuration, and has been indicated by viscosity,[7] light scattering[7] and sedimentation studies.[6,28]

c. Cu^{++} Ions Added to Denatured DNA

The complex formed by the addition of Cu^{++} ions to denatured DNA exhibits properties intermediate between those of the native DNA-Cu^{++} complex and the complex formed by denaturing DNA in the presence of Cu^{++} ions. The latter complex has been abbreviated to denatured

(DNA-Cu^{++}) for future reference.

The addition of Cu^{++} ions to single strand denatured DNA (abbreviated to (denatured DNA)-Cu^{++}) causes a small ultra-violet spectral shift to longer wavelengths[9,13] (not observed for the native DNA-Cu^{++} complex), and an enhanced hyperchromicity relative to denatured DNA.[7,9,13,28] These effects confirm the ability of Cu^{++} ions to interact with the base residues of denatured DNA, and indicate the essential differences between this complex and the native DNA-Cu^{++} complex.

As single strand heat denatured DNA is only partially reversible on cooling,[47-49] the addition of Cu^{++} ions capable of forming complexes with the base residues is expected to further diminish the ability of the (denatured DNA)-Cu^{++} complex to renature on cooling. The complete lack of reversibility of this complex on cooling[7] is similar to that of the denatured (DNA-Cu^{++}) complex (see Sect. II.2b). However, the denatured (DNA-Cu^{++}) complex can be completely renatured at high ionic strengths, whereas no hydrogen bonds are reformed in the (denatured DNA)-Cu^{++} complex by the addition of an electrolyte. A difference between these two DNA-Cu^{++} complexes is therefore apparent.

Few studies of the (denatured DNA)-Cu^{++} complex have been reported as the possible biological significance of the native DNA-Cu^{++} and denatured (DNA-Cu^{++}) complexes has confined most studies to these two types of complexes.

A discussion of Cu^{++} ion interaction with polynucleotides is relevant

to this section. However, as most of the information from these studies is directed more towards the specific site of the Cu^{++} ion interaction, they are best discussed in that context in Section II.3b.

Conformation studies indicate that the relatively open structure of single strand denatured DNA becomes more compact than native DNA when in the presence of Cu^{++} ions.[28]

3. SITES OF INTERACTION OF THE Cu^{++} ION COMPLEXES

The term "monomers" is used here to represent collectively the individual bases, their corresponding nucleosides and nucleotides (both ribo- and deoxyribo-). Any discussion of Cu^{++} ion interactions with the monomers requires a knowledge of the basic sites of the monomers, as these are the probable Cu^{++} binding sites.[54-56] With respect to the number of basic sites, the bases are chemically distinct from the nucleosides and nucleotides, as the acidic imino hydrogen on the bases is replaced by a ribose group in the nucleosides and nucleotides. Therefore, as they possess one less reactive site than the bases, substantial differences between these two classes of monomers may be expected with respect to the site and stability of Cu^{++} ion interactions. As the charged phosphate group is also a site of Cu^{++} ion interaction, similar differences of the site and stability of Cu^{++} ion interactions may also exist between the nucleosides and the nucleotides.

a. Monomers

For simplicity and clarity of comparison the Cu^{++} ion binding studies of the monomers have been tabulated with respect to the bases (Table II-1) and nucleosides and nucleotides (Table II-2). From Table II-1 it is apparent that the interaction of Cu^{++} ions with guanine, adenine, cytosine and xanthine has been established, and that the N_7 of the purines has been substantiated as the primary Cu^{++} binding site, especially by the direct studies of Harkins and Frieser[53] and Tu and Friederich.[61]

Many metal ion binding studies of the nucleosides and nucleotides have been undertaken to provide information on the interaction itself, while others have been primarily concerned with elucidating the nature of the metal ion interaction with DNA. For these reasons, many studies of the interaction of Cu^{++} ions with nucleosides and nucleotides have been reported (Table II-2).

Many different techniques have been used to confirm the interaction of Cu^{++} ions with guanosine, deoxy-guanosine, GMP and dGMP (see Table II-2), and indicate the N_7 position as a specific site of attachment.[11,13,61,80-83] Chelation of Cu^{++} to the enolised O_6 group of guanosine has often been indicated[10,11,32,61,78,82,83] and has been further supported by Sigel,[85] who detected that the N_1 protons of ITP and GTP were more acidic in the presence of Cu^{++} ions. However, apart from one brief and inconclusive infra-red absorbance

Base Interaction	Base No Interaction	Site	Technique	Reference
X' Th	Caffeine	N_7	kinetics	50,51
A',Hy			pH titration	52
A'		N_7	pH titration	53
A',G'			kinetics	21
G'		N_7,O_6 chelate	quantum mechanical calculations	54-56
substituted pyrimidines	T',C'		analysis of complex	57
A'			analysis of complex	58
A',purine			pH titration	59
C'			analysis of complex	60
Th	Caffeine	N_7	UV and conductometric	61
C'		N_3	X-ray diffraction	62
A',7MeA',9MeA'		N_7 and/or N_9		
Hy,7MeHy,9MeHy			pH titration, UV and visible difference	63
X',7MeX',1,3diMeX'			spectra	
Hy			ESR and analysis of complex	64

Table II-1. Interaction of Cu^{++} ions with the bases.

Nucleosides and Nucleotides		Site	Technique	Ref.
Interaction	No Interaction			
G,I	A		pH titration	52
G,dG,A,dA,GMP,dGMP, AMP,dAMP,CMP	TMP,dCMP		kinetics	21
	A		pH titration	53
$AMP^{=}$,ADP			pH titration	65
ATP		phosphates	pH titration	66
ADP,ATP		N_7 and phosphates	NMR	67
ADP,ATP			NMR and IR	68
	A,G,C,U		pH titration	69
A,ATP			UV,IR,NMR	70
ADP,ATP		N_7 and phosphates	kinetics and IR	71
A,C,G	T,U		pH titration	72
dG,dC,dA,dGMP,dCMP,dAMP	T		ESR	10
dG	dA		pH titration	73
ATP		phosphates	NMR	74
AMP,GMP,CMP,dAMP,dGMP,dCMP	UMP		chromatography	75
G			visible spectra	13
dA,AMP,dAMP,dG,dGMP		N_7	NMR (H)	13
C,dC,CMP,dCMP		N_3	NMR (H)	13
AMP		base and phosphate	pH titration	13
dAMP,TMP		phosphate	NMR (^{31}P)	13

Table II-2. Interaction of Cu^{++} ions with the mono-nucleosides and mono-nucleotides. (contd.)

Table II-2 (cont'd.)

Nucleosides and Nucleotides: Interaction	Nucleosides and Nucleotides: No Interaction	Site	Technique	Ref.
dG		N_7,O_6	IR	11
AMP(3')		N_3	NMR and UV	77
AMP(5')		N_7		
dG			IR and visible	78
$AMP^{=}$,ATP		N_7 and phosphate	pH titration, NMR	79
GMP		N_7	NMR and ESR	80
G,I,GMP,IMP	C,U,CMP,UMP	N_7,O_6	conductance, IR, UV	61
G,C,dGMP,dAMP	A,T,dCMP,TMP		UV	9
I,G,IMP,ITP		N_7,O_6	NMR and IR	82
dG	A,dA,U	N_7,O_6	pH titration	83
dG,dC	dA,T		IR	24
dG,dC	dA		UV	27
dG,dC,dA	T		visible	
ITP,GTP,UTP,TTP,ATP,CTP			UV and pH titration	85
	dGMP,dCMP,dAMP,TMP		UV	7
G,C,I,A	U		visible absorbance	86
G,C,T,A,U,GMP,CMP,TMP, AMP,UMP			ESR	20
A		N_7		
AMP(5'),AMP(3')		N_7,phosphate	NMR	87
IMP		N_7,O_6		
CMP,UMP		N_3	NMR	88
U		N_3		

Table II-2. Interaction of Cu^{++} ions with the mono-nucleosides and mono-nucleotides.

study,[61] there is no evidence to suggest an enol-type chelate in GMP. NMR ^{31}P relaxation studies indicate that Cu^{++} ions are associated with the phosphate groups of nucleotides,[13] and suggest that a Cu^{++} chelate between N_7 and the phosphate group may account for the stability of these complexes (Sect. II.4a).

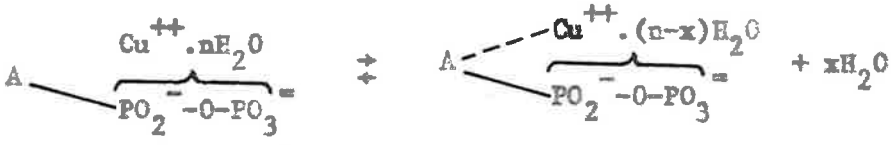
Much evidence has been presented which indicates the formation of a Cu^{++} complex with adenosine,[10,13,20,21,70,72,83,87] although many studies have failed to detect a complex.[9,24,27,52,53,69,73,83] However, there is clear evidence of a Cu^{++} complex with AMP and dAMP.[9,13,20,65,75,77,79,87] Eichhorn et al.[13] have concluded that Cu^{++} is chelated between N_7 of AMP (and dAMP) and the phosphate group, and have been supported by other studies.[67,70,71,74,79] This conclusion has also been indicated by the enhanced stability constants for the binding of Cu^{++} ions to AMP, as compared with adenosine (see Tables II-3 and II-4).

Sigel[77] detected a Cu^{++} chelate, of the type proposed above, with AMP(5'), but involving the N_3 group of AMP(3'). However, recent detailed NMR studies[87] have clearly indicated that the N_7 site is involved in both isomers, and has also indicated that for AMP(5') the N_7 site is chelated to the phosphate group of another nucleotide, and not with the phosphate group of the same nucleotide.

Cytidine and CMP (and the deoxy- compounds) form complexes with Cu^{++} ions.[10,13,21,24,27,72,75,86] The N_3 site has been indicated, as well as the phosphate group of the nucleotides,[13] as sites of attachment.

There has been only one[88] indication of a Cu^{++} complex involving the base residue of thymidine, uridine or the corresponding ribo- and deoxyribo-nucleotides (see Table II-2), although NMR and ESR techniques indicate that a Cu^{++} ion interaction with the phosphate group does occur.[13]

Cu^{++} ion interaction with any of the di- and tri-phosphate nucleotides appears to be essentially with the phosphate groups,[67,74] as there is a marked dependence of the stability constant on phosphate chain length.[76] However, some studies indicate the existence of a chelate between the base residue and the phosphate group.[70-72,79] Schneider et al.[70] have suggested that an equilibrium of the type



would explain the discrepancy of many reports regarding the site of attachment, as many measurable properties detect only one of the extremes of the equilibrium, depending on concentrations and other conditions.

Several studies have indicated that Cu^{++} ions do not bind to the deoxy-ribose residue of nucleosides or nucleotides.[53,61,83,84] However, more sensitive techniques (ESR and NMR) have recently detected a weak interaction.[20,88]

For comparison with studies of Cu^{++} ion binding to DNA, the interaction of Cu^{++} ions with the deoxy-mononucleotides are the most relevant if conducted at a pH at which the phosphate groups possess only

one negative charge, as in DNA. It appears that the N_7 group of dGMP, probably the N_7 group of dAMP and possibly the N_3 group of dCMP, are involved in Cu^{++} complexes. The phosphate group of all of the nucleotides has been implicated. The evidence suggests a Cu^{++} chelate between the base residue and the phosphate group. A chelate involving the N_7 of AMP with the phosphate group of another AMP molecule, has been established, suggesting that similar chelates may occur with GMP and CMP.

b. Polynucleotides

Several studies of the Cu^{++} ion interaction with the synthetic homopolynucleotides have recently been undertaken, as in either the single strand or double strand form these model polymers provide insight into the Cu^{++} ion interactions with DNA.

The presence of Cu^{++} ions inhibits the formation of double strand poly(A + U)[89] and poly(I + C),[90] as well as destroying the ordered structure of these co-polymers, indirectly indicating that the Cu^{++} ion complex may involve the hydrogen bonding sites. Bridging of Cu^{++} between the strands of poly A and poly U has been indicated by the NMR studies of Berger and Eichhorn.[88]

The interaction of Cu^{++} ions with poly A (essentially single strand near neutral pH)[29,30] has been shown by hyperchromicity,[38] ESR[20] and NMR[87,88] studies. The N_7 site of the adenine residue is clearly involved in an interaction with Cu^{++} ions.[87,88] The complex appears to

be of a mixed covalent-ionic type,[20] and suggests an N_7-phosphate chelate.

A Cu^{++} ion interaction occurs with poly I[38,88] and poly G.[20,91] The tertiary structure of these polymers is not well defined,[30,92] although poly I appears to exist as a triple helix near neutral pH.[30,93] The N_7 position of poly I is involved in a Cu^{++} complex[88] and a chelate with a phosphate group, similar to poly A, has been suggested.[88]

The Cu^{++} ion interaction with poly C (essentially single strand near neutral pH)[94,95] has been well substantiated,[20,28,38,86,88] and appears to be more involved than the other polynucleotides. The order of hyperchromicities, poly(5 methyl C) > poly C > poly(4,5 dimethyl C), for the interaction with both Cu^{++} ions and poly I, established that Cu^{+} ions interact with the hydrogen bonding sites of poly C.[86] Recent NMR studies indicate the N_3 group as the specific site of Cu^{++} interaction.[8] Two types of complex have been indicated by ESR studies.[20] One appears to be completely covalent in nature and similar to the covalent cytidine Cu^{++} complex detected.[20] The other appears to be partially ionic and covalent, and may represent the base residue-phosphate chelate discussed previously, (Sect. II.3a).

There is evidence to show that Cu^{++} ions do not interact with the base residues of poly U[38] or poly T.[96] However, a recent NMR study has indicated that Cu^{++} ions interact with the N_3 site of poly U, UMP and uridine, as well as with the ribose group.[88] An ESR study[20] has indicat that an essentially ionic complex of Cu^{++} is formed with thymidine,

uridine, poly U and ribose. This suggests a weakly associated complex, and may explain why a Cu^{++} ion interaction with the monomers (Table II-2) and polymers[38,96] of uridine, thymidine and ribosylthymidine has remained essentially undetected apart from a phosphate interaction.[13]

c. Cu^{++} Ions Added to Denatured DNA

The interaction of Cu^{++} ions with the base residues of denatured DNA produces hyperchromicity which is irreversible on cooling or on raising the ionic strength (Sect. II.2c). This suggests that Cu^{++} ions interact with the hydrogen bonding sites of denatured DNA. The similar ultra-violet difference spectra of apyrimidinic acid[27] and poly G,[91] when in the presence of Cu^{++} ions, suggests that Cu^{++} ion interaction with the guanine residues predominates.

No other direct studies have been reported of the interaction of Cu^{++} ions with single strand DNA. However, much information has been gained from Cu^{++} ion interaction studies of the synthetic homopolynucleotides (Sect. II.3b) as these polymers are essentially non-ordered near neutral pH, and as such are directly comparable with single strand DNA. Therefore, conclusions regarding the site of interaction of Cu^{++} ions with the non-ordered polynucleotides may also apply to single strand DNA.

d. DNA Denatured in the Presence of Cu^{++} Ions

Many visible and ultra-violet absorbance studies have indicated that

the base residues are involved in the denatured (DNA-Cu^{++}) complex (Sect. II.2b). Thermal studies (Sect. II.2a and Sect. II.2b), as well as dispersion analyses of the hyperchromic effect (Sect. II.2b), indicate that the guanine and cytosine residues are preferentially involved in the complex. This conclusion has also been reached recently from renaturation studies by Zimmer et al.[28] The N_7 position of the guanine residues has been indicated as a Cu^{++} binding site by thermal and difference spectra studies using DNA containing N_7 methylated guanine residues.[28]

The many studies confirming that DNA denatured in the presence of Cu^{++} ions is completely renatured on raising the ionic strength (Sect. II.2b) provide the most substantial evidence regarding this complex, and indicate that Cu^{++} in the complex holds the two strands sufficiently close together (and also close to the complementary base pairs), to allow a rapid and complete renaturation process to occur. As no renaturation occurs on cooling the complex, as indicated by the constant hyperchromicity, Cu^{++} ion interaction with the hydrogen bonding sites appears likely,[6,7,11,3] as this would prevent reformation of the hydrogen bonded double strand structure of DNA. The interstrand Cu^{++} chelate between the guanine and cytosine base pairs accounts for all of the facts mentioned above, and has previously been suggested by Eichhorn and Clark.[6] A similar complex has recently been proposed by Zimmer et al.,[28] who conducted extensive IR, ORD and CD studies in support of their model.

Kinetic studies of the renaturation process at high ionic strengths indicate that the reaction is essentially first order[97-100] (although a number of complicating factors make the results difficult to interpret) and suggest that the denatured (DNA-Cu^{++}) complex is an integral unit, in accordance with the models discussed above.

e. The Native DNA-Cu^{++} Complex

The question of the specific sites of interaction of Cu^{++} ions with DNA is not completely resolved. Many studies have indicated that only the phosphate groups interact with Cu^{++} ions,[1-20] while others indicate that only the base residues are involved.[4,5,10,12,14,16,17-28] Other studies indicate that both sites are involved.[4,5,10,12,14,16,17-20] The discrepancies apparent from many ultra-violet absorbance results have been attributed[19] to the high Cu^{++} ion and DNA concentrations required to produce small hyperchromic changes (Sect. II.2a).

Several lines of evidence suggest that the phosphate groups of DNA are involved in an interaction with Cu^{++} ions. The relative stability of T_m at low Cu^{++} ion to base residue ratios[6,7,9,11,37] has generally been attributed to screening of the phosphate charges (Sect. II.2a(ii)). Furthermore, the NMR relaxation of Cu^{++} ion water protons indicates that Cu^{++} ions are bound to the outside of DNA, apparently interacting with the phosphate groups.[2,3] This is supported by the detection by ESR[4,5,10,20] of ionic interactions between Cu^{++} ions and native DNA, and by the ^{31}P NMR relaxation studies of the nucleotides.[13] On the basis of several

unsubstantiated assumptions, kinetic[12,14] and pH titration[13] studies have also indicated that the phosphate groups are involved in the interaction.

Early kinetic inhibition measurements indicate that the adenine and guanine residues interact with Cu^{++} ions. Significant spectral shifts (both ultra-violet and visible) have clearly indicated that Cu^{++} ions interact with the base residues of native DNA.[12,14,16,19,32,33] The stability constants for the interaction, determined solely from absorbance changes,[16,19] agree with those determined by other techniques,[15-17] and confirm that base interaction occurs. The most convincing ultra-violet spectral evidence has been at high ionic strengths[19] where charge effects of the phosphate groups are diminished, and decisively implicate the base residues as Cu^{++} binding sites.

Using labelled Cu^{++} and Mg^{++} ions Altman et al.[101] have shown that these two ions do not compete with each other for binding sites on native DNA. As Mg^{++} ions appear to interact only with the phosphate groups,[11,18,35,42-44] Cu^{++} ion interaction essentially with the base residues is indicated.

The most sensitive techniques which confirm the interaction of Cu^{++} ions with the base residues are those of ORD[22,28] and CD.[28] These studies indicate that the guanine and cytosine residues are primarily involved in the interaction.[28]

Several ESR studies have indicated that both phosphate and base binding of Cu^{++} ions occur with native DNA,[4,5,10,20] supporting similar

findings from binding[16,17] and other studies.[26] Schrieber and Daune[19,102] have detected a complex independent of ionic strength and conclude the formation of an exclusively base residue chelate of Cu^{++} ions. This chelate appears to depend on the (G + C) content of the DNA.[102]

A similar (G + C) dependence has been found for the low ionic strength complex from infra-red studies.[27,28] Ultra-violet difference spectral studies of DNA methylated at the N_7 positions of the guanine residues confirm the interaction of Cu^{++} ions with that site.[28]

To summarise the results of these studies, it appears that a chelate of Cu^{++} occurs exclusively with the base residues at high ionic strengths. At low ionic strengths, both the phosphate groups and the base residues have been implicated. The N_7 of the guanine residue appears to be a specific site of Cu^{++} interaction. These conclusions are based only on experimental results of the native DNA-Cu^{++} system. Further indirect evidence is available from studies on the other DNA-Cu^{++} complexes, and the interaction of Cu^{++} ions with mono- and poly-nucleotides. These will be considered later (Sect. II.5).

4. STOICHIOMETRY AND STABILITY OF THE Cu^{++} ION COMPLEXES

a. Monomers

As mentioned earlier (Sect. II.3), there are fundamental differences between the abilities of the bases, the corresponding nucleosides and the nucleotides to accept protons. Therefore,

substantial differences may be expected, and are in fact observed, (Tables II-3 and II-4), in the ability of these three types of monomers to bind Cu^{++} ions. Typical apparent stability constants for adenine, adenosine and AMP^{2-} are log K = 8, 0.7 and 3 respectively (Tables II-3 and II-4). These trends correlate with Cu^{++} interaction at the basic imino site of adenine, negligible interaction when the basic site is not available (as in adenosine), and enhanced stability of AMP relative to the nucleoside due to the presence of a charged phosphate group.

The relative affinity of a range of monomers for Cu^{++} ions has long been established:[21] A,G,X,Hy > GMP,dGMP > G,dG,A,AMP,dAMP > dA,CMP > TMP,dCMP, and has been confirmed by ESR[10] and visible absorbance studies.[27,86] The great affinity of the bases A,G,X,Hy (log K ~ 6-7) for Cu^{++} ions has been well established (Table II-3).

Near neutral pH, log K values for the nucleoside-Cu^{++} interaction are 2.1 (guanosine), 1.6 (cytidine) and 0.7 (adenosine), in agreement with the series listed above. No stability constants are available for the interaction of Cu^{++} ions with GMP or CMP.

b. DNA and Polynucleotides

The interaction of various metal ions with DNA has recently been reviewed[105] and indicates that different functional groups of DNA may interact with Cu^{++} ions. Therefore, the stoichiometry and stability constants determined for interactions of metal ions with DNA represent averaged values for groups of similar types of sites. All stability

Base	Log K_1	Stoich. Cu^{++}:Base	Technique	I	pH	T(^{o}C)	Ref.
A'	2.5	1:1	pH titration	var.	-	20	52
A'	7.1 6.4 (K_2)	1:2	pH titration	0.01	6-8	25	53
A'	8.9						
Hy	7.6	1:2	pH titration	<0.01	3-6	25	59
Purine	6.9						
C'	-	1:1 and 1:2	analysis of complex	-	-	-	60
C'	-,1.4*		pH titration				
A'	7.0,2.7*	1:2	and UV	0.05	>6,<6*	25	63
Hy	6.5,1.9*						
X'	5.1						
C'	-	1:2	X-ray diffraction	-	-	-	62
Th	-	1:1	conductance and UV	var.	7	25	61
9Me Hy	5.4	1:2	pH titration	0.05	7-10	25	83

Table II-3. Apparent stability constants for the interaction of Cu^{++} ions with the bases.

K_1 and K_2 refer to the apparent stability constants for the addition of the first and second ligands to Cu^{++}.

Monomer	Log K	Stoich. Cu:Monomer	Technique	I	pH	T(°)	Ref.
G,I,X	~6,~5,3.4	-	pH titration	var.	var.	20	52
AMP^{2-}(3'),AMP^{2-}(5')	2.96,3.18	1:1	pH titration	0.1	3-9	25	65
ADP^{2-},ADP^{3-}	2.63,5.90						
ATP^{2-}	3.12	1:1	pH titration	0.1	3-9	25	66
A	0.84	-					70
ATP^{4-},$Me.TP^{4-}$	6.2,6.3	-	UV	var.	-	-	
A,C,G	0.7,1.6,2.1	-	pH titration	1.0	2-4	20	72
G,T,U	4.3,4.7,4.2	-		1.0	10	20	
AMP^{2-},ATP^{4-}	3.0,6.4	1:1	pH titration	0.1	<6	25	79
dG	5.6,5.4	-	pH titration	.05	7-10	25	83
G	-	1:1	IR	-	-	-	78
ATP^{3-},ATP^{4-}	3.12,6.13	1:1	pH titration	0.1	-	25	103
ATP^{3-},ATP^{4-}	3.97,6.83	-	pH titration	0.1	3-5.4	30	104
G,I,GMP,IMP	-	1:1	conductometric and UV	var.	7	25	61

Table II-4. Apparent stability constants for the interaction of Cu^{++} ions with mono-nucleosides and mono-nucleotides.

(association) constants, K, referred to are apparent stability constants as they have been calculated using concentration terms. No attempt has been made to allow for activity coefficients.

Kinetic studies of Cu^{++} ion catalysis of various reactions in the presence of DNA indicate that $K = 1.5.10^7$ for the native DNA-Cu^{++} interaction at low ionic strength, and has been attributed, without supporting evidence, to a phosphate-Cu^{++} interaction.[12,14] Cu^{++} ion interaction with the base residues has been detected by difference spectra studies at low ionic strength and indicates[14] that $K = 1.3.10^4$.

Using an experimentally determined value for the stoichiometry of the native DNA-Cu^{++} interaction at low ionic strength (n = 0.5 Cu^{++} ion binding sites per nucleotide residue), values of $K = 2.10^4$ and $K = 1.10^4$ have been calculated from gel-filtration and difference spectra studies respectively.[16] These two similar values indicate that all of the bound Cu^{++} is associated with the base residues, in contrast to that observed by others.[12,14] However, examination of the binding curve of Bryan and Frieden[16] indicates two types of interaction ($n \simeq 0.25$, $K > 1.3.10^5$ and $n \simeq 0.25$, $K = 2.10^4$) at low ionic strength.

At low ionic strength, similar values have been obtained for the native DNA-Cu^{++} association by polarographic analysis ($K = 6.10^4$),[15,17] while two sites have been indicated by difference spectra studies[19] ($n = 0.07$, $K = 1.10^6$ and $n = 0.4$, $K = 5.10^3$, calculated using the method outlined in Chapt. IV).

From polarographic studies at high ionic strength ($I \geq 0.1M$), only

one site has been indicated for the interaction of Cu^{++} ions with native DNA (n = 0.04, K = 2.10^5),[102] and a small (G + C) dependence of the stoichiometry was observed.[102] Others have also detected only one site of interaction at high ionic strength, although substantially different stability constants have been determined by ESR (0.07, K = $1.3.10^4$),[10] by equilibrium dialysis (assuming n = 1.0, K = $1\text{-}3.10^3$),[23] and by polarographic analysis (K = $1.5.10^3$).[15,17] It is important to note that the interaction occurring at high ionic strengths (n = 0.04, K = 2.10^5)[102] also appears to exist at low ionic strengths (n = 0.07, K = 1.10^6),[19] where at least one other complex has also been indicated.

The thermodynamic stability of the (denatured DNA)-Cu^{++} complex (K = 3.10^4 and 1.10^3) appears to be similar to the native DNA-Cu^{++} complex (K = 6.10^4 and 1.10^3) at both low and high ionic strengths respectively,[15,17] and is also similar to DNA denatured in the presence of Cu^{++} ions.[15,17] However, others have determined a different stability constant for an undefined denatured DNA-Cu^{++} complex (K = 1.10^5) in contrast to the native DNA-Cu^{++} complex (K = 1.10^4).[16]

No thermodynamic stability studies involving Cu^{++} ions and polynucleotides have been reported, although the dependence of the stability constant on the phosphate chain length for the interaction of metal ions with mononucleotides[65,66,76] and DNA[106] has been established. The interaction of Ag^+ ions with oligomers has been shown to depend on the nucleotide chain length,[107] and as interaction of Ag^+

ions with the base residues has been confirmed,[108-110] suggests that a similar chain length dependence may occur for Cu^{++} interaction with oligonucleotides.

5. MODELS OF THE DNA-Cu^{++} COMPLEXES

a. Cu^{++} Ions Added to Native or Denatured DNA

Three sites of Cu^{++} ion interaction with native DNA or single strand denatured DNA have been indicated by previous studies.

(i) Phosphate Site

This essentially electrostatic interaction has been suggested by direct studies of the binding of Cu^{++} ions to orthophosphate[79,111] and by ESR studies,[4,5,10,20] ^{31}P NMR relaxation studies of mononucleotides,[13] and by direct comparison of thermal studies of poly rT in the presence of Cu^{++} and phosphate specific ions.[96] Other less direct evidence has also been presented in Sect. II.3.

Studies on the interaction of other metal ions with polynucleotides[112] indicate that both ion-atmosphere (domain)[113] binding as well as site binding, may occur. NMR studies indicate that site binding of Cu^{++} ions predominates.[2,3,114] Co^{++} ions associated with the phosphate groups of RNA contain only five coordinated water molecules,[115] suggesting that a similar specific interaction of Cu^{++} ions with the phosphate groups of DNA may occur (Fig. II-4a). In view of the apparent stoichiometry of metal ions interacting with the phosphate groups (see Sect. II.6), such a complex may involve a rapid exchange of $Cu^{++}.5H_2O$

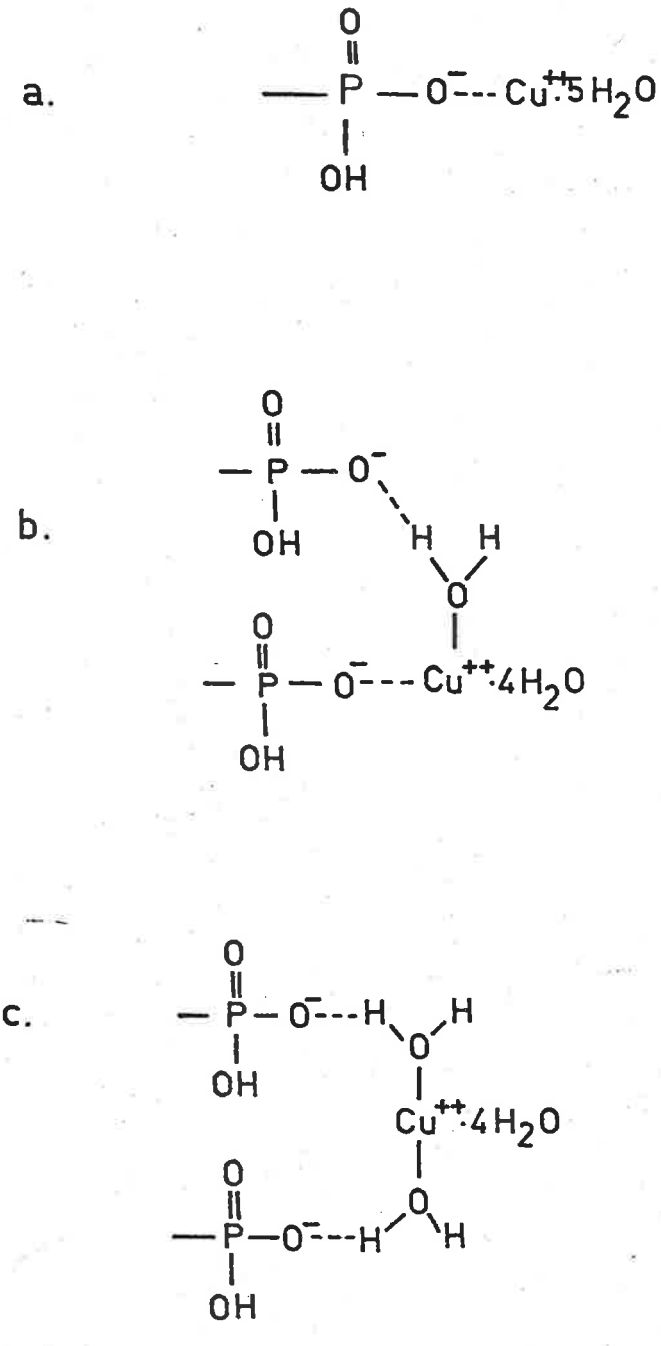


Fig. II-4. Possible Cu^{++} ion-phosphate complexes in DNA.

between two adjacent phosphate groups, as they are only 7 Å apart. DNA model studies have shown that other structures involving hydrogen bonds are possible (Fig. II-4b,c). Several of these structures have previously been suggested to explain the apparent binding of one Cu^{++} ion per two base residues.[116]

(ii) Base Residue-Phosphate Sites

The N_7 group of the guanine residue in native DNA has been implicated as a specific site of Cu^{++} ion interaction (Sect. II.3e) supporting a similar conclusion for GMP^- and AMP^- (Sect. II.3a). However, it is important to remember that the basicity of the base residues may be altered in DNA and, accompanied by the effects of a different stereochemical environment in DNA, may cause significant differences to the Cu^{++} ion binding ability of the monomers.

A chelate of Cu^{++} between N_7 of the guanine (also adenine) residue, and the phosphate group appears probable in the mononucleotides,[13,67,70,71,74,77,79] and has been supported by the enhanced stability constants of the nucleotides as compared with the nucleosides (Sect. II.4a). NMR broadening studies[117] using ^{35}Cl indicate a similar type of complex of Zn^{++} chelated between the base residue and the phosphate groups of ADP^-.

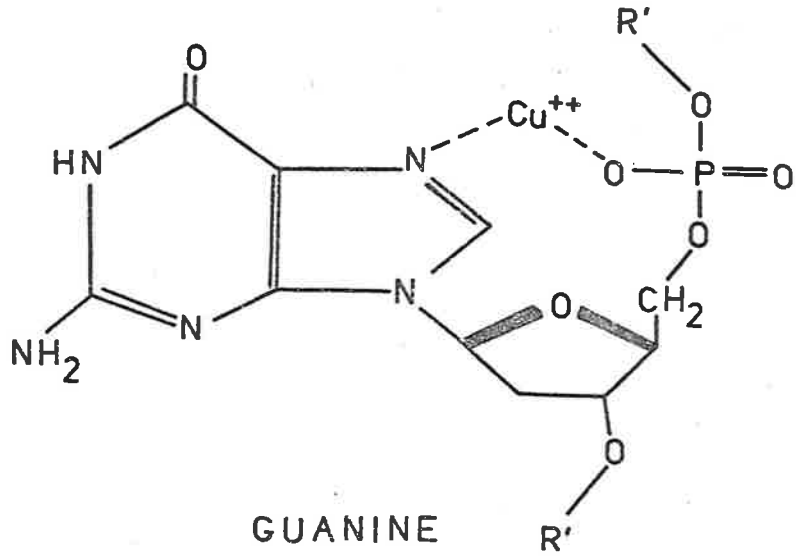
A similar type of chelate involving the N_3 position of the cytosine residues of CMP has been indicated from NMR proton broadening studies.[13] However, studies of the DNA model have shown that this type of complex involving the N_3 position of the cytosine residue is sterically

inaccessible in native DNA if Cu^{++} is chelated to the phosphate group of the same nucleotide. Substantial base re-arrangement may occur in the polynucleotides which are not orientated by hydrogen bonding or other forces, to accommodate such a chelate involving the phosphate groups of adjacent nucleotides. Such massive re-orientation of the base residues is unlikely in single strand DNA if Cu^{++} chelates involving the phosphate group and the guanine or adenine residues are present, as these are expected to supply a base orientating effect on the polynucleotide. On this basis, such a complex is not precluded for poly C where, apart from base stacking forces, no additional base orientating forces occur.

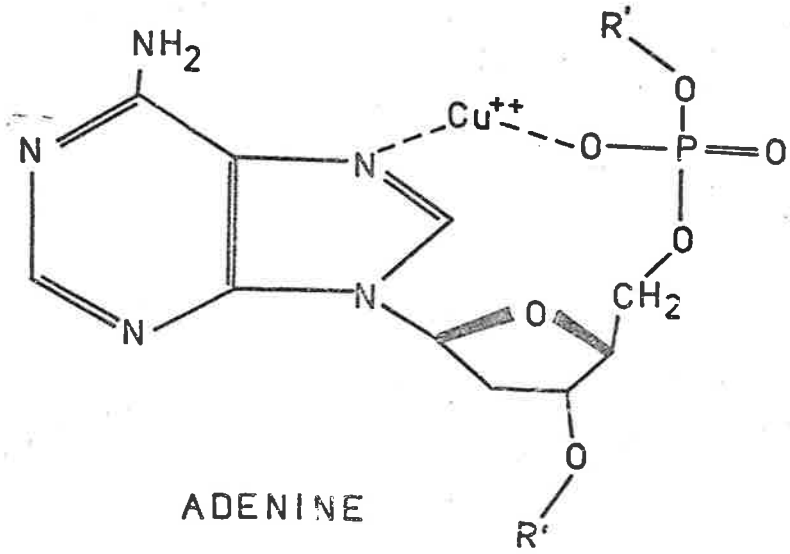
Cu^{++} complexes involving the thymine residue have not been indicated. The base residue-phosphate chelates proposed have previously been suggested[14,16,32,116,118] for the native DNA-Cu^{++} interaction at low ionic strength, and are indicated in Fig. II-5.

As such complexes involve an electrostatic interaction between the Cu^{++} ion and the phosphate group, an ionic strength dependence is expected for this interaction. Such a dependence has been observed for the native DNA-Cu^{++} interaction,[15,17,19] supporting the proposal for the occurrence of the above type of chelates in the native DNA-Cu^{++} complex.

At high ionic strengths, where the electrostatic effect of the phosphate group is diminished, a Cu^{++} ion interaction with the individual base residues may replace the base residue-phosphate chelate. Such complexes are expected to be similar to the nucleoside-Cu^{++} complexes



GUANINE



ADENINE

Fig. II-5. The base residue-phosphate Cu^{++} chelates proposed for the native DNA-Cu^{++} complex.

R' represents the continuation of the deoxyribose-phosphate chain.

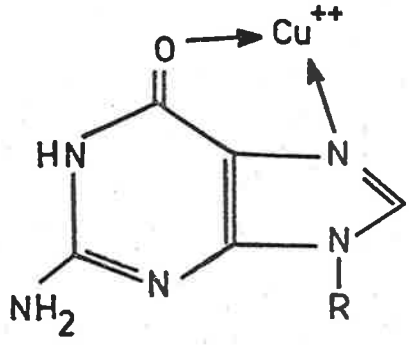
(Fig. II-6) discussed earlier (Sect. II.3a). The ionic strength independent complex at high ionic strength (log K = 3)[17] is expected to possess a slightly larger stability constant than the nucleoside-Cu^{++} complexes (log K ~ 2), as the presence of adjacent basic sites is expected to stabilise the same complexes in DNA. From a DNA model it appears that stereochemical factors may preclude the formation of a cytosine-Cu^{++} complex in native DNA, and possibly also in single strand DNA.

(iii) Base Residue Sites

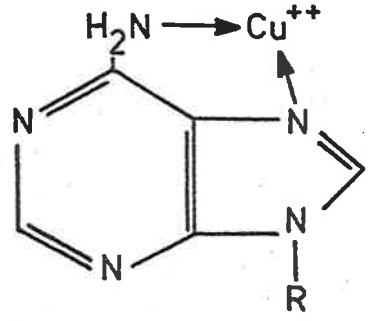
Two such complexes have been shown to exist at high ionic strengths. The relatively weak (log K ~ 3) Cu^{++} ion interactions with individual base residues (Fig. II-6) have been discussed above.

A more stable complex (log K = 5.3) at a few sites (n ~ 0.05) has been attributed to a base residue-Cu^{++} interaction[19,102] as it has been well substantiated[19,28] that the base residues are involved at low Cu^{++} ion to base residue ratios. A chelate of Cu^{++} between adjacent guanine residues has been suggested[19,116] (Fig. II-7) on the basis of the known reactivity of guanine residues with Cu^{++} ions, and from studies of the native DNA model which indicate that of all of the combinations of adjacent base residues, adjacent guanine residues provide the four most probable ligands capable of forming a terdentate complex with Cu^{++} ions. As such a 'sandwich' complex involves the N_7 position of guanine, the guanine-phosphate chelate, of lower stability, also involving the N_7 position, is probably excluded at the few sites involving adjacent

a. GUANINE



b. ADENINE



c. CYTOSINE

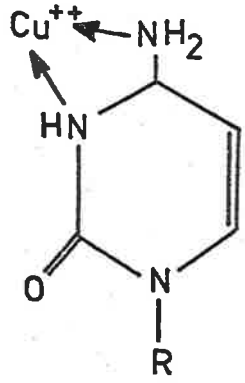


Fig. II-6. Possible individual base residue-Cu^{++} chelates in the native DNA complex (a,b) and the single strand DNA complexes (a,b,c).

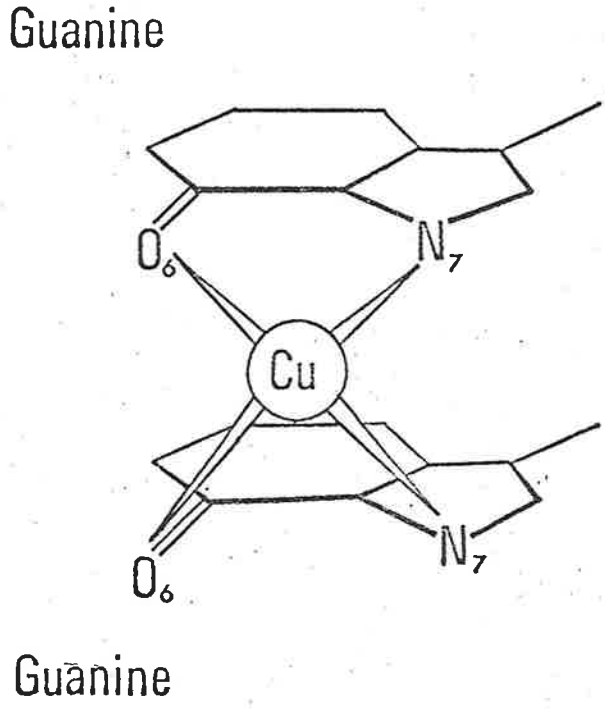


Fig. II-7. The 'sandwich' type Cu^{++} chelate proposed by Schrieber and Daune[19] for native DNA.

guanine residues.

In view of the complete covalent character of the poly C-Cu^{++} complex,[20] similar types of complexes involving adjacent cytosine residues may also be possible. However, much base residue re-orientation is envisaged and would only occur if no orientating forces were present. Therefore, such complexes may not occur in single strand DNA, where the Cu^{++} base residue-phosphate chelates of guanine and adenosine are expected to provide a base orientating effect. The number of adjacent guanine residues determined by nearest neighbour analysis[119] is of a similar order of magnitude to the number of sites determined by binding studies,[102] providing some support for the terdentate chelate proposed.

An alternative explanation assumes the formation of an inter-strand complex between G - C base pairs (to be discussed in Sect. II.5b) at a few sites only, and that some local strand separation occurs. Some of these complexes may exist at high ionic strengths (in contrast with the denatured (DNA-Cu^{++}) as Cu^{++} ions may be trapped by adjacent hydrogen bonded base pairs. However, as large ultra-violet spectral changes are not observed for the native DNA-Cu^{++} complex, such an interaction is unlikely.

The electrostatic phosphate interaction, the guanine and adenosine base residue-phosphate chelates (and also the guanosine, adenosine and perhaps cytidine type Cu^{++} ion complexes at high ionic strength) and the Cu^{++} chelate between adjacent guanine base residues, are all expected to

occur in single strand denatured DNA as well as in native DNA. The essential difference between these complexes and native DNA is that the base residues are no longer orientated by hydrogen bonding. Consequently, much larger spectral perturbations and conformational changes are observed in single strand denatured DNA on the addition of Cu^{++} ions (Sect. II.2a and Sect. II.2c).

b. DNA Denatured in the Presence of Cu^{++} Ions

The ability of the denatured (DNA-Cu^{++}) complex to be completely renatured at high ionic strengths, but not at all on cooling, whereas the (denatured DNA)-Cu^{++} complex is partially reversible in both cases, clearly indicates that Cu^{++} holds the complimentary strands together in some way in the denatured (DNA-Cu^{++}) complex, and that different complexes are involved in these two states.

The preferential destabilisation of the G - C base pairs[7,11,19,25] indicates Cu^{++} interaction with these residues. This is further supported by the reversible hyperchromicity (on cooling) of denatured (poly dAT-Cu^{++}),[7] as no G - C pairs can be involved. The hydrogen bonding sites of the cytosine residues have been decisively implicated in this complex,[86] supporting an inter-strand G - Cu^{++} - C type complex. However, the complex initially proposed[6] is not supported by molecular model studies.

Studies on DNA, methylated at the N_7 position of guanine residues, indicate that the N_7 position is involved in the Cu^{++} complex.[28] On this

basis, a model has been presented which involves "flipping" of the guanine residues to accommodate the N_7 position in a complex with the cytosine residue hydrogen bonding sites.[28] However, as the "flipping" must be reversed at room temperatures to allow renaturation at high ionic strengths, this model appears untenable.

The complex proposed (Fig. II-8) is significantly distinct from those suggested by others[6,28] and involves re-orientation of the guanine and cytosine residues, accompanied by a complete breakdown of hydrogen bonding and base stacking. Two stages of re-orientation are envisaged and have been outlined in Fig. II-8. At this stage it is not clear if the N_7 position of the guanine residues is directly involved in such a complex as its implication from N_7 methylation studies[28] may also be interpreted in terms of a stereochemical effect limiting base residue re-orientation.

The concomitant collapse of the secondary and tertiary structures, accompanied by aggregation at high temperatures, has been well documented (Sect. II.2b).

The other types of interactions proposed for the native DNA-Cu^{++} complex also occur in the denatured (DNA-Cu^{++}) complex. Cu^{++} ion interaction is expected with the phosphate groups, the guanine and adenine-phosphate sites, and the adjacent guanine sites. It is not readily apparent if the inter-strand G - Cu^{++} - C complex affects the formation of the other two complexes involving the guanine residues.

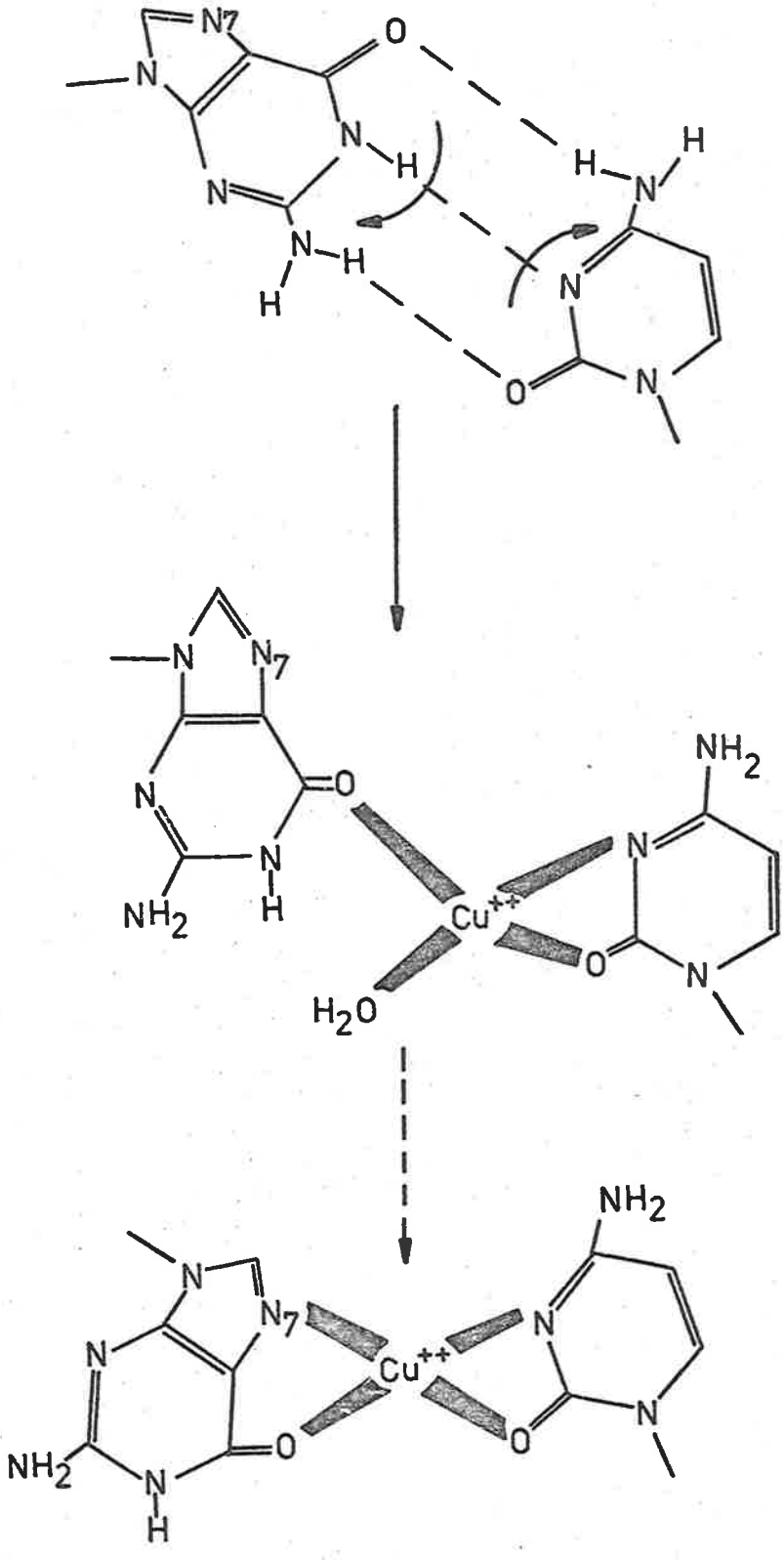


Fig. II-8. The inter-strand complexes proposed for DNA denatured in the presence of Cu^{++} ions.

c. The DNA-Cu^{++} Complex Reaction Scheme

A schematic representation of the formation of the three distinct types of DNA-Cu^{++} complexes is presented in Fig. II-9. Three types of Cu^{++} ion interaction with native DNA (complex II) have been indicated (Sect. II.5a), two of which may involve sterically hindered sites. Proton exchange studies have shown that the hydrogen bonds are constantly being broken and reformed at room temperatures.[120,121] This "breathing" process may be the mechanism involved in the interaction of Cu^{++} ions with partially hindered base residue sites of native DNA. Such complexes would account for the spectral perturbations observed (Sect. II.2a).

The "breathing" process increases at higher temperatures, and would allow the formation of both native DNA-Cu^{++} type interactions, and also some Cu^{++} interactions at the G - C hydrogen bonding sites, even at temperatures below T_m. Both types of complex are expected to disrupt hydrogen bonding and base stacking and so destabilise the helix. The hydrogen bonding character of the G - C pairs is lost above a critical Cu^{++} ion to base residue ratio, and a co-operative T_m transition occurs[7,9,11,35] corresponding essentially with that of only A - T base pairs.[7,11] The resultant product (Fig. II-9, complex III) possesses the three types of Cu^{++} complexes proposed for native DNA in addition to the inter-strand complex which is responsible for the unique renaturability properties (see Sect. II.2b) of complex IV.

At high ionic strengths the adenine-phosphate Cu^{++} may be

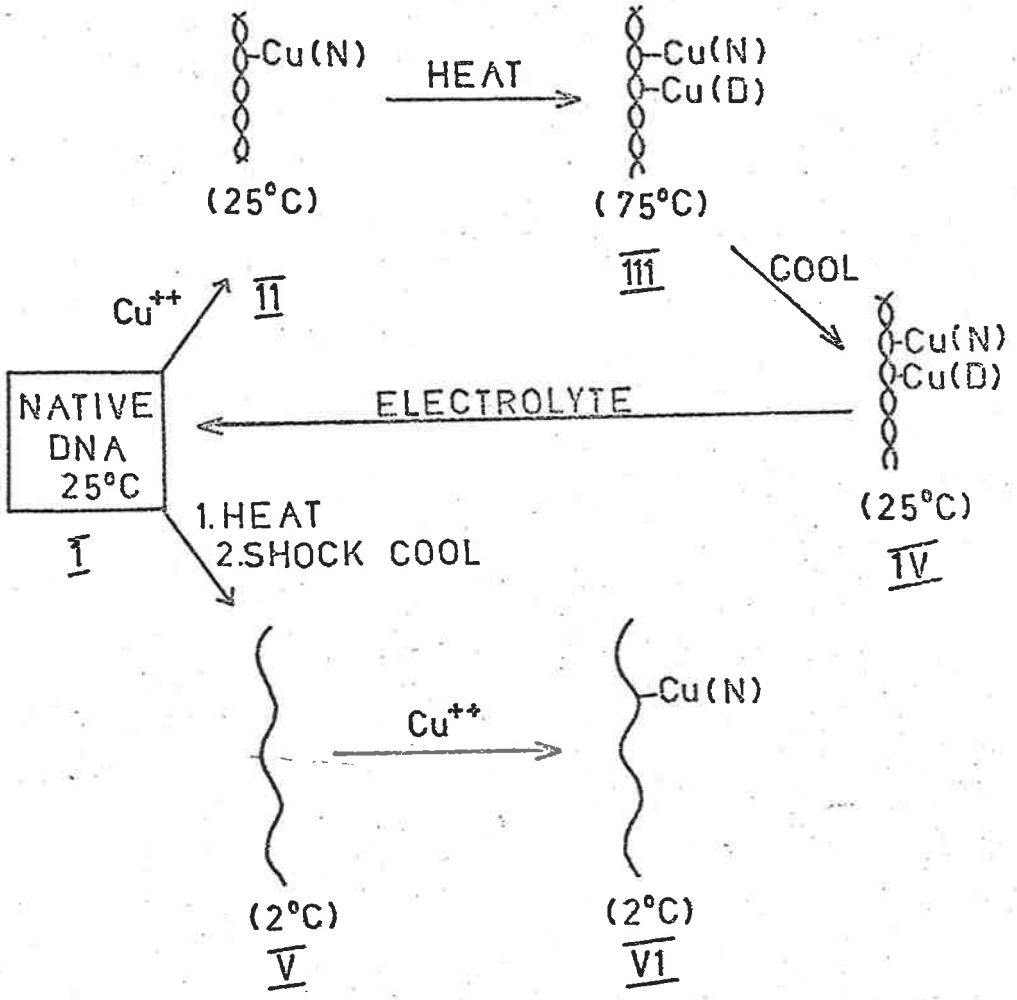


Fig. II-9. The DNA-Cu^{++} interaction scheme.

Cu(N) represents Cu^{++} complexes proposed for native DNA.

Cu(D) represents Cu^{++} complexes proposed for DNA denatured in the presence of Cu^{++} ions.

diminished, favouring the weaker adenine-Cu^{++} type chelate (Fig. II-6). The nucleoside-Cu^{++} complexes in guanine and cytidine (Fig. II-6) are not expected to occur if the same sites are occupied by the inter-strand complex.

In addition, the inter-strand type Cu^{++} complex is excluded as the helix stabilising forces become more dominant. The product then is DNA (structure I) possessing all of the properties of native DNA (Sect. II.2b), but perhaps containing some Cu^{++} complexes which are independent of ionic strength. The relatively weak guanosine-Cu^{++} type complex (Fig. II-6) may also occur, although there is no experimental evidence to suggest that this complex is able to reform when the DNA is in a substantially altered conformation at high ionic strength. The complexes remaining in renatured DNA have been ignored in recent renaturation kinetic studies[100] as few base residues appear capable of being involved, and because there is no direct evidence of their existence in the renatured DNA.

The Cu^{++} ion complexes with native DNA appear to be similar to those of single strand denatured DNA (complex VI) as the "breathing" effect of native DNA may simulate small regions of non-hydrogen bonded DNA.

The proposed scheme (Fig. II-9) contrasts with that suggested by Bryan and Frieden,[16] where complex II is assumed to form complex III at room temperature if left for a sufficient period of time at high Cu^{++} ion concentrations, a conclusion which has been contradicted by the failure of other studies to observe a large hyperchromic change associated with Complex II (see Sect. II.2a).

6. INTERACTION OF OTHER METAL IONS WITH DNA AND RELATED COMPOUNDS

a. Monomers

The interaction of metal ions (K^+, Na^+, Li^+, Mg^{++}, Ca^{++}, Sr^{++}, Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}) with adenosine and the adenine nucleotides has been reviewed by Phillips.[76] Metal ions which have little nitrogen chelating tendency (Mg^{++}, Ca^{++}) form essentially phosphate complexes. Those ions which interact electrostatically, and which also have a strong nitrogen chelating tendency, (Mn^{++}, Cu^{++}, Zn^{++}) tend to form bridge structures (or are in equilibrium between the two sites),[122] while those with a strong tendency to chelate nitrogen form essentially base residue complexes (Ag^+).

Other base residues interact in a similar manner with metal ions.[123-126] However, relatively few studies have been undertaken, as most studies utilise ATP and its related compounds because of their biological importance.

b. DNA and Polynucleotides

Effects of salts on the properties of DNA have been reviewed by Michelson,[127] while others have reviewed the interaction of metal ions with DNA in terms of the properties, structure and stability of the complex.[105] Some studies indicate that Mg^{++} and Mn^{++} ions are bound to the phosphate groups by a specific interaction.[3,115,128] However, others have obtained evidence indicating electrostatic ion atmosphere type binding.[43,129,130]

Many early metal ion-polynucleotide studies were conductometric,

and were complicated by the instability of polynucleotides and native DNA at the low ionic strengths required, and by the difficulty of interpreting results. These and other studies indicate approximately stoichiometric binding ($n \approx 0.5$) of Ca^{++}, Mg^{++} and Mn^{++} ions to poly A,[132] poly U[132] and native DNA.[1,43,131,133] The similar binding of Ca^{++} and Mg^{++} ions to synthetic polynucleotides and DNA (both native and denatured) indicates interaction with the phosphate groups. This has also been concluded from reviews of the interaction of monovalent[134] and divalent[134,135] ions with synthetic polynucleotides and DNA. These results suggest that one cation neutralises two phosphate charges. However, as the phosphate charges are 7 Å apart, and the exact nature of the interaction is not resolved, a detailed picture of this interaction is not yet possible.

Many studies of metal ion interactions with polynucleotides and native DNA have appeared in the literature since the last review[105] on this topic. These studies have been tabulated in terms of the metal ions and polynucleotides studied, and the technique of investigation used (Table II-5). Typical apparent stability constants range from $2.8.10^2$ for the native DNA-Mg^{++} interaction (at 0.2M ionic strength),[1,138] to $2.4.10^6$ for the native DNA-Ag^{+} interaction (at 0.1M ionic strength),[108] and represent the predominantly phosphate and base residue metal ion interactions respectively.

Eichhorn and Shin[18] have provided a unifying explanation of the various effects of a range of metal ion interactions with DNA. They

Metal Ion	Polynucleotide	Technique	Reference
Na^{+},Li^{+},Cs^{+},K^{+},Mg^{++},Ca^{++},Mn^{++},Zn^{++},Co^{++}	DNA,RNA	equilibrium dialysis	131,136-139
Nd^{+++},La^{+++},Co^{++}, Zn^{++},Mg^{++},Ca^{++},Mn^{++}	DNA,RNA	tracer studies	137,140
Mn^{++},Cr^{+++},Fe^{+++},Fe^{++},Ni^{++},Co^{++}	DNA,RNA,poly A, poly I, poly C, poly U	NMR proton relaxation	3,114
Mn^{++},Co^{++}	RNA	NMR ^{31}P relaxation	141
Co^{++}	RNA	NMR proton shift	115
Ag^{+},Hg^{++},Mg^{++}	DNA	viscosity	109,131,142
Ag^{+},Mg^{++},Hg^{++}, Zn^{++}	DNA	UV spectral shifts	109,110,142-145,151
Ag^{+},Cu^{+}	DNA	UV absorbance and difference spectra	107,108,144,146,147
Na^{+},Ba^{++},Ca^{++},Mg^{++},Zn^{++}	DNA,poly A,poly U	conductance	148
Mg^{++},Ca^{++},Ba^{++},Mn^{++},Co^{++},Ni^{++},Zn^{++} Cd^{++},Pb^{++},Fe^{++},Ag^{+},K^{+},Na^{+}	DNA,poly dAT	melting point and other thermal studies	18,35,43,129, 149-153
Fe^{+++}	DNA	infra-red	105
UO_2^{++},Mg^{++}	DNA	direct binding studies	42,106
Li^{+},Na^{+},K^{+},Ca^{++},Mg^{++},Mn^{++}	DNA	electrophoresis	154,155
Fe^{+++}	DNA	kinetics	156
Ag^{+},Mg^{++}	DNA	density gradient ultracentrifugation	110,128
Ag^{+},Cu^{+}	DNA	circular dichroism	157
Ag^{+}	DNA	pH titration	151
Mg^{++}	poly A,poly U	direct binding studies	158

Table II-5. Metal ion interactions with DNA and polynucleotides.

conclude that the change of properties resulting from metal ion interaction with DNA is due to the relative ability of the ions to interact with the charged phosphate groups compared with their ability to form complexes with the base residues. An order of increasing relative ability to interact with the base residues has been established:[18]

$$Mg^{++} < Co^{++} < Ni^{++} < Mn^{++} < Zn^{++} < Cd^{++} < Cu^{++}$$

Studies on the Ag^{+} ion[107,108,110,151] and Hg^{++} ion[142-144] interaction with DNA indicate that they complex strongly with the base residues and produce an ordered structure in which the ions are held between the complementary strands which do not appear to separate significantly. Therefore these ions have been placed to the right of Cu^{++} in the above series.[18]

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CHAPTER III

THE BINDING PARAMETERS

1. INTRODUCTION

The equations discussed in this section were formulated to describe the binding of small molecules and ions to macromolecules (and macro-ions) containing many binding sites. This was necessary because it becomes increasingly difficult to determine experimentally the stability constants for more than a few sites on macromolecules. The theory of multiple equilibria attempts to reduce the number of parameters required to describe such complex equilibrium interactions and is particularly successful when many of the binding sites are identical.

Two fundamental parameters can be deduced from such interactions by this technique: the stability constants for groups of identical sites and the number of interacting sites of each group on the macromolecule. This topic has been extensively reviewed by Edsall and Wyman[1] and by Tanford.[2]

2. THE BINDING PARAMETERS

a. Independent Sites

The system used in this study to represent the macromolecule-small molecule interaction, is the native DNA-Cu^{++} ion interaction, which may be represented by the equation

$$(nP)^{n-} + Cu^{++}.6H_2O \rightleftharpoons [Cu^{++}.(6-x)H_2O - nP]^{n-2} + xH_2O \qquad \text{III.1}$$

where one Cu^{++} ion binding site consists of n base residues.

For interactions of this type, the theory of Klotz[3-5] is applicable

and has been successfully used by Klotz,[3-5] Scatchard,[6-9] and others,[10-17] to determine the number of binding sites and the apparent[18] stability constants for the interactions of small molecules (dyes and metal ions) with polymers (proteins and nucleic acids).

If the association of one Cu^{++} ion with a site on native DNA does not alter the ability of other sites on the same or other molecules to bind Cu^{++} ions, such sites are said to be independent of each other. If these independent sites are also identical in their ability to interact with Cu^{++} ions, they are generally termed equivalent and independent sites.[1,2]

The apparent stability constant (K) for such an interaction may be represented as

$$K = \frac{r}{(n-r)C_f} \qquad \text{III.2}$$

where r represents the number of Cu^{++} ions bound per base residue, n is the total number of sites per base residue, and C_f is the free Cu^{++} ion concentration at equilibrium.

In principle, n should be directly obtained from the maximum value of r at high Cu^{++} ion concentrations. However, a clear plateau region from a plot of r against the total small molecule concentration is rarely observed,[2] and necessitates an extrapolation procedure to determine n.

Equation III.2 has been rearranged by Klotz,[3] to give

$$\frac{1}{r} = \frac{1}{C_f}\left(\frac{1}{K_n}\right) + \frac{1}{n} \qquad \text{III.3}$$

A plot of $\frac{1}{r}$ against $\frac{1}{C_f}$ yields n from the intercept $(\frac{1}{n})$ as $\frac{1}{C_f} \to 0$, and K from the slope $(\frac{1}{Kn})$.

An alternative re-arrangement has been presented by Scatchard,[6]

$$\frac{r}{C_f} = Kn - Kr \qquad \text{III.4}$$

and from a plot of $\frac{r}{C_f}$ against r, yields n from the intercept as $\frac{r}{C_f} \to 0$, and K from the slope (-K). The Scatchard plot is generally used in preference to the Klotz plot for several reasons.

(i) Experimental values are more evenly spaced graphically towards the limiting condition $\frac{1}{C_f} \to 0$.[1]

(ii) Curvature of the Scatchard plot can be readily interpreted in terms of interactions between sites, or by the presence of more than one type of site, or both.

However, in the case of Cu^{++} ion interaction with DNA and similar compounds, the stringent conditions of equivalent and independent sites are not expected to apply. If more than one type of independent site exists (i.e. m sites), the binding equation can be expressed[1] as

$$\frac{r}{C_f} = \sum_{i=1}^{m} \frac{n_i K_i}{1 + K_i C_f} \qquad \text{III.5}$$

Therefore,

$$\lim_{C_f \to 0} \frac{r}{C_f} = \sum_{i=1}^{m} n_i K_i \qquad \text{III.5a}$$

and

$$\lim_{C_f \to \infty} r = \sum_{i=1}^{m} n_i = n \qquad \text{III.5b}$$

Hence, for two types of independent sites,

$$\frac{r}{C_f} = \frac{n_1 K_1}{1 + K_1 C_f} + \frac{n_2 K_2}{1 + K_2 C_f} \qquad \text{III.6}$$

and from a Scatchard plot of $\frac{r}{C_f}$ against r,

$$\lim_{C_f \to 0} \frac{r}{C_f} = n_1 K_1 + n_2 K_2 \qquad \text{III.6a}$$

and

$$\lim_{C_f \to \infty} r = n_1 + n_2 \qquad \text{III.6b}$$

enabling the parameters n_1, n_2, K_1, K_2 to be determined by solving four simultaneous equations,[11] (equations III.6a, III.6b and two sets of experimental values of r and C_f in equation III.6), or by graphical analysis (see Sect. IV.5).

b. Interacting Sites

In many macromolecules it is probable that the interaction of one site with a small ion or molecule will produce either a co-operative or an anti-co-operative effect for subsequent additions to neighbouring sites because of stereochemical or conformational changes. If only one type of site is present, the variation of the apparent stability constant (K) with the extent of the interaction can be taken into account.[2]

$$\frac{r}{C_f} = K.e^{-\beta(r)}\ (n-r) = K_o\ (n-r) \qquad \text{III.7}$$

Thus,

$$K_o = K.e^{-\phi(r)} \qquad \text{III.7a}$$

where $\phi(r)$ is a function of the extent of binding, and K_o is the intrinsic stability constant, the stability constant at r = 0 where interaction between sites (as a result of binding), has not occurred.

If interaction occurs between different types of sites,

$$\frac{r}{C_f} = \sum_{i=1}^{m} \frac{n_i K_o^{(i)}.e^{-\phi_i(r)}}{1 + K_o^{(i)}.e^{-\phi_i(r)}.C_f} \qquad \text{III.8}$$

where $\phi_i(r)$ may be different functions of r.

For the case of Cu^{++} ion interactions with DNA or charged polynucleotides the interaction between different sites is expected to be largely electrostatic in nature. Therefore, the inter-site interaction parameter, $\phi(r)$, is a function of charge $\phi(\bar{Z})$, where $\bar{Z}$ is the average charge of the DNA molecule. For electrostatic interactions the intrinsic stability constant of a site is that due to the interaction, independent of the potential of the macro-ion (i.e. when $\bar{Z} = 0$). Therefore, electrostatic interactions can be allowed for by equations analogous with equations III.7, III.7a and III.8. For one type of site with an electrostatic interaction,

$$\frac{r}{C_f} = K_o.e^{-\phi(\bar{Z})}(n-r) = K(n-r) \qquad \text{III.9}$$

Thus

$$K = K_o.e^{-\phi(\bar{Z})} \qquad \text{III.9a}$$

where K_o and n are obtained from the slope and intercept as before (see

discussion of equation III.4). For more than one type of site involving electrostatic interaction,

$$\frac{r}{C_f} = \sum_{i=1}^{m} \frac{n_i K_o^{(i)} e^{-\phi_i(\bar{Z})}}{1 + K_o^{(i)} e^{-\phi_i(\bar{Z})} . C_f} \qquad \text{III.10}$$

and analogous with equations III.5a,

$$\lim_{C_f \to 0} \frac{r}{C_f} = \sum_{i=1}^{m} n_i K_o^{(i)} e^{-\phi_i(\bar{Z})} \qquad \text{III.10a}$$

It is important to note here that under these conditions of electrostatic interaction between more than one type of site,

$$\lim_{C_f \to \infty} r = \sum_{i=1}^{m} n_i = n \qquad \text{III.5a}$$

which is the same as the limiting condition for binding to more than one type of independent site (equation III.5). Therefore, the interpretation of binding data by either equations III.5, III.8 or III.10 will yield the same value of n, the total number of Cu^{++} ion binding sites per base residue of DNA.

Several methods have been presented in the literature for the evaluation of $\phi(\bar{Z})$ and these are discussed in the following sections.

3. THE ELECTROSTATIC FUNCTION

a. Theoretical Macro-ion Potential

Tanford[2] has presented a derivation of the equation for the electrostatic interaction parameter

$$\phi(\bar{Z}) = 2wz_i\bar{Z} \qquad \text{III.11}$$

by using a general equation for the electrostatic free energy of a single macro-ion (W_{el}).

$$W_{el} = A(\bar{Z})^2 \qquad \text{III.12}$$

where the constant

$$w = \frac{AN}{RT} \qquad \text{III.13}$$

where A is a proportionality factor which depends only on the model used to represent the macro-ion, and z_i is the charge of the interacting ion.

Using the infinitely long cylindrical model for DNA proposed by Hill[19] to compute the potential on the surface of DNA, and hence W_{el} (as $W_{el} = \int \psi.dq$),[20] a solution for $\phi(\bar{Z})$ is obtained,

$$\phi(\bar{Z}) = 33i \qquad \text{III.14}$$

where i is the charge per base residue of DNA. However, such an equation implies impossibly high Cu^{++} ion concentrations close to the DNA surface. This is due essentially to the assumption that the Boltzman distribution $\frac{e\psi}{kT} << 1$ which, as has been previously pointed out,[21,22] is not applicable to highly charged macro-ions. Therefore, other estimates of $\phi(\bar{Z})$ are required.

b. Zeta Potential

A different approach has been used by Ross and Scruggs[23,24] to allow for the electrostatic effect. The effective concentration of Cu^{++} ions, $(C_f)_{eff}$, near the DNA molecule can be given by the expressio

$$(C_f)_{eff} = C_f \cdot e^{-\frac{2e\psi}{kT}} \qquad \text{III.15}$$

Thus, in effect,

$$\phi(\bar{z}) = \frac{2e\psi}{kT} \qquad \text{III.16}$$

That the electrostatic potential (ψ) can be equated to the zeta potential (ζ) at the DNA surface,

$$\psi = \zeta \qquad \text{III.17}$$

has been shown theoretically[25] and has been experimentally confirmed.[26,27]

The zeta potential is given by Henry's Equation[28]

$$\mu = \frac{C.D.\zeta}{\eta} \qquad \text{III.18}$$

where μ is the electrophoretic mobility, D and η are the dielectric constant and viscosity of the solvent, and C is a constant which depends on the size of the macro-ion, its symmetry and the ionic strength, and is given by Gorin's Equation,[29,30]

$$C = \frac{1}{F'(\kappa a)\pi} \qquad \text{III.19}$$

If it is assumed that native DNA can be approximated by a randomly orientated cylinder of radius 8.8 Å,[29] $F'(\kappa a) = 6.58$.[28] For a uniformly charged cylinder randomly orientated with respect to the field, it has been shown[28] that

$$\mu = \frac{ie}{f} \qquad \text{III.20}$$

where i and f are the charge and friction factor per base residue.

Combining equations III.17 to III.20,

$$\psi = \left(\frac{F'(\kappa a)\pi\eta e}{Df}\right) i \text{ ergs/esu} \qquad \text{III.21}$$

Substituting the values $F'(\kappa a) = 6.58$,[28] $\eta = 0.00895$ poise,[31] $D = 78$,[32] (for a 1:1 electrolyte, the dielectric constant is the same as that of water),[32] $f = 2.0.10^{-9}$ gm/sec (an extrapolated value at 5mM ionic strength)[24] at 298°K into equation III.16, yields

$$\phi(\bar{Z}) = 13.4\ i \qquad \text{III.22}$$

Assuming $i = -0.20$ for native DNA in 5mM KNO_3 (see Sect. III.3d), $\phi(\bar{Z}) = -2.7$ in reasonable agreement with the value $\phi(\bar{Z}) = -3.4$ obtained from electrophoretic mobility studies by Mathieson and Matty[33] at pH 6 and 0.1M ionic strength.

It must be emphasised that equation III.22 applies only to native DNA, as several of the parameters vary with the DNA conformation. Other values of the friction factor per base residue $f = 0.70.10^{-9}$ gm/sec[33] and $f = 0.97.10^{-9}$ gm/sec[34] at pH 6 and 5mM ionic strength provide substantially different solutions to equation III.16, $\phi(\bar{Z}) = 27.5\ i$ and $\phi(\bar{Z}) = 38.1\ i$ respectively. As such estimates of the electrostatic effect imply impossibly high Cu^{++} ion concentrations near the DNA surface (see equation III.15) they can not be considered as acceptable descriptions of native DNA.

c. Other Electrostatic Corrections

A correction outlined by Abramson et al.[29] allows for the finite size of the interacting small ions, but suffers from the Debye-Hückel

limitations.

Several theoretical calculations of ion binding to rod-like macro-ions, without making the Debye-Hückel assumption that $\frac{e\psi}{kT} << 1$, have been published.[35-38] The most extensive treatment has been given by Lifson,[38] who considered nearest neighbour interactions as well as the electrostatic potential. Ross and Scruggs[23] have shown that stability constants for mono-valent ion interactions with native DNA are similar (approximately two thirds of the value) to those calculated using an electrostatic correction term derived from the zeta potential, and supports the validity of the zeta potential model used in the present study.

d. Variation of the Phosphate Charge of DNA with Cu^{++} Ion Binding

As no experimental information is available for the variation of DNA phosphate charge with Cu^{++} ion concentration, a model must be established in order to estimate this variation.

The DNA charge per base residue, i (i.e. the charge of each phosphate group), as determined by membrane potential and electrophoretic mobility studies[37] over a range of pH and ionic strengths normally employed in DNA studies, is generally in the range -0.2 to -0.4. At pH 6 and 5mM ionic strength, the conditions employed in this study, the data of Mathieson and Matty[33] suggests that i = -0.20. This value then provides a boundary condition for the phosphate charge in the absence of Cu^{++} ions (i.e. when r = 0).

As no other experimental boundary condition is available, this value must be estimated. If one Cu^{++} ion is bound per base residue of DNA (r = 1.0) the phosphate charge is neutralised, and the maximum value of i = +1.0. If a similar DNA counter-ion screening effect occurs for this positive charge as for the negative phosphate charge, then an effective charge at r = 1.0 is i = +0.20.

Assuming a linear dependence of charge (i) with Cu^{++} ions bound to DNA (r) between these two boundary conditions, the variation of i may be expressed as

$$i = 0.4\ r - 0.2 \qquad \text{III.23}$$

Therefore, substituting for i in equation III.22, and combining with equation III.9, produces an equation which describes the binding of Cu^{++} ions to one type of site on native DNA, and allows for the electrostatic interaction between these sites.

$$\frac{r}{C_f . e^{-13.4(0.4\ r - 0.2)}} = K_o n - K_o r \qquad \text{III.24}$$

is the expression used for calculation of K_o for the interaction of Cu^{++} ions with native DNA (Chapt. V).

Although equation III.23 is based on several assumptions, it is nevertheless considered to be a significant improvement on those studies which, by ignoring the counter-ion screening effect on the phosphate and metal ion charges, make the misleading approximations that i = -1.0,[15] or that each bound cation reduces the effective negative charge of DNA by its own charge.[15,40]

4. ALTERNATIVE DETERMINATION OF K_o

Assuming that the interaction between the Cu^{++} ion binding sites of DNA and polynucleotides is essentially electrostatic, then the general equation for the interaction (equation III.11) may be used in equations III.9 or III.10. Therefore, for one type of Cu^{++} ion binding site,

$$\frac{r}{C_f} = K_o.e^{-2wz_i\bar{Z}(n-r)} \qquad \text{III.25}$$

which can be re-arranged to

$$\log[\frac{r}{C_f(n-r)}] = \log K_o - w'i \qquad \text{III.26}$$

Thus, a plot of $\log[\frac{r}{C_f(n-r)}]$ against i yields the apparent intrinsic stability constant K_o (when i = 0), and -w' (from the slope).

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CHAPTER IV

THE INTERACTION OF Cu^{++} IONS WITH MONO-, OLIGO- AND POLY-NUCLEOTIDES

1. INTRODUCTION

To evaluate the parameters n and K (by means of the equations discussed in Chapt. III) for the interaction of Cu^{++} ions with nucleotidic materials, the fundamental experimental quantity C_f (the free Cu^{++} ion concentration at equilibrium), must be determined. This quantity has generally been determined in the past from equilibrium dialysis studies, the Cu^{++} ion concentration subsequently being determined by titrating with a complexing ion, polarographic analysis, or by spectrophotometric means after complexing with another species. However, the technique is complicated by the Donnan equilibrium and the difficulty of removing all traces of plasticiser from the membrane (this may be serious as DNA interacts with such materials[1]). Furthermore, the amount of DNA and Cu^{++} bound to the membrane is expected to vary with the concentration of each species, and as this is difficult to overcome or predict accurately, large errors from this source may be introduced. In the last few years the technique of gel filtration has been successfully used in various ways (see Sect. V.2) to overcome the difficulties mentioned above.

Until very recently, reversible Cu^{++} ion electrodes were limited by their response characteristics and the conditions necessary for their use,[2] and have rarely been used for dilute Cu^{++} ion systems.[3] However, the advent of solid state reversible Cu^{++} ion electrodes[4-6] which are convenient to use and have acceptable response characteristics for dilute Cu^{++} ion systems, has provided a direct means for measuring

C_f for a wide range of Cu^{++} ion systems. Membrane Cu^{++} ion electrodes are also available,[4-6] but are substantially inferior to the solid state electrode for two reasons. Their response is seriously limited by the presence of other ions (including H^+ and OH^- ions) and the stability of the response is poor,[1,7] sometimes being so erratic that any results are unuseable.[7] For these reasons the membrane Cu^{++} ion electrode has been superseded by the solid state Cu^{++} ion electrode.[4]

It is of interest to mention here that direct Cu^{++} ion studies can only be carried out in a limited pH range as the solubility product of $Cu(OH)_2$ forms an upper limit of pH 6 for a 10^{-3}M Cu^{++} ion solution.

2. THE SOLID STATE REVERSIBLE Cu^{++} ION ACTIVITY ELECTRODE

a. Response

The response of the electrode to Cu^{++} ion solutions in 5mM KNO_3 was found to depend on the stirring rate, the time of standing after stirring, and on the ambient light intensity. The dependence of the response on the stirring rate was not due to non-equilibration, as identical response characteristics could be observed for the system if left for several days. To completely overcome this effect, the potenti was recorded for the non-stirred solution five minutes after switching off the stirrer. This was found to produce a reproducible response ($\pm$ 0.4 mV) for the full range of Cu^{++} ion concentrations employed (10^{-6}M to 10^{-3}M), irrespective of the time of stirring. For details of the apparatus and method used for recording potentials, see Sect.

VIII.4.

Fluctuations of the light intensity caused changes of the electrode response up to 1-2 mV. This effect was eliminated by completely sealing off all windows in the room against extraneous light, and working in a constant light source provided by eighteen 40W commercial fluorescent tubes. Under these conditions, a reproducible response could be obtained for both day and night-time studies.

The stability of the response was $\pm$ 0.2 mV, and was quite adequate as the reading error of the expanded scale potentiometer was $\pm$ 0.1 mV. The Cu^{++} ion electrode was completely shielded from electrical interference (apart from the crystal at the base of the electrode), to ensure such a stable response.

To test the effect of the introduction of saturated KCl from the calomel electrode, the electrodes were allowed to stand in a stirred Cu^{++} solution and readings were taken one hour and twenty four hours after introducing the electrodes into the solution. The two potentials were identical (within the reproducibility range of $\pm$ 0.4 mV for individual readings) with respect to a standard solution, and confirmed that any changes of ionic strength were negligible and insufficient to affect the Cu^{++} activity at 5mM ionic strength. This test also indicated that large potential drifts did not occur in such a time period. Therefore, in the eight hours required for a Cu^{++} ion binding study, large drifts were not expected.

b. Calibration

The electrode was expected to provide a Nernst type response,

$$E = E_o - \frac{RT}{2F} \ln a_{Cu^{++}} \qquad \text{IV.1}$$

For the purposes of calibration, the most dilute Cu^{++} ion solution used was 1.10^{-6}M as contaminants from the air and glassware can significantly influence the response of more dilute solutions. Cu^{++} ion solutions less than 1.10^{-4}M were diluted from a stock solution on the day of use to minimise the adsorption of Cu^{++} ions to glassware.[1,8] Solutions, buffered with respect to Cu^{++} ions were used to estimate the electrode response at extremely low Cu^{++} ion activities. Cu^{++} ion activities were calculated from the data of Kielland.[2]

By re-writing equation III.1 as

$$E = E_o - \frac{2.303\ RT}{2F} \log a_{Cu^{++}} \qquad \text{IV.2}$$

it can be seen that the theoretical slope of such a response is $\frac{2.303\ RT}{nF}$, which is 0.02958 at 25°C. A calibration of the electrode response (Fig. IV-1) indicates a theoretical Nernst response in the Cu^{++} ion activity range $\sim 1.10^{-7}$M to $\sim 1.10^{-3}$M, as the slope of the line in this range is ~ 0.029. In the unbuffered Cu^{++} ion activity range fro 10^{-3}M to less than 10^{-5}M, the least squares slope of the line is 0.0295 volts per ten-fold change of Cu^{++} ion activity, in excellent agreement with that expected.

As all work with the electrode was conducted at a constant ionic strength, the electrode could be calibrated directly in terms of Cu^{++}

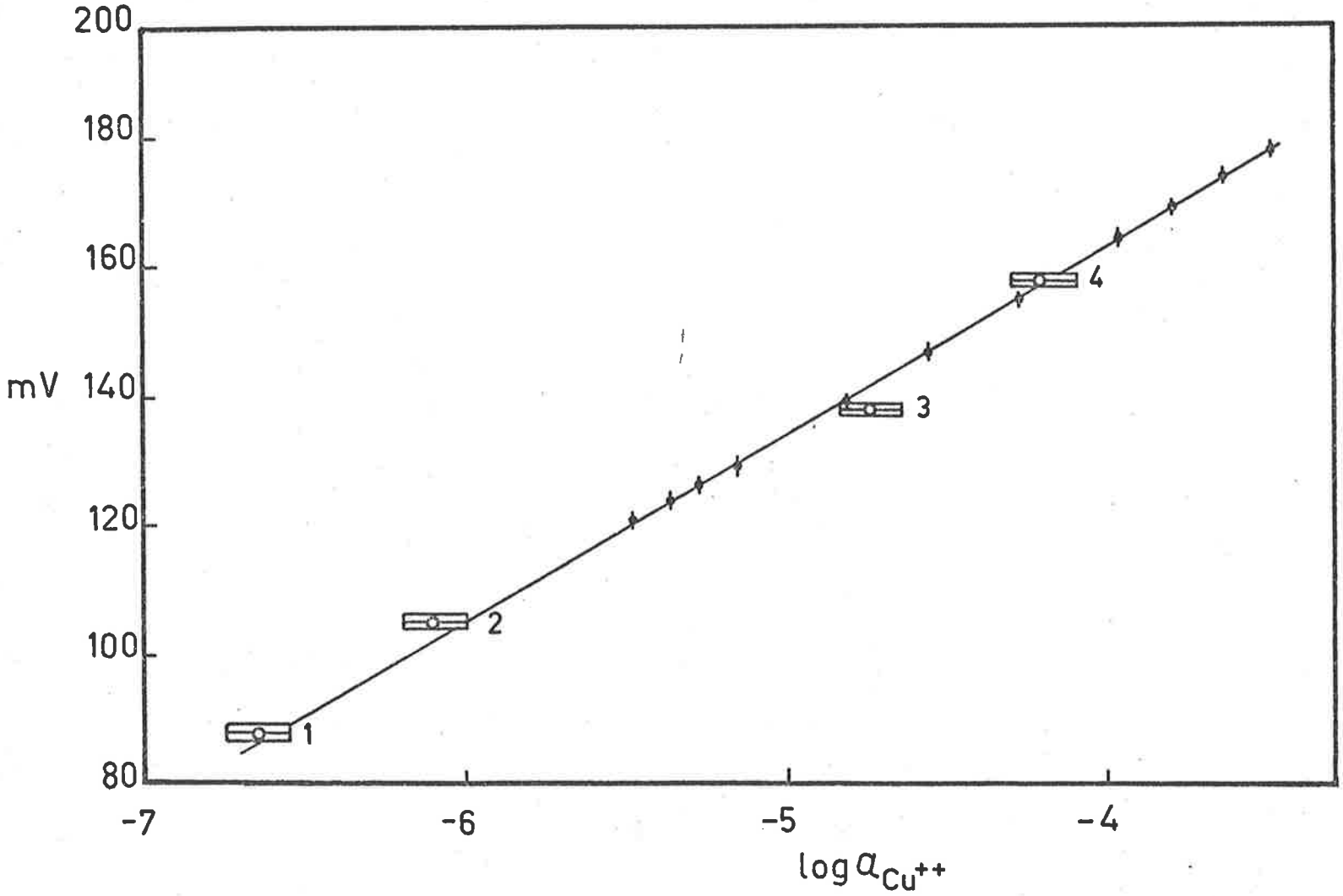


Fig. IV-1. Calibration of the Cu^{++} ion activity electrode at 25°C.

1,2 buffered by oxalic acid.

3,4 buffered by glycine.

ion concentration. Calibrations were carried out at a Cu^{++} ion concentration on each side of the range expected for each experiment, both before and after all binding studies.

c. Comparison with Literature Stability Constants

To confirm that the electrode was in fact responding to Cu^{++} ions, and that the same behaviour occurred in the presence of Cu^{++} ligands, a test binding curve was undertaken with malonic acid, as the stability constant with Cu^{++} ions is similar to that expected with native DNA. For the purpose of confirming the validity of the technique as applied to DNA and associated compounds, the conditions employed were those under which the electrode was to be subsequently used, namely 5mM ionic strength solutions at 25.0°C.

Cu^{++} ions were titrated into a malonic acid solution (both solutions at pH 6.4) using calibrated micro-syringes. To test the reversibility of the system, malonic acid was also similarly titrated into a Cu^{++} ion solution. The results have been plotted (Fig. IV-2) and evaluated in terms of equation III.4, and indicate that the apparent stability constant for the formation of a 1:1 $malonate^{--}$-Cu^{++} complex under these conditions is $\log K_{app} = 5.45 \pm 0.04$. From estimates of activity coefficients,[2] $\log K \sim 5.64$, which agrees well with values determined by other authors using different techniques under slightly different conditions (Table IV-1), and indicates the validity of the electrode as a technique for the determination of Cu^{++} ion stability constants.

log K	I	T(^{o}C)	Technique	Ref.
5.60	extrapolated to infinite dilution	25	conductance	9
5.86	0.02	25	conductance	10
5.54	0.035	25	pH titration	11
5.64	0.005	25	Cu^{++} ion electrode	this study

Table IV-1. Stability constants for the formation of a 1:1 malonate^{--}-Cu^{++} Complex.

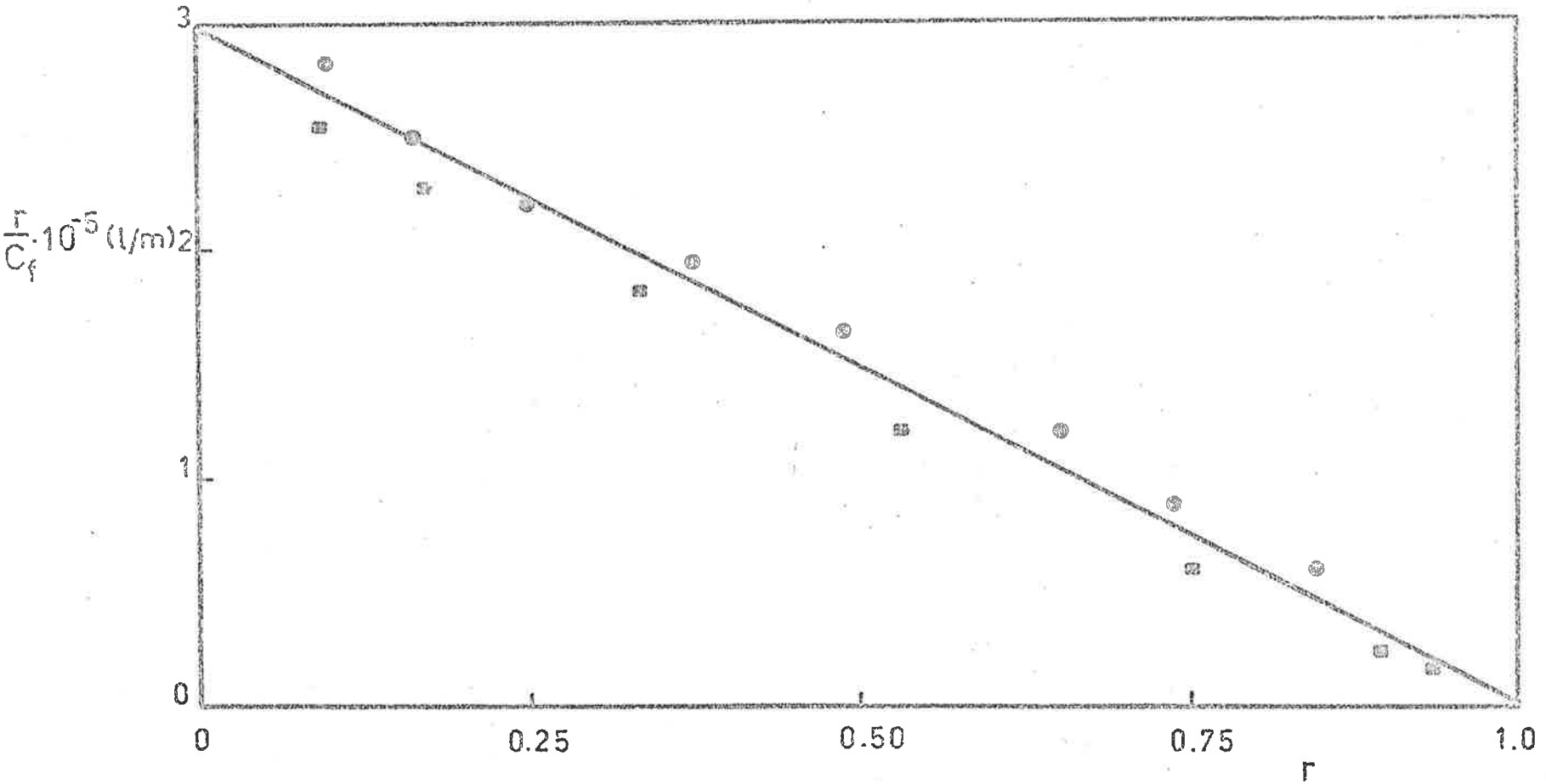


Fig. IV-2. Scatchard plot of the malonate-Cu^{++} interaction at pH 6.4, 5mM KNO_3. 25°C.

● Malonate into Cu^{++}.

■ Cu^{++} into malonate.

3. EXPERIMENTAL PROCEDURES

a. Cu^{++} Ion Potentiometric Studies

All nucleotide solutions were dissolved in 5mM KNO_3 to maintain a constant ionic strength for comparison with similar studies on DNA, and were adjusted to the appropriate pH (see Sect. IV.5) by addition of 0.1M NaOH to 100 or 250 ml stock solutions. Small portions were then transferred for pH measurements, to avoid contamination of the stock solution by KCl from the saturated KCl calomel electrode. The pH of all mono-, oligo- and poly-nucleotide solutions was found to be essentially constant during the Cu^{++} ion binding studies, thereby eliminating the need for a pH buffer which would have increased the difficulty of interpreting the results, as more potential Cu^{++} ion binding sites would have been present.

Immediately prior to all binding studies, the electrode was calibrated with two Cu^{++} ion solutions of known concentration. This procedure has been described in detail in Sect. VIII.4. One solution was that used in the titration, and normally provided the higher Cu^{++} ion concentration point (cal. 1). The more dilute solution was diluted on the day required from a separate stock solution, and provided a Cu^{++} ion concentration calibration point (cal. 2) at the other extreme of the Cu^{++} ion concentration range to be observed in the binding study, thereby checking the electrode response as well as providing a check on the Cu^{++} ion concentration used in the titration.

The nucleotide solution of known concentration (determined

spectrophotometrically; see Table VIII-3) was weighed into the titrating vessel (Fig. VIII-2) and titrated with known volumes of a Cu^{++} ion solution of known concentration, recording the cell potential after each addition of Cu^{++} ion (see Sect. VIII.4). After each binding study had been completed, the electrode was re-calibrated (cal. 3) using the Cu^{++} ion solution used for titrating. As the final Cu^{++} ion solution of the titrated nucleotide solution was usually similar in concentration to that used for cal. 3, this provided an accurate comparison.

All Cu^{++} binding studies were carried out by titrating Cu^{++} ions into the nucleotide solution, and then reversing this procedure to test the reversibility of the interaction.

b. Hyperchromicity Studies

The nucleotidic solution (2-3 ml) of known concentration was weighed into 1 cm spectrophotometer cells. The concentration was such that the absorbance of the solution itself was near 0.4 at the wavelength of maximum absorption. Small volumes of concentrated Cu^{++} ion solutions were added and the cell re-weighed. The quantity R, the total number of moles of Cu^{++} ion present per mole of base residue, was calculated from the weights and concentrations of both solutions used, assuming that the density of each solution was equal to that of water.

Knowing the dilution factor of the nucleotidic solution and the

ultra-violet spectrum of the nucleotidic solution in the absence of Cu^{++} ions, the expected absorbance at each wavelength (A_t) was calculated assuming that the absorbance was unchanged by the presence of Cu^{++} ions. From the actual absorbance of the nucleotidic-Cu^{++} solution at various wavelengths (A_{ob}), the hyperchromicity (H) was calculated at each wavelength studied in the same way as that used for the calculation of the hyperchromicity of denatured DNA.

$$H = \left(\frac{A_{ob} - A_t}{A_t}\right).100 \qquad \text{IV.3}$$

4. CALCULATION OF C_f AND r

Using the final calibration point (cal. 3) as the most accurate calibration point, a graph similar to Fig. IV-1 (but in terms of Cu^{++} ion concentration), was constructed for each binding study using the theoretical slope of 29.58 mV at 25°C, as confirmed earlier (Sect. IV.2). Any small drift of potential (usually less than 2 mV) determined by the calibrations before and after the titration (cal. 1 and cal. 3) was compensated for in all titration potential measurements by correcting the potential relative to the final calibration (cal. 3) to minimise errors. The other initial calibration point (cal. 2) was always within $\pm$ 0.8 mV of the theoretical response.

Cu^{++} ion concentrations (C_f) corresponding to the titration potentials were read directly from the calibration plot of cell potential against the logarithm of the free Cu^{++} ion concentration.

Knowing the total volume of the solution, the number of moles of Cu^{++} ions free at equilibrium can be determined. Therefore, by subtracting from the total number of moles of Cu^{++} ions added, the number of moles of Cu^{++} ions bound to the nucleotides (Cu_B^{++}) is known. As the density of dilute nucleotide solutions is effectively that of water,[12] the volume and hence the number of moles of nucleotide present is known (M_p). Therefore, the other fundamental binding parameter r (the number of moles of Cu^{++} ion bound per nucleotide) can be calculated as

$$r = \frac{Cu_B^{++}}{M_p} \qquad \text{IV.4}$$

Similar calculations are used for the reverse titration procedure. As C_f and r are known for a range of Cu^{++} ion concentrations, a Scatchard plot may be drawn for the interaction of Cu^{++} ions with each of the mono-nucleotides. As only one type of Cu^{++} binding site is expected and no interaction, electrostatic or otherwise, occurs between these sites, the plots may be interpreted in terms of equation III.4. The interaction of Cu^{++} ions with polynucleotides is more involved, as more than one type of Cu^{++} ion binding site may exist, as well as electrostatic interactions between the sites. However, such interactions are still readily interpreted from the same Scatchard plot of $\frac{r}{C_f}$ against r.

5. GRAPHICAL ANALYSIS OF SCATCHARD PLOTS

Fig. IV-3 represents a typical binding curve for the interaction of Cu^{++} ions with two types of binding sites on DNA or polynucleotides. The two slopes of the binding curve and their corresponding intercepts on the X-axis provide approximate estimates of the quantities K_1, K_2, n_1, n_2 (see Sect. III.2), where the subscripts 1 and 2 refer to the stronger and weaker of the two Cu^{++} ion interactions. However, this procedure is often subjective, particularly if the two slopes are not well defined. For this reason the more objective method presented by Danchin and Gueron[13] has been modified and used to analyse all Scatchard plots indicating the presence of two or more types of Cu^{++} ion interacting sites.

The points A, B and D of Fig. IV-3 are obtained directly by extrapolation of the linear portions of the binding curve fitted by the method of least squares of a polynomial (for computer program, see Appendix 1) to the experimental points. As $A \sim n_2K_2$ (see equation III.6a) is a good approximation if $K_1 > 10K_2$, and $B = n_2K_2 + n_1K_1$, then $B - A = B' = n_1K_1$. Using the experimental slope of the stronger interaction, C is obtained as the intercept on the X-axis and represents n_1 (see equation III.6b). Therefore, as $D = n_1 + n_2$, $D - C = D' = n_2$. The slope of $FD'(K_2)$ is determined by E, the intersection of B'C with the midpoint of OX_T, where X_T is a point on the binding curve. The intercept F is a more accurate determination than A of the value n_2K_2.

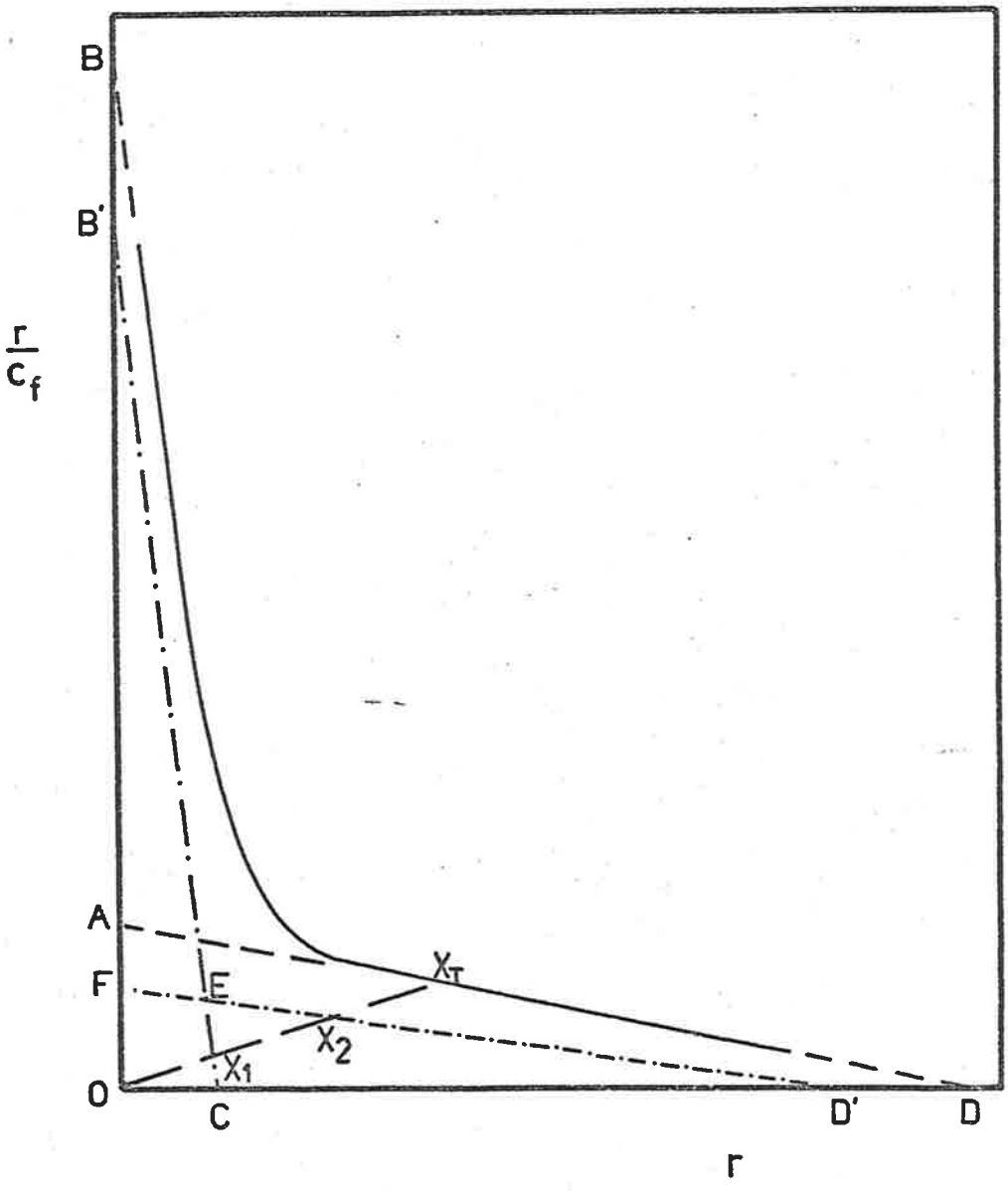


Fig. IV-3. Analysis of a typical Scatchard plot indicating two types of sites.

To appreciate this procedure, it must be understood that B'C and FD' represent the stronger and weaker Cu^{++} ion binding components of the total binding curve. All points on the slope of OX_T are at the same equilibrium free Cu^{++} ion concentrations, as the slope of $OX_T = \frac{1}{C_f}$. Therefore, at any particular C_f, $OX_1 + OX_2 = OX_T$, as the sum of both the r and $\frac{r}{C_f}$ components of the two types of binding must equal the total binding. Therefore, when $OX_1 = OX_2$, $OE = \frac{1}{2}(OX_T)$, and illustrates why E, the intersection of the two binding components, is a convenient point to determine the slope of FD'.

If more than two types of Cu^{++} ion binding sites are present, $OX_1 + OX_2 \neq OX_T$ over the whole binding curve, providing a completely objective basis for determining if more than two types of Cu^{++} ion binding sites are present.

6. RESULTS AND DISCUSSION

a. Mononucleotides

The mononucleotides were adjusted to a pH intermediate between the pK_a of the secondary phosphate group and the pK_a of the next most acidic group. The pK_a values and the pH of the nucleotide solutions used are listed in Table IV-2. The Cu^{++} ion solutions were adjusted to the pH of the nucleotide solution under study. It was necessary to adjust the pH of these solutions in order to study Cu^{++} ion binding to nucleotides containing single-charged phosphate groups, as in DNA.

Binding studies of Cu^{++} ions to these mononucleotides are represente

Monomer	pK_a Secondary Phosphate	pK_a Next Acidic Group	Ref.	pH	n	K
GMP	~5.9	~2.3	14	4.5	1.0 ± 0.1	$(1.0 \pm 0.2).10^{3}$
dGMP	6.4	2.9	15,16	4.7	0.97 ± 0.09	$(1.7 \pm 0.2).10^{3}$
dGMP*	6.4	2.9	15,16	4.5	1.0 assumed	$(0.8 \pm 0.2).10^{3}$
dAMP	6.4	4.4	15	5.4	1.0 assumed	300 ± 30
dCMP	6.6	4.6	15,16	5.6	1.0 assumed	220 ± 20
TMP	6.5	1.6	15,16	5.0	1.0 assumed	50 ± 10
ortho-phosphate	7.1	2.1	17	4.6	1.0 assumed	32 ± 10
guanosine	-	1.6	18	5.5	-	-
deoxy-guanosine	-	1.6**	-	5.3	-	-

Table IV-2. Binding parameters for the interaction of Cu^{++} ions with monomers in 5mM KNO_3 at 25°C.

* in 0.15M KNO_3

** assumed equal to guanosine

in the form of Scatchard plots in Fig. IV-4. The binding parameters n and K have been calculated in terms equation III.4 from a least squares plot of the data, and have been listed in Table IV-2. Electrostatic interaction parameters are not necessary for these studies as the monomers do not affect the binding of Cu^{++} ions to each other.

To obtain experimental points at high r values, high monomer and Cu^{++} ion concentrations were required, resulting in small increases (up to 2mM) of the ionic strength. As the Cu^{++} ion electrode responds to Cu^{++} ion activity, but was calibrated in terms of Cu^{++} ion concentrations, small activity coefficient corrections[2] were necessary at these higher ionic strengths.

Apart from the Cu^{++} ion interaction with GMP and dGMP, complete Scatchard plots of the other nucleotides could not be obtained without using extremely concentrated solutions, and these were prevented by the precipitation of nucleotide-Cu^{++} complexes. All earlier studies of Cu^{++} ion interaction with the mononucleotides (Table II-4) indicate a 1:1 complex (n = 1.0). As this has been confirmed for the Cu^{++} complexes of GMP and dGMP (Fig. IV-4), the stoichiometry of the Cu^{++} complexes with the other mononucleotides (and orthophosphate) has been assumed to be 1:1.

Many attempts failed to detect any significant interaction of Cu^{++} ions with either guanosine or deoxy-guanosine. As there is clear evidence of a complex with GMP and dGMP, a comparison of these studies

Fig. IV-4. Scatchard plot for the interaction of Cu^{++} ions with mono-nucleotides in 5mM KNO_3 (except where otherwise indicated *) at 25°C.

● dGMP, pH 4.7

X dGMP, pH 4.5, 0.15M KNO_3*

Δ GMP, pH 4.5

. dAMP, pH 5.4 (inset)

0 dCMP, pH 5.6 (inset)

■ TMP, pH 5.0 (inset)

▲ $H_2PO_4^-$, pH 4.5 (inset)

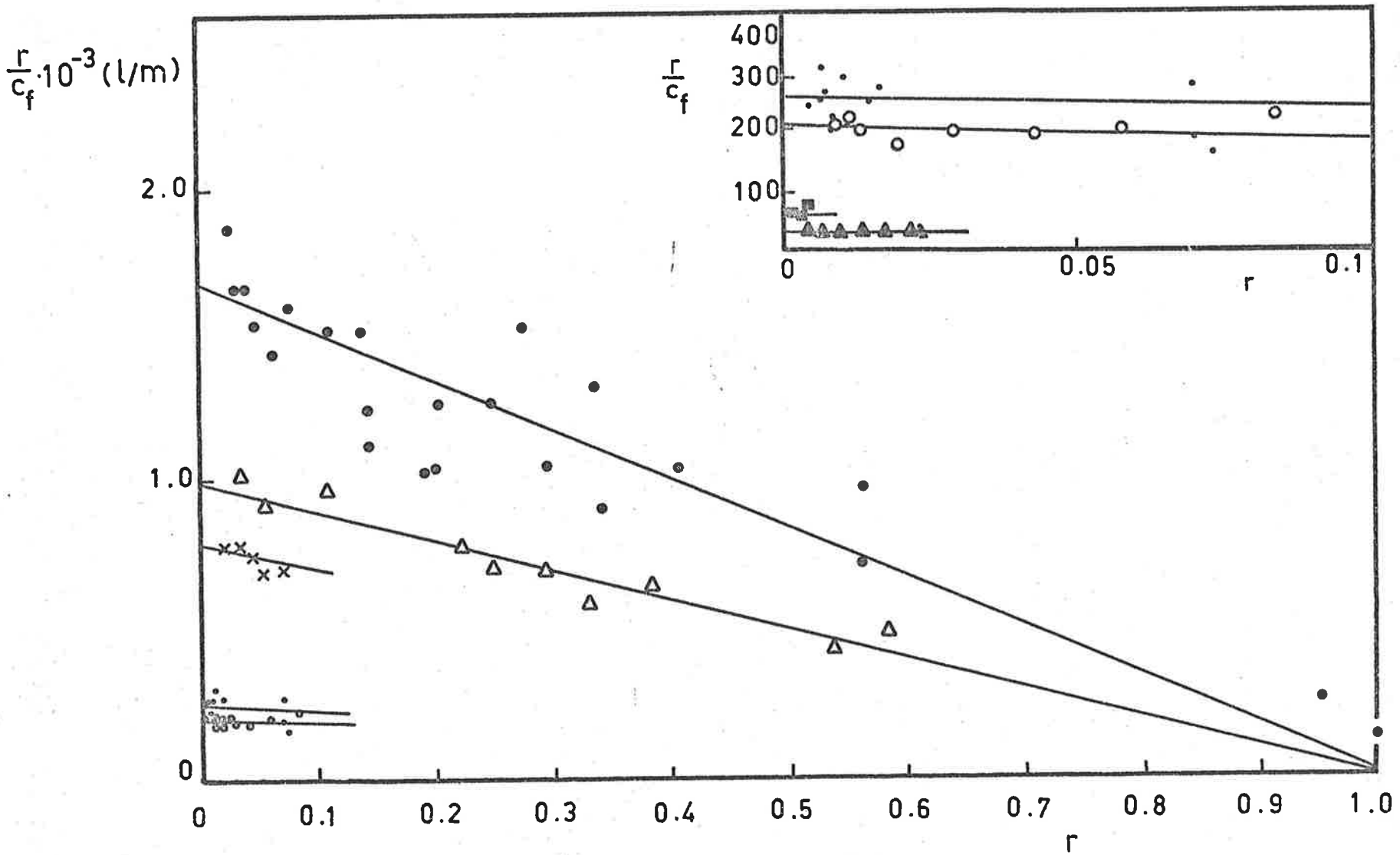

$\frac{r}{c_f} \cdot 10^{-3}$ (l/m)
2.0
1.0
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
r
$\frac{r}{c_f}$
400
300
200
100
0
0.05
0.1
r

Fig. IV-4.

implicates the phosphate group of the nucleotides in a complex with Cu^{++}, thereby providing further support for the guanine residue-Cu^{++}-phosphate chelate complex indicated by earlier studies (see Fig. II-5 and Sect. II.3a). Such a complex has recently been directly indicated[19] by NMR studies of AMP. A similar complex involving the N_3 position is expected for dCMP in contrast to the evidence suggesting the role of the N_7 groups in dGMP and dAMP (see Sect. II.3a). The inability to detect a complex with guanosine contrasts with the studies by Tu and Friederich[20] who detected what appeared to be a relatively strong complex. However, it appears that some doubt may be cast on these results[20] as the electrode used was extremely erratic in response and no interaction was detected on several occasions.[7]

The apparent stability constants are in the order $dGMP^- > GMP^- > dAMP^- > dCMP^- > TMP^-$, in agreement with the order for the stability of Cu^{++} ion interactions with monomers detected by other techniques.[21-24] However, no other studies have actually determined stability constants for these interactions, apart from the AMP^{2-}-Cu^{++} complex,[25] which is not directly comparable with the AMP^--Cu^{++} complex in the present study. The relative stabilities of the Cu^{++} complexes of dGMP($K = 1.7.10^3$) and GMP($K = 1.0.10^3$) supports a previous observation that the deoxy- series binds Cu^{++} ions more strongly than the ribo- series.[21] The important implication is that it appears that the presence of the 2'OH group in GMP does not enhance its stability

with respect to the Cu^{++} ion interaction with dGMP. In fact, the stability of GMP-Cu^{++} is slightly reduced but clearly of a similar order of magnitude to the dGMP-Cu^{++} complex. In view of the large errors associated with the determination of these stability constants, it may be concluded that the presence or absence of the 2'OH group appears to have little effect on the affinity of Cu^{++} ions for GMP and dGMP. Similar studies were not attempted with AMP and CMP as the order of the stability constants for the Cu^{++} ion interactions with these compounds are known to be similar to those for dAMP and dCMP,[21] the interactions of which border on the limit of detection by the method used.

Several other significant factors emerge from these values. The $dGMP^{-}$-Cu^{++} complex is significantly more thermodynamically stable than complexes of the other DNA monomer constituents, providing one explanation of the clearly established preferential interaction of Cu^{++} ions with the guanine residues of DNA (see Sects. II.2 and II.3). Also, the similar values of the apparent stability constants for the TMP^{-}-Cu^{++} and $H_2PO_4^{-}$-Cu^{++} complexes suggests that the TMP^{-}-Cu^{++} complex involves an exclusively phosphate interaction. This has also been concluded from proton and ^{31}P NMR relaxation studies.[26]

The apparent stability constant for the formation of a $dGMP^{-}$-Cu^{++} complex is halved by increasing the ionic strength from 0.005 to 0.15M. A similar decrease can be predicted for the $dAMP^{-}$-Cu^{++} complex on the basis of interpolation of the data collated by Phillips.[26a] The values

of K for the $dGMP^{-}$-Cu^{++} complex at the two ionic strengths used are particularly significant when compared with similar values involving the native DNA-Cu^{++} complex. These results are discussed further in that context in Sect. V.4a.

b. Oligo- and Poly-Nucleotides

(i) Direct Cu^{++} Ion Binding Studies

All polynucleotides studied were adjusted to a specific pH at which the structure and conformation have previously been investigated To eliminate any 'end effects' due to the presence of double charged phosphate groups on the end of the oligonucleotides, all such solutions were adjusted to the pH of the corresponding mononucleotides.

Scatchard plots of the Cu^{++} ion interaction with these oligo- and poly-nucleotides are illustrated in Figs. IV-5,6,7,8. As they are non-linear plots, it is necessary to determine if interaction (essentially electrostatic in the case of charged polynucleotides) between the sites accounts for the curvature, or whether it is due to the presence of more than one type of site. Poly I is used as an example as the Cu^{++} ion binding data for this interaction shows the least curvature (Fig. IV-5) of the polynucleotides studied. Therefore, if this small curvature is not eliminated by allowing for an electrostatic interaction, the presence of more than one type of site is indicated.

Fig. IV-5. Scatchard plots for the interaction of Cu^{++} ions with poly C and poly I in 5mM KNO_3 at 25°C.

● poly I, pH 5.9 (inset)

■ poly C, pH 5.6 (inset)

Δ poly C, pH 4.2

□ poly C, pH 6.0, I = 0.15M

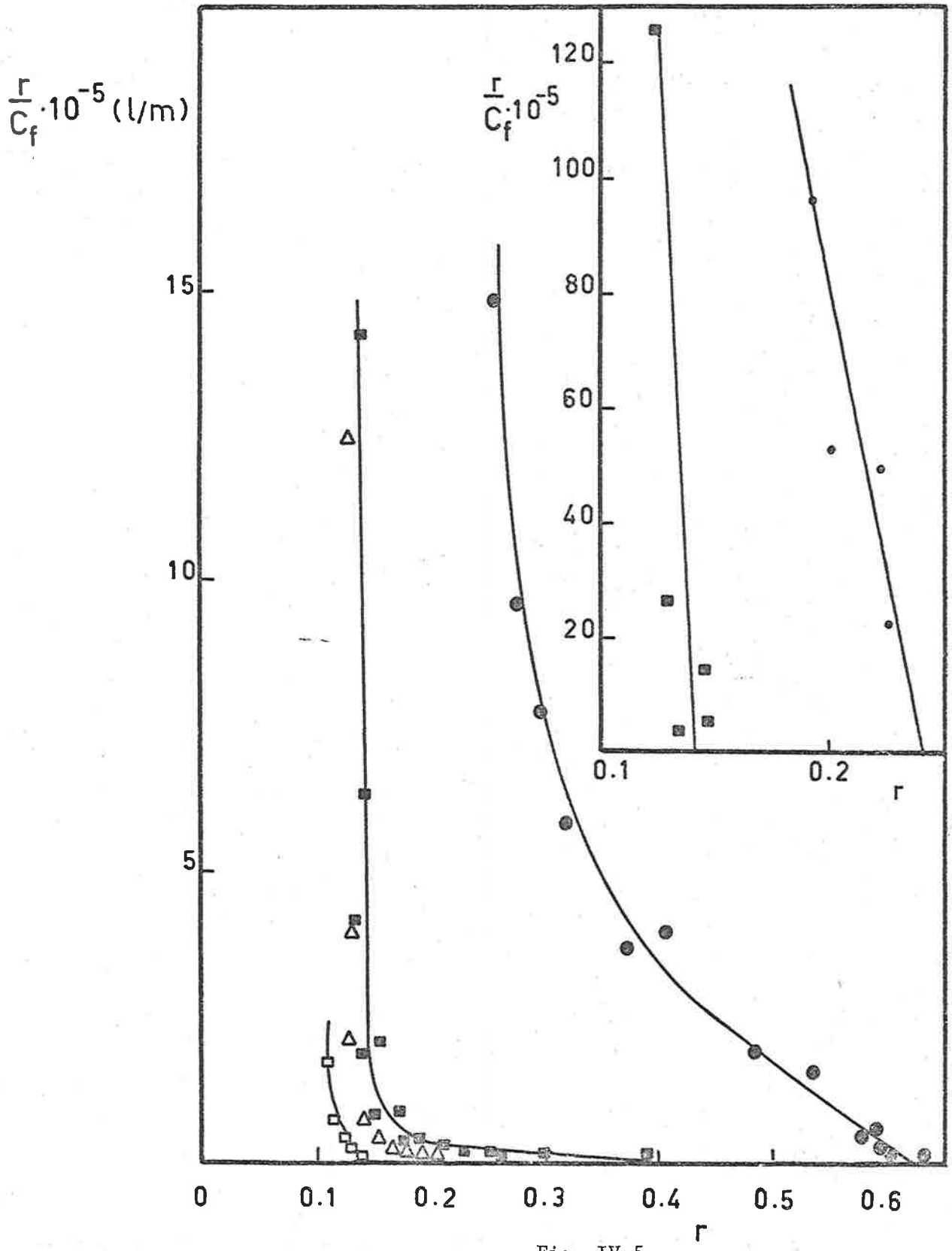

r/Cf·10^-5 (l/m)
15
10
5
0
0.1
0.2
0.3
0.4
0.5
0.6
r
r/Cf·10^-5
120
100
80
60
40
20
0.1
0.2
r

Fig. IV-5.

Fig. IV-6. Scatchard plots for the interaction of Cu^{++} ions with poly A in 5mM KNO_3 (except where otherwise indicated *) at 25°C.

● Cu^{++} into poly A, pH 6.4

■ poly A into Cu^{++}, pH 6.4

▲ poly A-Cu^{++}, pH 4.6 (inset)

○ Cu^{++} into poly A, pH 6.6, 0.15M KNO_3*

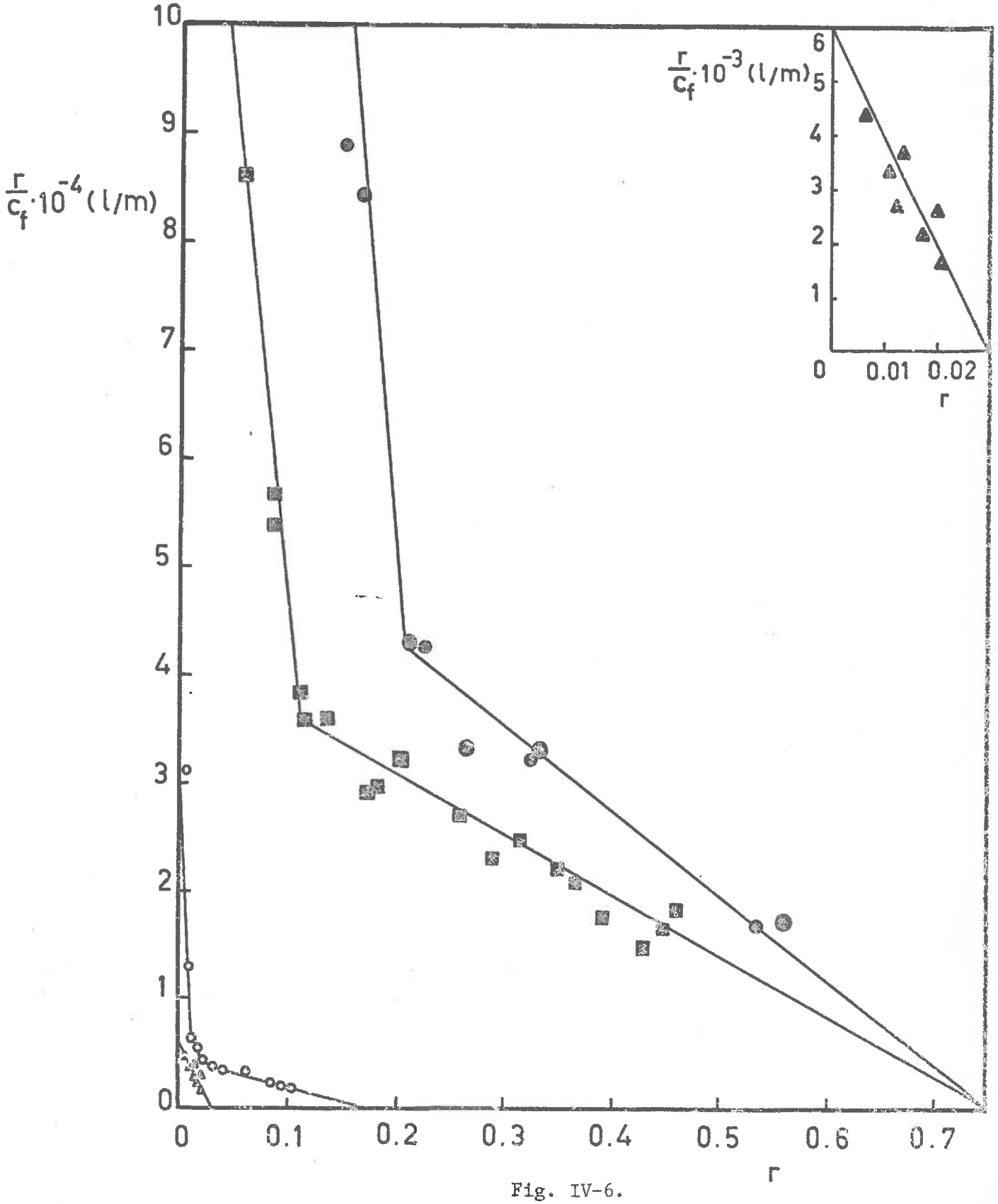

$\frac{r}{c_f}\cdot 10^{-4}$ (l/m)
$\frac{r}{c_f}\cdot 10^{-3}$ (l/m)
r
r
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.01
0.02
1
2
3
4
5
6
7
8
9
10

Fig. IV-6.

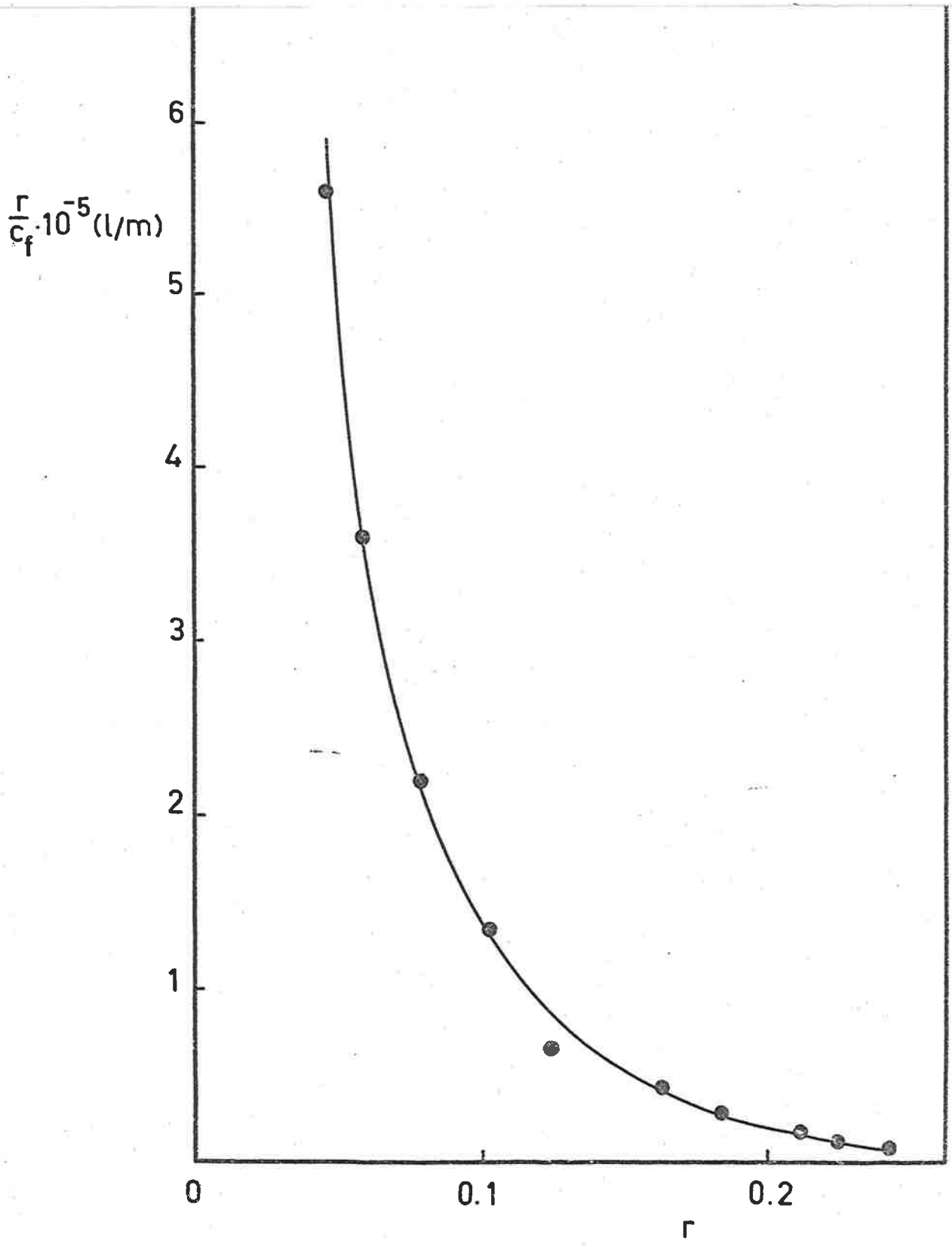


Fig. IV-7. Scatchard plot of the interaction of Cu^{++} ions with poly G in 5mM KNO_3 at 25°C, pH 5.5.

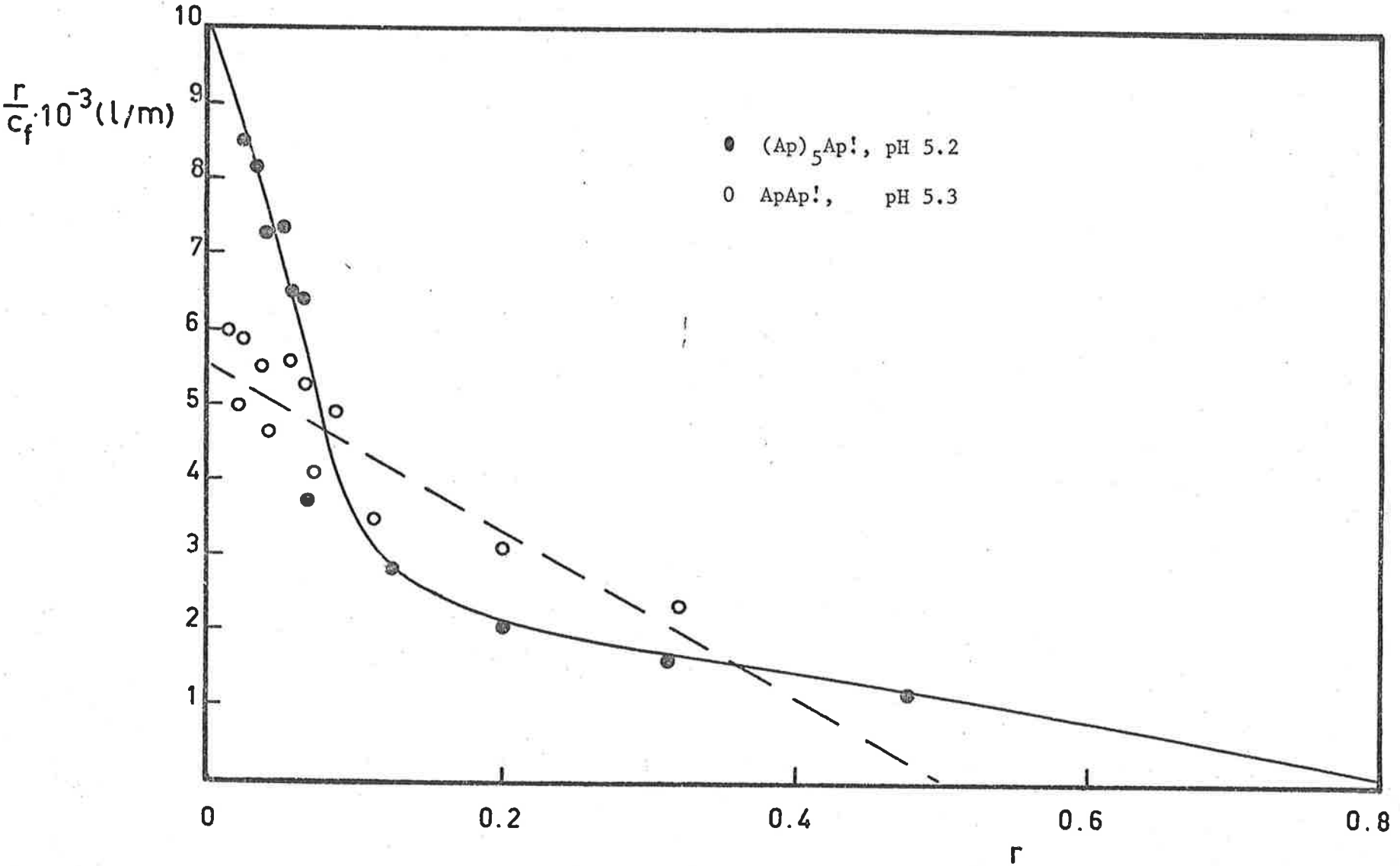


Fig. IV-8. Scatchard plots for the interaction of Cu^{++} ions with adenine oligonucleotides in 5mM KNO_3 at 25°C.

As poly I has an ordered structure near neutral pH,[27-29] and appears to be a triple helix,[28,29] the electrostatic interaction function (equation III.22) may be a reasonable approximation to that applicable to native DNA. Assuming only one type of interaction and applying this electrostatic correction to the binding data of poly I, it can be seen from Fig. IV-9 that a linear plot does not result. The presence of more than one type of site is therefore indicated, provided that the electrostatic correction used was a reasonable approximation and that conformational changes are not significant. The validity of this approximation can be tested by re-plotting the original binding data of Fig. IV-5 according to equation III.26, as shown in Fig. IV-10. From this graph, $w' = 6.3$ and $K_o = 7.5.10^5$. K_o is directly comparable with the value of $K_o = 3.6.10^5$ obtained assuming $w' = 13.4$ (Fig. IV-9), and confirms that the estimate of the electrostatic effect of poly I is acceptable. The experimental determination of w' is a measure of both electrostatic and other interactions, and may differ from the theoretical estimate of the electrostatic interaction alone. The same calculations can be applied to other polynucleotides. However, this is unnecessary as other studies have shown that w' does not vary greatly with the conformation of polynucleotides,[30] and indicates that for Scatchard plots exhibiting curvature greater than that of poly I, more than one type of Cu^{++} ion binding site exists on the polynucleotide.

The Scatchard plots of the Cu^{++} ion interaction with oligo- and

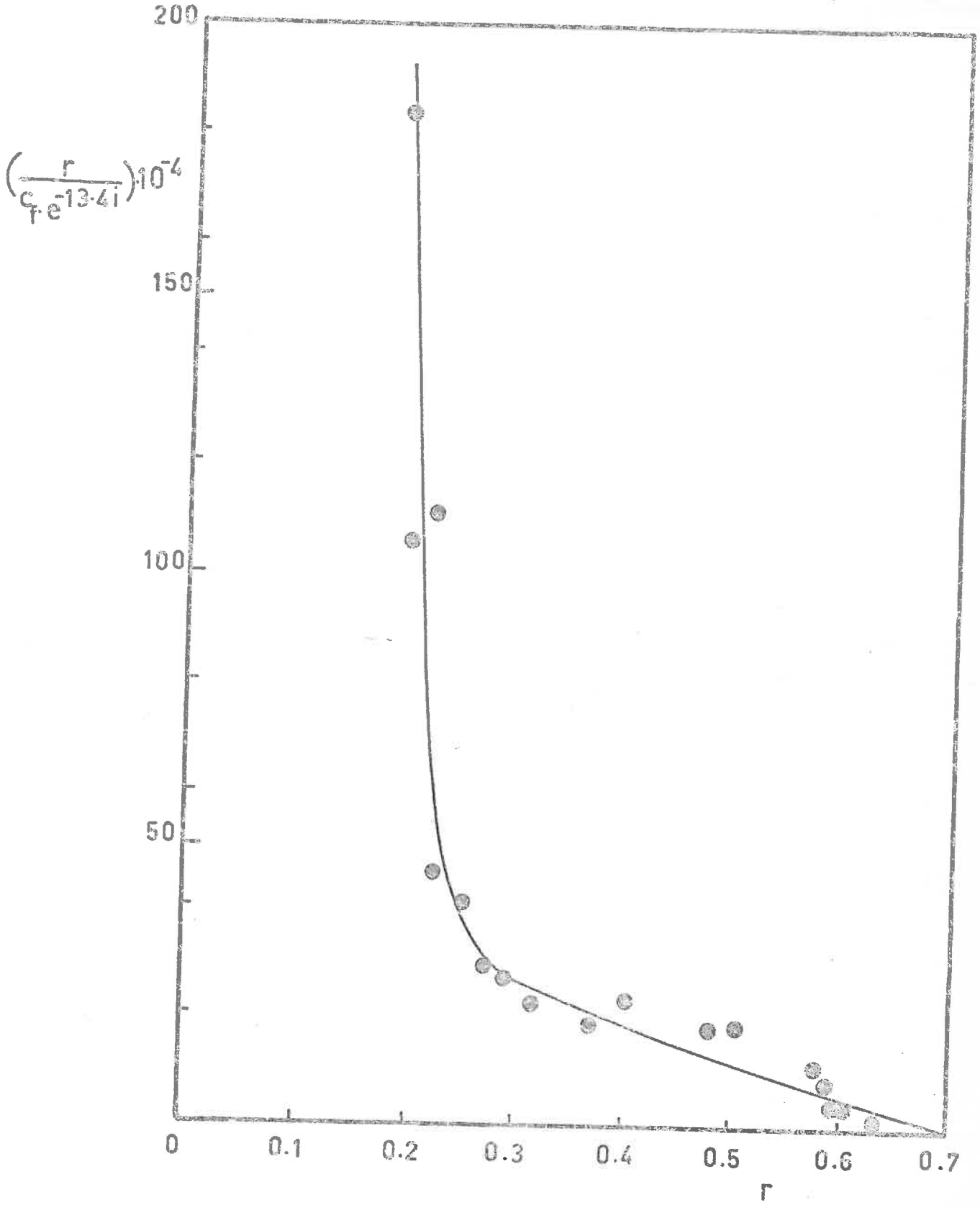
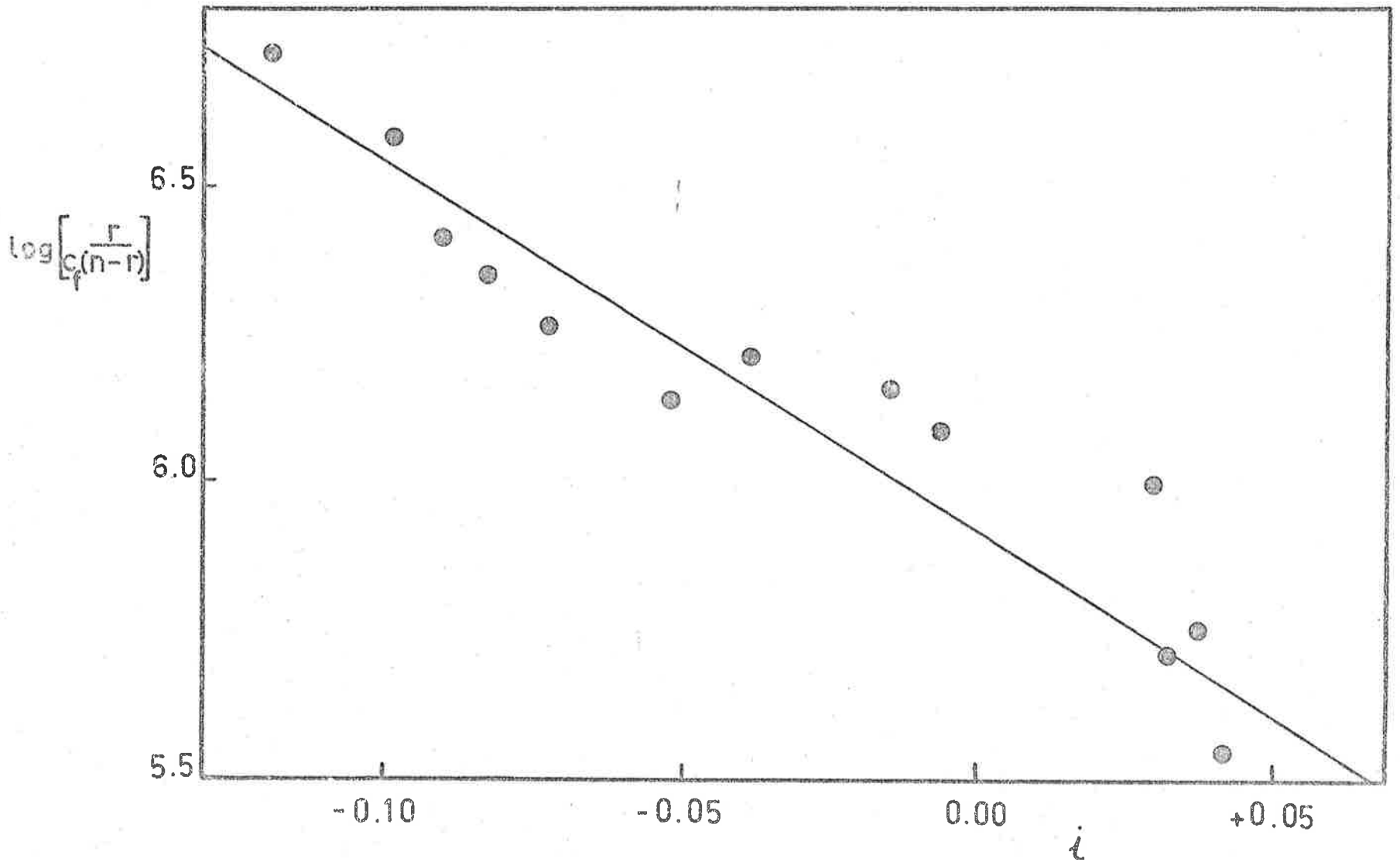


Fig. IV-9. Scatchard plot for the interaction of Cu^{++} ions with poly I, allowing for the electrostatic effect of poly I.

Fig. IV-10. Data for the interaction of Cu^{++} ions with poly I (Fig. IV-5) re-plotted to determine K_o for the weaker interaction.



poly-nucleotides have been analysed graphically as described earlier (Sect. IV.5), and indicate that in all cases the interaction is adequately described in terms of a maximum of two types of Cu^{++} ion binding sites. The interaction parameters are listed in Table IV-3. It is important to realise that the limitations of the technique are such that some weaker interactions may not be detected, especially if they occur in the presence of one or more stronger interactions. An example illustrating this point is the interaction involving poly C (Fig. IV-5).

The interaction of Cu^{++} ions with poly I and poly C was sufficiently strong for an actual titration end-point (Fig. IV-11) to be utilised in determining the stoichiometry of the interaction. This is often not possible as high stability constants for an interaction are required to obtain a sufficiently decisive end-point.[31] The values obtained for the stoichiometry of the interactions are slightly more accurate than those obtained from Scatchard plots, with which good agreement has been obtained (Table IV-3).

(ii) Hyperchromicity Studies

The hyperchromicities (H) of the polynucleotides were determined (see Sect. IV.3b) for a range of Cu^{++} ion concentrations to establish that the base residues were involved in the interaction, and to relate to the direct binding studies. For such a comparison, H must be determined as a function of r (moles of Cu^{++} ion bound per base residue) or R (total moles of Cu^{++} ion present per base residue).

Polynucleotide	pH	Titration End-point (n_1)	n_1	K_1	n_2	K_2
poly I	5.9	0.21 ± 0.02	0.24 ± 0.03	$(1.9 \pm 0.9).10^8$	0.40 ± 0.08	$(6.9 \pm 2.3).10^5$
poly I*	5.9	-	0.22 ± 0.03	$(6.5 \pm 3.0).10^7$	0.48 ± 0.07	$(3.6 \pm 1.0).10^5$
poly I**	5.9	-	-	-	-	$(7.5 \pm 1.6).10^5$
poly G	5.5	-	0.08 ± 0.02	$(1.0 \pm 0.3).10^7$	0.17 ± 0.03	$(2.4 \pm 1.0).10^5$
poly C	5.6	0.130 ± 0.007	0.14 ± 0.01	$(4 \pm 2).10^8$	-	$\sim 1.10^5$
poly C (I = 0.15M)	6.0	0.11 ± 0.01	0.12 ± 0.01	$\sim 1.10^8$	-	-
poly C	4.2	0.122 ± 0.004	0.13 ± 0.01	$\sim 1.10^8$	-	-
poly A†	6.4	-	0.20 ± 0.03	$(1.0 \pm 0.2).10^6$	0.55 ± 0.15	$(4.4 \pm 1.3).10^4$
poly A††	6.4	-	0.10 ± 0.02	$(1.0 \pm 0.2).10^6$	0.65 ± 0.15	$(3.3 \pm 1.1).10^4$
poly A (I = 0.15M)	6.6	-	~ 0.01	$\sim 3.10^6$	0.16 ± 0.02	$(3 \pm 1).10^4$
poly A	4.6	-	~ 0.03	$\sim 2.10^5$	-	-
$(Ap)_5Ap!$	5.2	-	0.10 ± 0.05	$(3 \pm 1).10^4$	~ 0.7	$\sim 1.5.10^3$
ApAp!	5.3	-	0.5 ± 0.2	$(1.3 \pm 0.6).10^4$	-	-
TpTpTpTp	4.1	-	-	-	-	-

Table IV-3. Binding parameters for the interaction of Cu^{++} ions with oligo- and poly-nucleotides in 5mM KNO_3 at 25°C.

* Allowing for a theoretical electrostatic interaction (see Fig. IV-9).

** Allowing for the actual total interaction (see Fig. IV-10).

† Titrating a Cu^{++} ion solution into poly A.

†† Titrating poly A into a Cu^{++} ion solution.

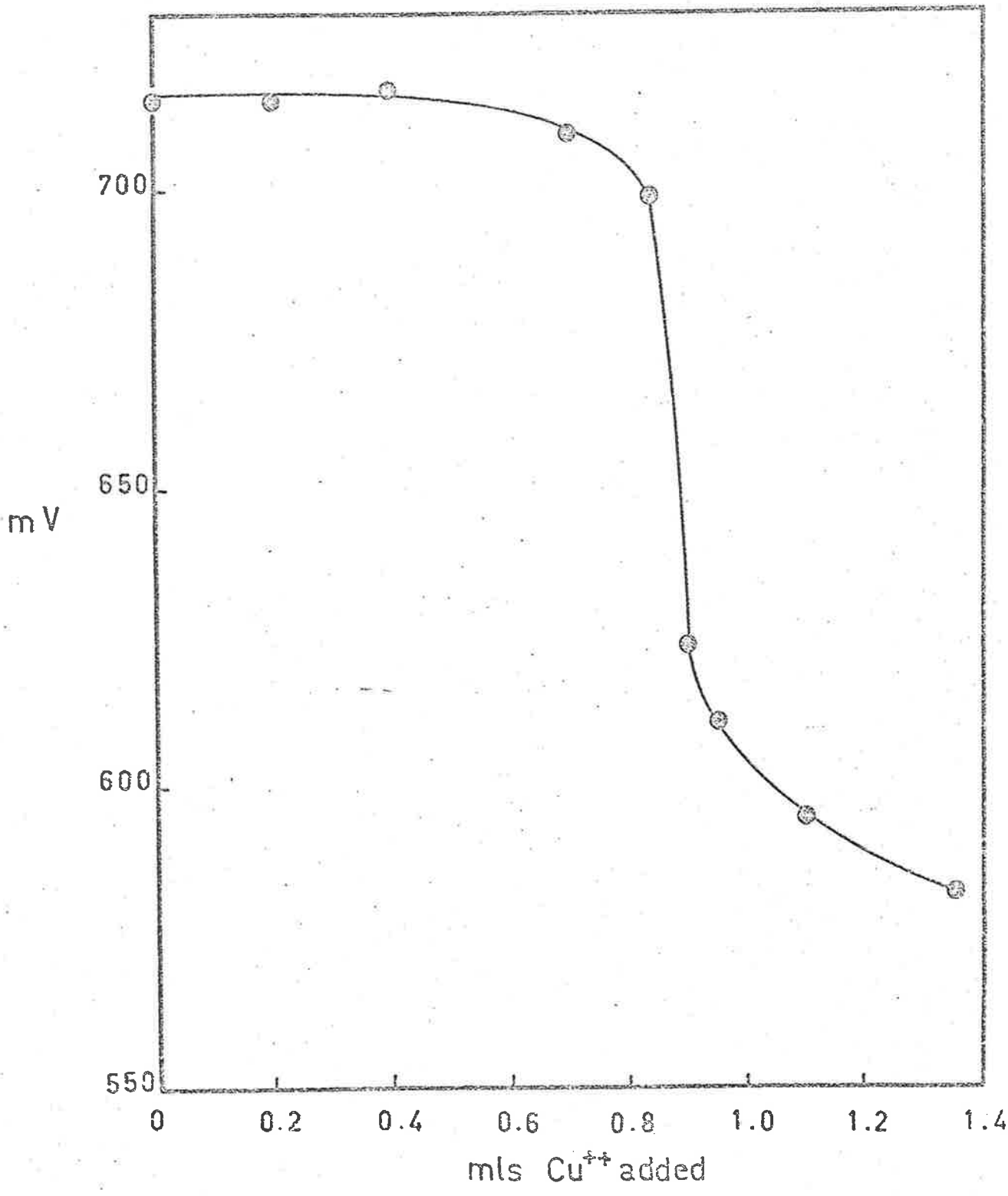


Fig. IV-11. Titration of 13.85 ml. of $5.84.10^{-5}$M$_p$ poly C, pH 5.6, with $1.23_7.10^{-4}$M Cu^{++}, pH 5.6, both 5mM KNO_3 and at 25°C.

It has been found more informative to relate the hyperchromicity of the polynucleotides to R (Figs. IV-12,13), and from a plot of r against R for each of the polynucleotides (Fig. IV-14), related to r and the binding parameters (Table IV-3). The range of these studies was limited by the formation of slowly precipitating but not visible aggregates when R > 1.4 for poly A, or by visible precipitation when R > 2.3 for poly C.

The trend of hyperchromicity and r with the total Cu^{++} ions concentration (Figs. IV-12,13,14) shows a marked similarity for all of the polynucleotides studied, and clearly indicates that the binding of Cu^{++} ions causes a simultaneous perturbation to the base residues. As the stronger of the two sites is completely occupied when $r = n_1 = 0.1 - 0.2$ (Table IV-3), then for R > 0.1 - 0.3, (from Fig. IV-14), only the weaker of the interacting sites are being titrated. Therefore, as the hyperchromicity of all four polynucleotides continues to increase after the stronger interacting sites have been titrated, it is apparent that the Cu^{++} ion interaction with the weaker sites is essentially responsible for this effect. However, base residue perturbation due to the stronger interaction may also contribute to the hyperchromicity, as at low r (hence low R) values, the hyperchromicity is small, and even using the method of difference spectrophotometry it would be difficult to detect a separate hyperchromicity dependence of the stronger interaction, as distinct from the weaker interaction.

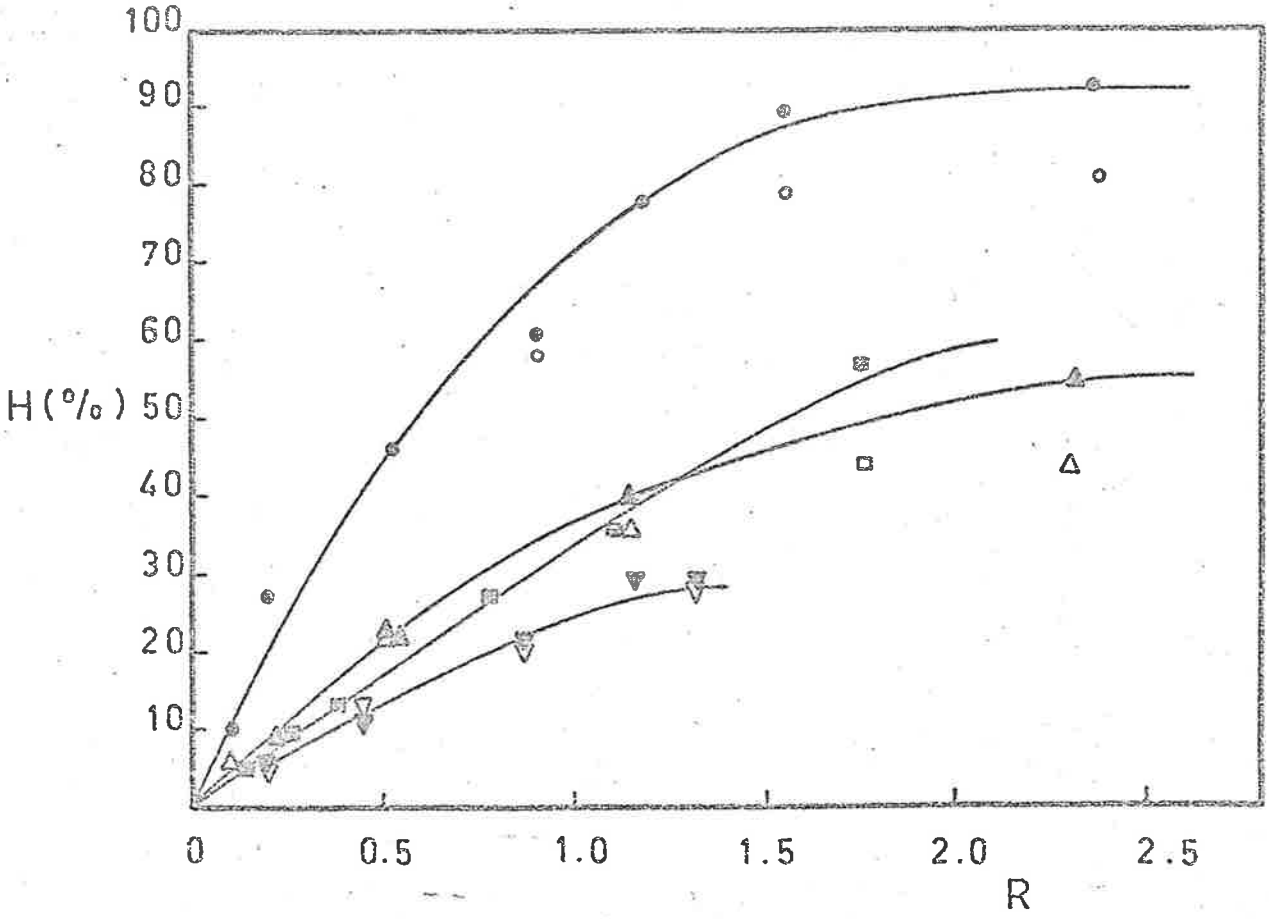


Fig. IV-12. Hyperchromicities of the polynucleotide-Cu^{++} complexes at the wavelength of maximum hyperchromicity. Open symbols, 1 hour after mixing. Closed symbols, 2 days after mixing.

● poly I, pH 6.1, 265 nm

▲ poly C, pH 5.9, 250 nm

■ poly G, pH 5.5, 235 nm

▼ poly A, pH 6.4, 270 nm

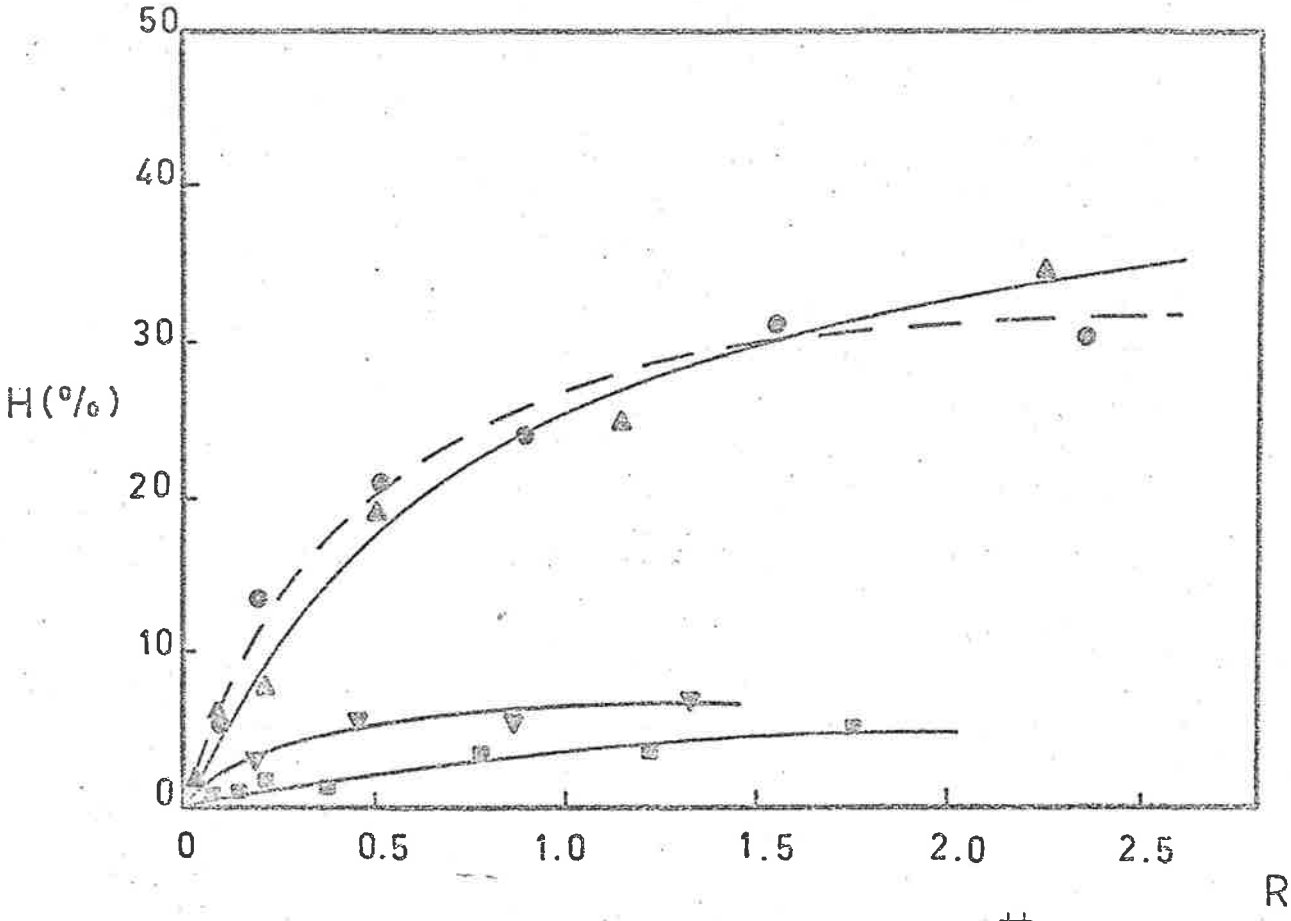


Fig. IV-13. Hyperchromicities of the polynucleotide-Cu^{++} complexes at the wavelength of maximum absorption of the polynucleotides in the absence of Cu^{++} ions (λ_{max}), two days after mixing.

--●-- poly I, pH 6.1, 248 nm

—▲— poly C, pH 5.9, 250 nm

—■— poly G, pH 5.5, 253 nm

—▼— poly A, pH 6.4, 270 nm

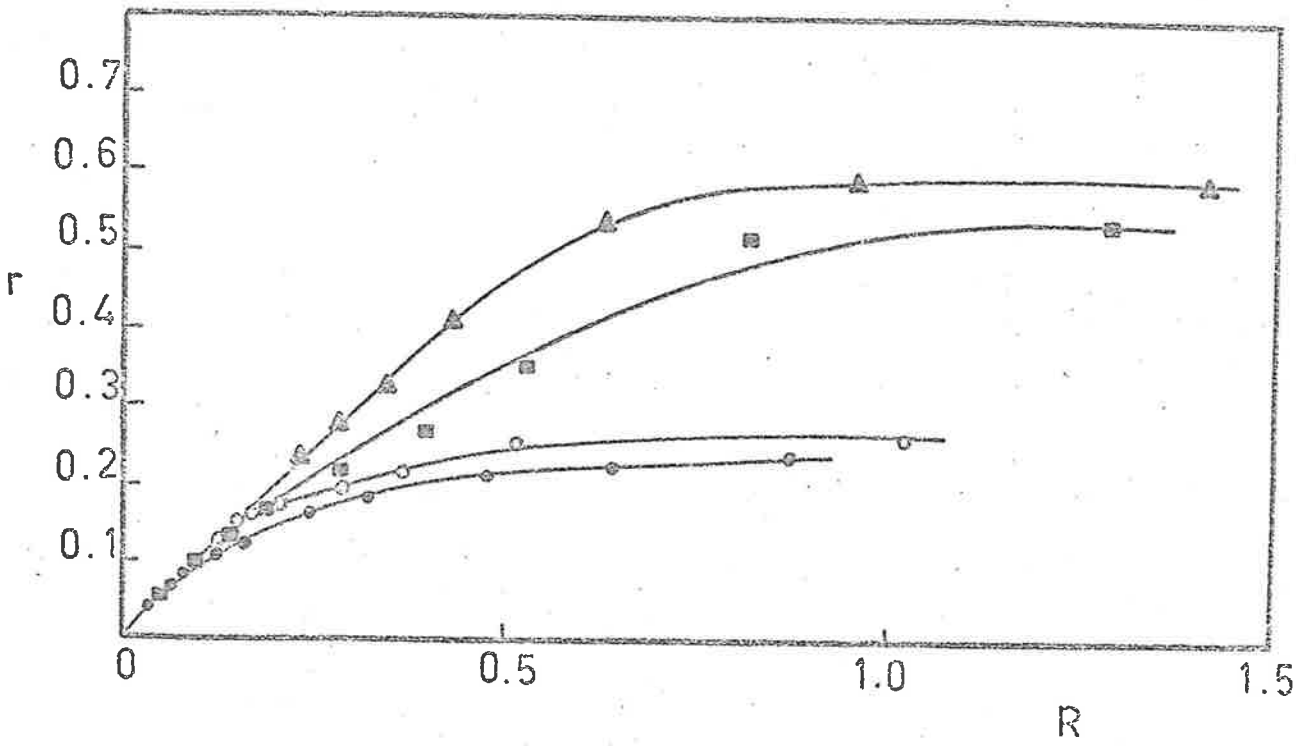


Fig. IV-14. Variation of the number of moles of Cu^{++} bound per residue with the total number of moles of Cu^{++} present per residue.

▲ poly I, pH 5.9

○ poly C, pH 5.6

● poly G, pH 5.5

■ poly A, pH 6.4

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A time effect of the hyperchromicity has been observed for poly I, poly G and poly C, but not with poly A (see Fig. IV-12). A similar time effect has been noted by others[32] with poly I and poly C, as well as the lack of any such effect with poly A. The absorbance was found to be constant after 48 hours for poly I, poly G and poly C. Attempts to relate this time effect to variations of the amount of Cu^{++} bound to the polynucleotides (r) during this time interval were negative as identical values of r were determined one hour and 48 hours after mixing the interacting species at 25°C. This indicates that the small change of hyperchromicity may be due solely to base residue re-orientation, perhaps due to slight stereochemical re-arrangement of the Cu^{++} ion binding sites during the base residue 'breathing' process (see Sect. II.5). Similar time effects have been observed by others with DNA.[33,34] No spectral shifts were observed with any of the polynucleotides.

The hyperchromicity studies of poly C differ substantially from those of Zimmer and Szer[23] who failed to detect any hyperchromicity until R = 3.5. However, they have observed a plateau region of the hyperchromicity (35-40% at 270 nm) similar to that indicated in Fig. IV-13.

(iii) Probable Complexes

From the binding parameters listed in Table IV-3, it appears that a terdentate chelate of Cu^{++} between adjacent inosine residues (similar to Fig. II-7) and an inosine-Cu^{++}-phosphate(5') type chelate

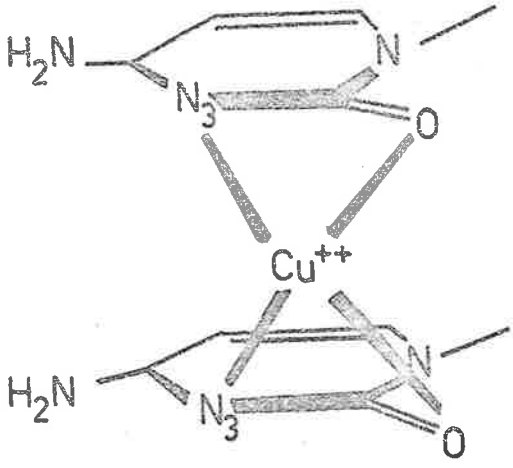
on the remaining 50% of the residues (similar to Fig. II-5) are probable as such complexes account for the observed stoichiometry and stability. The involvement of the N_7 position of triple-strand poly I has been confirmed from NMR relaxation studies[35] as a site of Cu^{++} ion interaction.

There is some evidence[36] that Cu^{++} is involved in a complex with the phosphate and 2'OH groups of poly I, poly A, poly C and poly U, the last three of which are degraded (at 64°C) much faster than poly I. It is extremely significant that several studies have failed to observe any interaction of Cu^{++} ions with UMP,[20,37,38] poly U[32] or poly rT[38] (although a weak ionic interaction has been detected with poly U[39]). This indicates that any complex involving the 2'OH group of UMP, poly U or poly rT is relatively thermodynamically unstable and, as the rate of degradation of poly A, poly C and poly U is similar, suggests small stability constants for the complexes that are responsible for the degradation of these polymers at room temperatures. It is therefore apparent that such interactions are unlikely to be measurable using the Cu^{++} ion activity electrode, especially in the presence of other stronger Cu^{++} interactions that occur with these polynucleotides. For this reason, such interactions are not discussed further with respect to poly C and poly A. As poly I is degraded much more slowly than poly C, poly A and poly U,[36] it is probable that the 2'OH-Cu^{++}-phosphate complex is weakened, probably as a result of Cu^{++} bound in such a complex being more involved in the residue-Cu^{++}-phosphate complex. Such a view

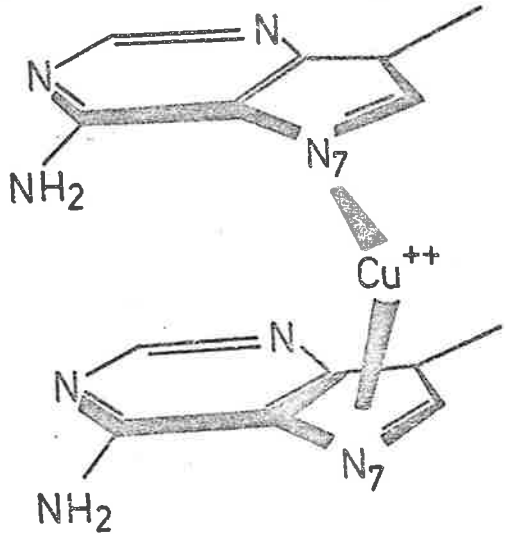
is supported by the observed stability constant of GMP-Cu^{++} relative to the other mononucleotides (Table IV-2).

The affinity of Cu^{++} ions for poly G (Fig. IV-7) is expected to be similar to that of poly I in view of the similarity of guanosine and inosine and the fact that poly G also exists in an ordered form near pH 5.5.[27,29] The order of stability for the two types of sites of poly G is similar to poly I. However, the number of sites is substantially reduced and may reflect differences of the secondary and tertiary structure of these polymers as the number of strands involved in the ordered form of poly G is not known.[27]

Poly C has been shown[40,41] to exist as a single strand near neutral pH. The large stability constant for this single strand poly C-Cu^{++} complex ($K_1 \sim 1.10^8$) suggests a terdentate complex (Fig. IV-15). The N_3 position of poly C has previously been implicated from NMR studies as a site of Cu^{++} ion interaction.[35] Furthermore, no interaction with the amino group has been detected.[35] Such a complex would involve a large degree of base residue re-orientation, and may account for the low stoichiometry observed (one Cu^{++} ion bound per eight residues). A weaker interaction also occurs, and has previously been detected by others.[23] However, the nature of this interaction is not clear as molecular model studies indicate that a cytosine-Cu^{++}-phosphate type complex (either 3' or 5' phosphate) is sterically unlikely. Furthermore, as discussed above, the affinity of Cu^{++} ions for the 2'OH group appears to be quite weak and unlikely to account for



a. POLY C



b. POLY A

Fig. IV-15. Probable 'sandwich' type Cu^{++} complexes of the homo-polynucleotides poly C and poly A.

the stability constant observed for the weaker interaction ($K \sim 1.10^5$).

The formation of the stronger complex with poly C appears to be largely unaffected by high ionic strengths or the double strand form of poly C (at pH ~ 4.5),[41-46] even though the double strand (acid) form contains a positive core.[47] The high ionic strength is expected to eliminate any weak cytosine residue-Cu^{++}-phosphate type chelates but may not affect the stronger interaction unless large conformational changes or base residue re-orientations occur. As the acid form of poly C contains a positive core,[47] and the sites available for the strong interaction of Cu^{++} ions with single strand poly C are involved in hydrogen bonding when in the acid form,[48,49] it is difficult to see why the number of stronger Cu^{++} ion interacting sites is not diminished in the double strand acid form of poly C compared with the single strand form.

The non-ordered form of poly A exists near neutral pH[50] and exhibits irreversible characteristics of the stronger binding site (Fig. IV-6). It is therefore unique among the polynucleotides in this respect. The reason for this irreversible behaviour is not clear, although one suggestion is offered below. There appears to be one strongly interacting site per four residues and a 1:1 complex, probably the adenine-Cu^{++}-phosphate(5') type chelate discussed earlier (see Fig. II-5), for the remaining residues. The stronger interacting site can be explained in terms of a chelate (Fig. IV-15). The N_7 position

has been demonstrated as a site of Cu^{++} ion interaction in poly A.[19] However, there has been no evidence to suggest that the amino group is involved in a terdentate complex. The complex suggested would involve substantial base residue re-orientations which may be the cause of the irreversibility of the titration.

High ionic strength eliminates 90% of the stronger interacting sites and supports the suggestion that significant base residue re-orientations are necessary for the formation of such sites, as the ionic strength is known to be capable of strongly influencing the conformation of charged polynucleotides.[50,51] The decrease in the number of weaker interacting sites with an increase of ionic strength agrees with the partially ionic complex proposed. The double strand form of poly A at low pH[50] precludes the formation of either complex, although a few sites still appear capable of interacting with Cu^{++} ions. The stability constant obtained probably represents an average of the two interactions because of the limited accuracy of such minimal binding. The few interacting sites may be attributed to small regions of single strand chain ends.

Such a result with poly A is not unexpected as the acid form contains a positive core.[47] In addition, hydrogen bonding between the strands[52] reduces the degree of base residue re-orientation required for formation of the bidentate complex and may also inhibit the formation of the weaker complex. These results contrast with poly C, both at high ionic strength and for the double strand form. The reason

for such a disparity of effects involving similar types of interactions is not understood but may involve conformational effects.

Binding data for the oligo A-Cu^{++} interactions supports the terdentate complex proposed for poly A as a completely different complex appears to be formed with the adenine nucleotide dimer ($n = 0.5$, $K \sim 1.10^4$) as compared with the monomer (n assumed to be 1.0, $K \sim 300$). Furthermore, a dependence of the stability constant on chain length is apparent ($K_1 = 1.10^4$, 3.10^4 and 1.10^6 for ApAp!, $(Ap)_5Ap!$ and single strand poly A respectively; $K_2 = 3.10^2$, 2.10^3 and 4.10^4 for AMP^-, $(Ap)_5Ap!$ and single strand poly A respectively). Such a dependence has also been observed for the binding of Ag^+ ions to an oligo A series.[30] The dependence is attributed to the effect of π interactions altering the basicity of the residues. This explanation for the oligo A-Ag^+ interactions has been supported by a similar dependence of λ_{max} of the ultra-violet absorbance, and T_m of the melting curves, on the chain length of various adenine oligonucleotides.[53]

As Cu^{++} ions do not interact with thymine bases, nucleosides or nucleotides (see Sect. II-3), an oligo T (the tetramer) was selected as a model compound to investigate the binding of Cu^{++} ions to phosphat groups under identical stereochemical conditions as in polynucleotides and DNA. No Cu^{++} ion binding could be detected at the concentrations used. Unfortunately greater concentrations could not be used as these

materials were only prepared in limited quantities. However, it is quite evident that the stability of any Cu^{++} ion interaction with the thymidine tetramer is not enhanced relative to that of the Cu^{++} ion interaction with individual single charged phosphate groups of TMP^{-} or $H_2PO_4^{-}$ (Table IV-2). Therefore, if a complex involving Cu^{++} ions bridged between two adjacent phosphate groups exists in the thymidine tetramer (or polynucleotides or DNA), it cannot be distinguished by an enhanced stability relative to that of the 1:1 type association.

The studies and conclusion presented in this chapter for the interaction of Cu^{++} ions with mono-, oligo- and poly-nucleotides have been discussed further in Chapt. VI in relation to the Cu^{++} ion interactions with DNA presented in Chapt. V.

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CHAPTER V

THE INTERACTION OF Cu^{++} IONS WITH NATIVE DNA

1. INTRODUCTION

Two of the most direct techniques possible for the study of Cu^{++} ion interactions at equilibrium have been used in this investigation of the formation of DNA-Cu^{++} complexes. The techniques involve the use of a specific Cu^{++} ion activity electrode (discussed earlier in Sects. IV.2 and IV.3) and gel exclusion chromatography, both enabling the determination of the binding parameters, n and K. The technique utilising gel chromatography enables more reproducible determinations of C_f, and therefore of n and K, but is an extremely time consuming process. For this reason, this technique has been used essentially as a comparison with the more efficient process involving the Cu^{++} ion electrode.

Some hyperchromicity studies were also undertaken to establish absolute values for spectral changes of native DNA in the presence of Cu^{++} ions, and to investigate the existence of isosbestic points under the conditions used in this study. As most of the evidence regarding the conformation of the native DNA-Cu^{++} complex is conflicting,[1,2] a more accurate investigation of conformational changes appeared desirable. By employing the conditions used for the binding and hyperchromicity studies, the base residue perturbation and the DNA conformation may be directly compared with the extent of Cu^{++} ion binding.

As the pH of DNA solutions was unaltered by the presence of Cu^{++} ions, it was not necessary to buffer these solutions. The extreme

desirability of being able to undertake any Cu^{++} ion interaction studies in the absence of potentially complicating buffers has been discussed earlier (Sect. IV.3a).

2. GEL EXCLUSION CHROMATOGRAPHY

a. Possible Methods of Use

A sephadex column may be used in three essentially different ways to study large molecule-small molecule interactions such as the DNA-Cu^{++} system.

(i) Pre-equilibration of the column with the solvent and subsequent addition of a DNA-Cu^{++} mixture enables the analysis of the leading boundaries of the plateau regions of the two separated species. This method has received substantial theoretical[3-5] and experimental[5-8] attention and has been used successfully for the study of the native DNA-Cu^{++} interaction.[9]

(ii) The method used by Hummel and Dreyer[10] involves pre-equilibration of the column with a known Cu^{++} ion concentration. As the elution of a small volume of DNA is accompanied by bound Cu^{++}, a peak, followed by a trough of Cu^{++} ion concentration, can be detected. This method has been used successfully by Bryan and Frieden[1] for the study of the native DNA-Cu^{++} interaction.

(iii) A modification of method (ii) involves the use of sufficient DNA to produce a plateau region instead of a peak of DNA.

For several reasons the third method was the one adopted for this study. Method (i) is complicated by the fact that sephadex retains Cu^{++} ions,[9,11] thereby introducing serious errors, especially at low Cu^{++} ion concentrations. Method (ii) involves the determination of the total number of moles of Cu^{++} ions associated with the DNA peak. (The accompanying trough will generally be of little assistance for an averaging procedure as the Cu^{++} ion concentrations will usually be small and difficult to analyse accurately.) In addition, a large number of total Cu^{++} ion concentrations (hence an accurate average total Cu^{++} ion concentration) can be determined in the DNA plateau region for various DNA concentrations. As the column is initially equilibrated with a constant Cu^{++} ion concentration, any Cu^{++} ion binding sites of the sephadex are saturated at that concentration, thereby eliminating the source of error encountered in method (i).

b. Spectrophotometric Determination of Cu^{++} Ion Concentrations

Before efficient reversible Cu^{++} ion electrodes became available (see Sect. IV.1), Cu^{++} ion concentrations were usually determined spectrophotometrically. The most recent spectrophotometric methods have been based on the formation of a complex with bis-(cyclohexane)-oxalyldihydrazone (cuprizon). The cuprizon-Cu^{++} complex has a larger extinction coefficient than other similar complexes,[12] and can therefore be used for the determination of lower Cu^{++} ion concentrations. This

method has been found to give anomalous results[13] unless both pH and ammonium ion concentrations are carefully controlled. Several modifications[12-14] of Williams' and Morgan's[15] method have been required to obtain the optimum conditions. The method used was essentially the same as that used by Somers and Garraway.[13]

Approximately 1 ml of Cu^{++} ion solution was weighed into a pre-weighed 1 cm spectrophotometer cell. 0.15 ml of 10% tri-ammonium citrate (BDH, AR grade) was then added, followed by one drop of 2M NaOH to neutralise the solution and then 0.5 ml of borate buffer, pH 7.96 (prepared by the addition of 100 ml of 0.5M NaOH to 500 ml of 0.5M boric acid). The cell was carefully shaken after each addition to assist mixing. Finally, 0.15 ml of freshly prepared 0.5% cuprizon (AG Fluka, Switzerland, puriss pa grade) in 50% ethanol was added and the solution made up to approximately 2.5 ml by the addition of 5mM KNO_3. The cell was then re-weighed, inverted twenty times with parafilm held over the top, and the absorbance read at 595 nm at 20°C, 10 minutes after mixing. (Maximum absorbance was achieved after 5 minutes and was constant for 15 minutes for all Cu^{++} ion concentrations measured.) The reference cell was prepared simultaneously in an identical manner starting from 1 ml of 5mM KNO_3. Usually six or seven determinations were carried out at the same time. Corrections were made to the absorbance measurements for cell path lengths and cell absorbances.

Using this technique to obtain the absorbance of known Cu^{++}

ion concentrations, the calibration graph (Fig. V-1) provided an accurate determination of the extinction coefficient of the complex, ϵ_{595} = (16, 460 ± 100) l. mole^{-1} cm^{-1}. The Cu^{++} ion concentration of the initial 1 ml of test solution can be readily calculated from the dilution factor, the absorbance and the extinction coefficient. The density of Cu^{++} ion solutions has been assumed equal to the density of the cuprizon-Cu^{++} solutions as, for the range of Cu^{++} ion concentrations studied, it has been confirmed[9] that errors from this assumption would be less than 0.1%. Evaporation from the spectrophotometer cells was checked and indicated a 0.45% loss of weight per hour. Therefore, for the time intervals used in this study, the maximum error from this source would be 0.1-0.2%.

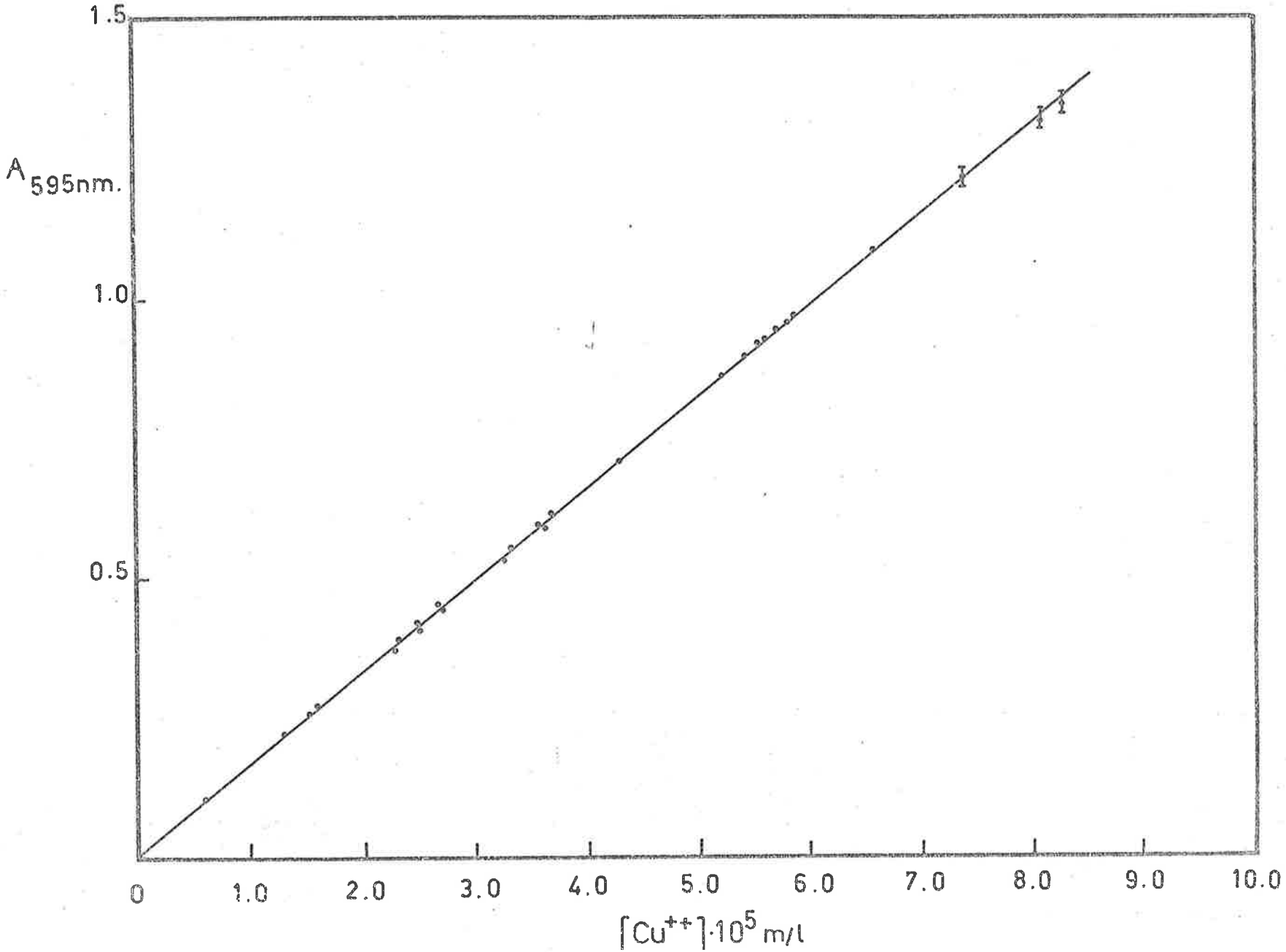
The above technique was used successfully for initial (undiluted) Cu^{++} ion concentrations in the range 2.10^{-4}M to 1.10^{-5}M. For initial concentrations less than 4.10^{-5}M, 2 cm and 4 cm cells were used, 1 cm cells being used for the more concentrated solutions. Absorbance measurements were accurate and reproducible to ±0.003 for all absorbances up to 0.4. Therefore, most concentrations were accurate to ±1%.

c. Spectrophotometric Determination of Cu^{++} in the Presence of DNA

The use of gel exclusion chromatography for the determination of binding parameters for DNA-Cu^{++} interactions required the determination

Fig. V-1. Calibration of absorbance at 595 nm of the cuprizon-Cu^{++} complex, with Cu^{++} ion concentration.

Fig. V-1.



of the total Cu^{++} ion concentration (both free and bound) in the presence of native DNA, as well as DNA denatured in the presence of Cu^{++} ions. It was therefore necessary to check that the cuprizon method detected the total Cu^{++} present in both systems.

Known amounts of Cu^{++} ions were added to DNA solutions of final concentration $1.10^{-4}M_P$, some solutions being denatured at 75°C for 10 minutes. The Cu^{++} ion concentrations of these solutions were then determined by the method outlined above. The results are summarised in Table V-1 and indicate that in both cases, the cuprizon method measures the total amount of Cu^{++} in solutions containing DNA, whether it be native DNA or DNA denatured in the presence of Cu^{++} ions.

d. Experimental Procedure

The sephadex column (see Sect. VIII.3) was eluted overnight at 20 ml/hr with 400 ml of degassed Cu^{++} ion solution of known concentration. Care was taken not to disturb the top of the sephadex bed by carefully pipetting the first 10 ml into the column. The remaining solution was added dropwise to the top of this 10 ml solution by means of a dropping funnel and pipette arrangement (see Fig. VIII-1). Using this method the top of the sephadex was continually maintained level, ensuring sharp elution profiles.

A binding run was initiated by stopping the column elution just as the last fraction of Cu^{++} ion solution entered the sephadex, and

1.10^{-4} M_P E. Coli	$[Cu^{++}].10^4$ m/l Initially	1 hour after mixing	24 hours after mixing
Cu^{++} added to native E. Coli, 25°	5.48	5.53	5.63
	1.03	1.05	1.03
	0.097	0.109	0.089
E. Coli denatured in the presence of Cu^{++}	5.42	5.62	5.68
	1.08	1.12	1.05
	0.098	0.101	0.099

Table V-1. Determinations of total Cu^{++} ion concentration in the presence of 1.10^{-4} M_P DNA.

carefully pipetting 10 ml of degassed DNA (usually approximately 1.10^{-4} M_p) onto the sephadex. Fifteen minutes was allowed for thermal equilibration to 25.0°C before elution was begun. As the last fraction of DNA entered the sephadex, 10 ml of the Cu^{++} ion solution was carefully pipetted onto the column, and the Cu^{++} ion reservoir connected. For all binding runs, the elution rate was controlled to approximately 20 ml/hr by the fine control 'Fluon' tap at the bottom of the column.

The column was connected by PTFE tubing to a continuously recording flow spectrophotometer (Isco, model UA, Instrumentation Specialists Co. Inc., U.S.A.) which analysed for DNA at 245 nm. This was in turn connected to a 'drop type' fraction collector monitored by a photo-electric cell. Usually about 1 ml of eluent was collected in each tube which was then immediately sealed with parafilm. All tubes in the DNA plateau region, as well as others immediately prior to and following this region, were analysed for the total Cu^{++} ion concentration. A typical elution profile is illustrated in Fig. V-2.

e. Calculation of r

The free Cu^{++} ion concentration (C_f) is the concentration of Cu^{++} in the pre-equilibrated column and was taken to be the concentration of the equilibrating Cu^{++} ion solution. This was checked by analysing three tubes on each side of the DNA plateau region after all experiments the concentrations always agreeing within 1%.

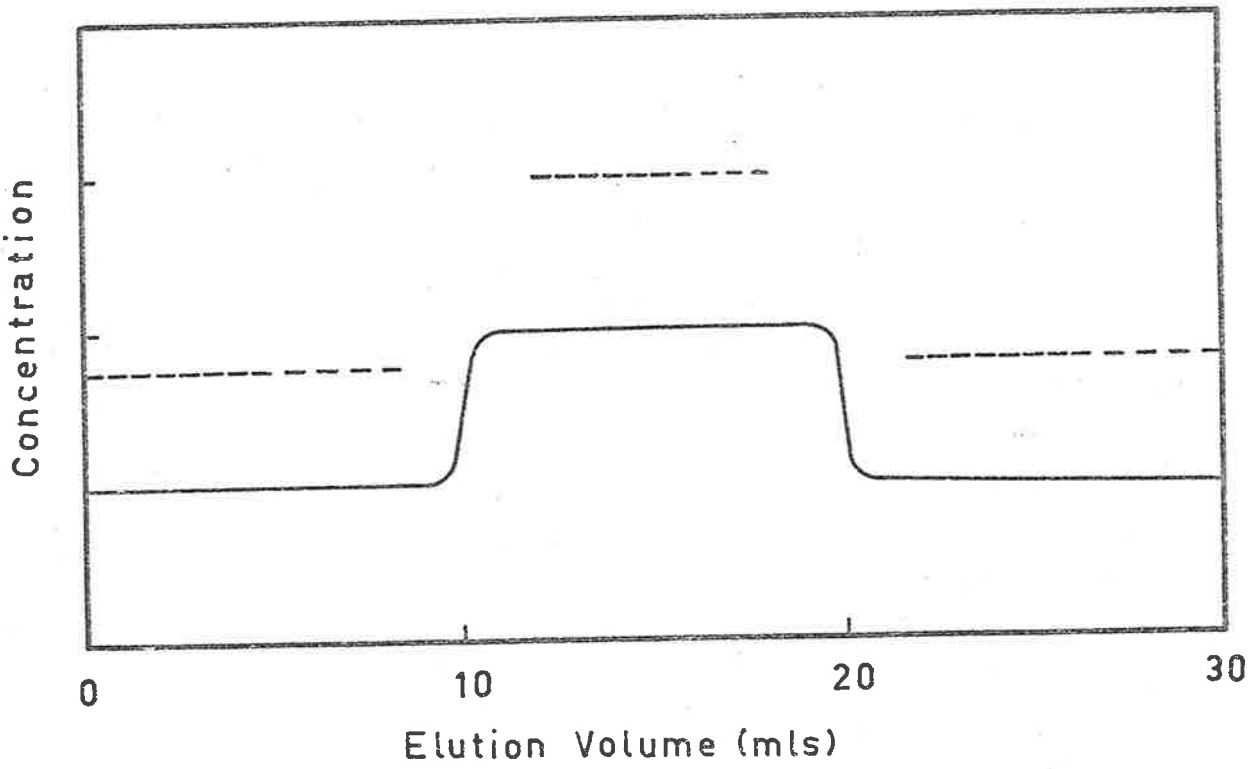


Fig. V-2. Diagrammatic representation of an elution profile for the DNA-Cu^{++} interactions.

—— DNA

--- Cu^{++}

From the total Cu^{++} ion concentration (C_T) in the DNA plateau region, the concentration of Cu^{++} bound to DNA, ($C_T - C_f$), can be readily evaluated. The concentration of DNA, (M_P), in the plateau region (determined by continuous analysis at 254 nm) was always within 0.5% of the concentration of DNA added to the column (determined spectrophotometrically at λ_{max}). The latter value was always used as this was determined with far greater accuracy and reproducibility using a Zeiss spectrophotometer.

Knowing the two quantities ($C_T - C_f$) and M_P, r, the number of moles of Cu^{++} bound per base residue can be calculated as

$$r = \frac{(C_T - C_f)}{M_P} \qquad V.1$$

3. ULTRACENTRIFUGATION

Experimental values of the sedimentation coefficient, S, are usually extrapolated to zero concentration (S^o) as the interpretation of terms in the equation

$$S = \frac{M(1 - \bar{\nu}\rho)}{Nf} \qquad V.2$$

is valid only under these conditions. M and f represent the molecular weight and frictional coefficient of the sedimenting particle, $\bar{\nu}$ is the thermodynamic partial specific volume of the solute and ρ is the density of the solvent. However, it is possible to conduct relative sedimentation studies of DNA if the DNA is maintained at a constant

concentration, thereby ensuring that there is no variation of any inter-strand interactions depending on concentration. The second procedure was the one adopted for the present study as this enabled comparisons with earlier studies and also provided greater accuracy for comparing actual experimental values, rather than comparing extrapolated values of S^o.

From a least squares plot of ln x against time the sedimentation coefficients were calculated directly (see computer program, Appendix 2) from the equation

$$S = \frac{1}{x\omega^2}\frac{dx}{dt} = \frac{1}{\omega^2}\frac{d(\ln x)}{dt} , \qquad V.3$$

where x is the distance of the sedimenting boundary from the centre of the rotor and ω is the angular velocity of the rotor. The sedimentation coefficients were corrected to standard conditions using the equation

$$S_{25} = S.\frac{\eta_{25}}{\eta_{25,w}} . \frac{(1 - \bar{\nu}\rho_{25,w})}{(1 - \bar{\nu}\rho_{25})} \qquad V.4$$

where η_{25} and $\eta_{25,w}$ are the viscosities of the solution and water at 25°C. It is necessary to emphasise here that as S^o values were not determined, the viscosity of the solution and not that of the solvent was required[16] (see Sect. VIII.5). The ratio

$$\frac{(1 - \bar{\nu}\rho_{25,w})}{(1 - \bar{\nu}\rho_{25})}$$

was assumed to be unity as the density of the DNA solutions used is

effectively that of water,[17] and $\bar{\nu}$ does not change appreciably with changes of the DNA conformation.[17]

Small volumes (0.05 to 0.10 ml) of concentrated Cu^{++} ion solutions were added with calibrated syringes to weighed amounts of 1.10^{-4} M_p E. Coli. Ultracentrifugation was instigated one hour after mixing the solutions. The sedimentation coefficients were calculated by substituting values for x, t, ω, η_{25} and $\eta_{25,w}$ into equation V.4. To calculate x, it is necessary to know the magnification factor, M, of the ultracentrifuge optics. As the scanner (see Sect. VIII.5e) moves down the cell, the relative absorbance of the cells is automatically recorded against the distance travelled by the scanner. The magnification factor must be determined from the recorded distances through which the scanner passes for each chart speed, as this distance is obviously a function of the chart speed. The most accurately defined distances, both actual and magnified on the chart (see Figs. V-3a,b) are those between the edge of the outer reference hole (X_0) and the outer edge of the inner reference hole (X_I). The value supplied by Beckman for this distance is 15.0 mm. Therefore, the magnification factor is

$$M = \frac{(X_I - X_0)}{15.0} = 5.20$$

for a chart speed of 1 cm/min at scanner speed of "4.75".

The values for x were determined from the 50% concentration point of the sedimenting boundary. The actual distance x was calculated by reading from the chart (see Fig. V-3b) the distance

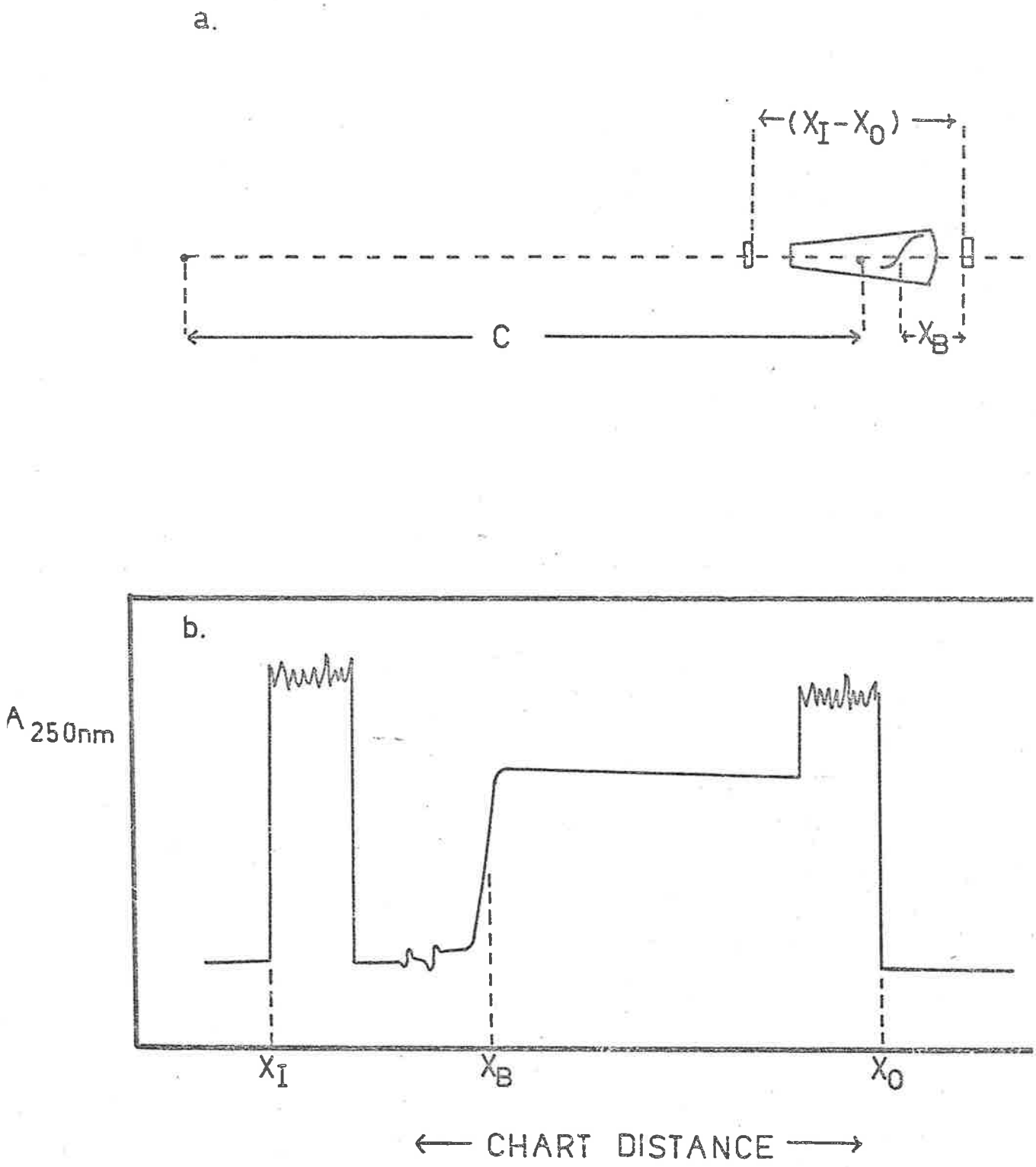


Fig. V-3. a. Diagrammatic representation of the position of the cell, the sedimenting boundary and the inner and outer reference holes with respect to the centre of the rotor.

b. A compacted representation of a scan of a cell containing DNA Cu^{++} complex in 5mM KNO_3 sedimenting at 25°C at 40,000 rpm.

of the boundary from the inside edge of the outer reference hole (X_B), and substituting this value into the equation

$$x = C + \left[\frac{\left(\frac{X_I - X_O}{2}\right) - X_B}{M}\right] \qquad V.5$$

Therefore,

$$x = 6.50 + \left[\frac{3.90 - X_B}{5.20}\right] \text{ cm}$$

where the distance from the centre of the rotor to the mid-point of the cells, C, was supplied by Beckman.

4. RESULTS AND DISCUSSION

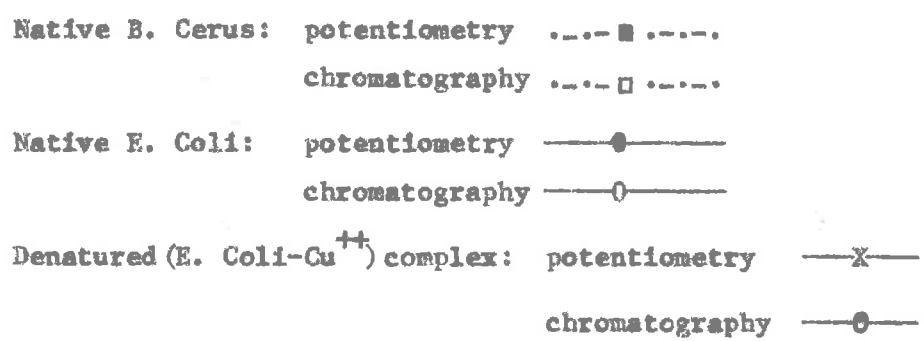
a. Comparison of Several Techniques Used for Binding Studies

As the technique of equilibrium dialysis can be applied to the study of Cu^{++} ion interactions with DNA,[18-20] the method was to be used for some high temperature studies and for the purpose of comparison with binding studies by other techniques. Preliminary studies were conducted to determine the extent of Cu^{++} binding to the dialysis bag. The total Cu^{++} ion concentration was measured both inside (~5 ml) and outside (~20 ml) the dialysis bag by complexing with cuprizon and analysing spectrophotometrically (see Sect. V.2b) after rocking for 96 hours at 25°C. The extent of Cu^{++} ions bound to the dialysis tubing varied from 22% at a Cu^{++} ion concentration of $1.5.10^{-4}$ M, to 38% for a 3.10^{-5} M solution. As studies of the binding of Cu^{++} ions to DNA involve the difference between the total and equilibrium Cu^{++} ion concentrations, this difference often being small, attempts

to allow for the extent of Cu^{++} bound to the dialysis tubing would obviously limit the range of Cu^{++} ion concentrations which could be used and the accuracy of any values obtained. For these reasons, and those discussed in Sect. IV.1, this technique was discarded as a means of determining binding parameters.

Two other techniques used in this study to investigate the native DNA-Cu^{++} interactions were gel-exclusion chromatography and Cu^{++} ion potentiometry. The Cu^{++} complexes of native B. Cerus and E. Coli DNA, as well as the denatured (E. Coli-Cu^{++}) complex were studied by both techniques, and are compared in Fig. V-4. It is apparent that the two techniques yield similar results, although the chromatographic studies involving native B. Cerus and native E. Coli show a consistently smaller extent of Cu^{++} ion interaction over the range of free Cu^{++} ion concentrations studied. This difference is further illustrated in Figs. V-5,6 by Scatchard plots of the interaction of Cu^{++} ions with native DNA. Studies of the denatured (DNA-Cu^{++}) complex by either technique yields similar results (Fig. V.5), although any possible disparity between the two techniques is masked by the large scatter of results. The reason for the small but detectable difference between the binding studies determined by the two techniques is not clear, but may be due to Donnan equilibrium effects which occur in gel-exclusion chromatography, even though calculations indicate that this effect is negligible at the concentrations and conditions used in these studies.

Fig. V-4. Binding of Cu^{++} ions to DNA in 5mM KNO_3, at 25°C.



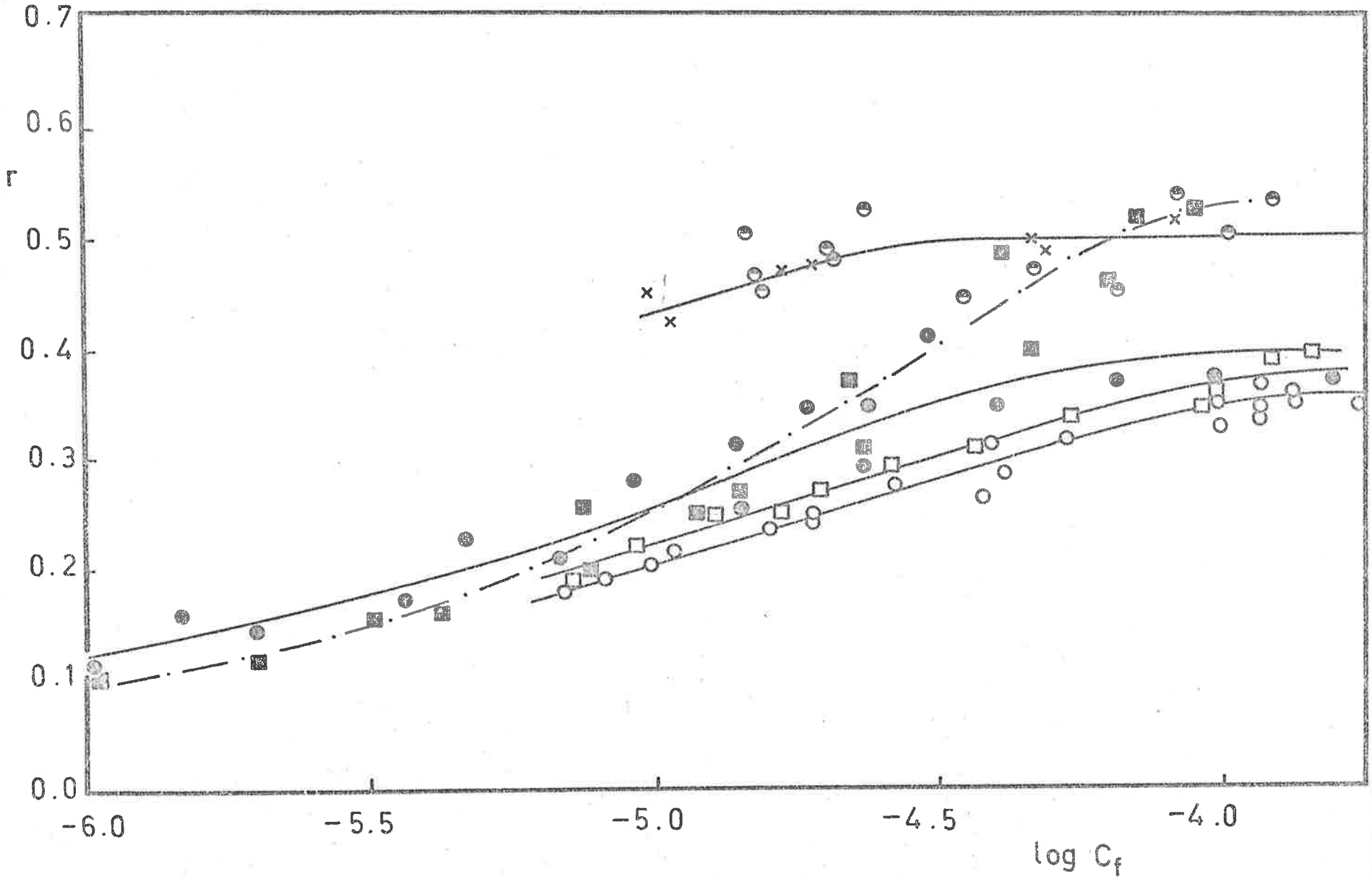


Fig. V-4.

Fig. V-5. Scatchard plots for the interaction of Cu^{++} ions with E. Coli DNA in 5mM KNO_3 at 25°C.

Potentiometric studies of native E. Coli, ●, and single strand denatured E. Coli (heat denatured, studied at 2°C, ○; dilution denatured, studied at 25°C, ◐).

Inset a. Comparison of potentiometric, ●, and chromatographic, X, studies of native E. Coli DNA.

b. Comparison of potentiometric, ▲, and chromatographic, +, studies of the denatured (E. Coli-Cu^{++}) complex.

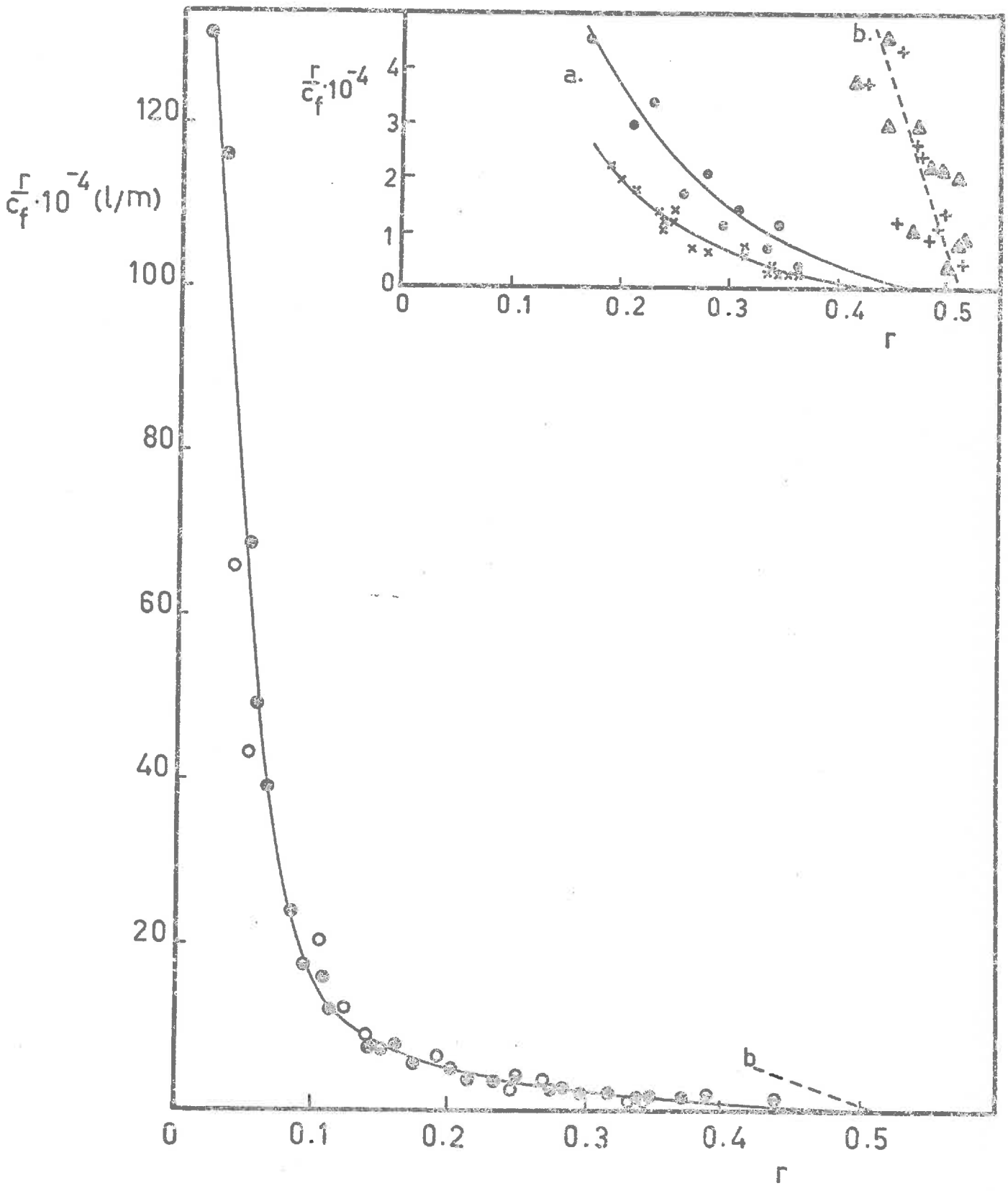

$\frac{r}{c_f}\cdot 10^{-4}$ (l/m)
120
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
r
b
$\frac{r}{c_f}\cdot 10^{-4}$
4
3
2
1
0
a.
b.

Fig. V-5.

Fig. V-6. Scatchard plot for the interaction of Cu^{++} ions with native B. Cerus DNA in 5mM KNO_3 at 25°C,
●, studied by Cu^{++} ion potentiometry,
+, studied by gel exclusion chromatography and single-strand B. Cerus DNA in 5mM KNO_3 at 2°C (0), studied by Cu^{++} ion potentiometry.

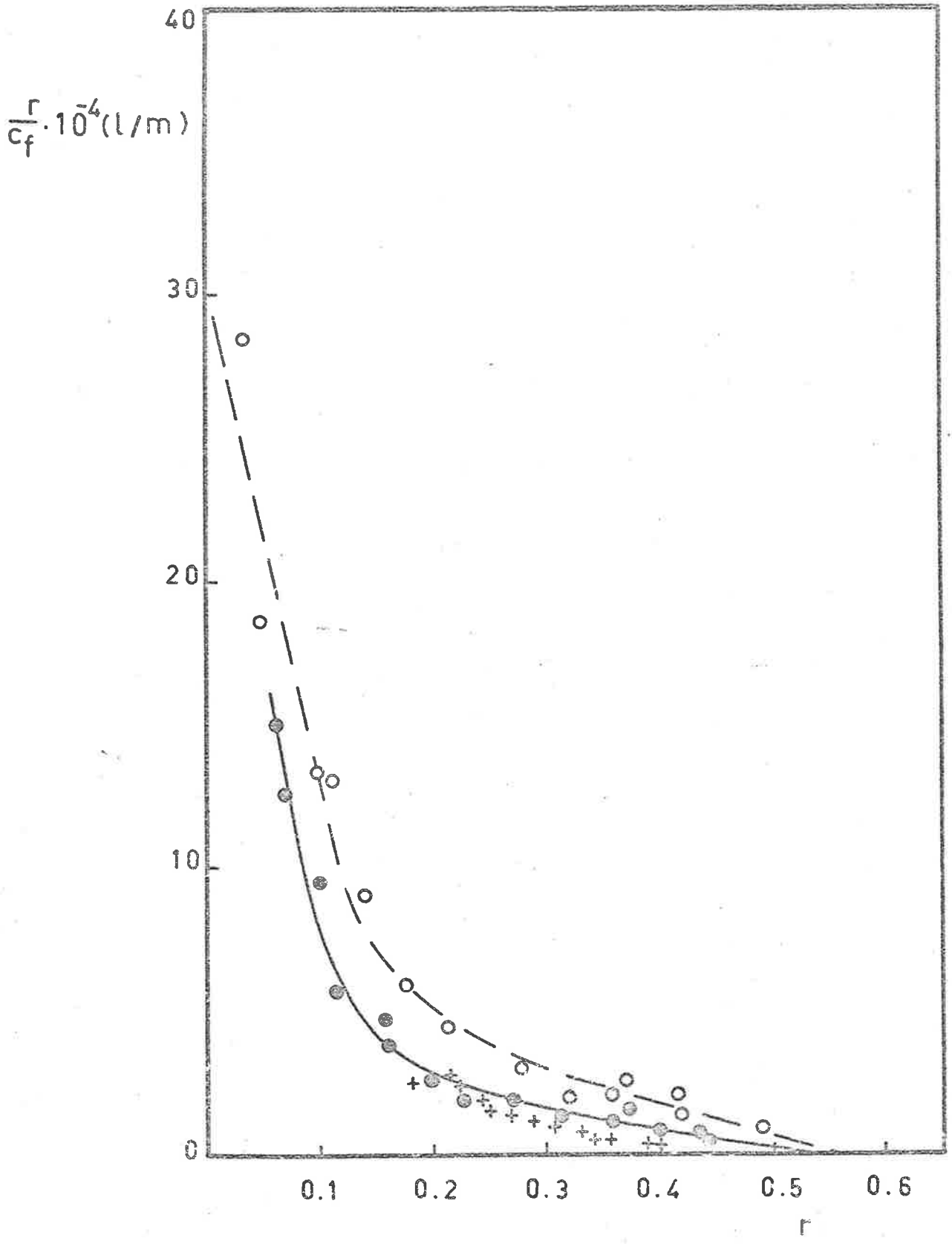


Fig. V-6.

Cu^{++} ion potentiometric studies were used in preference to chromatographic studies for several reasons. The method of analysis for total Cu^{++} ions formed a lower limit of Cu^{++} ion concentrations of 1.10^{-5}M using the chromatographic method, while the Cu^{++} ion electrode extended this lower limit to 1.10^{-7}M of free Cu^{++} ions, thereby enabling a more complete Scatchard plot to be obtained (see Figs. V-5,6). This is an important point as the accuracy of analysis of these Scatchard plots increases significantly the wider the range of binding that is studied. Efficiency was the second deciding issue as 20-30 days were required to obtain chromatographic binding data comparable with that obtained in one day using the Cu^{++} ion electrode.

b. The Binding Parameters

Potentiometric studies of the extent of Cu^{++} ion binding to a range of various (G + C) content DNA's have been presented in Fig. V-7. It can be seen that the native DNA's may be broadly classified as poly dG:dC, M. Lysodeikticus > E. Coli, B. Cerus > poly dAT, with respect to their affinity for Cu^{++} ions.

The Cu^{++} ion binding data for the different DNA's has been presented in terms of Scatchard plots in Figs. V-5,6,8,9. Where there is any curvature of a Scatchard plot, it is necessary to establish whether this is due to an interaction between non-independent sites, or to the presence of more than one type of site. For this reason the data for the native E. Coli-Cu^{++} and native poly dG:dC-Cu^{++}

Fig. V-7. Comparison of the potentiometric binding studies of Cu^{++} ions to DNA, in 5mM KNO_3 and at 25°C unless otherwise stated.

Native DNA-Cu^{++}

poly dAT ———▲———
B. Cereus* -.-.-.-.-
E. Coli* - - - - -
M. Lysodeikticus -..-◆-..-
poly dG:dC ———▼———

(Single strand DNA)-Cu^{++}

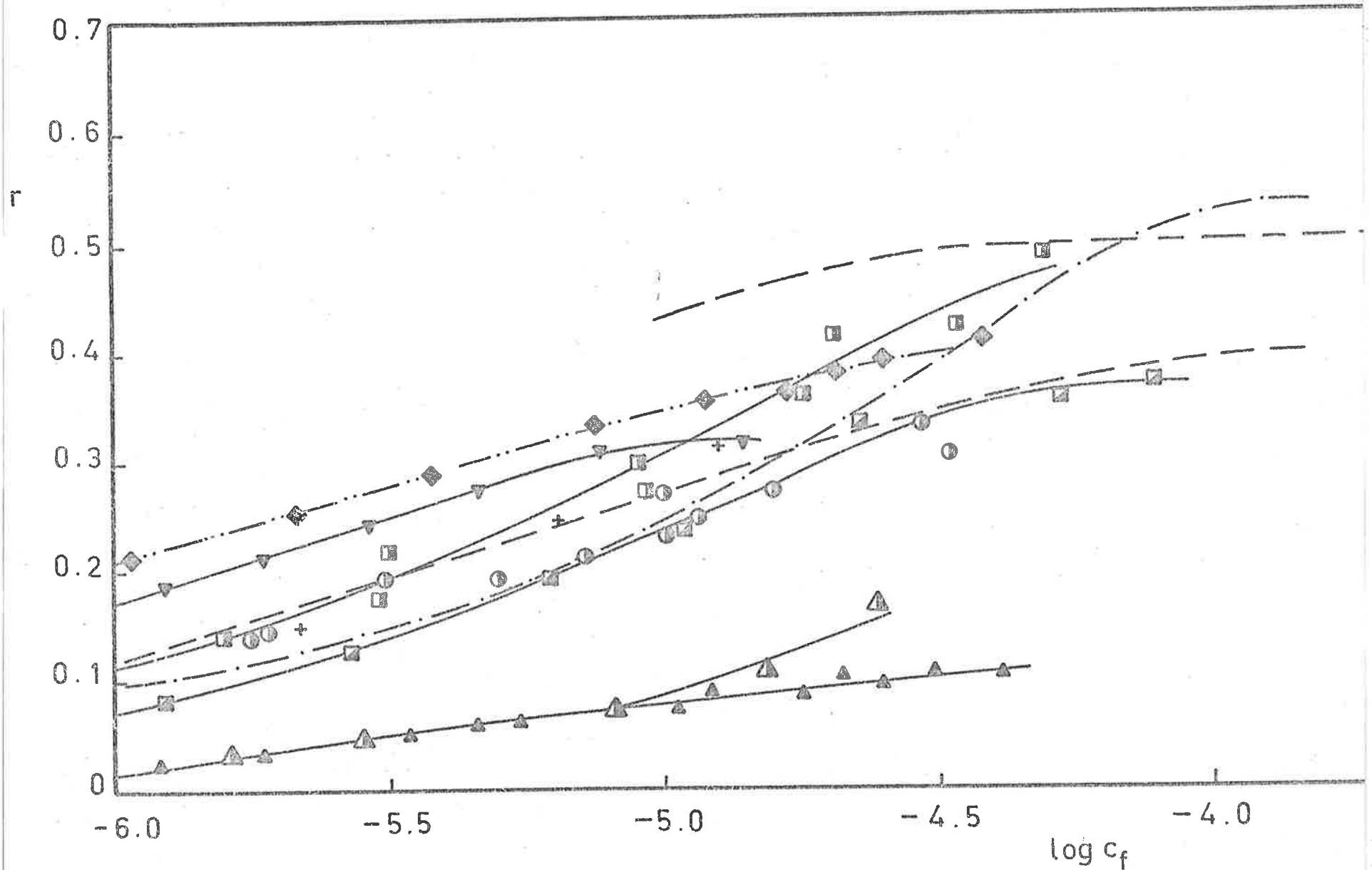
poly dAT, ——▲——, studied at 50°C
B. Cereus ——□——
E. Coli, + dilution denatured, studied at 25°C
0 heat denatured, studied at 2°C
ϕX174, ——◪——

Denatured (DNA-Cu^{++})

E. Coli* — - — - — -

* See Fig. V-4 for experimental points.

Fig. V-7.



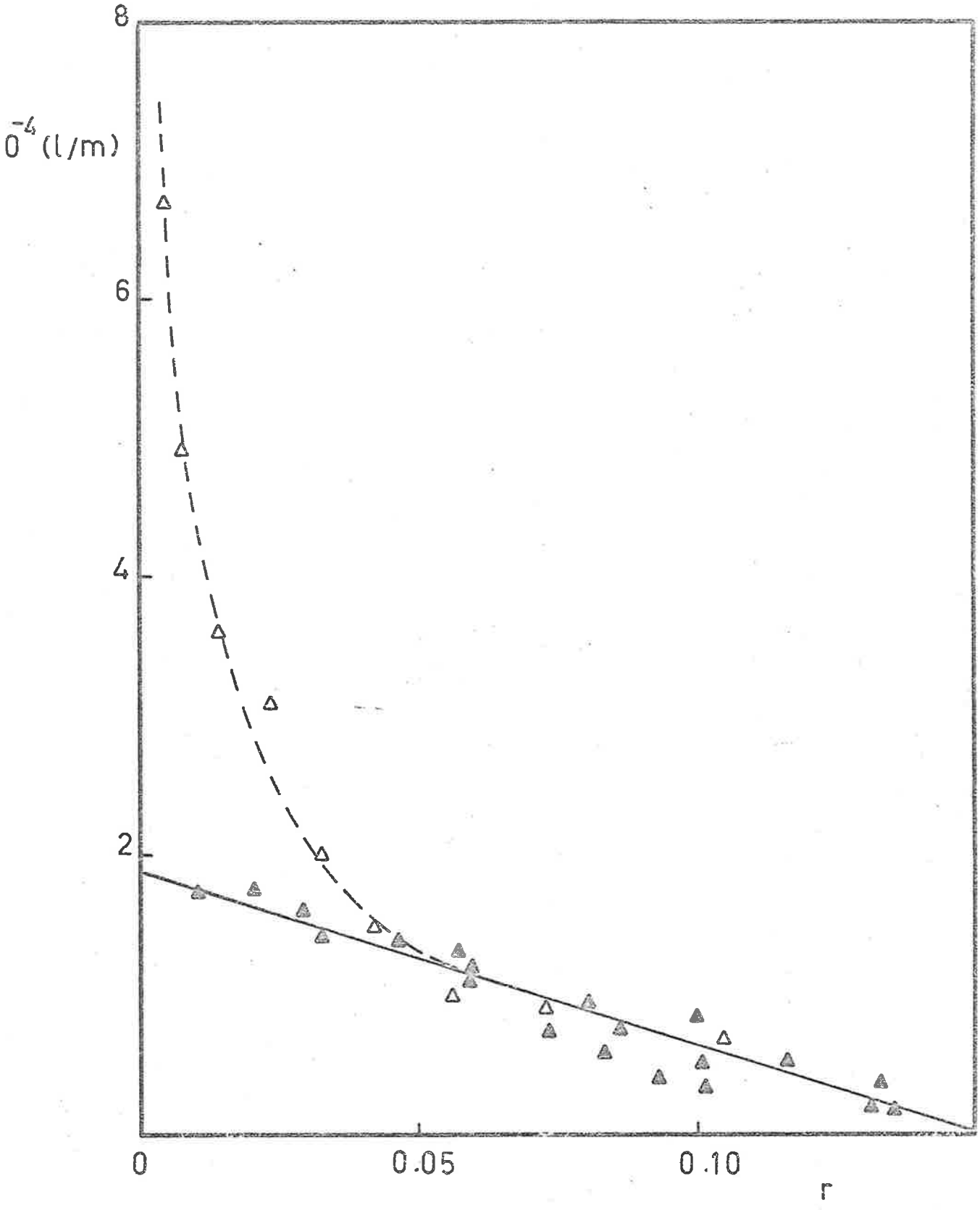


Fig. V-8. Scatchard plots for the interaction of Cu^{++} ions with poly dAT in 5mM KNO_3.

▲ Native poly dAT, 25°C.

Δ Single strand poly dAT, 50°C.

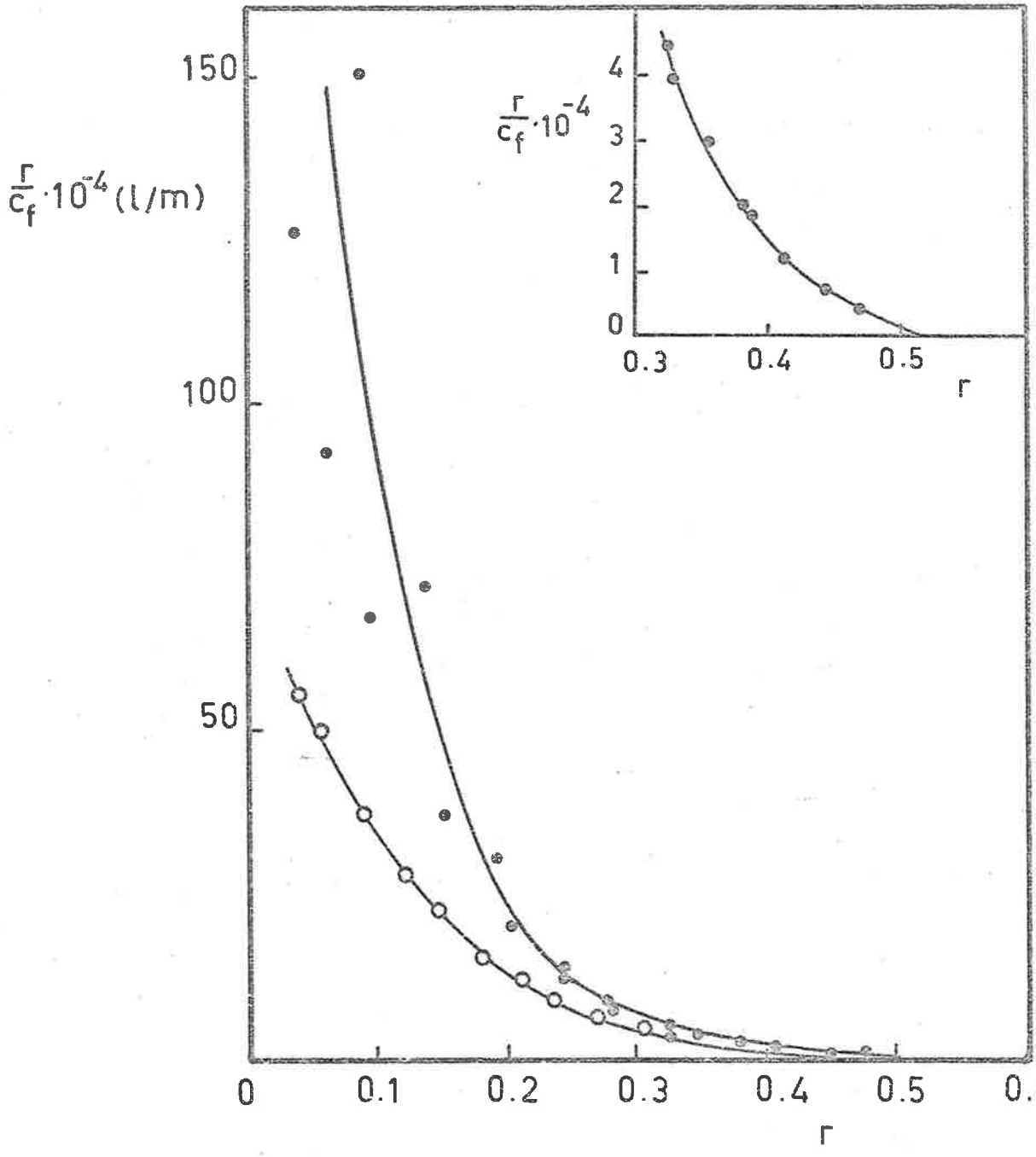


Fig. V-9. Scatchard plots for the interaction of Cu^{++} ions with native M. Lysodeikticus DNA (●) and native poly dG:dC (○) in 5mM KNO_3 at 25°C.

interactions in 5mM KNO_3 (Figs. V-5,9) have been re-plotted (Fig. V-10) with an electrostatic correction function (see Chapt. III), assuming that any interaction between the sites is completely electrostatic in nature.

The electrostatically corrected Scatchard plot for E. Coli is clearly non-linear, while the plot for poly dG:dC is linear. If the initial assumptions regarding the interaction are adequate (i.e. that the interaction between the sites is solely electrostatic in nature and that the electrostatic function used is accurate), then Fig. V-10 indicates that poly dG:dC contains only one type of Cu^{++} ion interacting site, while E. Coli contains two or more types of sites. To check these assumptions, the same data for E. Coli and poly dG:dC (Figs. V-5,9) have been re-plotted in Fig. V-11 according to equation III.26. At $i = 0$, $K_o = 1.6.10^4$ and $1.6.10^5$ for E. Coli and poly dG:dC respectively, in excellent agreement with the values $K_o = 2.2.10^4$ and $1.3.10^5$ determined by analysing the electrostatically corrected data (Fig. V-10) according to the method outlined in Sect. IV.5. The slope of the linear plots in Fig. V-11, w', (8.0 and 5.5 for E. Coli and poly dG:dC respectively) is a parameter representing all of the types of interactions between the sites, and can be compared with the value of 13.4 used assuming an approximate expression for electrostatic interaction exclusively. The excellent agreement of the two determinations for K_o, and the approximate agreement for the order of magnitude for the interaction parameter confirms that the Scatchard

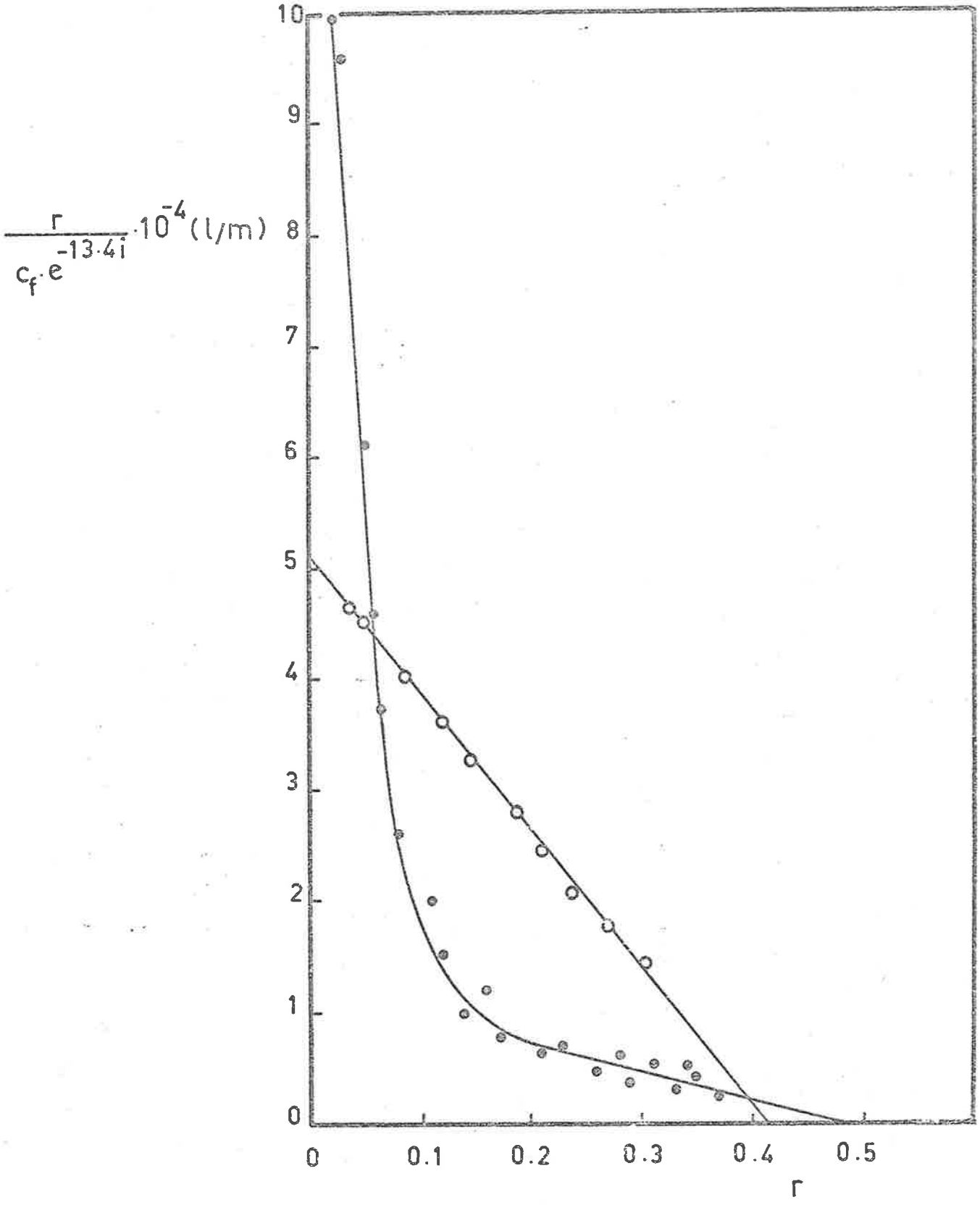


Fig. V-10. Scatchard plots containing an electrostatic correction, for th interaction of Cu^{++} ions with E. Coli DNA (●) and poly dG:dC (○) in 5mM KNO_3 at 25°C.

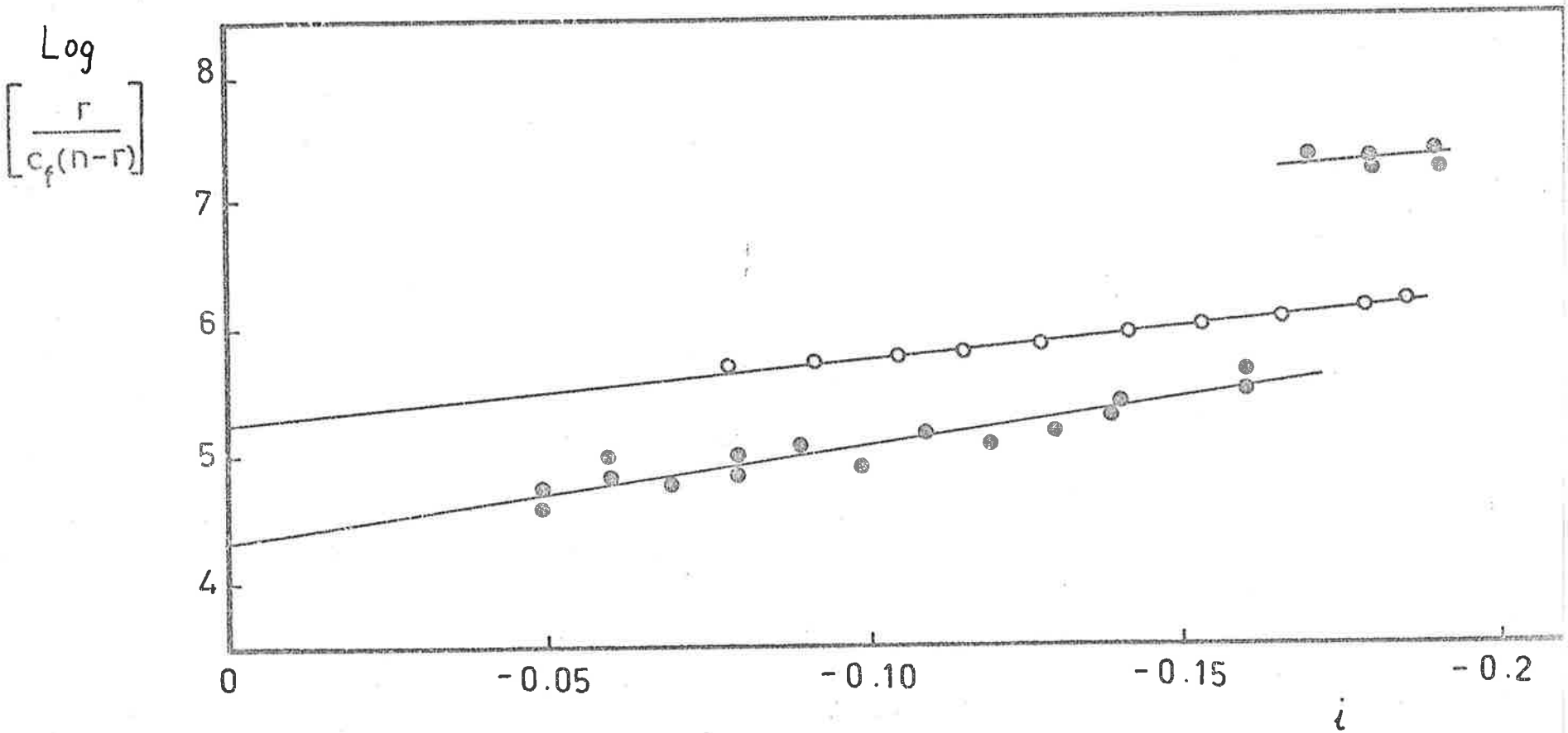


Fig. V-11. Data for the interaction of Cu^{++} ions with E. Coli DNA (●) and poly dG:dC (0) in 5mM KNO_3 at 25°C plotted according to equation III.26.

plots of native poly dG:dC and native E. Coli may be interpreted in terms of one and more than one type of Cu^{++} binding site respectively when in 5mM KNO_3 at 25°C. A similar analysis has been undertaken to confirm the presence of more than one type of Cu^{++} ion interacting site on B. Cerus and M. Lysodeikticus, both of which exhibit marked curvature of the Scatchard plot.

The Scatchard plots were analysed according to the method outlined in Sect. IV.5 and indicate that all of the native DNA-Cu^{++} interactions can be accounted for by a maximum of two types of sites. The binding parameters for these interactions have been listed in Table V-2. The most accurate determination for n, the number of sites per residue, is obtained from the electrostatically corrected Scatchard plots as they possess less curvature than the uncorrected plots, thereby enabling a more accurate extrapolation to obtain n. The approximation for the electrostatic function does not influence the number of sites determined from such plots (see Chapt. III). A K_o value for the stronger E. Coli DNA-Cu^{++} complex could not be obtained as the range of the phosphate charge is limited by the range of r values for which this interaction occurs (Fig. V-11).

These studies clearly indicate that native DNA in 5mM KNO_3 contains two types of Cu^{++} binding sites, supporting the conclusion reached by some earlier studies,[1,2,21-25] but in conflict with other evidence indicating only one type of site.[26-28] The total number of sites determined for E. Coli (n = 0.48) confirms the approximate value

DNA	n_1	K_1	n_2	K_2	Σn
poly dAT	-	-	0.15	12.10^4	0.15
B. Cerus	0.10	$\sim 3.10^6$	0.43	$5.5.10^4$	0.53
E. Coli, Fig. V-5	0.08	23.10^6	0.40	$8.0.10^4$	0.48
Fig. V-10	0.08	-	0.40	K_o, $2.2.10^4$	0.48
Fig. V-11	-	-	-	K_o, $1.6.10^4$	-
M. Lysodeikticus	0.15	15.10^6	0.36	$8.0.10^4$	0.51
poly dG:dC, Fig.V-9	0.40	$\sim 10.10^6$	-	-	0.40
Fig.V-10	0.42	K_o, $1.3.10^5$	-	-	0.42
Fig.V-11	-	K_o, $1.6.10^5$	-	-	-

Table V-2. Binding parameters for the interaction of Cu^{++} ions with native DNA in 5mM KNO_3 at 25°C.

determined by Bryan and Frieden[1] (n ~ 0.5). The apparent stability constant for the weaker E. Coli complex ($K = 8.10^4$) is also similar to values determined by Bach and Miller[27,28] ($K = 6.10^4$), by Bryan and Frieden[1] ($1\text{-}3.10^4$) and by Yatsimirskii et al.[24] ($K = 1.3.10^4$), all using different techniques. The one determination for the stronger interaction[24,29] ($K = 1.5.10^7$) agrees particularly well with the value determined from the present study ($K = 2.3.10^7$).

The interpretation of the complexes associated with these interactions is discussed in detail in the following section.

c. Dependence of the Binding Parameters on the (G + C) Content of Native DNA

The number of sites per residue, n, for the interaction of Cu^{++} ions with native DNA (Table V-2), and the fraction of adjacent guanine residues in the DNA (determined by Josse et al.[30] by nearest neighbour analyses), have been plotted in Fig. V-12 against the (G + C) content of the DNA. The similarity of the fraction of stronger interacting sites and the fraction of GpG groups suggests that one Cu^{++} binding sites per residue corresponds to every GpG group, indicating a 'sandwich' type complex proposed by Schrieber and Daune (see Fig. II-7). Such complexes are represented below for poly dG:dC, for which n would be 0.5. However, from an examination of a molecular model of DNA, it appears that it is stereochemically unfeasible for

Fig. V-12. Variation of the number of Cu^{++} binding sites per DNA residue with the (G + C) content and the GpG content of DNA.

Stronger interaction, ——●——

Fraction of GpG residues, ^{30} ——○——

Weaker interaction; experimental data, ▲
calculated, ————

Total number of sites; experimental data, □
calculated, -•-•-•-

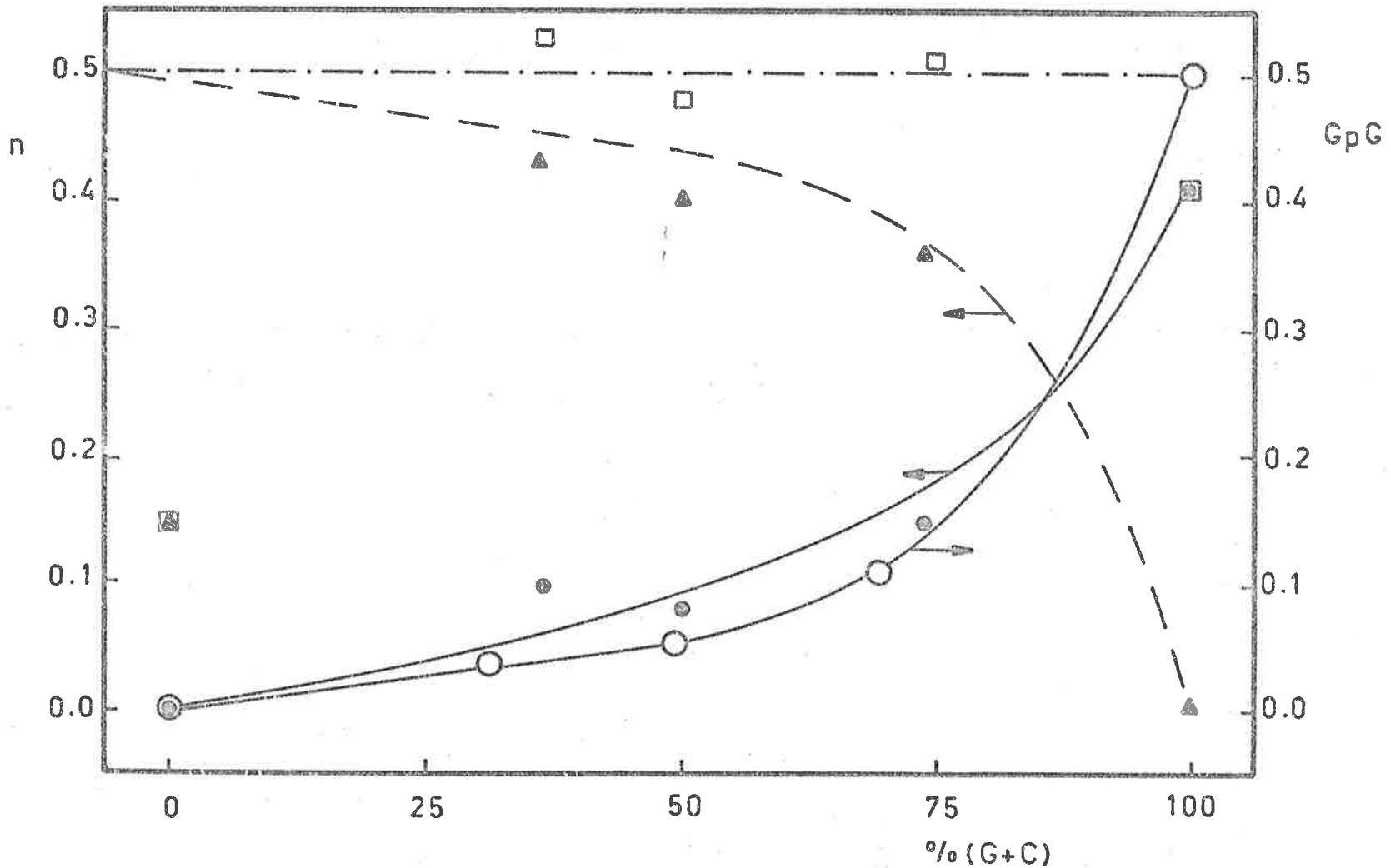
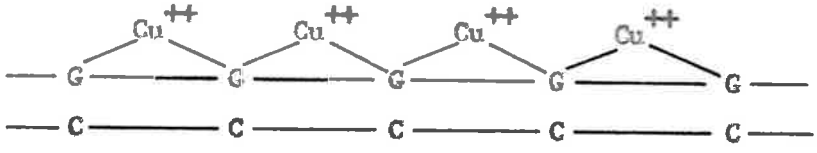
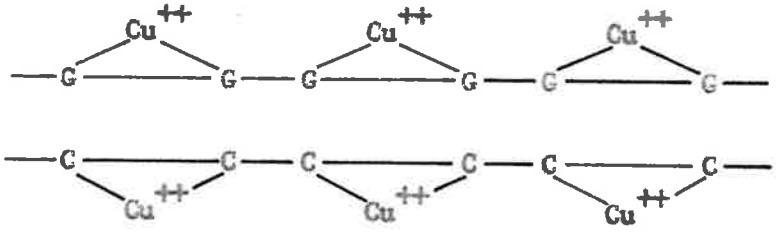


Fig. V-12.



such Cu^{++} complexes to form on both sides of a guanine residue where GpG groups are adjacent to each other. The maximum number of stronger interacting sites of poly dG:dC is therefore 0.25 per residue if each guanine residue is involved in only one 'sandwich' type complex. As Cu^{++} complexes between adjacent cytosine residues have been indicated from Cu^{++} binding studies of poly C (Sect. IV.6b), it appears probable that a similar 'sandwich' type complex involving CpC groups would account for an additional 0.25 sites per residue of poly dG:dC, the total number of such strong interactions being 0.5 per residue, similar to that determined experimentally (0.42) for poly dG:dC. The Cu^{++} binding sites therefore proposed for poly dG:dC are represented below.



The number of similar complexes in other (G + C) content DNA has been listed in Table V-3, assuming selected hypothetical DNA's containing repeating groups of twenty base pairs (i.e. 40 residues), in which occur the GpG sequences indicated in the table. These values

Selected sequences per twenty base pairs	Fraction of GpG groups	Number of sites per residue
----------	0	0
----GG------ ----CC------	0.025	0.05
----GGG----- ----CCC-----	0.05	0.05
----GGGG---- ----CCCC----	0.075	0.10
--GG--GG-- --CC--CC--	0.05	0.10
----GGGGG--- ----CCCCC---	0.10	0.10
GGGGGGGGGG etc. CCCCCCCCCC (poly dG:dC)	0.50	0.50

Table V-3. Relation between the fraction of GpG groups and the number of 'sandwich' type Cu^{++} complexes predicted.

show that there is a close similarity, but not a direct correlation between the fraction of GpG groups and the number of sites with a large affinity for Cu^{++} ions. The small excess of the number of these sites than the fraction of GpG groups, especially for DNA's containing few GpG residues, has also been detected experimentally (see Fig. V-12), supporting the interpretation above of two types of 'sandwich' complexes.

Whether one or two types of 'sandwich' complexes occur in native DNA may later be unambiguously resolved from Cu^{++} binding studies of pGpG and pCpC, as compared with pGpGpG and pCpCpC, the latter two of which are expected to contain the same number of strongly interacting complexes as the first two, as well as one guanine-Cu^{++}-phosphate type complex for each guanine trimer. If a Cu^{++} complex forms between all GpG groups, the pGpGpG group contains twice as many strongly interacting complexes as the pGpG group.

The interpretation above of two types of 'sandwich' complexes for the stronger interaction is acceptable only if the two interactions have similar affinities. If this is not the case, separate interaction would be evident from the Cu^{++} ion titrations (if one stability constan was much greater than the other) or from the Scatchard plots. Studies of the Cu^{++} ion interaction with polynucleotides indicate that the two stability constants are of a similar order of magnitude (from Table IV-3, K ~ 10^8 and 10^7 for the complexes of poly C and poly G respectively), supporting the above interpretation of the stronger Cu^{++}

interacting sites of DNA.

An inter-strand complex bridged between each G - C base pair is excluded for two reasons. Such a complex is not consistent with the observed number of stronger interacting sites as the complex would form between all G - C base pairs, and would therefore not be proportional to the number of adjacent guanine residues. Secondly, complexes with affinities similar to that observed for the strong Cu^{++} ion interaction with DNA occur with single-strand poly G and poly C (Chapt. IV), indicating the formation of intra-strand and not inter-strand complexes.

The weaker interaction may also be interpreted in terms of the (G + C) content of DNA. Since Cu^{++} ions interact with native (double strand) poly dAT (Fig. V-8) with an affinity similar to that for the weaker interaction with other DNA's (Table V-2), and since it is known that Cu^{++} ions fail to interact significantly with the thymine or uridine residues of TMP, UMP, poly U or poly rT (see Sect. II.3a,b), it appears that the interaction with poly dAT is due exclusively to an interaction with the adenine residues. As poly dAT is an alternating co-polymer of adenine and thymine residues, the complex appears to be the adenine residue-phosphate type chelate (Fig. II-5) indicated by studies of the interaction of Cu^{++} ions with dAMP (Sect. IV.6a). The same monomer studies indicate the predominant affinity of Cu^{++} ions for dGMP (Table IV-2), suggesting that a similar guanine residue-phosphate type chelate (Fig. II-5)

may also occur in DNA. Examination of a molecular model of DNA shows that such complexes involving the N_7 groups of the guanine and adenine residues, and the 5' phosphate groups, are possible. There is no evidence from any source to suggest that individual cytosine residues are involved in the weaker Cu^{++} interaction with DNA. In fact, molecular model studies indicate that a similar cytosine residue-phosphate (3' or 5') type chelate involving the N_3 group (see Sect. II.3a) is unable to form due to the stereochemistry of the groups involved.

If the guanine and adenine complexes suggested by the above evidence constitute the weaker native DNA-Cu^{++} complex, then the observed interaction from the Scatchard plot represents the average affinities of the two interactions which must therefore possess similar stabilities. The apparent stability constants for the complexes of Cu^{++} with dGMP and dAMP are $1.7.10^3$ and $0.3.10^3$ respectively. An enhanced stability of such complexes in DNA ($K \sim 8.10^4$) is not unexpected in view of the electrostatic effect of the DNA polymer, the effect of π bonding groups of the base residues both above and below the Cu^{++} complex, and changes of the basicity of the reactive sites of the monomers when in DNA.

If the Cu^{++} ion interactions occurring with native DNA are the four types of complexes discussed above, then the expected number of Cu^{++} binding sites can be calculated as a function of the (G + C) content of the DNA (Table V-4). Fig. V-12 shows that the

(G + C)%	Fraction GpG	Fraction of Sites				n_s	n_w	$n_s + n_w$
		GpG	CpC	G	A			
0	0	0	0	0	0.5	0	0.5	0.5
25	0.04	0.02	0.02	0.085	0.375	0.04	0.46	0.5
50	0.05	0.025	0.025	0.20	0.25	0.05	0.45	0.5
75	0.15	0.075	0.075	0.225	0.125	0.15	0.35	0.5
87.5	0.25	0.125	0.125	0.1875	0.0625	0.25	0.25	0.5
100	0.5	0.25	0.25	0	0	0.5	0	0.5

Table V-4. Calculations of the strong (n_s), weak (n_w) and total ($n_s + n_w$) number of Cu^{++} binding sites of native DNA at low ionic strength, assuming that all* GpG and CpC groups are able to form strong complexes with Cu^{++}.

* See discussion on p. 129.

experimentally determined parameters fit the theoretical curve for all of the DNA's studied except poly dAT. Molecular model studies indicate that the methyl groups of the thymine residues both above and below each adenine residue provide a stereochemical barrier to the formation of a Cu^{++} complex at the N_7 position of the adenine residues. Complexes are therefore likely to form only if accompanied or preceded by sufficient base residue re-orientation to remove the thymine methyl groups from their position adjacent to the N_7 group of adenine. Such a limitation probably accounts for the presence of only a fraction, (0.15), of the expected number of sites, (0.50), per residue of native poly dAT.

The fit of the experimental data to the calculated number of sites provides direct support for the four types of Cu^{++} complexes suggested from these studies for the native DNA-Cu^{++} interaction. The apparent inability of Cu^{++} to form a 'sandwich' type complex between adjacent adenine residues in native DNA may be accounted for by the inability of such complexes to form with double strand poly A (Sect. IV.6), and the fact that conformational changes accompanying the other interactions may also preclude the formation of such complexes.

The previous indications of the importance of the (G + C) content of DNA in DNA-Cu^{++} complexes (IR,[31-34] UV,[32] ORD[33,35] and CD[33]) appear to be due to the apparent dependence of the weaker interaction on the guanine residue content of DNA in the (G + C) content range from 25

to 75% (see Table V-4), the region usually investigated because of the (G + C) range of DNA's normally available. In addition, the stronger interaction appears to involve both guanine and cytosine residues.

The interpretation of the stronger and weaker types of sites contrasts with the assignment by Yatsimirskii et al.[24] of the complex associated with each of these interactions. On the basis of kinetic studies they have concluded that the stronger interaction involves the phosphate groups, but agree with the present study that the weaker interaction involves the base residues. It is significant that their interpretation of the stronger interacting complex was based on several assumptions without any supporting verification. Their values for the apparent stability constants for the two types of complex formed with native DNA, $1.5.10^7$ and $1.3.10^4$, agree reasonably well with the values determined in the present study for native E. Coli DNA, $2.3.10^7$ and 10.10^4, irrespective of the assignment to particular types of complexes.

These results support the observation by Altman[36] that labelled Cu^{++} and Mg^{++} ions does not appear to compete with each other for binding sites on native DNA. It is now apparent that almost all Cu^{++} interaction with native DNA involves the base residues, while only a minute amount (apparent K ~ 50, see Sect. IV.6) competes ineffectively with Mg^{++} ions (apparent K ~ 2.10^4)[37] for the phosphate sites as Mg^{++} ions are known to interact essentially with the phosphate groups

(see Sect. II.6).

d. Ionic Strength Dependence of the Binding Parameters

As some evidence has been presented in this and other studies indicating the importance of the charged phosphate groups in Cu^{++} ion interactions with native DNA (see Sect. II.3), it was necessary to investigate the variation of the binding parameters with ionic strength. Potentiometric studies of the Cu^{++} ion interaction with native DNA at various ionic strengths have been illustrated in Fig. V-13. The binding parameters determined from the present and other investigations are listed in Table V-5, and are plotted against ionic strength in Fig. V-14.

It is apparent that one type of site appears to be independent of the ionic strength, confirming similar results obtained using other techniques.[2,28] However, the number of sites at high ionic strength differs considerably from that determined by Schrieber and Daune[2] and, although the ionic strength dependence is similar (Fig. V-14), the apparent stability constants differ markedly (Table V-5).

From Table V-5 it can be seen that the limiting values of K determined by different procedures at high ionic strengths vary from 1.10^3 to $\sim 2.10^5$, the value determined in the present study being $\sim 1.10^4$.

It has been concluded in the previous section that the stronger interaction consists of 'sandwich' type complexes involving adjacent

Fig. V-13. Scatchard plots for the interaction of Cu^{++} ions with native E. Coli DNA in various ionic strengths at 25°C.

○ 0.005M KNO_3

■ 0.02M KNO_3

Δ 0.05M KNO_3

◆ 0.15M KNO_3

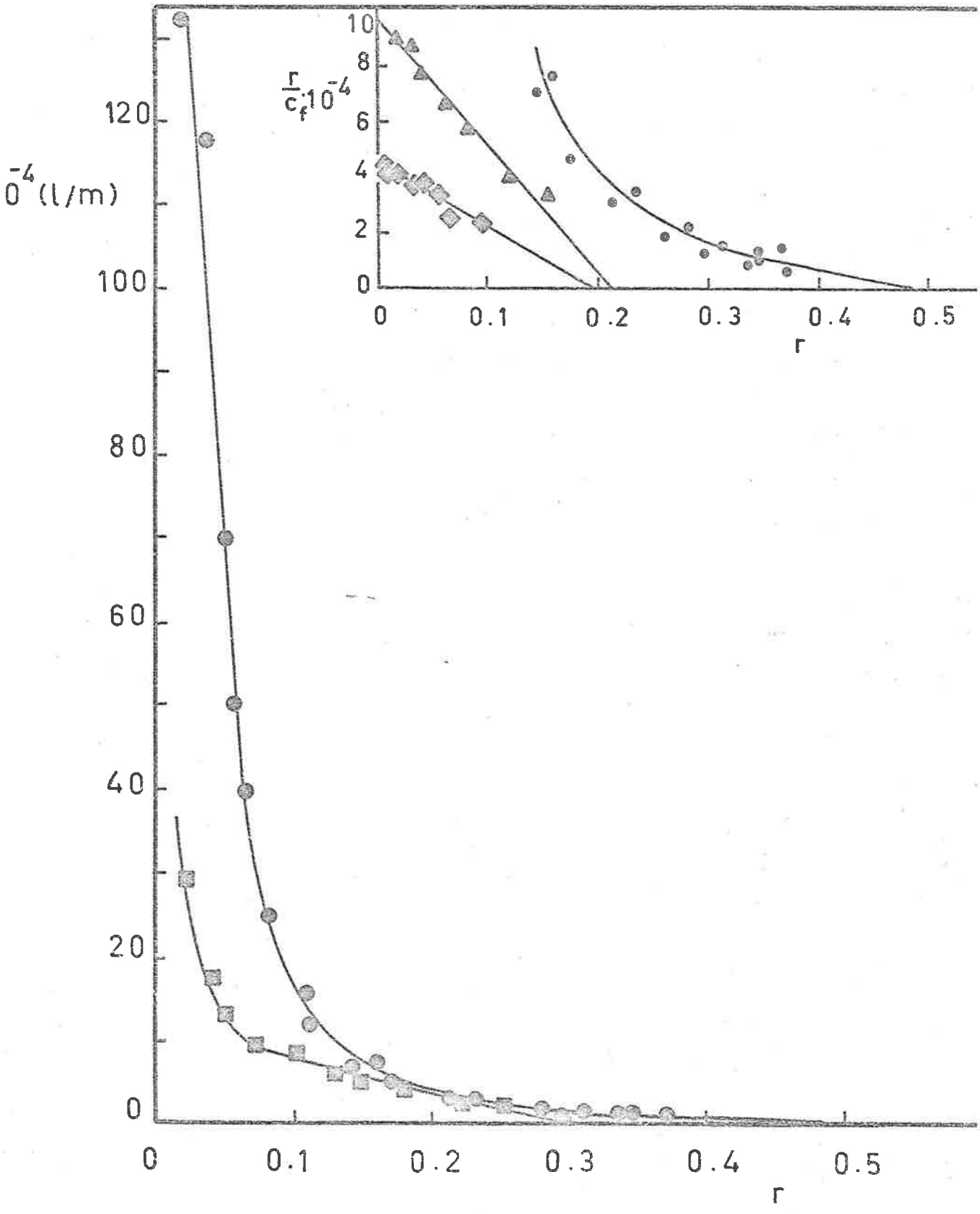

10^{-4} (l/m)
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
r
$\frac{r}{c_f}.10^{-4}$
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
r

Fig. V-13.

Fig. V-14. Variation of the number of Cu^{++} ion binding sites of E. Coli with ionic strength.

O Total number of sites.

□ Number of 'weaker' interacting sites.

Δ Data of Schrieber and Daune.[38]

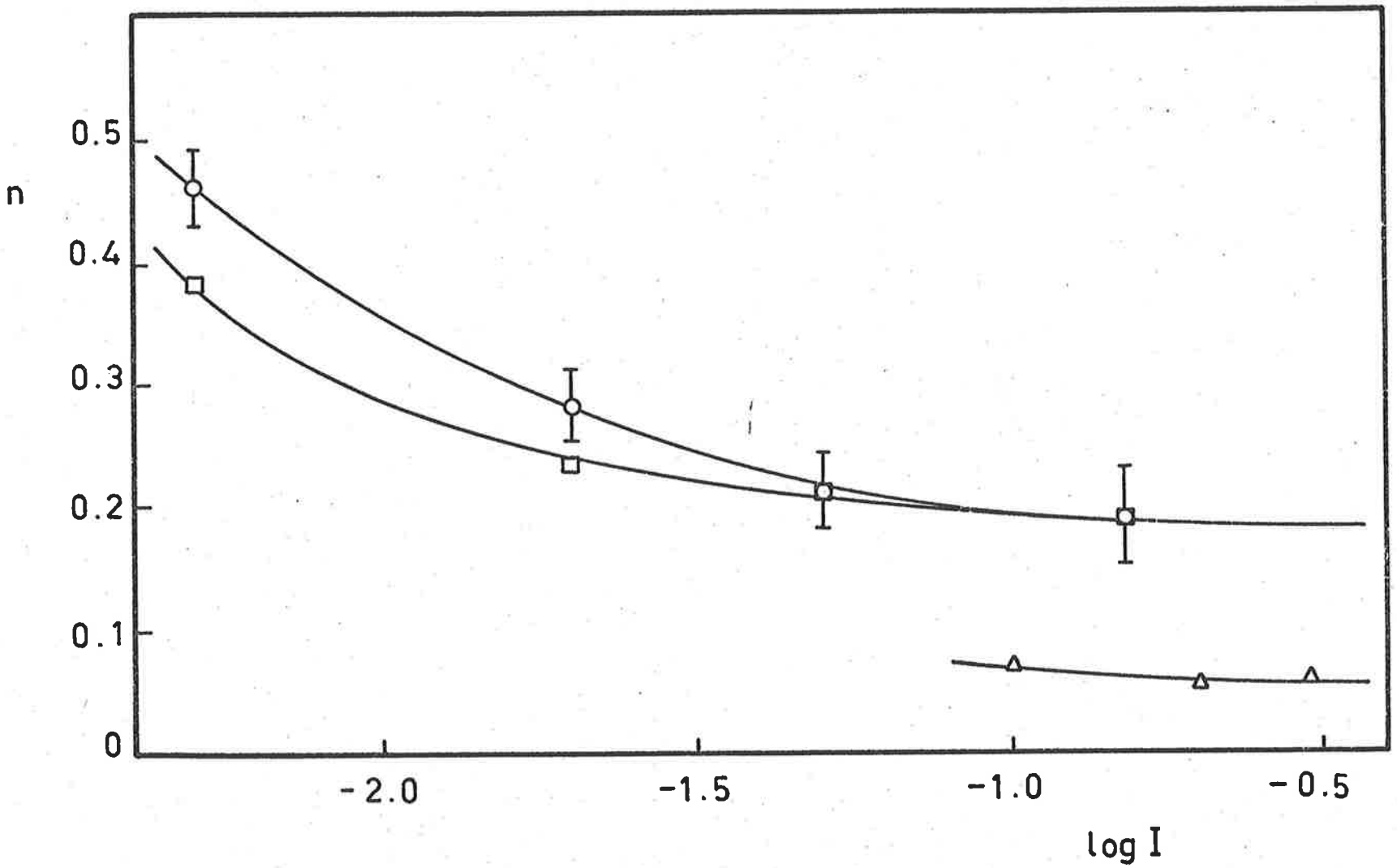


Fig. V-14.

I	n_1	K_1	n_2	K_2	Reference
0.005	0.08	$2.3.10^7$	0.40	$8.0.10^4$	this work
0.02	0.05	$7.5.10^5$	0.23	$4.5.10^4$	this work
0.05	-	-	0.21	$4.6.10^4$	this work
0.15	-	-	0.19	$2.4.10^4$	this work
0.1	-	-	0.069	$\sim 23.10^4$	38
0.2	-	-	0.055	$\sim 23.10^4$	38
0.3	-	-	0.055	$\sim 23.10^4$	38
0.005	-	-	-	$6.3.10^4$	28
0.002	-	-	-	$1.4.10^4$	28
0.05	-	-	-	$5.0.10^3$	28
0.15	-	-	-	$2.0.10^3$	28
0.3	-	-	-	$1.3.10^3$	28
0.14	-	-	<0.1	$1.8.10^3$	39*

Table V-5. Variation of the binding parameters with ionic strength for the interaction of Cu^{++} ions with native DNA (E. Coli) at 25°C.

* Calf Thymus DNA.

guanine residues and adjacent cytosine residues, while the weaker interaction is attributed to guanine-Cu^{++}-phosphate and adenine-Cu^{++}-phosphate chelates. As the stronger interaction is eliminated at high ionic strengths (Table V-5), only the weaker type of complexes exist. This is probably due to conformational changes that occur to DNA on increasing the ionic strength,[33] as studies of the DNA model indicate that the 'sandwich' type complex will be more susceptible to conformational changes than the monomer type interactions. Such a conclusion is opposite to that proposed by Schrieber and Daune[2] who infer that the 'sandwich' type complex remains at high ionic strengths. However, the above proposal can be supported by several lines of evidence. Electrophoretic mobility studies[40] of native DNA have shown that the effective charge on the phosphate groups is not altered appreciably on increasing the ionic strength from 0.02 to 0.2M. It is therefore probable that the base residue-phosphate sites will possess similar affinities for Cu^{++} ions at both low and high ionic strengths as it has been shown that the phosphate groups appear to be involved in such complexes (see Sect. II.3a). The observed stability constants for the $dGMP^{-}$-Cu^{++} interaction, $1.7.10^{3}$ and $0.8.10^{3}$ in 0.005 and 0.15M KNO_3 respectively support the proposals above. In addition, estimates from the data collated by Phillips[41] indicate that a similar decrease of the stability constant occurs for the $dAMP^{-}$-Cu^{++} complex. The relative decrease of K for these mononucleotides is similar to the decrease of K from $8.0.10^{4}$ to $2.4.10^{4}$ for the weaker interaction of

Cu^{++} ions with native DNA in 0.005 and 0.15M KNO_3, clearly supporting the proposal that the weaker native DNA-Cu^{++} interaction involves the base residue-phosphate type Cu^{++} chelates. The reason for the difference of the affinity of Cu^{++} ions for the mononucleotides, compared with the same site in native DNA, has been discussed earlier in Sect. V.4c.

As the phosphate charge of DNA does not vary appreciably with ionic strength in the ionic strength range studied,[40] the stability constant for the weaker interaction will vary only with the electrostatic potential of DNA, provided that no conformational changes occur to DNA as a result of ionic strength changes. In this specific case, it can be seen from equation III.9a that as $I \to \infty$, $K \to K_o$. Bach and Miller[27] have determined a K_o value using this procedure. However, as it is known that the DNA conformation is altered by an increase of the ionic strength,[33] the accuracy of such a K_o value ($1.5.10^3$) is unknown, but does not compare well with the value ($2.2.10^4$) determined using an electrostatic function correction (Fig. V-10) and a value ($1.6.10^4$) determined by a graphical procedure allowing for all types of interactions between sites (Fig. V-11).

e. Hyperchromicity Studies

These studies were undertaken in an attempt to relate the binding parameters for the native DNA-Cu^{++} interaction to hyperchromic and sedimentation changes. The procedure used for the determination of the

hyperchromicities was identical to that described for similar studies involving homo-polynucleotides (Sect. IV.3b). The data has been summarised in Fig. V-15 in the form of a plot of the hyperchromicity, H, against R. The hyperchromicity study involving poly dG:dC was limited by aggregation (when R > 1) which was detected by a decreasing absorbance on standing, but which could be regenerated at any time by shaking the solution. Aggregation was also detected by this method for E. Coli and M. Lysodeikticus DNA when R > 4.

The hyperchromicities of these interactions were independent of time, in agreement with all earlier reports (see Sect. II.2a) except for that of Bryan and Frieden.[1] As a time dependence does occur with the single strand polynucleotides (see Sect. IV.6b), it may be that the DNA used by Bryan and Frieden possesses some single-strand character, a possibility also indicated by anomalously high hyperchromicities of their "native" DNA-Cu^{++} complex (discussed in Sect. II.2a).

There does not appear to be any obvious relation between the hyperchromicity of the plateau region and the (G + C) content of DNA in contrast with that observed by Zimmer and Venner.[32] The value of 10-20% for the plateau region of M. Lysodeikticus is of a similar order of magnitude to that observed by others[32] at 260 mm with the same DNA in 6mM KNO_3 at 25°C. The native E. Coli-Cu^{++} complex exhibits an isosbestic point at 259 nm, confirming earlier reports of this phenomenon.[2]

Fig. V-15. Hyperchromicity of DNA in 5mM KNO_3 for various total Cu^{++} ion per base residue ratios, 30 minutes and 48 hours after mixing at 20°C.
DNA concentration $\sim 1.10^{-4}$ M_p. λ is the wavelength at which maximum hyperchromicity was observed.

poly dAT -·-▲-·-, λ_{max} = 262.5 nm

-·-△-·-, λ = 235 nm (0 to 100% scale)

E. Coli ——■——, λ_{max} = 259 nm

——□——, λ = 275 nm

M. Lysodeikticus --●--, λ_{max} = 257 nm

--○--, λ = 275 nm

poly dG:dC ——— + ——— (λ_{max} = 276 nm; λ = 252 nm)

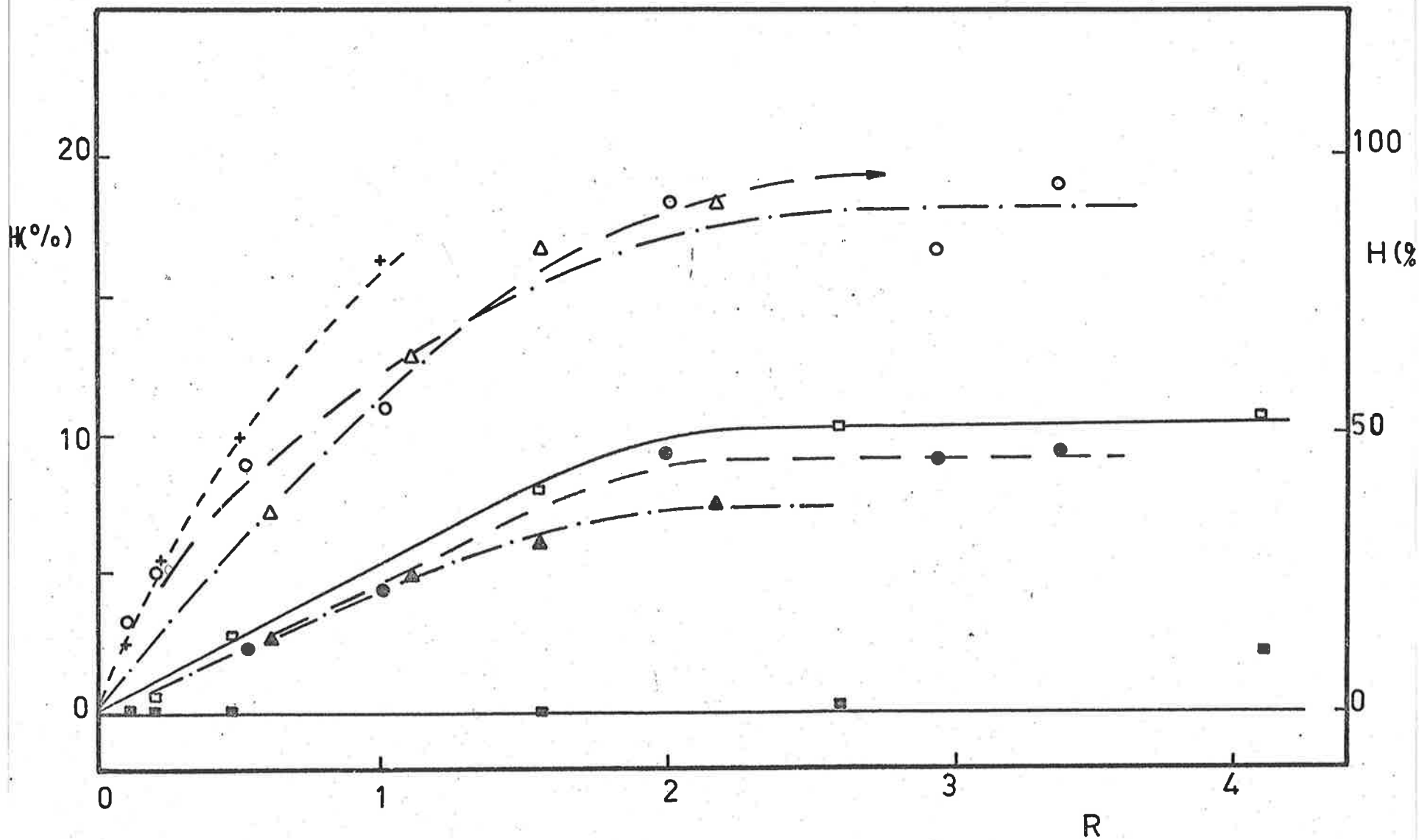


Fig. V-15.

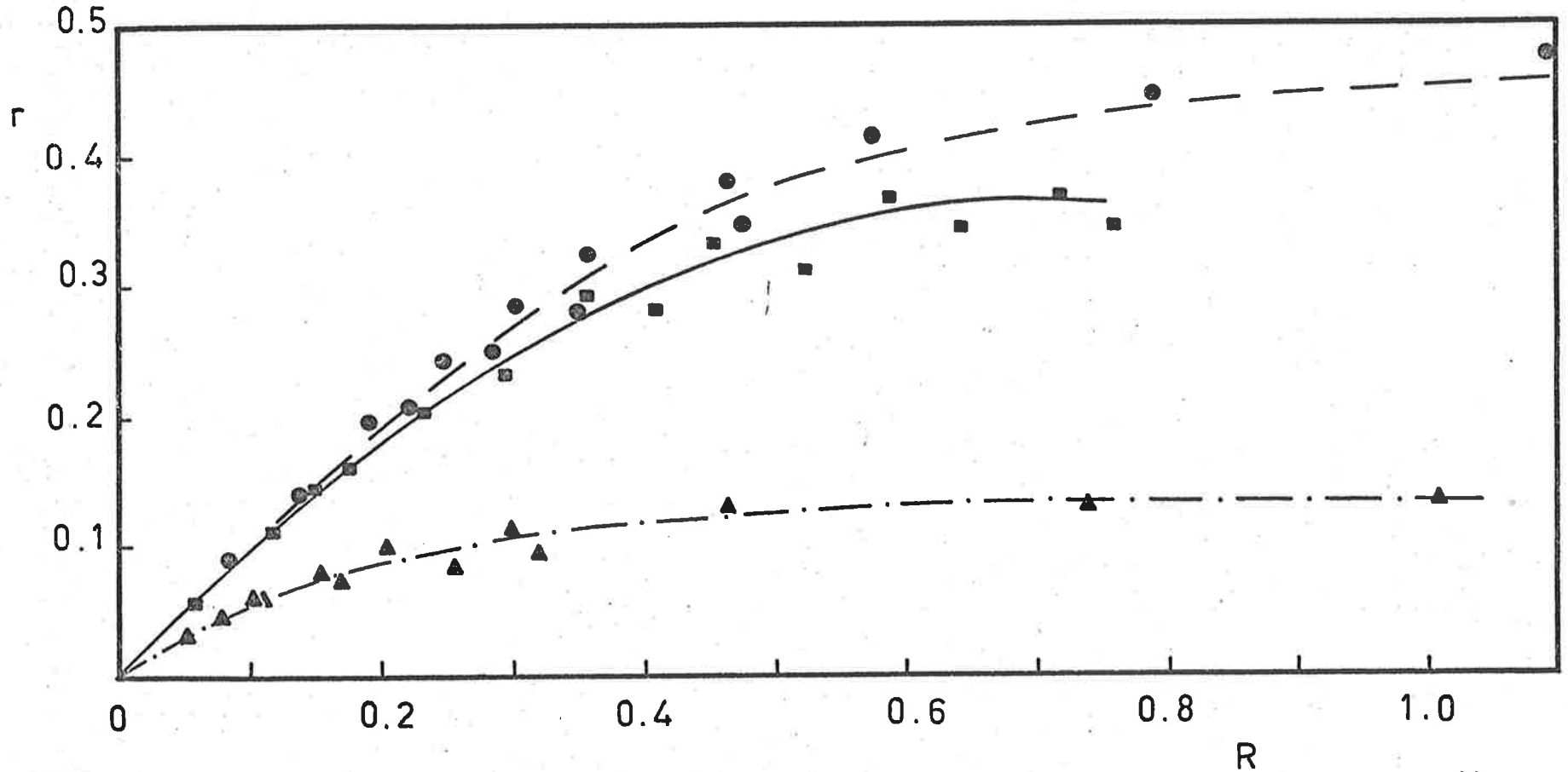


Fig. V-16. Variation of the amount of Cu^{++} bound to DNA per residue (r) with the total Cu^{++} ions present per residue (R) in 5mM KNO_3 at 25°C.

poly dAT -·-▲-·-

E. Coli ——■——

M. Lysodeikticus ---●---

The data in Fig. V-15 indicates that the hyperchromicity of the DNA's studied increases continuously as R varies from 0 to 2. As the number of stronger interacting sites is 0, 0.08 and 0.15 for poly dAT, E. Coli and M. Lysodeikticus respectively (Table V-2), it is clear from Fig. V-16 that for R greater than 0, 0.09 and 0.16 the weaker interacting sites are being titrated, and are therefore responsible for the observed increase of hyperchromicity up to R = 2. This conclusion is consistent with previous observations that the stability constants determined for the native DNA-Cu^{++} interaction by direct binding studies[1] were similar to those determined from ultra-violet difference spectral measurements,[1] indicating that essentially all of the Cu^{++} ions bound to DNA interact with the base residues in some way. A similar conclusion has also been reached in Chapt. IV from studies of the polynucleotide-Cu^{++} interactions.

f. Sedimentation Studies

Sedimentation coefficients of native E. Coli DNA in the presence of a range of Cu^{++} ion concentrations have been plotted in Fig. V-17 against R, the total number of moles of Cu^{++} ion present per base residue. The sedimentation coefficients were corrected to a water standard at $25^{o}C$ (equation V.4).

The $S_{25,w}$ values increase linearly as R varies from 0 to 2.2 and increase rapidly when R > 2.5. The initial small increase of $S_{25,w}$ at low R values has previously been observed by Schrieber and Daune,[2]

Fig. V-17. Viscosity (■) and sedimentation (immediately after mixing, ●; one week after mixing, ○) data for the E. Coli DNA-Cu^{++} interaction in 5mM KNO_3 at 25°C. DNA concentration $\sim 1.10^{-4}$ M_P.

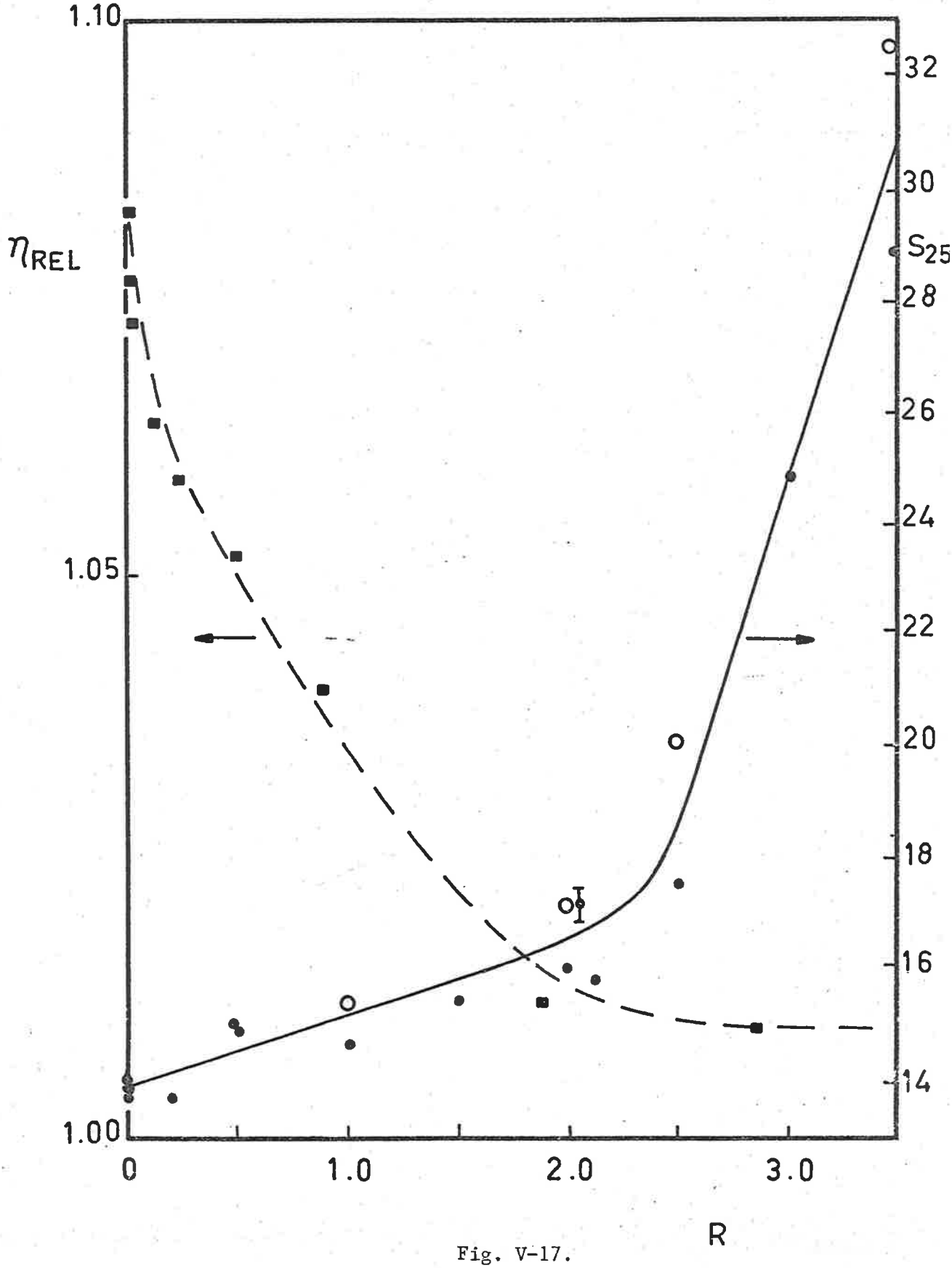


Fig. V-17.

but contrasts with that observed by Bryan and Frieden,[1] who failed to detect any increase of S_{25} when R < 1. As the partial specific volume of DNA, $\bar{\nu}$, does not change appreciably even with massive changes of DNA conformation,[17] the increase of $S_{25,w}$ may be attributed to either a change of the molecular weight of the DNA molecules, or to a change of the frictional coefficient (see equation V.2). As the molecular weight of DNA remains effectively constant at all Cu^{++} ion concentrations used, and strand separation does not occur (indicated in Fig. V-15 by a lack of hyperchromicity of E. Coli at 259 nm), any changes of $S_{25,w}$ appear to be due solely to conformational changes of the native DNA.

As R increases from 0 to 1, the extent of bound Cu^{++} increases up to r ~ 0.4 (see Fig. V-16), resulting in a decrease of the effective phosphate charge per residue from -0.2 to -0.04 (see equation III.23). An increase of the sedimentation coefficient can not be explained in terms of the primary salt effect, as this would produce a decrease of S as the DNA phosphate charge is reduced. It appears therefore that the increase of $S_{25,w}$ is due to a compacting of the DNA molecules as the repulsion between the phosphate groups is reduced when Cu^{++} is bound to DNA, decreasing the phosphate charge. This conclusion is supported by recent CD and ORD studies[33] which detected conformational changes of native DNA when in the presence of Cu^{++} ions at R values as low as 0.02, and by the light scattering and viscometry studies of Schrieber and Daune,[2] when R > 0.01.

A conformational change is also indicated by the change of relative viscosity of the native E. Coli-Cu^{++} solution in the region in which small sedimentation changes occur (Fig. V-17).

The small increase of $S_{25,w}$ occurs in the same range of R for which hyperchromicity changes occur (Fig. V-15), indicating that the presence of Cu^{++} ions not only induces base residue re-orientations, but also causes small conformational changes. Furthermore, both of these changes can be associated with the weaker of the two types of interaction detected by the binding studies discussed in this chapter, as both H and $S_{25,w}$ are proportional to R in the range of R in which the weaker interaction occurs. A direct comparison of these four studies (binding parameters, hyperchromicity, viscosity and sedimentation) is possible as all were conducted under the same conditions (5mM KNO_3 at 25°C).

The rapid increase of $S_{25,w}$ when R > 2.5 can be attributed to aggregation between DNA molecules, and has previously been detected by viscometry,[18] UV absorbance[42] (R > 4), hyperchromicity[1] (R > 2.5), sedimentation[1] (R > 1.5), NMR[43] and direct chromatographic binding studies[1,9] (R > 2). This effect is of little intrinsic interest to the present study as all binding and sedimentation studies were conducted at R values less than 2 to avoid any misleading results from the presence of such aggregation, although visible precipitation does not occur until much larger R values. As expected, aggregation and precipitation appear to depend on both the DNA and total Cu^{++} ion

concentrations.

Some $S_{25,w}$ values were determined one week after mixing the native E. Coli and Cu^{++} ion solutions. From Fig. V-17 it is evident that for low R value solutions the $S_{25,w}$ does not change significantly with the time after mixing, although aggregation does appear to increase with time of standing for the more concentrated Cu^{++} solutions when R > 2.5.

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CHAPTER VI

THE DENATURED DNA-Cu^{++} COMPLEXES

1. INTRODUCTION

A full understanding of the DNA-Cu^{++} interaction requires a knowledge of all the types of complexes involved: the native DNA-Cu^{++} complex, the (single strand DNA)-Cu^{++} complex and the complex formed when DNA is denatured in the presence of Cu^{++} ions. Binding parameters for the latter two complexes are reported in this chapter and are compared with values for the native DNA-Cu^{++} complex.

2. RESULTS AND DISCUSSION

a. The Single Strand DNA-Cu^{++} Complex

Scatchard plots for these complexes involving poly dAT (Fig. V-8), E. Coli (Fig. V-5) and B. Cerus DNA (Fig. V-6) are compared with the interaction involving the corresponding native DNA. The binding parameters have been analysed according to the method outlined in Sect. IV.5 and have been listed in Table VI-1, together with the values for ØX174 DNA (Fig. VI-1).

The single strand form of E. Coli DNA was prepared immediately prior to use by heat denaturation[1] (10 minutes at 75°C, followed by shock cooling) and by dilution denaturation (also heated to 75°C for 10 minutes, then shock cooled, thereby ensuring complete strand separation as distinct from the denatured but still partially double-strand DNA formed by dilution denaturation alone[2]), and was titrated with Cu^{++} ions at 25°C and 2°C respectively. The absorbance of all single-strand DNA was followed throughout the time of the titration (4-8 hours), and no significant strand

DNA	n_1	K_1	n_2	K_2	K_n
poly dAT*	0.02	$3.2.10^6$	0.13	12.10^4	0.15
B. Cerus	0.13	$1.3.10^6$	0.40	$8.4.10^4$	0.53
E. Coli	0.08	$\sim 20.10^6$	0.40	$8.0.10^4$	0.48
ØX174	$\sim$0.1	$\sim 1.10^6$	$\sim$0.4	$8.0.10^4$	0.50

Table VI-1. Binding parameters for the interaction of Cu^{++} ions with single strand DNA in 5mM KNO_3 at 25°C.

* Titrated at 50°C.

re-combination was detected in this time interval.

The Cu^{++} ion titrations of single strand E. Coli DNA at 2°C and 25°C are identical with each other and with the titration with native E. Coli DNA (Fig. V-5) indicating that the single strand DNA produced by either denaturing process is the same, and that the same number and types of Cu^{++} interacting sites are available in either the single-strand or double strand forms of E. Coli DNA, both of which were studied in 5mM KNO_3 at 25°C. Such a conclusion is confirmed by the similarity of the Cu^{++} ion interactions with single strand and native B. Cerus DNA (Fig. V-6 and Tables V-2 and VI-1). The similarity of the titration at 2°C (where any strand re-combination is a minimum) with the single strand E. Coli titration at 25°C not only provides a further test of the validity of the single-stranded nature of E. Coli DNA in the 25°C study, but also indicates that ΔH for the reaction is negligible. A similar conclusion has also been reached from more extensive studies of the temperature independence of the apparent stability constants for the native DNA-Cu^{++} interaction.[3]

As a further test of the single-stranded nature of the denatured DNA, ØX174 DNA, which is known to be single stranded,[4] was titrated with Cu^{++} ions (Fig. VI-1). As the native DNA-Cu^{++} interaction has a known (G + C) dependence (Sect. V.4c), and the number and stability of these sites appears to be unchanged for the single-strand DNA, the number and stability of these sites for ØX174 DNA (43% (G + C) content), may be predicted from Fig. V-12 and Table V-2. It is expected that two

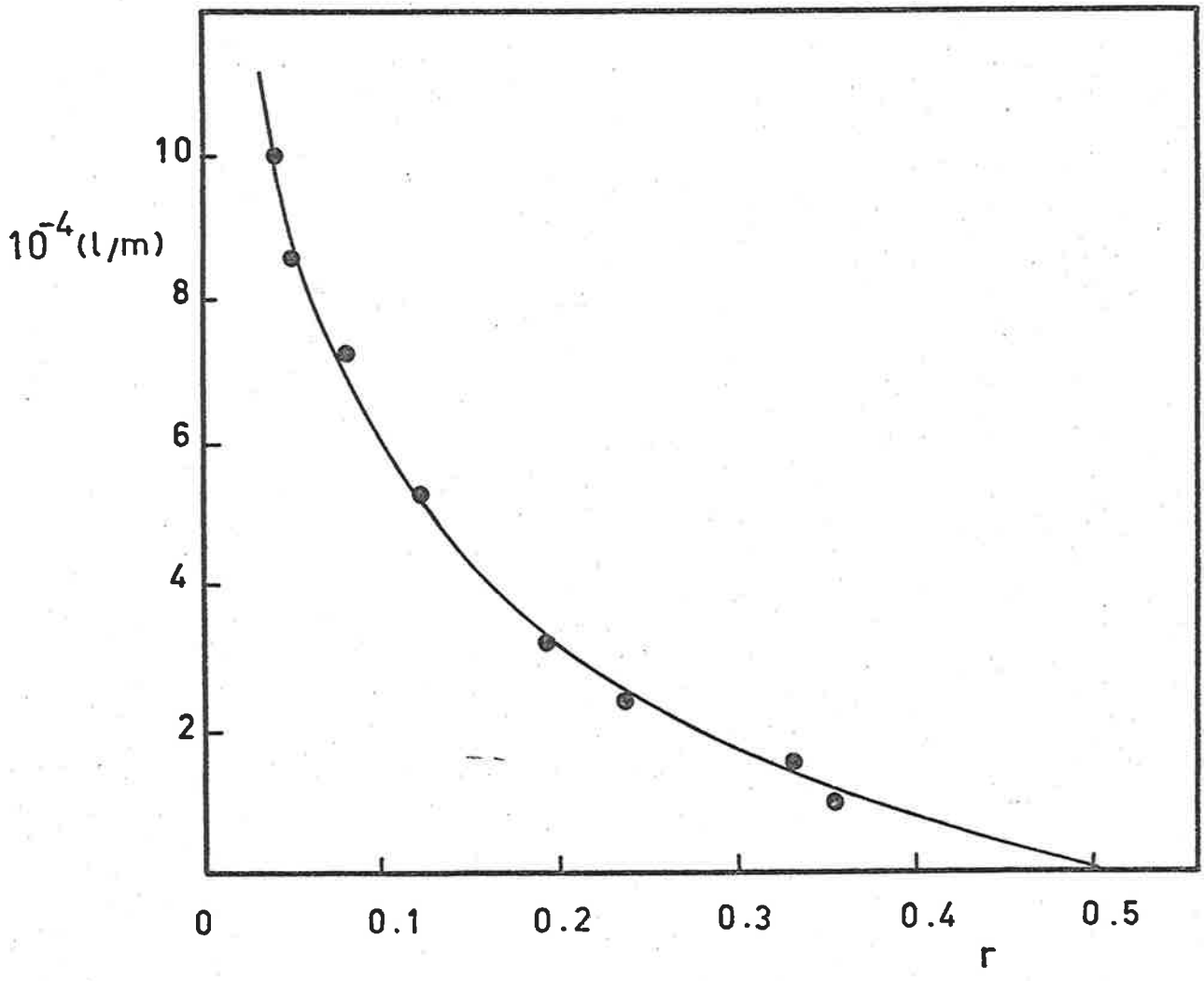


Fig. VI-1. Scatchard plot for the interaction of Cu^{++} ions with ØX174 DNA in 5mM KNO_3 at 25°C.

types of sites will be available with stabilities intermediate between those for E. Coli and B. Cerus DNA, and that the total number of sites will be 0.5 per DNA residue. This is in fact observed (Table VI-1), supporting the proposal that the number and stability of Cu^{++} binding sites of native and single strand DNA are identical.

The binding parameters for the poly dAT-Cu^{++} interactions support the above proposal as $K = 12.10^4$ for both the native ($n = 0.15$) and single-strand complex ($n = 0.13$). However, the anomalous behaviour of poly dAT detected previously (Sect. V.4c), is further illustrated by the formation of a few stronger complexes ($n = 0.02$, $K = 3.10^6$) not observed with native poly dAT. Any interpretation of the complex involved would be conjectural at this stage, but may be related to the loss of base stacking forces at $50^{\circ}C$, the temperature of the single strand poly dAT-Cu^{++} titration.

The similarity of the number and stability of the Cu^{++} binding sites for single-strand and double-strand DNA further supports the assignment of the stronger interacting sites of native DNA to 'sandwich' type complexes on individual strands (indicated by the binding parameters for the single-strand polynucleotides), rather than inter-strand complexes involving the guanine and cytosine residues.

b. The Denatured (DNA-Cu^{++}) Complex

It is known that strand separation of the denatured (DNA-Cu^{++}) complex may occur at low Cu^{++} ion concentrations,[5] although it has been

well documented that there is no decrease of the hyperchromicity of the complex if R > 2.[6-12] Therefore, prior to investigating the binding parameters of the complex formed by denaturing DNA in the presence of Cu^{++} ions, it was necessary to establish the minimum total Cu^{++} ion concentration required to maintain the complex at 25°C. E. Coli DNA (1.10^{-4} M_P) was denatured at 75°C for 10 minutes in the presence of varying total Cu^{++} ion concentrations. The minimum ratio of Cu^{++} ions to base residues which maintained a constant hyperchromicity on cooling from 75°C to 25°C, was R = 0.75, significantly lower than the value of 1.3 determined by Ivanov and Minchenkova.[12] A constant hyperchromicity on cooling was maintained for all R values up to 2.0, which was selected as the upper limit for binding studies in view of the formation of aggregates and precipitates of native DNA-Cu^{++} when R is much greater than 2.[13,14]

The denatured (E. Coli-Cu^{++}) interaction was studied using the techniques of gel exclusion chromatography and Cu^{++} ion potentiometry, described in Sects. V.2 and IV.3, using individually denatured (75° for 10 minutes) DNA-Cu^{++} solutions of known concentrations. Working within the limits of R from 0.75-2.0, it was just possible to obtain Scatchard plots over a sufficient range to establish the binding parameters. The data has been presented in Fig. V-5 for comparison with the native E. Coli-Cu^{++} interaction. It is apparent from Fig. V-5 that the data determined by the two techniques agree particularly well, and that one type of site is indicated, the binding parameters of which are n = 0.51

and $K = 6.4.10^5$.

As a result of the limited range of Cu^{++} ion concentrations attainable for this study, it was not possible to determine whether any stronger interacting sites were present, thereby increasing the difficulty of interpreting the interaction in terms of actual sites of complex formation. The 'sandwich' type of complex between adjacent guanine and adjacent cytosine residues in native DNA may be able to form, but can not be detected as such complexes form below the lower limit of $R = 0.75$. However, as small conformational changes appear to prevent such interactions in native DNA (see Sect. V.4d), it is probable that these complexes are incapable of forming in the denatured ($DNA-Cu^{++}$) complex as large conformational changes are known to accompany this process.[5,8]

The maximum number of inter-strand $G - Cu^{++} - C$ type complexes per residue for E. Coli DNA is therefore expected to be 0.25, although this value could possibly be 0.20 with the other 0.05 sites per residue occupied by the 'sandwich' type complexes. It is expected that all adenine residues in E. Coli DNA are capable of forming adenine-phosphate type chelates with Cu^{++} as in native DNA (see Fig. II-5 and Sect. V.4), the number of sites being 0.25 per residue. The predicted total number of sites per residue ($n = 0.50$) agrees with that determined for E. Coli DNA ($n = 0.51$), although the stability constant of the adenine-phosphate chelate of Cu^{++} is $6.4.10^5$, which can not be readily reconciled with the value of $(0.5-1.2).10^5$ for the same complex in the native E. Coli-Cu^{++}

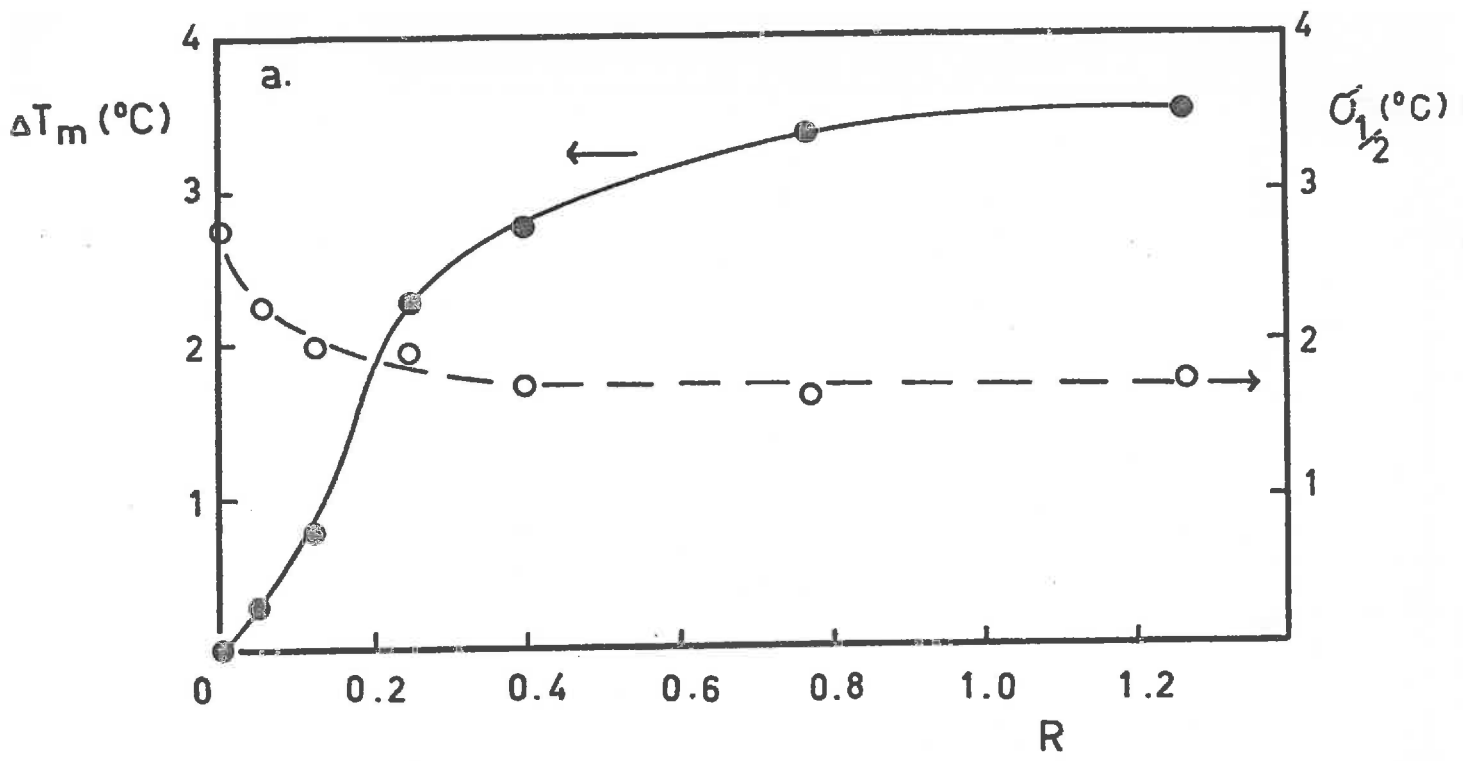
complex. Only if both this and the inter-strand complex possess similar stabilities of the order of 6.10^5 can these results be interpreted in terms of two distinct types of sites.

It is clear that the above interpretation is tentative and that the denatured (DNA-Cu^{++}) complex must be investigated with a range of (G + C) content DNA's to determine the exact nature of the complexes involved. However, any other interpretation must also take into account the known dependence of the complex on the (G + C) content of the DNA, indicated by hyperchromicity dispersion,[15,16] thermal denaturation[8,10] and renaturation[5] and conformational studies,[5] all of which have been discussed in detail in Sect. II.2b and Sect. II.3d.

The model for the inter-strand Cu^{++} complex indicated in Fig. II-8 has been suggested from earlier investigations of the complex and has been discussed in Sect. II.5b.

c. Dependence of the T_m of poly dAT on the Presence of Cu^{++} Ions

The T_m of poly dAT is known to increase in the presence of Cu^{++} ions.[8,10] The actual variation of T_m with the total Cu^{++} ion concentratio has been plotted in Fig. VI-2a in the form of ΔT_m against R (the ratio of total Cu^{++} ions per base residue), similar to the presentation of T_m data for other DNA's in the presence of Cu^{++} ions.[7,10,17] However, the interpretation of the data presented in this way can be misleading as the T_m of DNA is expected to vary with the extent of Cu^{++} bound to DNA, but not with the total Cu^{++} ion concentration. For this reason the data



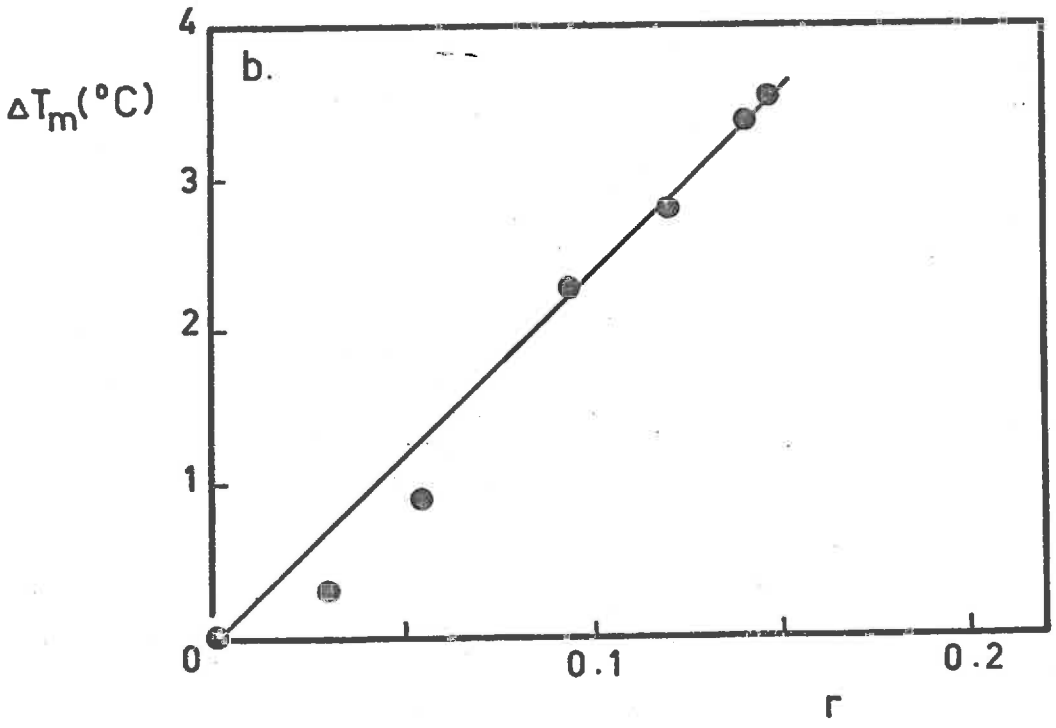


Fig. VI-2. Melting temperature data for poly dAT in the presence of Cu^{++} ions and 5mM KNO_3 (r determined at 25°C). T_m of poly dAT in 5mM KNO_3 is 35.8°C.

has been re-plotted in Fig. VI-2b against r, where r has been determined from the binding data in Fig. V-16 assuming that the sites of Cu^{++} interaction are similar to those in native poly dAT and that the extent of binding does not alter appreciably in the small temperature range indicated. Such an assumption is reasonable as the only types of Cu^{++} interactions with poly dAT that have been indicated from the present study are an adenine residue-phosphate chelate of Cu^{++} (Sect. V.4) and a small number of undefined complexes. Furthermore, the hyperchromicity of poly dAT denatured in the presence of Cu^{++} ions was found to be instantly and completely reversible on cooling (in contrast with that noted by Hiai[8]), indicating the lack of any inter-strand or other such complexes responsible for the irreversibility of the hyperchromicity on cooling other denatured (DNA-Cu^{++}) complexes (see Sect. II.5b).

The breadth of the melting point transition, represented by $\sigma_{\frac{1}{2}}$ (the breadth of the 25% to 75% hyperchromicity change), has also been plotted against R (Fig. V-2a). The transition breadth decreases to a limiting value nearly half that in the absence of Cu^{++} ions, indicating a co-operati transition which has also been detected for other DNA's in the presence of Cu^{++} ions.[8,10,17]

The linear relation between ΔT_m and r in Fig. VI-2b implies that the Cu^{++} interaction with the adenine residue is responsible for the increase of thermal stability as the binding and hyperchromicity data presented in Chapt. V has indicated the presence of such interactions with poly dAT. This conclusion is not in agreement with that of Eichhorn et al.[6,7,11] who

suggest that the stabilisation of DNA is due to a phosphate charge neutralisation, whereas the de-stabilisation is attributed to a base residue-Cu^{++} interaction. This interpretation may be further challenged because in 5mM KNO_3, where the DNA phosphate charge is screened to a large extent,[18] any effect on the DNA charge due to a purely electrostatic phosphate-Cu^{++} interaction is negligible at low R values because of the small affinity for such interactions (K ~ 50, see Sect. IV.6).

An alternative explanation is possible for the variation of T_m of DNA in the presence of small concentrations of Cu^{++} ions. If the predominant interactions in the denatured (DNA-Cu^{++}) complex are the adenine-phosphate chelate of Cu^{++} and the inter-strand guanine-Cu^{++}-cytosine complexes, and it appears from the poly dAT-Cu^{++} T_m data that an adenine-Cu^{++} complex stabilises the helix, the inter-strand complex may well de-stabilise the helix, accounting for the known (G + C) dependence of ΔT_m.[8,10] Future investigations on this topic can readily test this proposal as the T_m of poly dG:dC is not expected to be stabilised at all when in the presence of Cu^{++} ions at low R values. However, in the presence of large concentrations of Cu^{++} ions a screening effect of the phosphate groups is expected, the effect being comparable with other cations. Such an effect has been shown to occur with poly rT.[19]

3. T_m DEPENDENCE OF DNA ON Cu^{++} ION CONCENTRATION

Assuming that the extent of Cu^{++} bound to E. Coli DNA is largely

invariant of temperature, the melting temperature data of Eichhorn and Clark[7] has been re-plotted in Fig. VI-3 against the extent of bound Cu^{++} (from the binding data at 25°C, illustrated in Fig. V-16). It appears that the helix is stabilised when $r \tilde{<} 0.2$, supporting the proposal in Sect. VI.2c that the stabilisation may be due to Cu^{++} complexes involving the adenine residues (n = 0.25 for E. Coli).

Furthermore, the break of the curve[7] at high Cu^{++} ion concentrations when plotted against R is no longer evident, indicating that such a break is not related to stoichiometric binding of Cu^{++} to DNA. This confirms a similar observation by Bach and Miller.[20]

4. RENATURATION OF THE DENATURED (DNA-Cu^{++}) COMPLEX

Renaturation of the denatured (DNA-Cu^{++}) complex at high ionic strengths appears to produce DNA exhibiting all of the properties of native DNA (see Sect. II.2b). However, evidence has been presented in Chapt. VI suggesting that the denatured (DNA-Cu^{++}) complex contains an adenine-phosphate chelate of Cu^{++} as well as an inter-strand complex. The inter-strand complex appears to be eliminated at high ionic strength (as the hyperchromicity reverts to that of the native DNA), allowing the formation of a guanine-phosphate chelate of Cu^{++}, as in native DNA. Such complexes do exist in native DNA as the weaker interaction persists in 0.15M KNO_3 (see Sect. V.4d) as well as in a GMP^{-}-Cu^{++} complex in 0.15M KNO_3 (see Table IV-2), and they are expected to be accompanied by hyperchromic and conformational changes of DNA, as in 5mM KNO_3. The

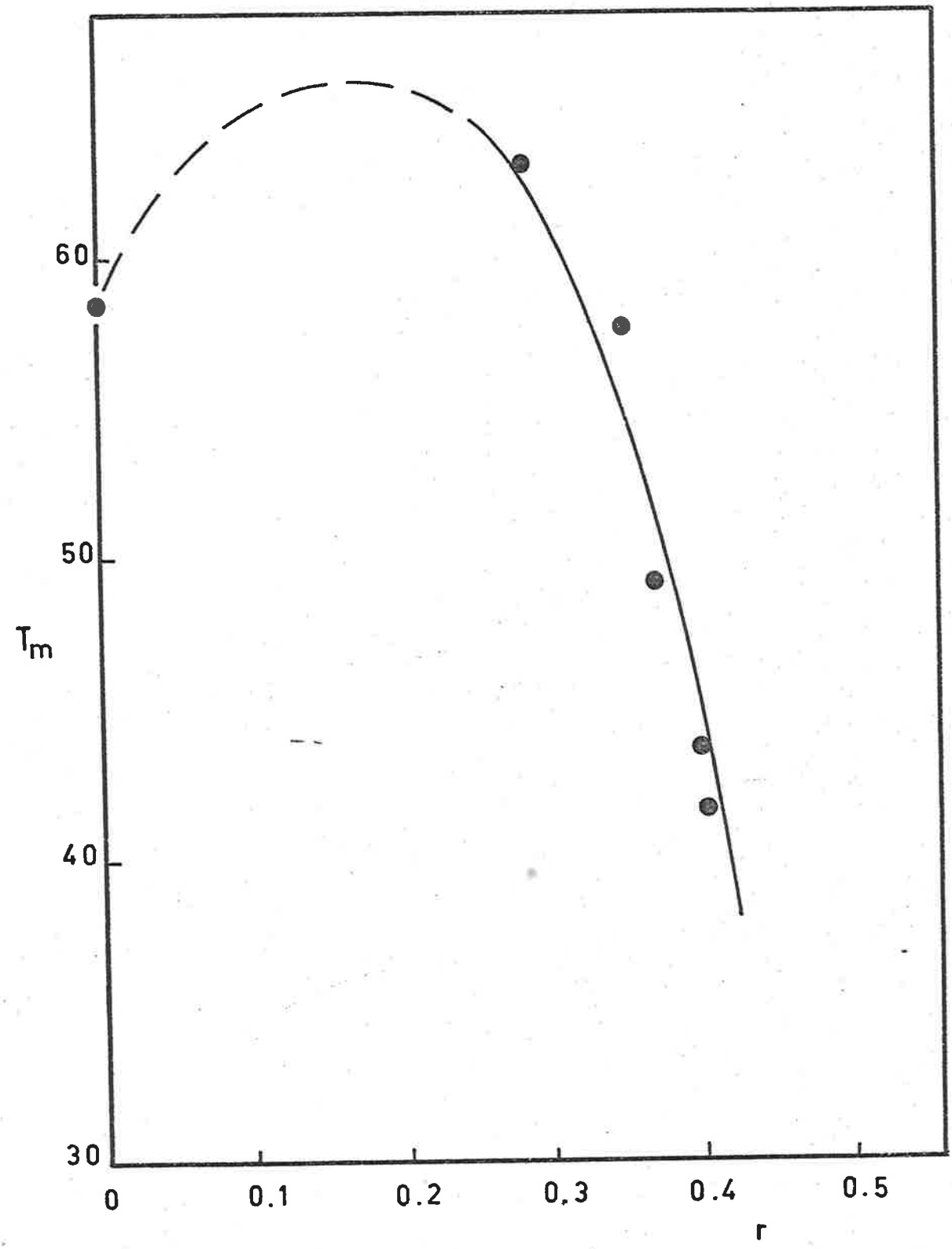


Fig. VI-3. T_m data of Eichhorn and Clark[7] for E. Coli DNA in the presence of Cu^{++} ions and 5mM $NaNO_3$ re-plotted against r (determined at 25°C).

'sandwich' type complexes which occur in native DNA-Cu^{++} at low ionic strength are not expected to be present in the DNA renatured at high ionic strengths as such complexes have not been observed with native DNA at high ionic strengths (see Sect. V.4d).

As hyperchromic and conformational changes have not been reported for DNA renatured at high ionic strengths from the denatured (DNA-Cu^{++}) state, further investigations are required on this aspect of DNA-Cu^{++} interactions as a better understanding of such interactions is desirable under these physiological conditions.

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CHAPTER VII

GENERAL CONCLUSIONS

1. SUMMARY OF CONCLUSIONS

The investigations presented in this thesis have led to some definite conclusions regarding the native DNA-Cu^{++} complex, and have provided the basis for several theories regarding the single-strand DNA-Cu^{++} and the denatured (DNA-Cu^{++}) complexes.

Three distinct types of Cu^{++} complexes have been determined in the native DNA-Cu^{++} complex. A strong 'sandwich' type complex of Cu^{++} between adjacent guanine and adjacent cytosine residues ($K \sim 3\text{-}23.10^6$) has been indicated by a direct relation between the fraction of GpG sites and the number of sites possessing a large affinity for Cu^{++} ions. Weaker type complexes ($K \sim 6\text{-}12.10^4$) appear to involve the 5' phosphate group and the N_7 sites of the guanine and adenine residues, and cause hyperchromic and conformational changes. A third extremely weak interaction ($K \sim 50$) has been detected from mononucleotide-Cu^{++} studies, and appears to be an essentially electrostatic interaction between Cu^{++} ions and the phosphate groups. This electrostatic interaction appears to be the only type which involves the thymidine groups. A 'time effect' of the native DNA-Cu^{++} interaction[1] was not detected by either hyperchromicity, sedimentation or direct binding studies, although a small increase of the sedimentation coefficient with time of standing was detected at high Cu^{++} ion concentrations.

The total number of the two stronger types of interactions occurring in native DNA is 0.5 per residue for all (G + C) content DNA. At high ionic strength only the base residue-phosphate type chelates

were detectable, indicating the probable dependence of the 'sandwich' type complexes on DNA configuration. The limiting values for the interaction at ionic strengths greater than 0.1M were $n \sim 0.2$ and $K \sim 1.10^4$.

Single-strand DNA appears to be identical with native DNA in respect to the number and stability of the sites of interaction, the stability constants for the strongest interaction being similar to those for the single-strand homopolynucleotides poly G and poly C.

Preliminary studies of the denatured (DNA-Cu^{++}) complex indicate the presence of completely different Cu^{++} complexes which are assumed to involve the inter-strand bridging of Cu^{++} between G - C base pairs, as well as an adenine-phosphate chelate of Cu^{++}. The number of sites available is again 0.5 per DNA residue.

As the poly dAT-Cu^{++} interaction appears to be anomalous in several respects an extensive study on this interaction is obviously required. The number of Cu^{++} binding sites in poly dAT is substantially lower than expected, although steric hindrance by adjacent methyl groups may account for this observation. However, there is no obvious explanation for the small number of sites in single-strand poly dAT exhibiting a high affinity for Cu^{++} ions. Furthermore, the T_m increase of poly dAT in the presence of Cu^{++} ions is difficult to relate to the decrease of the stability of poly (A + U), although the possible importance of the primary structure of these biopolymers is suggested.

It has become clear from these and other studies that further investigations are also required on the denatured ($DNA-Cu^{++}$) complexes for a wide range of (G + C) content DNA, as well as at high ionic strengths where they may be related to physiological processes.

2. BIOLOGICAL ASPECTS OF THE $DNA-Cu^{++}$ INTERACTIONS

As the total amount of Cu^{++} detected in human adults is approximately 0.1 gm,[2] the mean concentration of Cu^{++} in the body is near 2.10^{-5}M, in agreement with the concentration of 3.10^{-5}M in blood serum[2] and 2.10^{-5}M as a mean concentration in various adult organs (brain, muscle, blood serum, liver, intestine, lungs, kidney, heart and spleen).[2] From the DNA concentration in blood serum,[2] 3.10^{-5} M_p, the total Cu^{++} to base residue ratio, R, appears to be near 1. However, due to the presence of RNA (4.9 mgm/100 ml)[2] and total proteins (7 mgm/100 ml)[2] in blood serum the effective value of R with respect to DNA is of the order of 0.1-0.2, the amount of Cu^{++} bound per DNA base residue therefore being approximately 0.05 to 0.15, similar to that detected by Altman et al.[3] (0.05) in the nucleic acids of yeast.

Such a value indicates that for DNA near physiological conditions ($I \sim 0.1$, $pH \sim 7$), all of the available sites may be binding Cu^{++} ($n = 0.06$ to 0.2, see Table V-3) and may account for the many changes induced in DNA in vivo when in the presence of Cu^{++} ions (see Chapt. I). Eisinger et al.[4] have made similar calculations for bacterial DNA, and conclude

that only the strongest interacting sites would be complexed by divalent ions.

No attempt was made in the present study to investigate the proposal by Ivanov[5] that Cu^{++} bound to DNA in vivo provides a switching mechanism for DNA strand duplication by means of a Cu^{++} redox reaction. Melting temperature and binding studies of the denatured (DNA-Cu^{++}) complex at high ionic strength are obviously required to test such a proposal.

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CHAPTER VIII

EXPERIMENTAL

1. GENERAL TECHNIQUES

a. Precautions Taken to Avoid Contamination of Solutions

As the aim of the project was to investigate the interaction of Cu^{++} ions with DNA and related compounds, it was necessary that the nucleotidic compounds themselves be initially free of any species capable of interfering with such studies and that no contamination be allowed to occur in the subsequent use of these solutions. For this reason all DNA and various synthetic polynucleotides were treated with EDTA, as discussed in Sect. VIII.2a. All mono-, oligo- and poly-nucleotides were characterised spectrophotometrically before use (Tables VIII-1,2,3), and were checked for any Cu^{++} ion contamination with the specific Cu^{++} ion electrode.

The following precautions were adhered to throughout the investigation.

(i) All reagents which came into contact in any way with any of the solutions under study were of analytical reagent grade or better.

(ii) All water used was distilled, deionised water with a specific conductivity less than 2.10^{-6} ohm^{-1} cm^{-1} at $20^{o}C$, as measured by a Philips conductivity bridge, type PR 9500.

(iii) All apparatus that came into contact with any of the solutions under study was always rigorously cleaned. All volumetric and optical glassware was cleaned with a special acid solution (see Sect. VIII.5a). All other glassware was left overnight in an alkaline potassium permanganate bath, rinsed repeatedly with

deionised water and immersed in a 0.01% metabisulphite solution for three hours before being rinsed and left overnight in deionised water. Finally, they were rinsed repeatedly with distilled, deionised water and drained overnight. All stock solutions were stored in glass vessels cleaned by the above procedure and further cleaned by steaming for one hour to remove any traces of ionic contaminants.

(iv) All tubing which was to come in contact with nucleotidic compounds was PTFE tubing which had been boiled clean. Polythene tubing containing plasticisers which interact with DNA[1] was not used.

(v) All thymidine and cytidine mono-, oligo- and poly-nucleotides were protected from photochemical activation[2,3] by handling in a darkened room illuminated only by a low wattage tungsten lamp.

b. Thermal Denaturation

Three spectrophotometers were used for the determination of DNA melting temperatures, depending on the temperature range required. A Carl Zeiss PMQ 11 spectrophotometer was used up to 50°C for poly dAT determinations. A detailed description of the temperature controls for this instrument is given in Sect. VIII.5a.

A Gilford Model 2000 spectrophotometer fitted with a Beckman DU monochromator and equipped with a temperature chart recorder with built-in temperature calibrations was used up to 80°C for T_m determinations of B. Cerus, E. Coli and M. Lysodeikticus. Two sides of the cell compartment

were circulated with thermostatted water and were surrounded with other layers which were maintained at 20°C to protect the detector and other electronic equipment from prolonged exposure to high temperatures. The cell compartment was surrounded with one inch thick polystyrene to minimise heat losses. By using an accurate Wheatstone bridge (Eilco, Type 6401) to measure the resistance of a calibrated thermistor (see Sect. VIII.5a) set inside a spectrophotometer cell, thermal equilibrium was found to be complete ten minutes after constant absorbances were obtained. The cell solution temperatures recorded at this time were accurate to $\pm$0.2°C.

A Unicam SP 500 spectrophotometer with a Lab-gear power supply, Type 115 D, equipped with an electronically heated cell block was used for thermal studies of poly dG:dC up to 100°C. The sensing device was a bead thermistor housed in the cell block and was calibrated using a calibrated thermistor (see Sect. VIII.5a) set inside a spectrophotometer cell. The cell solution temperatures were accurate to $\pm$0.2°C, fifteen minutes after constant absorbances were obtained.

For temperatures greater than 50°C the most effective method of sealing the cells to prevent vapour losses was found to be the use of PTFE stoppers. As the coefficient of expansion of PTFE is greater than that of quartz, the stoppers were ridged to allow for vertical expansion. Because of the different expansion rates, the higher the temperature the more efficient was the seal obtained. Even after prolonged heating at 95°C solution losses were less than 0.5% by weight.

All solutions which were to be heated to greater than 50°C were degassed with helium (see Sect. VIII.1c). The conditions employed were found to be satisfactory for reducing the formation of air bubbles for all thermal studies up to 95°C.

c. Degassing of Solutions

All solutions to be used for thermal denaturation studies above 50°C and for all chromatographic work were degassed with helium. This technique was found to be effective if the helium was bubbled through the solutions for a total of 2 minutes per ml of solution. Immediately prior to use the helium was bubbled through two containers of distilled, deionised water to saturate the gas with water vapour.

2. PREPARATION OF SOLUTIONS

a. KNO_3 Solutions

All solutions used in this study were made up in 5mM KNO_3 unless stated otherwise. The KNO_3 used was of analytical reagent grade (British Drug Houses Ltd.) and was used without further purification. Distilled, deionised water was used for all KNO_3 solutions.

b. Preparation, Purification and Characterisation of DNA Solutions

The bacterial DNA's B. Cerus, E. Coli K12, and M. Lysodeikticus were prepared in this laboratory by Mr. I.F. Cullen using the method of Marmur.[4] This method involves several precipitations of DNA from EDTA

solutions, thereby ensuring that the DNA is free of any ionic contaminants. These DNA's were further precipitated twice from a phenol solution to remove any traces of protein.

Stock solutions of approximately 1.10^{-3} M_p were prepared by dissolving the DNA in 5mM KNO_3 in a conical flask and rocking at 50 rpm at 4°C for one week. The solutions were then centrifuged for one hour at 18,000 g to sediment any undissolved DNA and the top 90% of each tube was carefully pipetted off. Chloroform (spectrophotometric grade solvent, Mallinckrodt Chemical Works, St. Louis, Missouri, U.S.A.) was added (approximately 0.2%) and the solutions stored at 4°C.

All DNA's were characterised by thermal denaturation studies which provided T_m, the hyperchromicity H, and $\sigma_{\frac{1}{2}}$, the breadth of the transition between 25% and 75% hyperchromicity. These values have been listed in Table VIII-1. The DNA's were further characterised spectrophotometrically using the method of Hirschman and Felsenfeld[5] in combination with a computer program for the analysis of the data (Appendix 3). This analysis provided the (G + C) content of the DNA as well as the concentration. The values for the (G + C) content have been listed in Table VIII-1 and are accurate to ±3%.

The synthetic polynucleotides poly dAT and poly dG:dC were obtained from Miles Laboratories, Inc., Elkhart, Indiana, U.S.A. Stock solutions were prepared as described above. Thermal denaturation analyses indicated the need for purification as the T_m for each polynucleotide was substantially higher than the literature values, indicating the presence

DNA	λ_{max} (nm) observed	(G + C)%	T_m (°C)	H(%) at λ_{max}	$\sigma_{\frac{1}{2}}$ (°C)
poly dAT	263	-3	35.6	35.8	3.0
B. Cerus	260	37	56.2	34.9	4.0
E. Coli	259	48	62.0	34.2	3.4
M. Lysodeikticus	257	74	70.0	33.0	2.8
poly dG:dC (52% G)	252	100	81.0	50.0	3.2
ØX174	260	(43)*	-	-	-

Table VIII-1. Characterisation of the various DNA's used.

* Value stated in reference 6.

of ionic impurities. The solutions were therefore made up to 0.01M in EDTA and 20-40 ml of this solution dialysed exhaustively against 2.5 litres of 5mM KNO_3 for eight days, changing the external solution every two days. Precautions were taken with the dialysis tubing ($^{18}/_{32}$ Visking Company, Union Carbide, Chicago, U.S.A.) to prevent any contamination of the solutions by any species which might be capable of binding to the polynucleotides (see Sect. VIII.5f). The concentration of EDTA in the external solution was determined by potentiometric titration with a known Cu^{++} ion solution using the specific Cu^{++} ion electrode described in Section VIII.4. The EDTA concentration was determined every two days, enabling a direct estimate of the time when all EDTA could be assumed to have been removed from the polynucleotide solution. Subsequent spectrophotometric and thermal denaturation studies (Table VIII-1) indicated that the impurities had been removed. Poly dAT was further characterised by its ability to completely renature on cooling, whereas poly dG:dC did not renature at all when cooled slowly overnight. These two effects have previously been observed by Inman and Baldwin[7,8] and further characterise the purified poly dG:dC sample used.

The ØX174 DNA was a generous gift from Professor R.L. Sinsheimer, California Institute of Technology. The sample was treated with EDTA in the same way as described above for poly dAT and poly dG:dC.

All stock solutions were diluted with 5mM KNO_3. The concentration of all DNA solutions was determined spectrophotometrically using the

extinction coefficient or method listed in Table VIII-2.

Immediately prior to use, all solutions were tested with the specific Cu^{++} ion electrode and were found to contain negligibly small quantities of Cu^{++} ion.

c. The Monc-, Oligo- and Poly-Nucleotide Solutions

The monomers dGMP(5'), dAMP(5') and dCMP(5') were purchased from Schwarz Bioresearch, Inc., Mount Vernon, New York, U.S.A. GMP(5'), TMP(5'), guanosine, deoxy-guanosine and poly G were purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin, U.S.A. ApAp!, $(Ap)_5Ap!$, poly A, poly C and poly I were purchased from Miles Laboratories, Inc., Elkhart, Indiana, U.S.A. The thymidine tetramer TpTpTpTp was prepared by B.S. Chandler[10] in these laboratories using the method of Tener et al.[11]

All of these compounds were dissolved in 5mM KNO_3 at 4^oC for periods of time varying up to one week. pH adjustments were made by additions of 0.1M NaOH or 0.1M HNO_3. All pH determinations were performed on small aliquots of the stock solutions to avoid the introduction of KCl to the nucleotide solutions.

The concentrations of all solutions were determined using the conditions and values of the extinction coefficients listed in Table VIII-3. The wavelength of maximum absorption was determined under the same conditions and was found to be within 1 nm of the literature value for all solutions used. All solutions were found to contain negligible

DNA	ε(P) at λ_{max} (nm)	T(°C)	Ref.
poly dAT	6650 262	25	7
B. Cerus	6360 260	25	Calculated*
E. Coli	6740 260	25	9
M. Lysodeikticus	7040 257	20	Calculated*
poly dG:dC	7400 253	25	8
ØX174	9500 260	37	6

Table VIII-2. Values and conditions used for concentration determinations of DNA in 5mM KNO_3.

* Calculated from the concentration determined by the spectrophotometric analysis of reference 5.

Nucleotide	ε(P) at λ_{max}(nm)	λ_{max} observed	pH	Ref.
dGMP(5')	13,500	252.5	4.7	12
dGMP(5')	9,300	272	7.8	12
dAMP(5')	15,000	260	7.0	12
TMP(5')	9,500	267.5	4.6	12
GMP(5')	13,700	252	7.0	P-L*
ApAp!	11,800	258	5.4	13†
$(Ap)_5Ap!$	11,800	258	5.3	13††
TpTpTpTp	8,985	266.5	6.1	10
Guanosine	13,700	252.5	5.5	12
deoxy-guanosine	13,700	252.5	5.4	12
poly G	9,800	253	7.0	P-L*
poly C	6,320	272.5	6.1	14
poly A	10,100	257	6.6	15
poly I	5,630**	260**	7.0	Miles*

Table VIII-3. Values and conditions used for the characterisation and concentration determination of nucleotides in 5mM KNO_3.

† Taken same as $d(pA)_2$.

†† Taken same as ApAp!

* Certificate of analysis from manufacturer.

** At 260 nm instead of λ_{max}.

quantities of Cu^{++} ion as detected by the specific Cu^{++} ion electrode, and were used without further purification.

d. The Cu^{++} Ion Solutions

Analytical reagent grade $Cu(NO_3)_2.3H_2O$ (May and Baker Ltd., England) was twice recrystallised from distilled, deionised water and dried under vacuum to approximately constant weight. Two litres of a stock solution of approximately 0.02M concentration was prepared in 5mM KNO_3, using distilled, deionised water.

An accurate determination of the Cu^{++} ion concentration of the stock solution was made by the method of electrogravimetric deposition on to a platinum electrode. A potential of 1 V and current of 0.2 amps was applied for two hours to a weighed solution of approximately 100 ml of stock solution. The solution was stirred continuously by a rotating platinum anode. Taking the density of the solution as that of water, three determinations of the Cu^{++} ion concentration (0.02111, 0.02106 and 0.02100M) provided an average concentration of 0.02105M.

The solution was stored at 20°C in a two litre volumetric flask, the polythene stopper being sealed with parafilm. All dilutions were made by weight using 5mM KNO_3 and were stored in polythene containers to avoid concentration changes due to Cu^{++} ion adsorption onto glass. Such effects reach detectable proportions for small volumes of Cu^{++} ion solutions[1] below 10^{-3} to 10^{-4} M. Solutions of 1.10^{-5} M or less were

prepared and used on the day required to minimise any contamination from the air or glassware.

3. GEL EXCLUSION CHROMATOGRAPHY

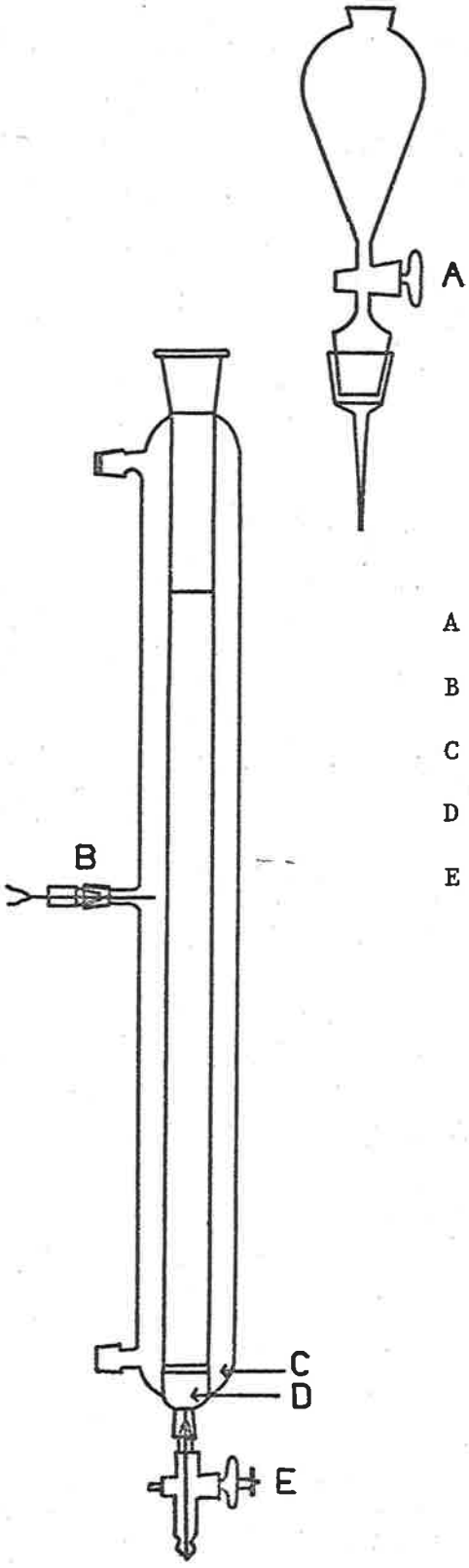
a. The Column

A jacketed glass column 70 cm long, 1 cm internal diameter, with a sintered glass support (Fig. VIII-1) was used for all chromatographic studies. A thermistor was inserted half-way along the column to record the column temperature. The resistance of the thermistor was determined using a Sargent thermistor bridge, Catalogue No. S-81601, and the temperature determined from a calibration graph of the logarithm of the thermistor resistance against temperature. The thermistor was calibrated as described in Sect. VIII.5a. Water was circulated around the column from a thermostatted bath and maintained the column at a temperature of 25.0 $\pm$ 0.05^{o}C for all studies.

The volume immediately below the sintered glass support was filled with glass chips to minimise the mixing volume. The elution rate was controlled by a glass impregnated PTFE ('Fluon') tap with a fine flow rate control (G. Springham and Co., Ltd., Temple Fields, Harlow, Essex, England) which was capable of maintaining a constant flow rate for several days.

b. Packing the Column

A dilute suspension of Sephadex G25, coarse (Pharmacia, Uppsala,



A PTFE tap

B Thermistor

C Sintered glass support

D Mixing volume packed with glass chips

E Fine control 'Fluon' tap

Fig. VIII-1. Schematic diagram of the sephadex column used.

Sweden) was equilibrated in degassed 5mM KNO_3 (see Sect. VIII.1c) for two hours, as suggested by Floden.[16] The supernatant was decanted and a similar volume of degassed 5mM KNO_3 introduced, the Sephadex stirred and then allowed to equilibrate and sediment for another two hours. This procedure was repeated a further three times. The Sephadex suspension was then poured into the column (thermostatted at 25°C) which had been extended three-fold by the use of glass tubing of the same diameter, and connected by a quickfit ground glass joint. Care was taken with a plumb-line to ensure that the column was perfectly vertical. After 1 cm had been allowed to settle out the column was allowed to elute slowly. Small additions of the Sephadex suspension was added continuously to the large volume of sedimenting Sephadex. This technique produced a very evenly packed column bed.

The column was then eluted slowly for 72 hours with degassed 5mM KNO_3 to remove any ultra-violet absorbing species and other soluble contaminants and to allow the column to 'bed in'. The absorbance of the eluent was measured at intervals in this time at 240 to 280 nm, and was found to be zero after a short eluting period. The final bed height was 60 cm.

All solutions used for packing the column and all solutions subsequently passed through the column for binding studies were degassed to prevent the formation of air bubbles in the Sephadex bed.

4. Cu^{++} ION POTENTIOMETRY

The electrodes used were a reversible, solid state specific cupric ion activity electrode (Orion Research Inc., Cambridge, Massachusetts, U.S.A., Type 94-29) and a saturated KCl porous pin-type calomel electrode (Radiometer, Type K 401) as the reference electrode. The junction potentials were sufficiently constant to enable accurate readings over many hours. Factors affecting the electrode response and the methods taken to ensure reproducible potentials have been discussed in Sect. IV.2a.

The vessels used for potentiometric analysis (Fig. VIII-2) were designed as a compromise between the desire to stir minimal volumes of solution, and the need to have the reactive section of both electrodes well immersed to allow good thermal contact with the thermostatted bath. The electrodes were housed in PTFE sleeves tapered to fit the ground glass joints of the analysis vessels. The sleeves could be moved along the electrodes to enable the height of the electrodes to be adjusted while still maintaining an efficient seal to minimise vapour losses.

Prior to a titration study the analysis vessel with electrodes immersed in the test solution was thermostatted in one hour to $\pm 0.05^{\circ}C$, the solution being stirred continuously by a small PTFE coated magnetic stirrer in conjunction with an underwater magnetic stirrer. The solution was then allowed to stand unstirred for five minutes before recording the cell potential from a Radiometer pH meter, Type 25SE, fitted with a scale expander. The solution was stirred for a further five minutes, left

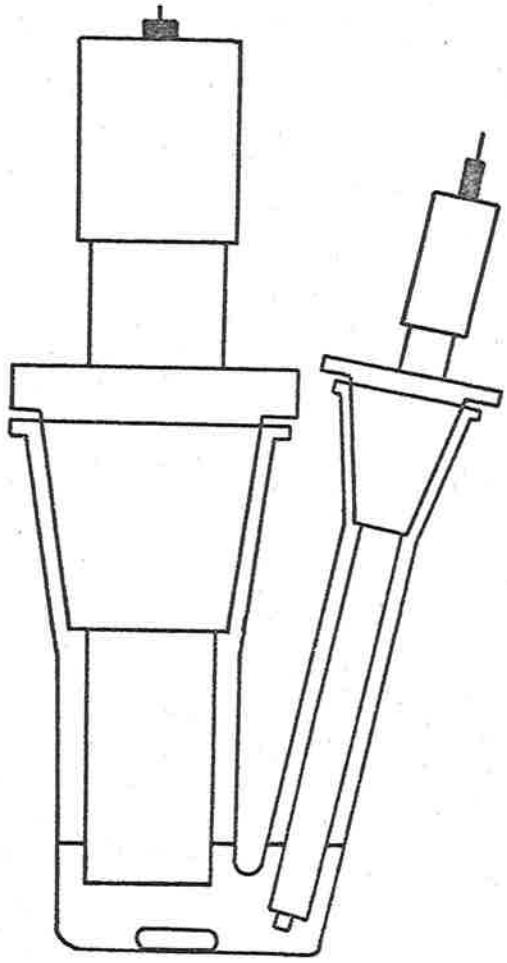


Fig. VIII-2. The type of vessel used for the determination of cell potentials of Cu^{++} ion solutions of known concentration.

unstirred for five minutes then a duplicate cell potential noted. Successive measurements by this procedure were always within $\pm$0.5 mV and often within $\pm$0.1 mV, the reading error of the potentiometer. The calomel electrode was pre-equilibrated at 2°C for two days[17] preceding any measurements at that temperature.

For studies of the extent of binding of Cu^{++} ions to nucleotidic materials, the nucleotide of known concentration was weighed into the titrating vessel. This was similar to the vessel in Fig. VIII-2 but contained two side arms, the second being used for the addition of known Cu^{++} ion concentration solutions from calibrated syringes (Hamilton and Co. Inc., Whittier, California, U.S.A.). The syringes used (0.05, 0.25 and 0.5 ml) were fitted with adjustable stoppers which enabled the volume of solution transferred to be accurate and reproducible to $\pm$0.0002 ml. After each addition the solution was stirred for 10-20 minutes to allow thermal equilibration and then allowed to stand unstirred for a further five minutes before recording the cell potential. A duplicate measurement was also determined as described above.

5. PHYSICO-CHEMICAL TECHNIQUES

a. Ultra-Violet Absorption Spectrophotometry

A Carl Zeiss PMQ II manual spectrophotometer was used exclusively for accurate ultra-violet absorption measurements. Also used for thermal denaturation studies were a Gilford Model 2000 and a Unicam

SP 500 manual spectrophotometer, both of which have been described in detail in Sect. VIII.1b.

The wavelength scale of these spectrophotometers was checked annually using the emission spectrum of a low pressure mercury lamp positioned for maximum intensity at the entrance slits of the instruments At regular intervals a check on the wavelength scale was carried out using the emission spectrum of the inbuilt deuterium lamp of the Zeiss spectrophotometer.

The accuracy of the photometric scale of these instruments in the range 220 to 400 nm was checked annually using freshly prepared standard alkaline potassium chromate solutions.[18] Analytical reagent grade KOH (Univar) and potassium chromate (Standard Laboratories Pty. Ltd., Melbourne, Australia) solutions were made with freshly distilled, deionised water which was also used as the reference.

The optical paths of both quartz and glass 1 cm spectrophotometer cells were measured to $\pm$0.0002 cm using Starrett small hole gauges and a calibrated micrometer.

The cells were cleaned and used in a similar way for all absorbance studies to ensure reproducible results. After use the cells were rinsed well with distilled, deionised water and dried in a warm oven. The dry cells were then immersed for several hours in a specially prepared cleaning mixture of concentrated sulphuric acid (AR grade, Adelaide Chemical and Fertilizer Co. Ltd.), 2% sodium nitrate (AR grade, Univar) and 2% anhydrous sodium perchlorate (AR grade, Univar). After being

rinsed and drained repeatedly with deionised then distilled, deionised water, they were left to soak overnight in the latter. Immediately before use the cells were rinsed several times with distilled, deionised water and dried in a warm oven. The absorbance near the maxima was recorded immediately the solution and reference were placed in the spectrophotometer and, if after 30 minutes the absorbance was unchanged, the appropriate values were recorded. After measurements were completed the cells were rinsed repeatedly with distilled, deionised water and the cell corrections determined with distilled, deionised water in both cells.

The concentrations usually employed were such that an absorbance of about 0.4 was measured. Under these conditions the absorbance measurements were accurate and reproducible to ± 0.002.

The Zeiss spectrophotometer was used in the temperature range $0^{\circ}C$ to $50^{\circ}C$. A 25% ethylene glycol and water mixture was circulated through the cell block used. A silica gel compartment adjacent to the cell block prevented condensation of moisture on the cells at low temperatures. For low temperature studies, the cell compartment was covered with one inch thick polystyrene foam to prevent moisture condensing on the outside of the spectrophotometer and to minimise the transfer of heat to the cell compartment.

The temperature of the cell compartment was calculated from the resistance of a bead thermistor (STC FS2) which was permanently fitted through a PTFE stopper into a cell filled with distilled, deionised water

The resistance of the thermistor was measured to $\pm$ 1Ω with an accurate Wheatstone bridge (Eilco, Type 6401). From a calibration graph of the logarithm of the thermistor resistance against temperature (measured with Bomb Calorimeter thermometers, Brooklyn Thermometer Co., 10°C range in 0.01°C graduations), the temperature of the cells could be read off to $\pm$0.02°C. Thermal equilibrium was assumed if the resistance remained constant over 15 minutes, and physico-chemical equilibrium was assumed if the absorbance remained constant for a further 15 minutes.

b. Visible Absorption Spectrophotometry

The Carl Zeiss PMQ II spectrophotometer was used exclusively for all visible absorption spectral measurements. A specially constructed transistorised DC power supply producing a very low ripple voltage was used to improve the stability of these readings.

A check of the wavelength scale and measurement of the cell path lengths were determined as described in Sect. VIII.5a. The accuracy of the photometric scale was checked annually using standard neutral glass filters (Chance-Pilkington Optical Works) which had been calibrated at the wavelengths 505 nm, 595 nm, and 640 nm by the National Standards Laboratory, Division of Physics, C.S.I.R.O., Sydney, Australia. The accuracy of calibration was stated to be $\pm$0.002 on the transmittance scale. The Carl Zeiss spectrophotometer operated within these limits for the period of this study.

c. pH Determinations

All pH measurements were performed with a Radiometer pH 25SE meter. The scale calibration was carried out using two standard buffer solutions which encompassed the expected pH value to be determined.

Because most of the solutions to be tested were not buffered, it was found useful to carry out pH measurements to a time schedule, recording the value two minutes after the electrodes were immersed. Using this technique, readings were reproducible to ± 0.05 of a pH unit on successive samples of the same solution.

A combined glass and calomel micro-electrode (Titron Instruments, Victoria, Australia) was used for all measurements and allowed as little as 0.5 ml of solution to be used. As the thymidine tetramer was required for re-use because of the minute quantities available, contamination from the small but finite leakage of saturated KCl from the calomel electrode had to be considered. Conductometric studies by Chandler[10] with the same combined electrode showed that less than 1 µg of KCl enters the solution in the two minutes required for pH determinations. Therefore, any increase of the ionic strength was negligible.

d. Viscometry

The viscosity of native DNA-Cu^{++} solutions was determined at $25.000^{\circ}C \pm 0.002^{\circ}C$ in a specially designed low shear suspended level Ubbelohde viscometer in which the capillary (1 metre of 1 mm internal

diameter) was wound in a spiral. The flow time of distilled, deionised water was 200.07 $\pm$ 0.05 seconds.

The viscometer was cleaned overnight with an acid solution used for cleaning optical glass surfaces (see Sect. VIII.5a), then rinsed repeatedly with distilled, deionised water in which it was allowed to soak overnight. Finally, it was drained and then dried with AR acetone by attaching to a vacuum pump. All solutions were filtered through a fine sintered glass funnel attached to the viscometer to minimise the entry of any dust particles into the capillary. In a similar way, all air sucked into the viscometer when under vacuum passed through a fine sintered glass filter.

For the viscosity study of DNA in the presence of Cu^{++} ions a DNA solution was transferred to the viscometer with a calibrated 5 ml syringe fitted with a large internal diameter needle (gauge 20). The DNA solution was pumped slowly into the top, centre bulb where increments of concentrated Cu^{++} ion solution were added with a calibrated 0.05 ml syringe. The solutions were then pumped slowly five times from the top, centre bulb, through the capillary to the bulb above the capillary. This process ensured the complete mixing of all of the solution. To measure the flow time, each solution was pumped slowly into the capillary until it just reached the bulb above. Using a 3-second per 360^{o} stop-watch, flow times were then recorded in triplicate in the normal manner, and were always within $\pm$0.2 second.

e. Ultracentrifugation

A Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics was used to measure sedimentation coefficients of the native DNA-Cu^{++} system. All measurements were made at 30,000 rpm in a 12 mm double sector 'Filled Epon' cell using sapphire windows. The An-F analytical rotor used was thermostatted at 25.0 $\pm$ 0.1°C and enabled three cells to be analysed simultaneously. The advantage of the multi-cell rotor is that the cells are exposed to the same conditions of temperature and centrifugal force, and enable strict comparisons to be made. The reference solution in each cell was 5mM KNO_3.

The ultracentrifuge was equipped with a high intensity Xenon arc lamp which enabled a Beckman photoelectric scanning system (scanner) to be used. The scanner recorded the absorbance of the test solution in the leading sector with respect to the reference solution in the trailing sector of the cell. The absorbance was plotted automatically on a chart as a function of the distance the scanner moved along the cell

A Beckman multiplexer accessory for the scanner was used to automatically control the successive scanning of cells. Using the minimum time for re-scanning, each cell could be analysed about eight times before the sedimenting boundary reached the centre of the cell. This point was considered a convenient time to stop recording results as the accuracy and reproducibility of further readings decreased after that time.

f. Dialysis

The method of dialysis was used for purifying poly dAT and poly dG:dC as well as for a preliminary study of the native DNA-Cu^{++} interaction. In both cases it was necessary to ensure that the dialysis tubing ($^{18}/_{32}$ Visking Company, Union Carbide, Chicago, U.S.A.) did not contain any plasticiser or ultra-violet absorbing species as such compounds are expected to interact with DNA[1] and would, even in the absence of any interaction, introduce an error in any ultra-violet absorbance measurements. For this reason all dialysis tubing was boiled in a 5% bicarbonate solution for 30 minutes, twice boiled in distilled, deionised water after rinsing repeatedly, and soaked at 4°C in distilled, deionised water for a further four days, the water being changed daily. In a test experiment the spectral properties of E. Coli DNA were found to be completely unaltered after dialysing against 5mM KNO_3 for one week at 25°C. This indicated that the tubing treated in the manner described above appeared to be free of any complexing or ultra-violet absorbing contaminants.

Each end of the dialysis bag was tied with two separate knots as this was found to provide a most simple and effective seal. The dialysis vessel was rocked continuously in a see-saw manner at 4°C throughout the purification process.

6. REFERENCES

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APPENDICES

APPENDICES

APPENDIX 1

Fortran Computer Program: Least Squares of a Polynomial.

```
      PROGRAM LSQP(INPUT,OUTPUT)
      DIMENSION X(50),Y(50),W(50)
      COMMON A,C(21),D(200),TITLE(8)
   99 READ 19,(TITLE(J),J=1,8)
   19 FORMAT (8A10)
      IF (TITLE(1).EQ.10H          )STOP
C     NO IS THE NUMBER OF VALUES OF X AND Y
   20 READ1,NO
    1 FORMAT(I2)
      READ2,(X(I),Y(I),I=1,NO)
    2 FORMAT(2E10.2)
      DO 21 I=1,NO
   21 W(I)=1.0
      DO 30 NORDER=1,4
      CALL LSQPOL (NO,X,Y,W,0,30,4,1,NORDER,0)
      CALL QIKPLT(X,Y,-1*NO,1,14H*1/C    EXPTL.* ,5H*1/R*)
      CALL QIKPLT(X,D,-1*NO,1,14H*1/C    THEOR.* ,5H*1/R*)
   30 CONTINUE
      GO TO 99
      END
```

APPENDIX 2

Fortran Computer Program: Calculation of $S_{25,w}$.

```
      PROGRAM SEDMN(INPUT,OUTPUT)
      DIMENSION XB(50),T(50),XL(50),A(50),B(50), COM(8),DL(50),DY(50),W(50
     *50),WR(50),R(50),CD(50),RN(50),S(50),SB(50),PE(50),SE(50),SXS(50),
     *SPS(50),RV(50)
      READ3,NF
      DO16L=1,NF
      READ2,COM
    2 FORMAT(8A10)
      READ3,N
    3 FORMAT(I2)
      READ4,(XB(I),T(I),I=1,N)
    4 FORMAT(F10.0,F10.0)
      XLR=39.00 $ CE=5.20
      DG5I=1,N
      A(I)=(XB(I)-XLR)/CE
      A(I)=A(I)/10.0
      B(I)=6.5-A(I)
    5 XL(I)=ALOG(B(I))
      READ6,W(L),R(L),CD(L),RN(L),RV(L)
      WR(L)=(W(L)**2)*0.010974
      CALL LSQ(T,XL,N,Z,E)
      SES=0.0
      DO7I=1,N
      DL(I)=E*T(I)+Z
      DY(I)=XL(I)-DL(I)
      SE(I)=DY(I)**2
    7 SES=SES+SE(I)
      ZW=FLOAT(N)
      SDF=SES/(ZW-1.0)
      SDF=SQRT(SDF)
      PRINT8,COM
    8 FORMAT(1H1,////,40X,*SEDEMENTATION PROGRAM A*,//,30X,8A10)
      PRINT9
    9 FORMAT(///,20X,*X BOUNDARY*,10X,*TIME (SECS)*,10X,*C VALUE*,10X,*X
     * (=6.5-C)*,10X,*LN X*,//)
      PRINT10,(XB(I),T(I),A(I),B(I),XL(I),I=1,N)
   10 FORMAT(22X,F6.2,13X,F7.1,13X,F8.5,10X,F7.4,11X,F7.5)
      PRINT13
   13 FORMAT(    ////,20X,*LN X*,10X,*TIME (SECS)*,10X,*CORRECTED LN X*,10X,*
     *10X,*RESIDUAL LN X*//)
      PRINT14,(XL(I),T(I),DL(I),DY(I),I=1,N)
```

(contd.)

APPENDIX 2 (contd.)

```
   14 FORMAT(20X,F7.5,10X,F7.1,12X,F7.5,17X,F8.5)
      S(L)=E/WR(L)
      SXS(L)=(((DL(N)+(1.0*SDF))-DL(1))/(T(N)*WR(L)))-S(L)
      SXS(L)=(SXS(L)/1.0E-13)*RV(L)
      S(L)=S(L)*RV(L)
      WR(L)=SQRT(WR(L))
      SB(L)=S(L)/1.0E-13
      SPS(L)=(SXS(L)*100.0)/SB(L)
      PRINT15,RN(L),W(L),WR(L),E,Z,SDF,S(L),SB(L),SXS(L),SPS(L)
   15 FORMAT(///,20X,*RUN NO.*, A5 ,//,20X,*ROTOR SPEED WAS *,F10.1,*(RP
     1M)        OR *,F10.1,* RADIANS/SEC*,//,20X,*SLOPE IS *,E12.5,10X,*
     2INTERCEPT IS *,F10.5,//,20X,*S. M. D. OF FIT is *,E12.5,//,20X,*SE
     3DIMENTATION COEFFICIENT IS *,E12.5,*            OR *,F12.7,* SVEDBERGS*
     4ERGS*,//,20X,*STANDARD ERROR OF SEDMN COEFFICIENT IS + OR - *,E12
     5.5,*     SVEDBERGS    OR *,F7.3,* P/C*)
      CALL QIKPLT(T,XL,-N,1,13H*TIME (SECS)*,6H*LN X*)
   16 CONTINUE
      PRINT18,NF
   18 FORMAT(*1*,////,30X,*SUMMARY OF RESULTS*,//,30X,*NUMBER OF RUNS IS
     1*, I3,////,10X,*RUN NO*,10X,*CONCN DNA*,10X,*ROTOR SPEED*,10X,*R VA
     3ALUE*,10X,*SVEDBERGS*,10X,*STANDARD ERROR*,/,26X,*(MOLES/L*,12X,*(
     4RPM)*,29X,*(10-13 SECS)*,14X,*OF S*,/)
      PRINT19,(RN(I),CD(I),W(I),R(I),SB(I),SXS(I),I=1,NF)
   19 FORMAT(//,10X, A5 ,11X,F9.7,11X,F10.1,11X,F7.4,10X,F8.5,12X,F10.7)
      STOP
      END
```

APPENDIX 3

Fortran Computer Program: Spectrophotometric Analysis of DNA.

```
   PROGRAM GC DNA  (INPUT,OUTPUT)
   DIMENSION  OD(15),TITLE(8)
   READ  1,(TITLE(I),I=1,8),N,(OD(I),I=1,N)
 1 FORMAT (8A10/I2,15F5.3)
   CALL NATIV3(OD,TITLE)
   READ  1,(TITLE(I),I=1,8),N,(OD(I),I=1,N)
   CALL  DENAT3(OD,TITLE)
   READ  1,(TITLE(I),I=1,8),N,(OD(I),I=1,N)
   CALL  HYPER2(OD,TITLE)
   READ  1,(TITLE(I),I=1,8),N,(OD(I),I=1,N)
   END


   SUBROUTINE   NATIV3(A,TITLE)
   DIMENSION  A(12),W(12),TITLE(8)
   W(1)=235.
   DO  1  I=1,11
 1 W(I+1)=W(I)+5.
   S1=9.329E-08 $ S2=2.0631E-07 $ S3=6.198E-08 $ S4=2.792E-08
   S5=2.124E-08 $ S6=8.513E-08
   U1=-2026.*A(1)-1889.*A(2)-1390.*A(3)+43.*A(4)-319.*A(5)-608.*A(6)+
  22515.*A(7)+871.*A(8)-386.*A(9)+1159.*A(10)+1797.*A(11)+1187.*A(12)
   U2=-656.*A(1)-1251.*A(2)-1917.*A(3)-2830.*A(4)-1807.*A(5)-1141.*A(
  26)-3379.*A(7)-1409.*A(8)-154.*A(9)-1558.*A(10)-2424.*A(11)-2099.*A
  3(12)
   U3=3952.*A(1)+5031.*A(2)+6338.*A(3)+7480.*A(4)+7616.*A(5)+7307.*A(
  26)+7052.*A(7)+5740.*A(8)+4587.*A(9)+3938.*A(10)+3164.*A(11)+2188.*
  3A(12)
   CONC=U1*S4+U2*S3+U3*S5
   THETA=U1*S1+U2*S2+U3*S3
   THETA=THETA/CONC
   GC=(1.-THETA)*100.
   B=U1*S6+U2*S1+U3*S4
   B=B/CONC-THETA*THETA
   DELTA=B/THETA
   PRINT  2, (TITLE(I),I=1,8),((W (I), A(I)),I=1,12),GC,CONC,DELTA
 2 FORMAT  (1H1,55X,*DNA CALCULATIONS*/55X,17(1H*),//26X,8A10///50X,*
  2WAVELENGTH : ABSORBANCE*/12(55XF3,3X*:*5XF6.3/)
  2              ///45X,*MOLE. PERCENT GC = *,F6.1,///45X,*CONCENTRAT
  +ION DNA = *,F10.9*MOLES. NUCLEOTIDE/LITRE*//45X,*DELTA = *,F7.3)
   RETURN      $       END
```

(contd.)

APPENDIX 3 (contd.)

```
      SUBROUTINE DENAT3(A,TITLE)
      DIMENSION  A(5),W(5),TITLE(8)
      W(1)=240. $ W(2)=250. $ W(3)=260. $ W(4)=270. $ W(5)=280.
      S1=5.473E-08 $ S2=1.2806E-07 $ S3=3.897E-08 $ S4=8.97E-09
      S5=1.55E-08 $ S6=7.62E-08
      U1=-2754. *A(1)+2354.*A(2)+1090.*A(3)+3095.*A(4)+1705.*A(5)
      U2=-1545.*A(1)-4786.*A(2)-1263.*A(3)-3849.*A(4)-4421.*A(5)
      U3=7210.*A(1)+10154.*A(2)+9606.*A(3)+8980.*A(4)+6942.*A(5)
      CONC=U1*S4+U2*S3+U3*S5
      THETA=U1*S1+U2*S2+U3*S3
      THETA=THETA/CONC
      GC=(1.-THETA)*100.
      B=U1*S6+U2*S1+U3*S4
      B=B/CONC-THETA*THETA
      DELTA=B/THETA
      PRINT  2, (TITLE(I),I=1,8),((W (I), A(I)),I=1, 5),GC,CONC,DELTA
    2 FORMAT  (1H1,55X,*DNA CALCULATIONS*/55X,17(1H*),//26X,8A10///50X,*
     2WAVELENGTH . ABSORBANCE*/ 5(55XF3,3X*.*5XF6.3/)
     2              ///45X,*MOLE.PERCENT GC = *,F6.1,///45X,*CONCENTRAT
     +ION DNA = *,F10.9*MOLES. NUCLEOTIDE/LITRE*//45X,*DELTA = *,F7.3)
      RETURN      $       END


      SUBROUTINE  HYPER2(D,TITLE)
      DIMENSION  D(4),W(4),TITLE(8)
      W(1)=250. $ W(2)=260. $ W(3)=270. $ W(4)=280.
      S1=3.4359E-08 $ S2=4.6556E-08 $ S3=1.47455E-07
      U2=43.*D(1)+1388.*D(2)-629.*D(3)-2422.*D(4)
      U3=2264.*D(1)+1981.*D(2)+2892.*D(3)+2920.*D(4)
      CONC=U2*S1+U3*S2
      THETA=(U2*S3+U3*S1)/CONC
      GC=(1.-THETA)*100.
      DELTA=1.0
      PRINT  2, (TITLE(I),I=1,8),((W (I), D(I)),I=1, 4),GC,CONC,DELTA
    2 FORMAT  (1H1,55X,*DNA CALCULATIONS*/55X,17(1H*),//26X,8A10///50X,*
     2WAVELENGTH : ABSORBANCE*/ 4(55XF3,3X*:*5XF6.3/)
     2              ///45X,*MOLE. PERCENT GC = *,F6.1,///45X,*CONCENTRAT
     +ION DNA = *,F10.9*MOLES. NUCLEOTIDE/LITRE*//45X,*DELTA = *.F7.3)
      RETURN      $       END
```