INTERACTION OF DNA WITH Cu(II) IONS

by

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Contents

SUMMARY

This thesis first describes the thermal denaturation of DNA from Calf Thymus and E. Coli. From the measurements of U-V spectra of DNA both in the presence and absence of cupric ions, direct evidence is presented that cupric ions interact with the nitrogen atoms of the DNA bases at higher temperatures and also at lower temperature when the DNA helix is exposed to them after the hydrogen bonds present in the intact molecule have been severed. Visible absorption spectra measurements produce an additional evidence for such binding.

Absorbance and sedimentation velocity measurements before and after denaturation and after addition of solid potassium nitrate to the denatured solution, show that the denaturation of DNA caused by cupric ions is reversible.

It is observed that no bonding of cupric ions to heterocyclic nitrogen atoms occurs until the thermal denaturation process occurs. At 25°C, determination of the extent of binding of cupric ions to DNA, by the equilibrium dialysis method, show that binding increases

with concentration and that there is only one type of binding site. The same is true for DNA denatured in the absence of cupric ions and subsequently placed in an environment containing these ions. At 55°C, however, the number of binding sites is found to be greater.

Spectrophotometric and polarographic studies have been made for the binding of cupric ions to different nucleosides and nucleotides as present in the DNA double helix, in order to find out to which of these cupric ions are more bound.

General discussion of the present study to an understanding of the chemistry of possible sites for binding of cupric ions to DNA is presented in the concluding chapter.

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 written by another person, except when due reference is made in the text.

V. K. Srivastava

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CHAPTER I

INTRODUCTION

Nucleic acids, like proteins and polysaccharides, belong to a major group of natural organic macromolecules which are essential constituents of living organisms. In all, nucleic acids commanded tremendous interest during the past few decades and hold a key position in cell physiology and biochemistry[1,2]. This is because they carry and transcribe the genetic messages for the life processes[3]. The genetic information in the chromosomes in the cell nucleus is contained in DNA whereas the transcription of the message to the various parts of the cell seems to be carried out by RNA[4].

It has been earlier reported that nucleic acids isolated from various sources contain or may interact with several metals[5-18]. Many of the biochemical reactions of nucleic acids which occur in living cells, require divalent metal ions. When protein synthesis is studied in vitro, it is necessary to provide the ribosomal medium with Mg^{+2}, and also there is a magnesium requirement both in the enzymatic synthesis of DNA and in the function of ATP-ase[19]. Certain steps of proteins synthesis can utilise Mn^{+2} ions instead of Mg^{+2} [20].

It has now been demonstrated that under a variety of conditions, metal ions will react with and produce different effects on the structure of polynucleotides[21-25].

These metal ions have been shown to stabilise the secondary and tertiary macromolecular structures[26], labilise these structures[27] or even bring about the degradation of the primary structure[28]. Recently, Eichhorn[27] has found that different bivalent metal ions exert different effects on the melting temperature of DNA. Amongst the various metal ions studied, he showed that Mg^{+2}, Ca^{+2}, Ba^{+2}, Co^{+2}, Mn^{+2}, Ni^{+2}, and Zn^{+2} increase the melting temperature (Tm) whereas Cu^{+2}, Cd^{+2}, and Pb^{+2} decrease it. The increased Tm was attributed to the stabilisation of the DNA structure by metal binding to phosphate. At the commencement of this investigation it had been suggested that the decrease in Tm resulted from coordination of the metal to electron donor groups on the nucleosides and that such coordination would displace the hydrogen bonds that hold together the two strands of the DNA helix[29]. Direct experimental proof of this hypothesis was however lacking.

The main aim of this present investigation was to study the interaction of cupric ions with DNA in particular with E.Coli and Calf Thymus DNA, and to compare these interactions with those of other divalent metals. It was also hoped that these studies might provide information about the macromolecular configura-

tion of DNA in solution in presence of cupric ions. Cupric ion was chosen in this study because amongst all the bivalent metal ions which lowered the Tm of DNA, it was found to be the most effective.

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CHAPTER II

THERMAL DENATURATION OF DNA IN THE PRESENCE OF CUPRIC IONS

1. Introduction
2. Temperature - absorbance curves of different DNA's with varying concentrations of cupric ions.
3. Variation of Tm with cupric ion concentration.
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6. Sedimentation velocity and absorption values at different temperatures and their reversal after adding solid KNO_3.
7. Kinetics of denaturation.
8. Discussion.
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1. Introduction

Eichhorn and Clark[1] and Hiai[2] have shown that copper has a specific effect in decreasing the stability of the DNA helix to thermal denaturation whereas the presence of most bivalent metals will stabilize the helix[3,4,7]. The decrease of Tm with increase of copper concentration at constant ionic strength is thus markedly different from the effect observed with other ions. Furthermore, the denaturation at relatively low temperature induced by the presence of copper ions has been shown by Eichhorn and Clark[1] to be reversible by the addition of electrolyte $NaNO_3$ which produces a high ionic strength renaturing the denatured DNA. Hiai[2] has reported that quenched samples of thermally denatured DNA, obtained by addition of concentrated salts, EDTA, or by dialysis, possess similar properties to native DNA including the optical density- temperature profiles, chromatographic patterns and transforming activity. The assumption that the loss of hypochromicity at relatively low temperature in the presence of copper ions is consequential on a denaturation process, is supported by the evidence that under the same conditions there is observed a marked decrease in viscosity, a loss of biological activity and a decrease of molecular asymmetry.

Thermal denaturation studies of Calf Thymus and E.Coli DNA in the presence of copper ions are reported in this chapter confirming and further extending the findings of Eichhorn and Clark[1] and of Hiai[2]. Direct evidence is presented showing that there is interaction between copper ions and nitrogen atoms of DNA bases after denaturation in the presence of copper ions at elevated temperatures but not at room temperature. Sedimentation velocity measurements are reported to show that renaturation by adding excess of solid KNO_3 to the denatured DNA apparently regenerates the native DNA.

2. Temperature - absorbance curves of different DNA's with varying concentrations of cupric ions

Heating DNA solution brings about a cooperative transition to flexible, coiled chains, which in turn changes several properties such as viscosity, optical rotation, optical density, etc. The mid-point of this transition has been termed the melting or denaturation temperature which has been denoted by Tm[5,6].

The results of the denaturation of E.Coli DNA as obtained in the presence of different concentrations of copper is shown in Fig. 1. As is observed,

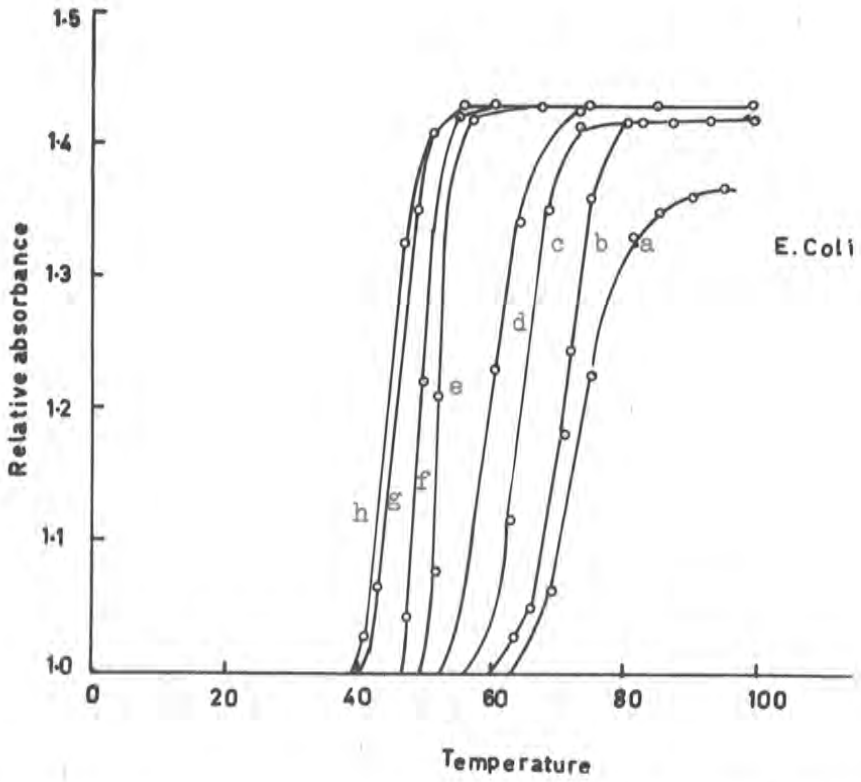

Fig. II-1. Melting curves of E.Coli DNA (3.69×10^{-5} Mp) in the presence of various concentrations of Cu(II) ions. Curves a, 0mM; b, .01mM; c, .02mM; d, .03mM; e, .05mM; f, .07mM; g, .09mM; h, .1mM.

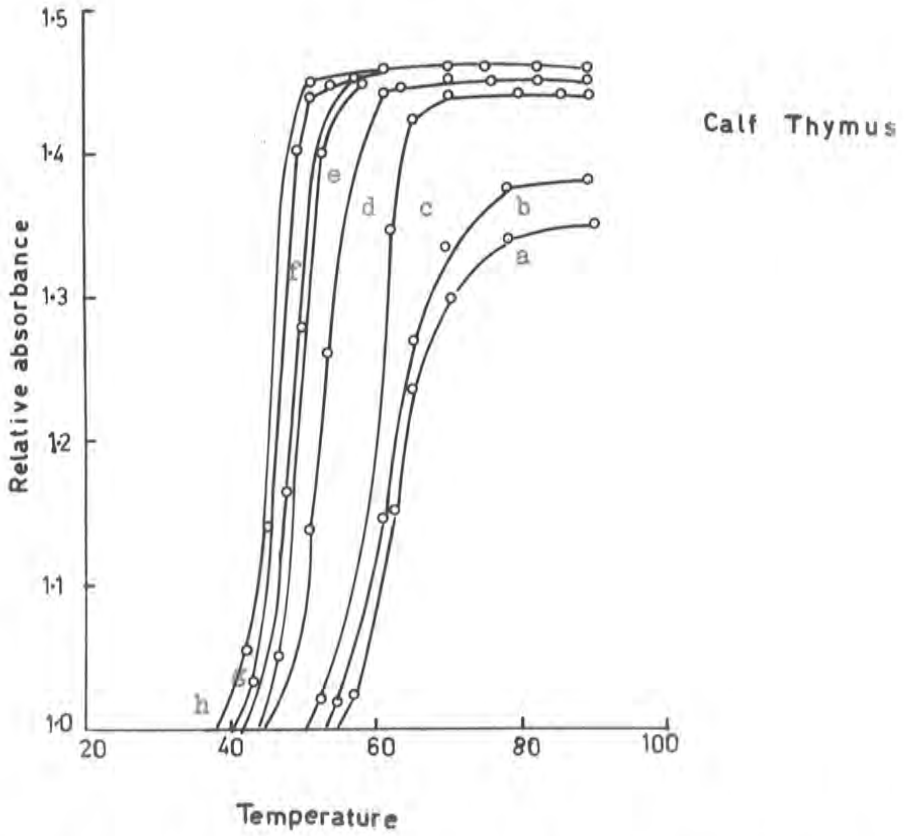

Fig. II-2. Melting curves of Calf Thymus DNA($2.99 \times 10^{-5} M_P$) in the presence of various concentrations of Cu(II) ions. Curvesa, 0mM; b, .01mM; c, .02mM, d, .04mM; e, .05mM; f, .06mM; g, .08mM; h,.1mM.

the melting curves gradually shift towards lower temperatures with increase in copper concentration. The curve h was obtained with a 0.1 mM. concentration of copper. Higher concentrations of copper were avoided because of the probability of precipitating a Cu-DNA complex. The curves become steeper as compared to curve a (native DNA) with the increase in copper concentration. The hyperchromicity also increases gradually but finally attains a constant value (from curve d onwards).

The profiles for the thermal denaturation of Calf Thymus DNA are similar to those of E.Coli DNA. However, the maximum shift of the melting curves to lower temperatures with 0.1 mM. copper concentration is less and the maximum hyperchromicity is more as compared to E.Coli DNA (Fig. 2).

3. Variation of Tm with cupric ion concentration

From the melting curves of Fig. 1 and 2 of the foregoing section, the high temperature limit (h_{max}) is estimated. Tm is now defined as the temperature for which

$$h = \tfrac{1}{2}(h_{max} + 1)$$

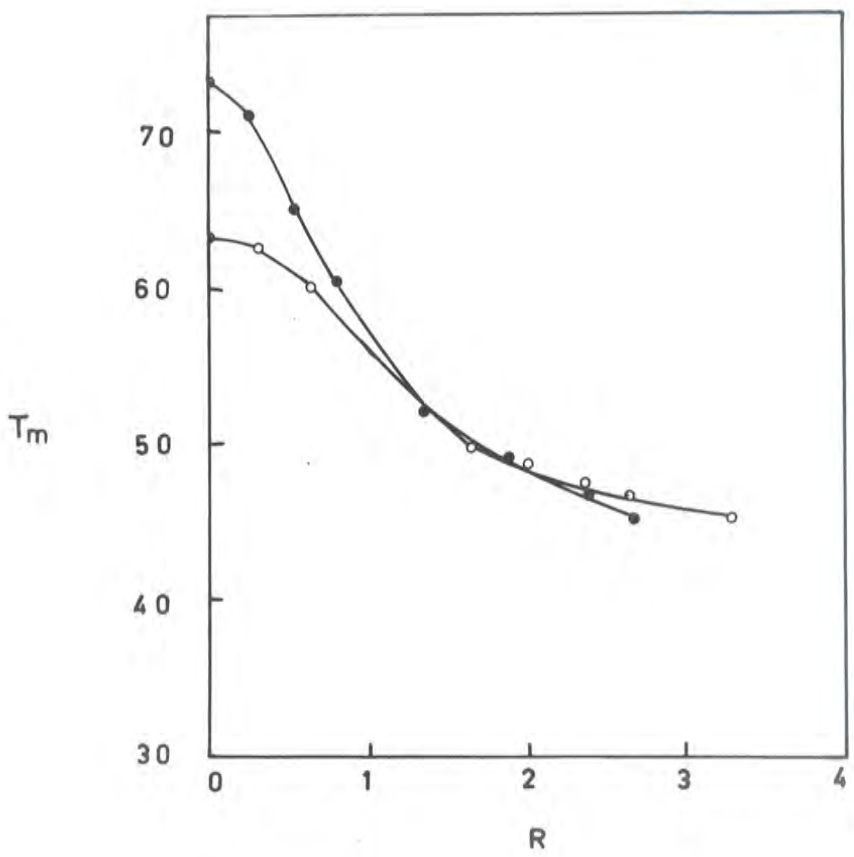

Fig. II-3. Variation of Tm with R
● ● E. Coli DNA.
○ ○ Calf Thymus DNA

as given by Dove and Davidson.[7] These calculated Tm values have been plotted against R (R = total amount of copper per nucleotide in the solution, both free and bound) and are shown in Fig. 3. From the figure it is evident that with R = 0.3, Tm of E. Coli DNA is decreased to 71.0 and this decrease in Tm value becomes very rapid until R = 1.3 approximately whereafter it decreases more slowly. At the highest value of R = 2.7, the Tm is lowered to 45°C from 73.5°C, the value obtained in absence of copper.

Similar behaviour is observed with Calf Thymus DNA, at the maximum value of R = 3.3. The Tm value decreases to 45.0° from 63.0°C obtained in absence of copper.

4. Hyperchromicity of DNA at different temperatures

Solutions of Calf Thymus DNA (2.99×10^{-5} M) were heated in the presence of $Cu(NO_3)_2$ (1.0×10^{-4} M) at 25°, 35°, 45° and 55°C for 10 minutes and then cooled by plunging them into ice cold water. The spectra were recorded at 25°C and are shown in Fig. 4. The U-V spectrum of DNA gives a maximum at 258 mμ (curve a). The position of this maximum remains unchanged by the addition of copper at 25°C (curve a) but on increasing

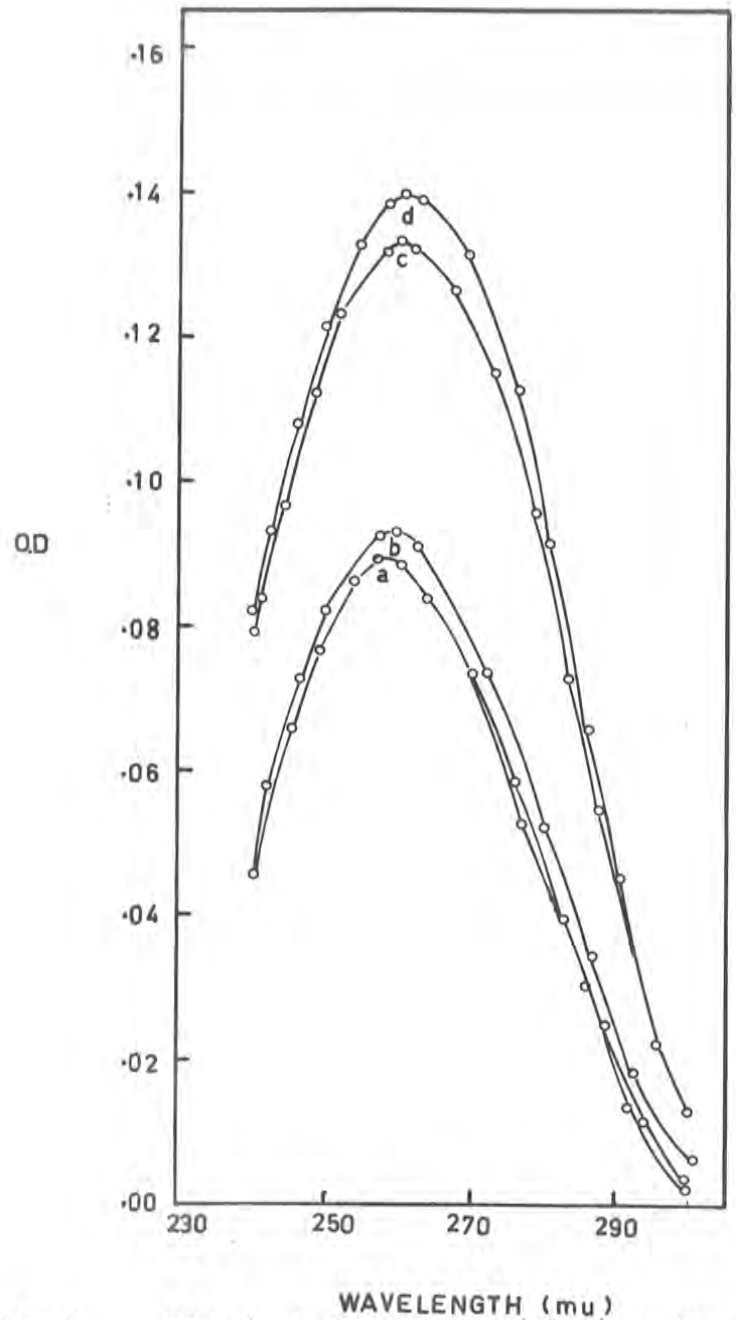

Fig. II- 4. Effect of Cupric ions (.1mM) on the spectrum of Calf Thymus DNA ($2.99 \times 10^{-5} M_P$) when heated together at various temperatures and quenched. Curves a, 25°C(also native DNA without cupric ions); b, 35°C; c, 45°C; d, 55°C.

the temperature at which heating was carried out there is observed a small shift in the maximum towards longer wavelength (261 mμ, curve d) and an increase in absorbance with the increase in temperature, as is shown in Table I. In all cases broadening of shoulders has been observed towards longer wavelengths.

TABLE I

Shift of the U-V absorption maximum of Calf Thymus DNA after heating at various temperatures in presence of cupric ions

Temperature	Position of maximum absorbance	$O.D._{max\ T}/O.D._{max\ 25}$
25	258	1.00
35	259	1.04
45	260	1.45
55	261	1.55

Heating a DNA solution to 90°C for 45 minutes and subsequently cooling by the method as described above, it was observed that the wavelength of the maximum did not change (Fig. 5, curve b), although there was about a 40% increase in absorbance. On adding copper to this denatured solution, to give a solution of 1.0×10^{-4} M

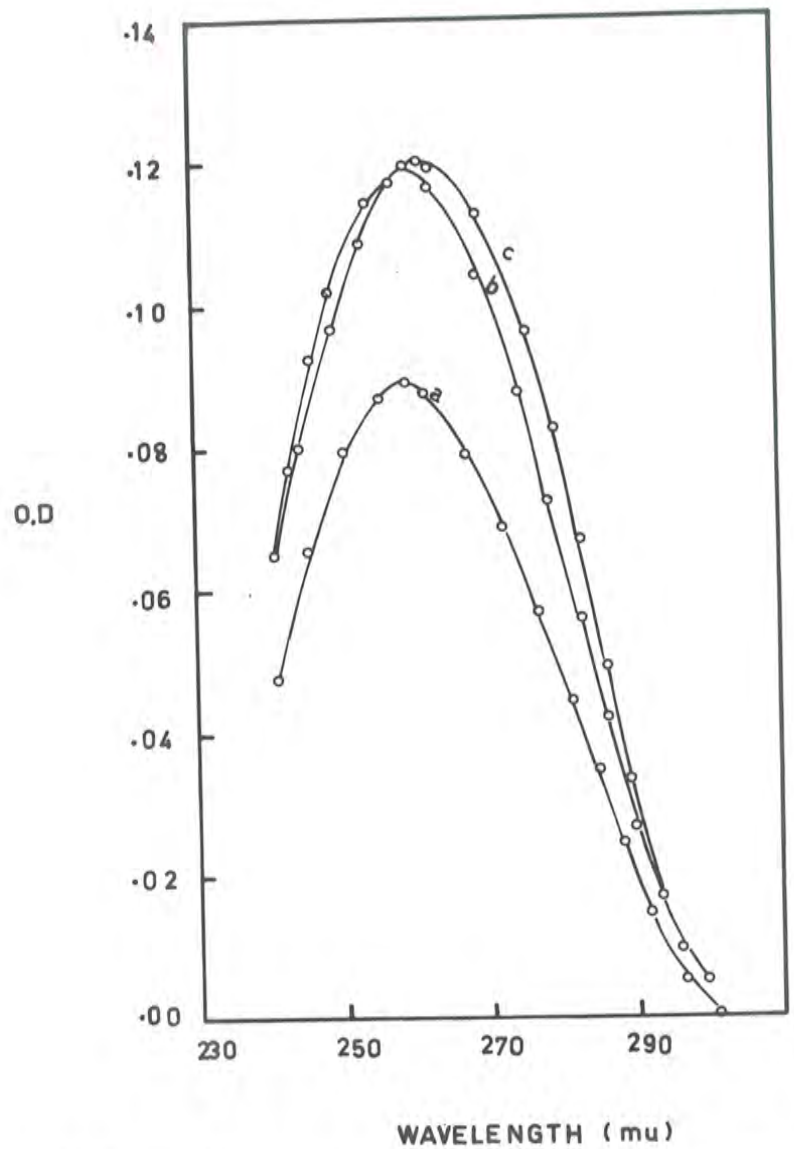

Fig. II-5. Effect of Cupric ions (.1mM) on the spectrum of Calf Thymus DNA (2.99×10^{-5} M$_p$). Curves a, native DNA and also when copper added to the native DNA; b, DNA heated at 90°C for 45 minutes and quenched; c, copper added to DNA treated as in b.

concentration, the spectrum showed a shift in the maximum (261 mμ, curve c) with a little broadening of the shoulders towards longer wave-length.

5. Renaturation by adding solid KNO_3 to the denatured solutions

Under certain conditions when heated, double stranded hydrogen bonded DNA molecules are separated into two tangled single chains in which the hydrogen bonds have been broken and which when cooled under optimal conditions, are reformed to bring recovery of the original structure and also of the biological activity. This process is known as the renaturation of DNA.

In the present case it has been observed that the hyperchromicity of DNA denatured by heating in the presence of copper is irreversible upon cooling. Curves b, c and d of Fig. 4 all behave in the same way. However, when solid KNO_3 is added to make the solutions 0.1 M in KNO_3, the absorbance decreases and after 15 hours all the spectra followed the curve a (Fig. 4), which was obtained before heating the solution.

Similar observations have been recorded in the next section (6) where after adding solid KNO_3 to

solutions of DNA heated in the presence of copper at different temperatures, it was noted that the absorbance values gradually decrease and in the course of 15 hours all solutions attain the value which they had before heating. Similar observations were also noted by Eichhorn and Clark[1].

6. Sedimentation velocity and absorption values at different temperatures and their reversal after adding solid KNO_3

Any changes in size or shape of a macromolecule in solutions may be followed by the measurement of the sedimentation coefficient (S) and the sedimentation coefficient distribution in turn characterizes the distribution of molecular weights. In the present case sedimentation velocity measurement has been used to find if there is any configurational change when DNA is denatured by heating in the presence of copper. This experiment was necessitated because reversal of absorbance values by adding solid KNO_3 does not necessarily mean that after apparent renaturation of the denatured DNA, native DNA has been regenerated. Care was taken to see that all the experiments were done on portions of the same solution as different solutions may have

different degrees of shear degradation.

All the sedimentation coefficients reported herein were corrected to a standard basis according to the equation given by Svedberg and Pederson[8]

$$S_{20,w} = S \left(\frac{\eta\ H_2O,t}{\eta\ H_2O,20} \right) \left(\frac{\eta\ \text{solvent}}{\eta\ H_2O} \right)_t \frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)_t}$$

where $S_{20,w}$ is the corrected sedimentation coefficient,

S is the sediment coefficient under the conditions of the experiment,

$\eta\ H_2O$, η solvent, ρ are respectively the viscosity of water and solvent, the density of the solvent at the temperature of the experiment (t^oC),

$\eta\ H_2O$, $\rho_{20,w}$ are the corresponding quantities for water at 20^oC,

$\bar{v}$ is the partial specific volume of DNA in the solvent used.

The values of η and ρ were taken from the International Critical Tables. The figure used for $\bar{v}$ was 0.556 for the sodium salt of DNA[9].

Equal volumes of DNA (1.07×10^{-4} M) and copper (2×10^{-4} M) heated separately for 10 minutes in

a water bath, were mixed in a preheated flask and left for 4 minutes. The flask was then taken out, cooled by plunging in ice cold water for nearly 5 minutes and the $S_{20,w}$ and absorbance values were then recorded. Solid KNO_3 was added to the rest of the solution to make 0.1 M solution of KNO_3 and the measurements were repeated. The same operation was carried out at each temperature.

The values so obtained are shown in Fig. 6. It can be seen that both the absorbance and the sedimentation values increase with the increase in the heating temperature and are changed drastically at the highest temperature of 51°C (at this temperature the absorbance begins to form a plateau). When solid KNO_3 is added to these solutions, both of these values do not revert exactly to the values for native DNA. There is not very much deviation in the absorbance values but the sedimentation values are lower by a few units at the higher temperatures.

7. Kinetics of denaturation

The extent of denaturation was followed by measuring the increase in the absorbance as a function

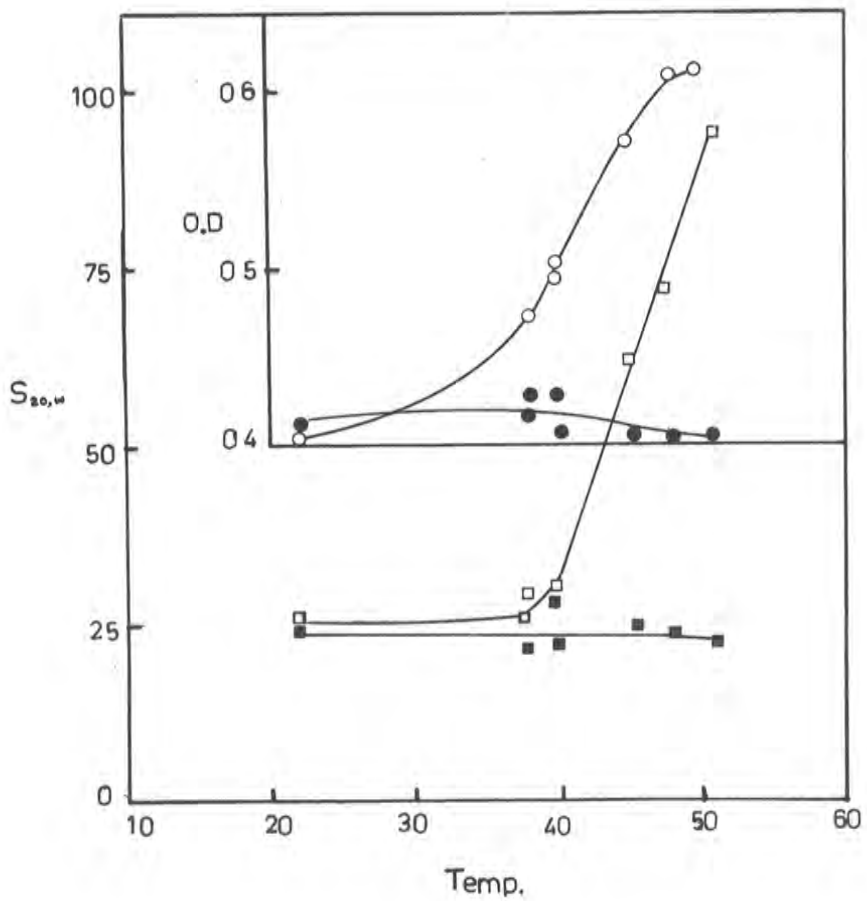

Fig. II-6. Changes in $S_{20,w}$ and absorbance of Calf Thymus DNA ($.54 \times 10^{-4} M_p$) and Cupric ions (1×10^{-4} M) heated to various temperatures and quenched and also when renatured by adding solid potassium nitrate (.1M)

□ □ ○ ○ Heated and quenched.

■ ■ ● ● Heated, quenched and potassium nitrate added.

of time of the solutions of Calf Thymus DNA (0.30×10^{-5} M) and copper (1×10^{-4} M) at 38.6°, 41.0°, 45.4°, 48.0°, 50.2° and 55.0°C. This denaturation reaction was done with a thermoregulator chamber so as to maintain a constant temperature during the time of the experiment.

The results so obtained are shown in Fig. 7 from which it is evident that in the beginning there is a sudden increase in the absorbance values which is marked with the increase of temperature. This reaction is so fast that at the temperatures of 50.2 and 55.0°C, within 30 seconds, the absorbance reaches to the point wherefrom it begins to form a plateau. Though this reaction was carried out for a further 5½ hours, a small increase in the absorbance was observed. In other cases also there is a sudden increase in the absorbance in the beginning for 1 minute and then increases gradually up to 2 hours, after which period they follow the same pattern as above. There is observed a very little difference in the final absorbance values obtained after 5½ hours at 50.2° and 55°C and it is presumed that after 50.2°C the denaturation of DNA is completed.

8. Discussion

The behaviour of cupric ions in lowering the

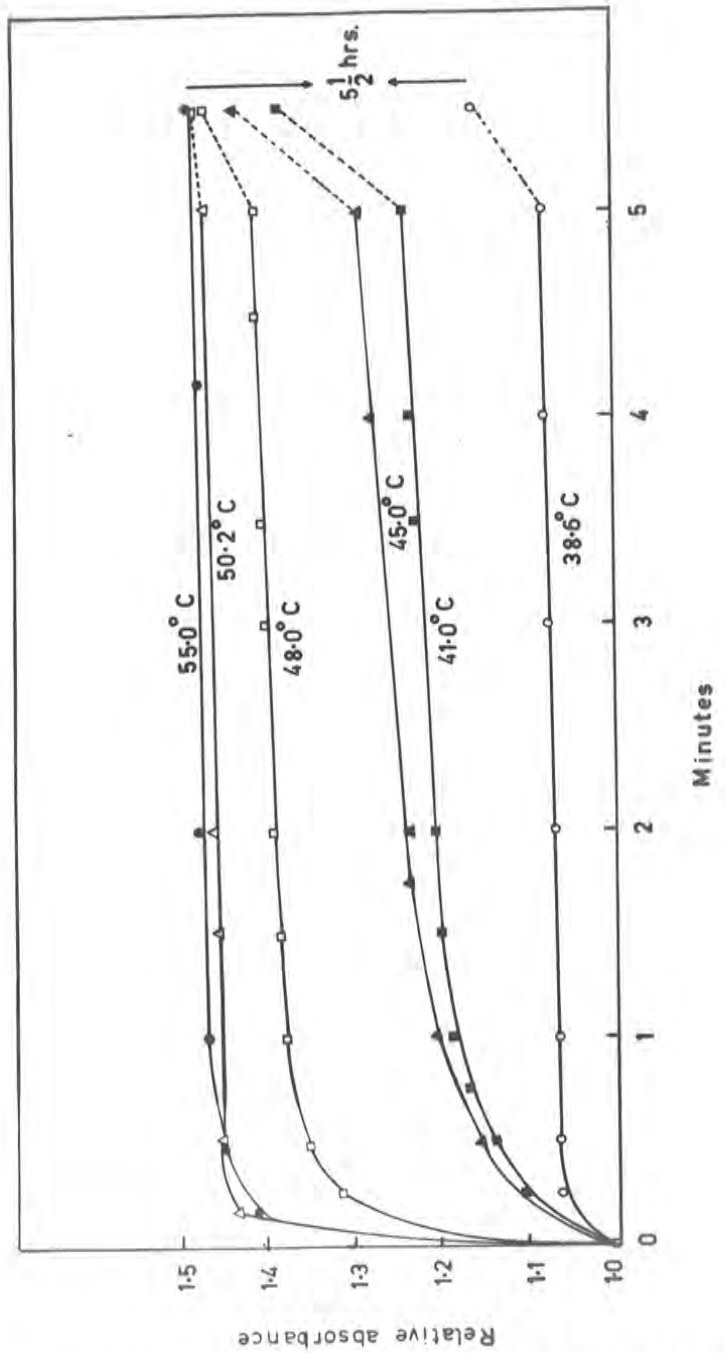

Fig. II-7. Changes in the relative absorbance with time for Calf Thymus DNA (8.30×10^{-4} M$_P$) and cupric ions (1×10^{-4} M) in 10^{-2} M KNO_3 at different temperatures.

melting temperature of DNA remained undetected previously because measurements were made in higher concentrations of electrolyte solutions (0.1 M) under which condition the Tm is not lowered. Presumably either there was greater competition with sodium ions for binding sites or it was difficult for the metal to approach binding sites due to shielding effects produced by sodium ions. However, in low concentrations of electrolyte, they are less shielded by the counterions. It is also known that lowering of the salt concentration at constant temperature below a critical point brings about dilution denaturation[10-13], but since the double stranded helical configuration of DNA is known to remain stable in solutions as low as 0.001 M solution of electrolyte[14] all the measurements in the present case were done in 0.01 M solution of KNO_3.

All the experiments were carried out in the absence of any buffer to maintain constant pH because of possible complications which may arise due to contribution of added cations to the binding with DNA. Under these conditions, however, there is a possibility that the copper denaturation is affected by pH changes produced by cupric ions. Following the suggestion of Cavalieri et al[15] that the pH differences between solutions of DNA with and without cupric ion accounts for a little change

in the conformation of DNA at room temperature and the additional experimental verification which has been put forward by Eichhorn and Clark,[1] small pH differences have been neglected in these studies.

The behaviour of DNA from E. Coli and Calf Thymus is quite similar with regard to the increase and decrease in absorbance and Tm values. The Tm of DNA depends entirely on the concentration of copper used and the steepness of the curve suggests the independence of Tm with respect to G-C content of DNA. The fact that Tm is lowered suggests that destabilisation which is due to phosphate binding as happens with Mg^{+2} and Mn^{+2} where the Tm was found to be increased.[7,16] But Lyons and Kotin[17] have stated that the magnetic resonance studies do not correspond to the concept of specific interaction with the bases as the destabilising effect. There is the possibility that the large quantities of some simple salts may cause destabilisation through an indirect influence on the structure of water around and within the helix thereby reducing the hydrophobic bonding between the base pairs.[18]

The decrease in Tm has been noted in the

presence of small amounts of copper. At R = 0.27, the Tm decreases significantly which is in direct contrast to what Eichhorn[1] has reported. Eichhorn found that with R = 0.4 there is observed an increase in Tm initially which decreases with increasing value of R. Eichhorn also suggested that a break occurred in the curve at R = 1.5 for Calf Thymus DNA leading to the possibility of binding of one copper to phosphate and the rest to the nucleosides or vice versa. However, in view of the possibility of the fact that some of the copper may not be bound at all, discussion of this question has been left until after this chapter where equilibrium dialysis technique has been employed to work out the possible stoichiometry of the reaction.

The wave-length of the absorbancy maximum shifted to 261 mμ from 258 mμ when heated to 55°C in presence of copper. The appearance of the shoulder in the absorption curves in the region 265-280 mμ (Fig. 4) may be attributed to the perturbation of the allowed energy levels of the bases by the coordinating copper ions. This behaviour is similar to that observed by Yamane and Davidson[19] for the spectral shift associated with the interaction of DNA with mercuric ions, which was interpreted as the interaction between the mercuric

ions and the ring nitrogen atoms of the heterocyclic bases.

When copper was added, at 25°C, to the denatured solution of DNA obtained by heating to 90°C in absence of copper, the increase in absorbancy was about 10% less than when denaturation was carried out in presence of copper. At 55°C, there is a marked increase in the absorption compared with the value at 25°C (Table I). It is thus concluded that at higher temperatures, when some relative motion of the two strands in the DNA helix is possible such as occurs at the "annealing temperature",[20] penetration of the helix by the copper ions can occur which results in binding of copper ions to the nitrogen atoms of bases such as to bring about marked distortion or partial disruption of the helix to produce denaturation.[21]

The irreversibility of the hyperchromicity of DNA denatured in presence of copper ions indicates that at higher temperatures, the copper complex becomes strong enough to compete effectively and at this low ionic strength the stability of the complex overcomes that of the double helix and hence the reformation of the native DNA does not take place. But after increasing the ionic strength by adding solid KNO_3, the stability of the complex becomes less and the absorbance decreases and approaches the original

value. There is always the possibility that in this renatured DNA, the conformation and other properties might have been affected. In normal cases of renaturation the strands which unite are not necessarily of equal length and one chain may extend beyond each end in an unpaired manner and consequently these unpaired ends become sites for further base pairing which may lead to aggregation making it impossible to reform the native DNA.[22,23]

To observe further whether complete renaturation has taken place or not, the sedimentation measurements were done before and after denaturation and after renaturation. On renaturation the return of the $S_{20,w}$ values to almost the same as those obtained before heating indicates the fact that native DNA has been regenerated.

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CHAPTER III

BINDING OF CUPRIC IONS TO DNA

1. Introduction.
2. Theory and method of binding.
3. Number of bound counterions.
4. Binding at 5°C.
5. Binding at 55°C.
6. Variation of Tm with r.
7. Visible absorption spectra of cupric ions and DNA.
8. Discussion.
9. References.

1. Introduction

It is well known that nucleic acids bind metal cations on the negatively charged sites of their phosphate groups. Mn^{+2} binds to the phosphate groups of nucleic acids[1] and also to the phosphate groups of ATP[2] which was confirmed by quantitative equilibrium[3,4] and kinetic studies[5]. Eisinger et al, by N.M.R. technique, also confirmed the binding of Mn^{+2} to phosphate groups of nucleic acids[6]. Shack[7] proposed the same site of DNA for the binding of Ca^{+2} and Mg^{+2}. The binding with Cu^{+2} has been studied by many workers[8-14] and they proposed the binding sites to be either bases or phosphate groups of nucleic acids. By this time evidence had been brought forward that not only the phosphate groups are potential binding agents but the binding occurs to a large extent at the nitrogenous bases also[15-19].

Binding of Cu^{+2}, Mg^{+2}, Hg^{+2}, Ca^{+2}, Zn^{+2} and Mn^{+2} metal ions to DNA has been studied previously by conductimetric[17,20,21], spectrophotometric[20,22], electrophoresis[23], ultra-violet rotary dispersion[24], ion-exchange[25] and equilibrium dialysis[11,25] methods. Zubay and Doty[11] reported that the break in the conductimetric titration occurs when 0.83 equivalent copper per nucleotide has been added and that cupric ions chelate

with phosphate groups on the adjacent DNA molecules. But this method involves considerable experimental difficulties and also the interpretations are ambiguous as the contribution of the partially complexed molecules to the conductivity is neither negligible nor constant. As already has been reported in Chapter II, Eichhorn and Clark and Hiai, from their spectrophotometric observations suggested that cupric ions bind DNA at the phosphate and the bases. However, no definite conclusion about the stoichiometry of the reaction was given by these workers.

The investigation in the present Chapter deals with the equilibrium dialysis method to find out the possible stoichiometry of the reaction. This has been done at $5^{o}C$ and also at $55^{o}C$, all in 0.01 M solution of KNO_3. The shift of the absorption band from the far red towards the middle red region of the visible spectrum gives additional proof of the fact that there is a direct interaction between copper ions and nitrogen atoms of DNA at the elevated temperature.

2. Theory and method of binding

In principle, the same theory and methods as

suggested by Scatchard[26] and Scatchard, Coleman and Shen[27] for analysing the interaction of large polymeric molecules with small molecules has been followed to characterize the binding process in these studies. If r represents the number of ligands bound per polymer molecule (P) at a free ligand concentration of C_f moles per litre, and n shows the maximum number of binding sites per P, then the ion association constant (K), for the interaction can be expressed as

$$K = \frac{r}{(n-r)(C_f)} \qquad \ldots\ldots (1)$$

which on slight rearranging, gives

$$\frac{1}{r} = \frac{1}{C_f}\left(\frac{1}{K}\cdot\frac{1}{n}\right) + \frac{1}{n} \qquad \ldots\ldots (2)$$

This simple relation between r and C_f holds good only under the condition that all the binding sites have the same association constant (K) and that aside from the statistical factor, the free energy of binding to any particular site is independent of binding to other sites[28,29]. The plot of $^1/r$ vs $^1/C_f$ yields a straight line with an intercept at $^1/C_f = 0$, which will give n the number of binding sites thus the value of K can be calculated from the slope. The same method has been

followed by others in finding these values[30,31]. The presence of more than one class of binding sites results in the curvature of $^1/r$ vs $^1/C_f$ plots and thus makes accurate determination of intercept and slope more difficult.

The factors which may cause the non-linearity in such type of plots could be due to the possible interaction between bound molecules and also due to the electrostatic factors because of complex formation. If all these factors are eliminated, the characterisation of binding can be accomplished by the least square method with the assumption that all random errors occur in $^1/r$ only. The solution thus obtained represents one set of calculated values of n and K which fits into the experimental data.

3. Number of bound counterions

The solutions of copper and DNA inside the dialysis bag was equilibrated against the copper solution of equivalent concentration. After this equilibration period, the concentration of the copper solution outside the dialysis bag was determined by means of polarography. It is known from the Ilkovic equation[32] that for the

total polarographic diffusion current (i_d) at the dropping mercury electrode.

$$i_d = 709\ n\ C\ D^{1/2}\ m^{2/3}\ t^{1/6} \quad \ldots\ldots \quad (3)$$

where n is the number of electrons required for the reduction of one ion, C the concentration (mM.) of the reducible ion, D the polarographic diffusion coefficient of the reducible ion ($Cm^2\ sec^{-1}$), m the rate of mercury flow ($mg\ sec^{-1}$), t the drop time (sec.). Since all the other factors are constant,

$$i_d = K\ C \quad \ldots\ldots \quad (4)$$

The diffusion current (i_d) values of the copper solutions before dialysis were determined and after making correction for the residual current, a linear relationship was obtained between these determined i_d values and the concentrations of the copper solutions (Fig. 1). All (i_d) measurements were made at -1.0 volts against S.C.E. (other experimental details and the theory of polarography have been discussed in Chapter IV).

The i_d values of the solution after the dialysis were determined by the same method as described above. Quantitative analysis in this case is based on the change in the magnitude of the diffusion currents and since these are proportional to the copper concentrations, the

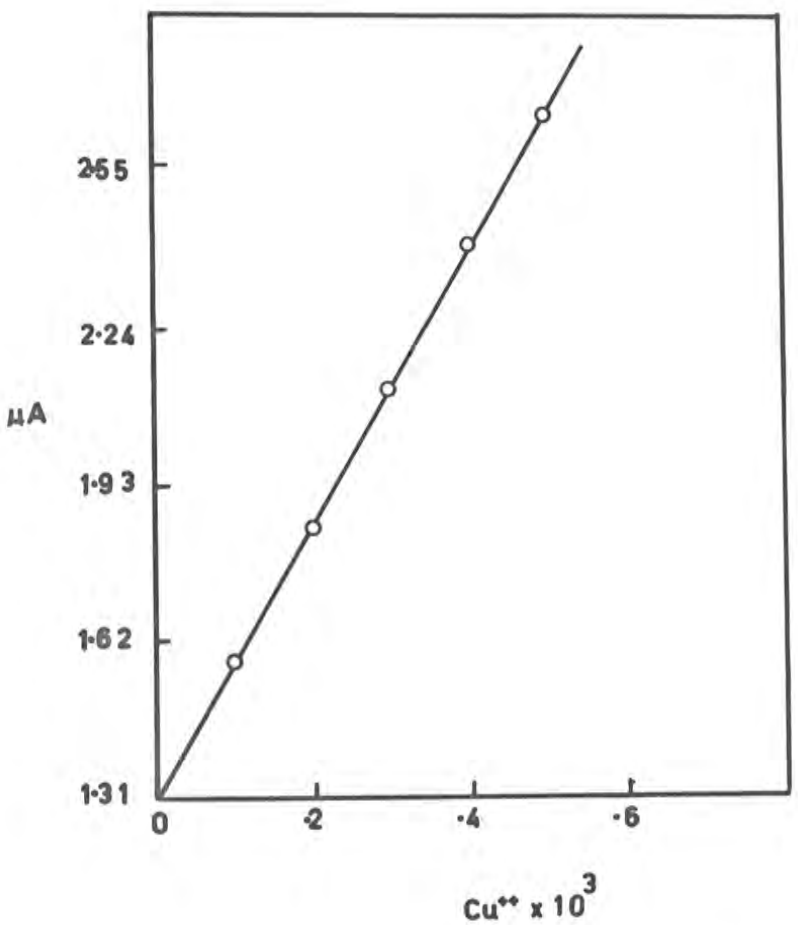

Fig. III-1. Diffusion current as a function of cupric ion concentration for a solution containing 0.01M KNO_3. The points represent the average current and were corrected for the residual current.

unknown copper concentrations were calculated directly from the height of the waves. After knowing the concentrations of copper, the number of bound counterions r (r now defined as ratio of the moles of copper bound per mole of DNA phosphorus was calculated by the following relationship:

$$r = \frac{V (C_1 - C_2)}{P} \qquad \ldots\ldots (5)$$

where C_1 and C_2 are the initial and final molar concentrations of copper solutions before and after the dialysis, V the total volume of the solution (both inside and outside) and P the total molar concentration of DNA (P).

4. Binding at 5°C

Dialysis bags, containing solutions of DNA (10^{-4} M_p) and Cu^{++} (10^{-5} M) all prepared in KNO_3 (10^{-2} M), were placed in pyrex tubes with identical concentrations of copper solutions outside the bags. There was no detectable binding of copper by the bag and also since the difference in the copper concentration both inside and outside the bag was found to be very small, great care was taken to achieve equilibrium between these two solutions. These tubes were then stoppered and placed on a

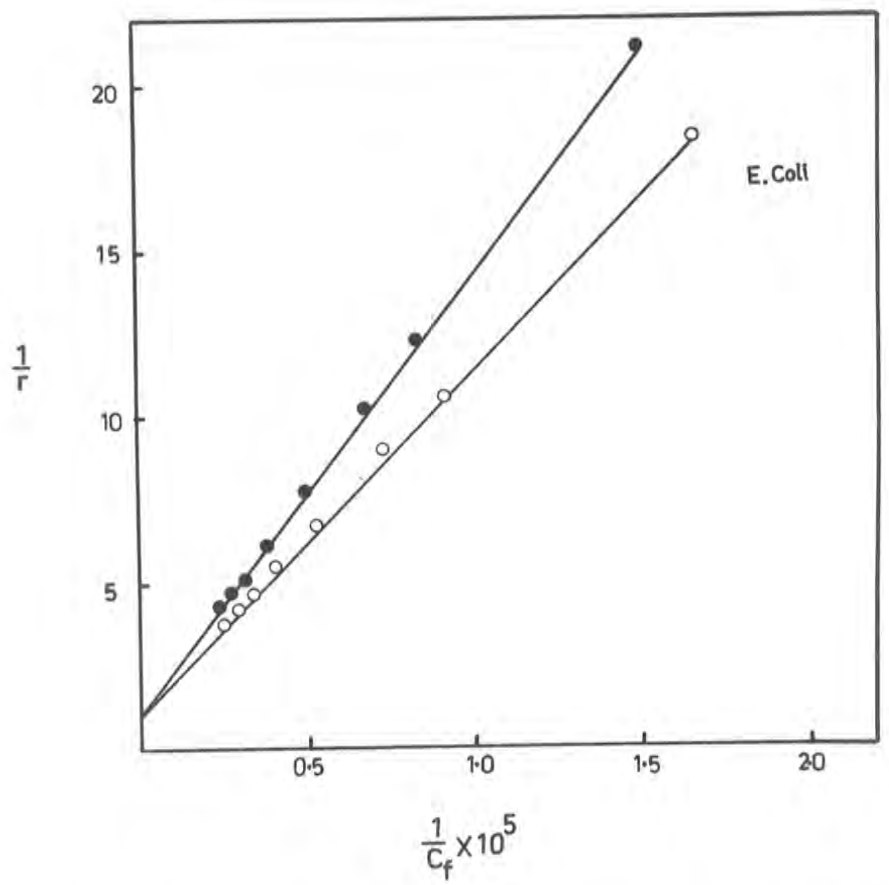

Fig. III-2. The plot of $1/C_f$ versus $1/r$ for E. Coli DNA at 5°C. ○ ○ native, ● ● denatured.

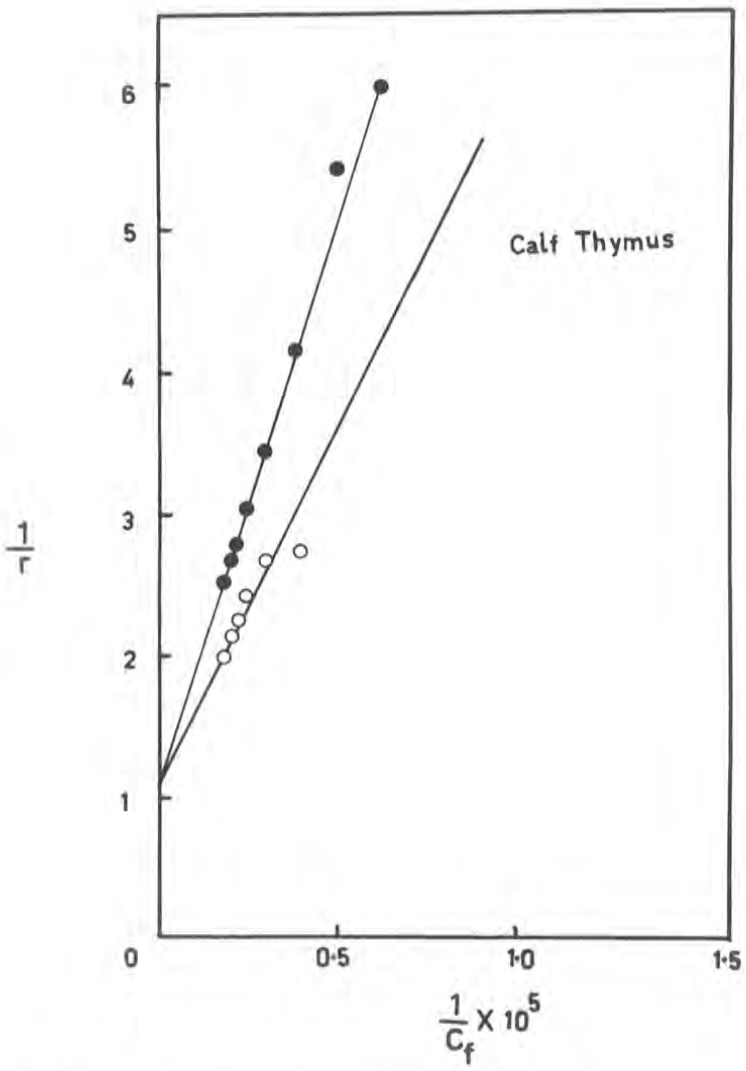

Fig. III-3. The plot of $1/C_f$ versus $1/r$ for Calf thymus DNA at 5°C. ○ ○ native, ● ● denatured.

moving shaker placed in the dark at 5°C. The unbound copper was allowed to equilibrate across the membrane for nearly 72 hours. After this period the measurement of the concentration of the copper solution outside the bag was made by the same method which has been described in Section 3. These experiments were done with native DNA and DNA denatured in the absence of copper by heating at 90°C for 45 minutes and then adding copper to the cooled solution of denatured DNA.

The results of such experiments are shown in Figs. 2 and 3 which show the plot of $^1/r$ vs $^1/C_f$ for native and denatured solutions of E.Coli and Calf Thymus DNA. It is evident from the figure that the number of binding sites remains the same under two different conditions and with two different types of DNA. With $n = 1$, it is concluded that copper binds at only one site of DNA and presumably to the phosphodiester groups.

5. Binding at 55°C

Essentially, the method of dialysing the solutions of copper and DNA (E.Coli) was the same as has been described in the earlier section excepting the fact that in this case the stoppered tubes were kept in a water

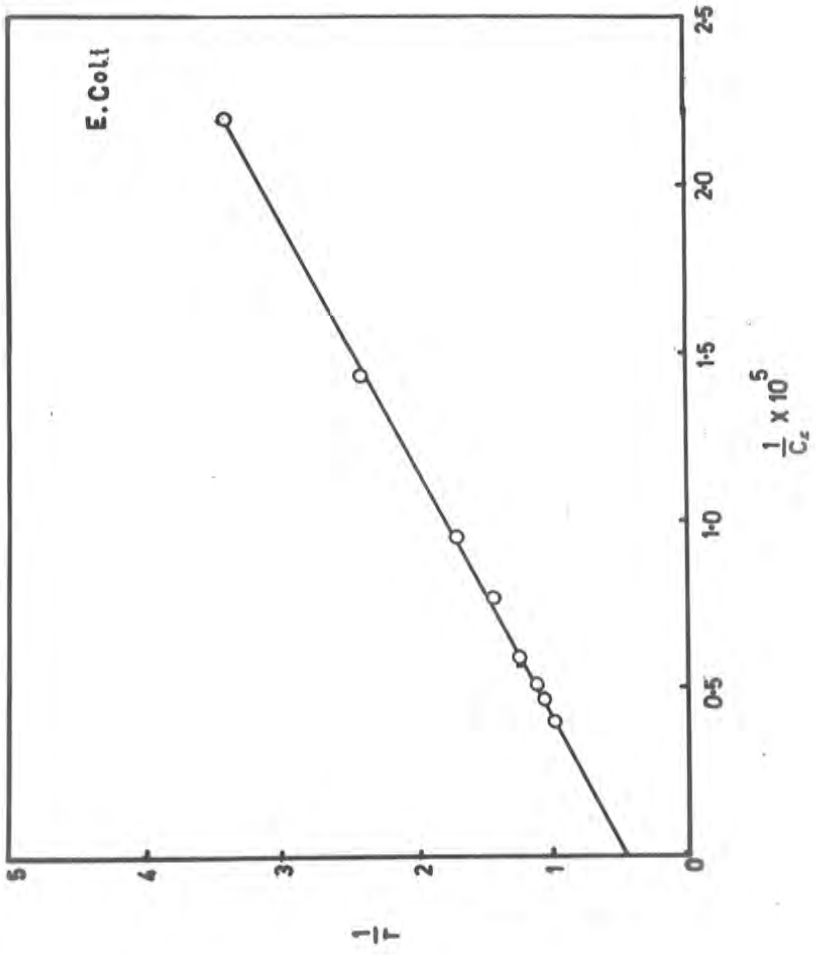

Fig. III-4. The plot of $1/C_f$ versus $1/r$ for E. Coli DNA at $55^{\circ}C$.

bath thermostated at 55°C for nearly 48 hours. The tubes were then taken out and the concentrations of the solutions outside the bag were determined as before.

The results so obtained are shown in Fig. 4 from which it is evident that the number of binding sites (n) has been increased from 1.0 to 2.30 in this case and also the value of the association constant ($K = 13.84 \times 10^4$) and is much higher than the value obtained with E.Coli DNA for binding at 5°C. The results have been summarised in Table I.

TABLE I

Binding Parameter

	5°C				55°C
	E.Coli DNA		Calf Thymus DNA		E.Coli DNA
	Native	Denatured	Native	Denatured	
n	1.00	1.00	1.00	1.00	2.30
k	1.12×10^4	0.84×10^4	3.81×10^4	1.25×10^4	6.02×10^4
K = nk	1.12×10^4	0.84×10^4	3.81×10^4	1.25×10^4	13.84×10^4

6. Variation of Tm with r

Fig. 5 shows the plot of r (as obtained from the binding experiments at 5° and 55°C) versus Tm (calculated from melting curves of Chapter I) for E. Coli DNA. This shows the relationship between Tm and the number of bound counterions, r. The Tm decreases rapidly with increasing values of r.

It is also observed from Fig. 5 that r at 55°C is always much larger than at 5°C. This arises because at this higher temperature, the number of binding sites and also the association constant is larger than at 5°C (Table I).

There are several approximations implicit in the plot of Tm against r. The values of r were determined not at Tm but at 5° and 55°C. It is clearly not possible to determine r at Tm for the DNA double helix alone as the denatured form will also be present in the solution. A possible approach would have been to determine r at a series of temperatures and then extrapolate to give the value of r for the DNA double helix at Tm. Some of the values of r at higher copper concentrations used in the graph were calculated by the interpolation method from the binding curves at 5° and 55°C.

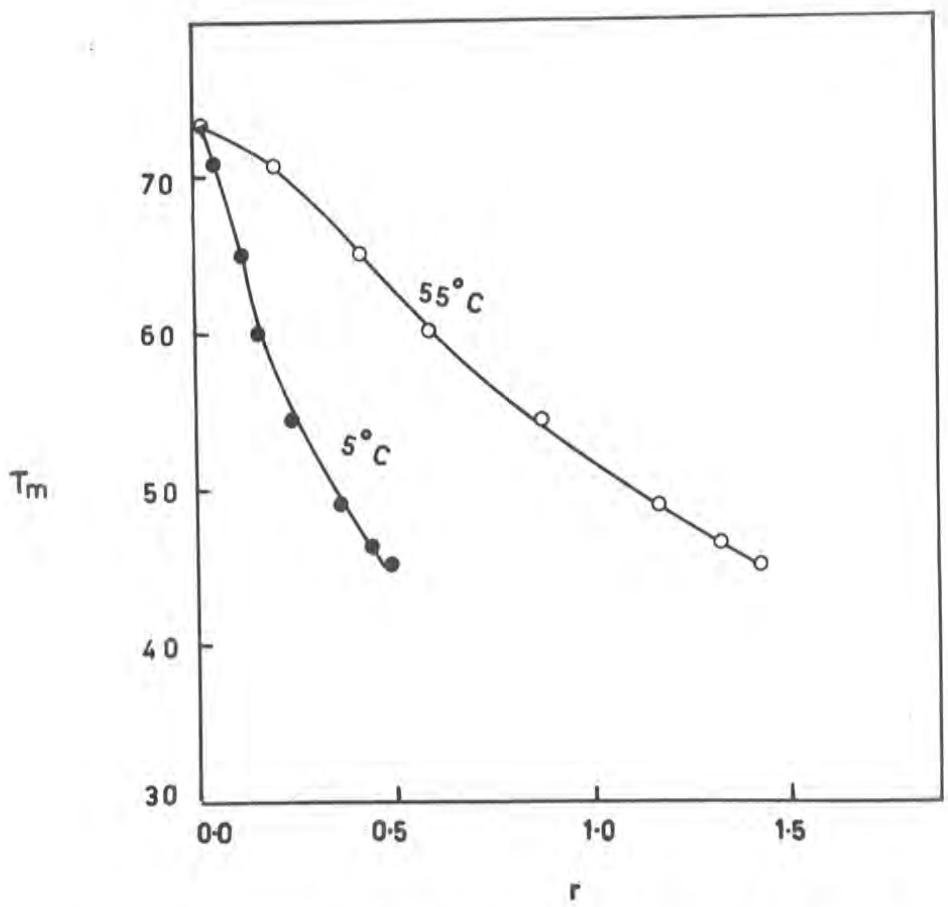

Fig. III-5. The plot of r, measured at 5° and 55°C, versus Tm for E. Coli DNA.

The concentrations of copper and DNA were arranged to be the same (approximately) in both thermal denaturation and binding experiments because if the dialysis is carried out at higher concentrations of DNA, dilution of the DNA solution will become necessary for the determination of Tm in the suitable optical density range and this will bring about a reduction in r values.

7. Visible absorption spectra of cupric ions and DNA

It has been mentioned earlier that heating DNA solution in the presence of copper results in an increase in the absorbance, a bathochromic shift in the maximum and also increase in the number of binding sites. All these results indicate that under these conditions, copper is bound to the nitrogen bases. To obtain more direct evidence to the fact that bonding of copper to the nitrogen atoms does take place, visible absorption spectra of the solutions of copper and DNA heated at different temperatures were recorded against DNA solution of identical concentration.

For this purpose 30 mls. each of copper (8.40×10^{-4} M) and DNA (14.88×10^{-4} M_p) all prepared in

10^{-2} M KNO_3 solution, were heated separately for ten minutes in a water bath, kept constant at the temperature of the experiment, and then mixed in a preheated flask. After mixing, the heating was continued for another four minutes and then cooled by plunging into ice cold water. When cooled, the spectra were recorded against a solution of DNA (14.88×10^{-4} M_p) in 10^{-2} M KNO_3 in a matched cell.

Solid KNO_3 was then added to the rest of the solutions to make 0.1 M solution of KNO_3 and again the spectra were recorded 15 hours after this addition.

It is observed from Fig. 6 that the solution at 25°C gives a maximum of 800 mμ (curve a) but shift in the maximum towards shorter wavelength takes place in gradual steps with the increase in the heating temperature and finally it comes down to 740 mμ at the temperature of 51.2°C. After adding solid KNO_3, all the solutions follow the same curve as obtained at 25°C which again confirms the previous findings that native DNA has been regenerated.

8. Discussion

From Fig. 6 it is observed that copper ions in the presence of non-complexing anions (e.g. NO_3^-) exhibit

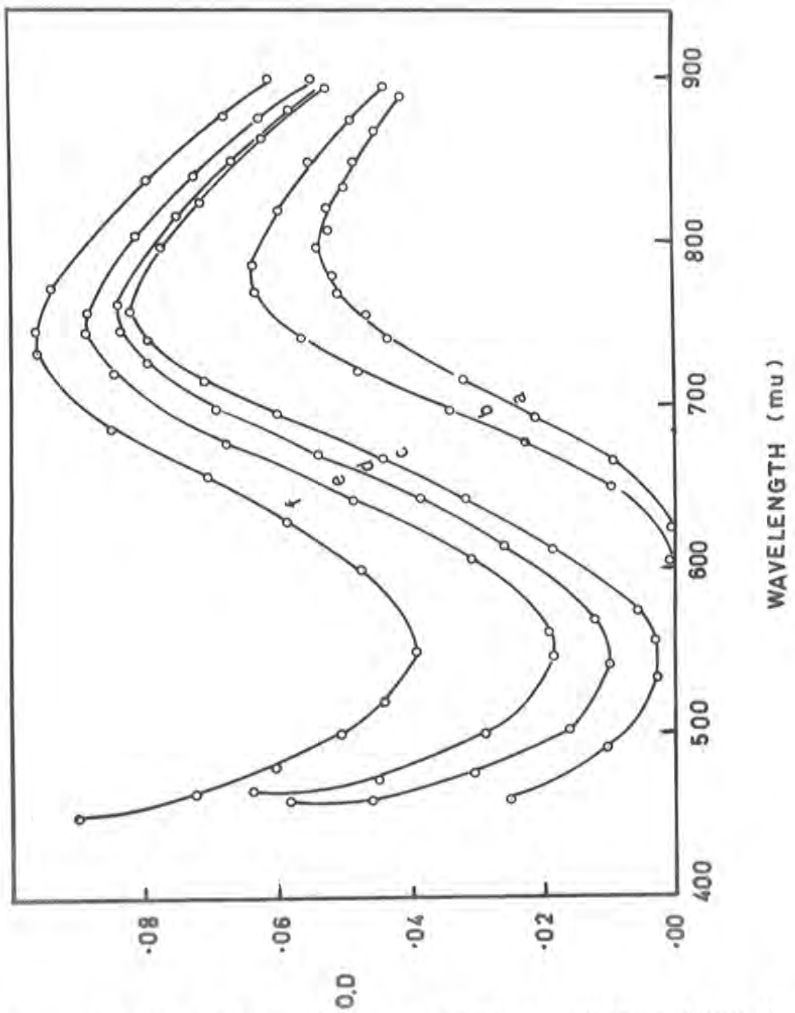

Fig. III-6. Visible absorption spectra of cupric ions (.42mM) in the presence of Calf Thymus DNA (.74mM$_P$) heated at different temperatures for 4 min. and quenched. Curves a, 25°C (and also cupric ions without DNA); b, 38.5°C; c, 40.4°C; d, 45.2°C; e, 48.4°C; f, 51.2°C. After adding solid KNO_3 (.1M) all the above curves followed the curve a.

an absorption spectrum in the region 470 to 900 mμ with a maximum at 900 mμ (Fig. 6 a) which is characteristic of $[Cu(H_2O)_6]^{++}$ ion. This spectrum is little changed on addition of DNA at 25°C. But after heating the solutions of DNA and copper at different temperatures, for four minutes, the maximum shifts to shorter wavelengths with a marked increase in the absorbance in all the cases. At the highest selected temperature of 51.5°C, the maximum is shifted to 740 mμ (Fig. 6 f). This increase in absorbance and shift of the maximum to shorter wavelength is similar to the effect observed on the coordination of copper with ammonia and ethylene-diamine, the stronger the ligand field due to the nitrogen derivative, the stronger the shift and increase in the intensity of the absorption band. The maximum for the ion $[Cu(NH_3)(H_2O_5]^{++}$ occurs at 740 mμ and for $[Cu(NH_3)_2(H_2O)_4]^{++}$ at 680 mμ.[33] These spectral data thus afford further additional direct evidence for the bonding of the copper ions to the nitrogen atoms in the heterocyclic bases after denaturation but not at the room temperature.

In addition, there is also observed an increase in absorbance in the region 470 to 520 mμ, as is

observed in the U-V region of 265-280 mμ (Chapter II), such behaviour may again be described as the formation of shoulder in the absorption curve.

In the binding studies which have been done by the equilibrium dialysis method, attempts were made to keep the ionic strength constant to a reasonable degree, by making all the solutions in 0.01 M solution of KNO_3. The correction for the Donnan effect has not been taken into account because at such low DNA concentrations relative to KNO_3, the Donnan effect should be minimum. Also, it has been shown practically to be negligible under such experimental conditions[34].

At 25°C, the determination of the extent of binding of copper to DNA shows that there is only one type of binding site (Figs. 2A and 3A), the same is true for DNA denatured in the absence of these ions and subsequently placed in an environment containing these ions. (Figs. 2B, 3B). However, at 55°C, the number of binding sites increases and reaches up to 2.3 as has been shown in Fig. 4. The value of the association constant (K) also increases and is much higher than the value obtained at 25°C (Table I).

Increase in the number of binding sites from one to two, bathochromic and hypsochromic shifts and

other spectral evidence which have been reported in the second and this Chapter, support strongly the view that copper binds to the electron donor groups of the nucleoside bases at higher temperatures.

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CHAPTER IV

BINDING OF CUPRIC IONS TO NUCLEOSIDES

1. Introduction
2. Polarographic studies
   - (a) General theory
   - (b) Diffusion current or kinetic current
   - (c) Effect of copper concentrations on diffusion current depression at constant nucleoside concentrations
   - (d) Relation between $E_{\frac{1}{2}}$ and nucleoside concentrations
3. Spectrophotometric studies
   - (a) U-V absorption spectra
   - (b) Visible absorption spectra
4. Conclusions
5. References

1. Introduction

It was first shown by Albert[1] that nucleosides can form complexes with cupric ions when he made a potentiometric study of the stability of a copper-guanosine complex. He also suggested the existence of the complexes of transition metal ions with basic sites of the nucleosides. Frieden and Alles[2] observed that nucleoside and nucleotide bases inhibit the catalysis by copper of the enzymatic oxidation of ascorbate and this inhibition was interpreted by them in terms of complex formation by copper with these inhibitors. An interesting observation was made by Harkins and Frieser[3] who, following the Calvin[4] - Bjerrum[5] technique, observed that the titration curve of adenosine and copper was practically identical with the hydrolysis curve of copper. They also concluded that the ribose group of adenosine is not involved in the reaction as the titration curve of D-ribose and copper was found to be identical with that of copper and adenosine. This view is also held by Eichhorn et al[6] who failed to observe a shift in the potentiometric titration of nucleosides and copper. These observations show that such complex formation proceeds without the removal of the proton from the ligands. But the observation of Fiskin and Beer[7] appears to be in conflict with this conclusion since they

reported the stabilities of such complexes based on proton displacement in acid solution. Such proton displacement has also been observed in cases of complex formation of the nucleosides with mercury[8,9,10].

There has been a continuous interest regarding the possible sites of the nucleosides for complexing with copper. Bryan and Tomita[11], with the crystallographic method, confirmed copper binding at N_1 for adenosine, whereas binding at N_3 for cytidine and N_7 for guanosine were proposed and confirmed on the basis of crystallographic and proton magnetic resonance studies[12,13,14,15]. In a very recent publication, Eichhorn et al[6], with nuclear magnetic resonance studies, suggested that copper binds to N_7 position of guanosine and adenosine and to N_1 of cytidine.

This Chapter describes spectrophotometric and polarographic studies of the binding of copper with different ribonucleosides and deoxyribonucleosides in order to determine to which nucleosides these ions become more attached. To observe further the role of phosphate groups on such bindings, some spectrophotometric observations were also made with ribonucleotides and deoxyribonucleotides both in the presence and absence of copper.

2. Polarographic studies

The application of polarographic method to study the metal-nucleoside interaction has so far received very little attention, primarily because of the involved nature of the reduction of the nucleosides at the dropping mercury electrode (d.m.e.). Controlled experiments were done in the present case so that the nucleosides were not reduced at d.m.e. and for this purpose polarograms were obtained extending only up to -0.5 volts as it is known that beyond this limit, they were found to be reduced[16-19].

(a) General theory

The interpretation of the polarographic waves of complex metal ions has been discussed by Lingane[20]. By complex formation, the reduction potentials of the metal ions are usually shifted to more negative values and that by measurement of this shift, both the formula and the dissociation constant of the complex can be determined very easily provided that the reduction of the metal ion is reversible at the dropping mercury electrode. The general discussion is restricted to the case where the complexing agent is present in excess.

Lingane[20] has considered the case where the

reduction of a complex of a metal soluble in mercury may be represented by the net reaction

$$MX_q^{(n-qb)} + ne + Hg \rightleftharpoons M(Hg) + q\,X^{-b} \quad \ldots\ldots (1)$$

which for convenience may be regarded as the sum of two partial reactions

$$MX_q^{(n-qb)} \rightleftharpoons M^{n+} + q\,X^{-b} \quad \ldots\ldots (2)$$

and $$M^{n+} + ne + Hg \rightleftharpoons M(Hg) \quad \ldots\ldots (3)$$

(where X^{-b} is the complex forming substance and M(Hg) is the amalgam formed at the surface of the d.m.e.). This division is merely an artifice which assists in clarifying the thermodynamic relations involved and is not intended to indicate an actual kinetic mechanism.

If the rates of all of the possible intermediate steps in the electrode reaction are more rapid than diffusion rates, the dropping electrode is only subject to concentration polarization. When this condition is fulfilled the electrode reaction will proceed reversibly and the current at any point on the wave will be diffusion controlled and can be expressed in terms of the diffusion processes around the dropping electrode. The potential of the d.m.e. at any point on the wave should be given by

$$E_{d.m.e.} = \epsilon + \frac{RT}{nF} \ln \frac{K_c f_{mx}}{f_a (f_x)^q} - \frac{RT}{nF} \ln \frac{C^o_a (C^o_x)^q}{C^o_{mx}} \quad \ldots (4)$$

where ϵ = a constant

K_c = dissociation constant of the complex

C^o_a = concentration of the metal in the amalgam formed at the electrode surface

C^o_x = concentration of complex forming ion at the electrode surface

C^o_{mx} = concentration of the metal complex at the electrode surface

f_a, f_x, f_{mx} = corresponding activities.

The values of $E_{d.m.e.}$ and the various concentrations at the electrode surface, vary periodically during the life of each mercury drop, because of the periodic change in the electrode area as each drop grows and falls.

In order to express the various concentrations at the electrode surface in terms of the current it is assumed that the diffusion layer is thin and thus the concentration gradients may be considered linear, consequently

$$C^o_{mx} = \frac{i_d - i}{K_c} \quad \ldots\ldots (5)$$

where i is the average current (μA) during the life of a

drop at a given value of $E_{d.m.e.}$, i_d is the limiting diffusion current and K_c is the constant defined by the Ilkovic equation as

$$K_c = 607\ n\ D_{mx}^{1/2}\ m^{2/3}\ t^{1/6} \qquad \ldots\ldots (6)$$

where D_{mx} is the diffusion coefficient of the complex ($cm^2\ sec^{-1}$), m is the mass of the mercury (mg.) flowing from the electrode per second and t is the drop time (sec.).

Since the concentration of amalgam formed at the surface must be directly proportional to the current,

$$C^o_a = {}^1/K_a \qquad \ldots\ldots (7)$$

where K_a is defined in the same way as K_c except that the diffusion coefficient of the metal in the amalgam is used.

Now since the complex forming substance X^{-b} is a product of the electrode reaction, its concentration at the electrode surface increases with increasing current.

$$C^o_x = C_x + q\,\frac{1}{K_x} \qquad \ldots\ldots (8)$$

where K_x is proportional to the square root of the diffusion coefficient of X^{-b}, and C_x is the concentration of X^{-b} in the body of the solution. If it is assumed that the solution originally contains an excess of X^{-b} at a concentration that is relatively large compared to the

concentration of the complex metal ion, and when this condition is fulfilled, the quantity $q\ ^{i}/K_x$ in equation (7) will be negligibly small and C^{o}_{x} can be regarded as virtually a constant and equal to C_x. On substituting these relations into equation (4) the equation of the wave at 25°C with an <u>excess of complex forming substance present</u> in the solution can be written as

$$E_{d.m.e.} = E_{\frac{1}{2}} - \frac{0.0591}{n} \log \frac{i}{i_d - i} \qquad \ldots\ldots (9)$$

$E_{\frac{1}{2}}$ is given by

$$E_{\frac{1}{2}} = \epsilon + \frac{0.0591}{n} \log \frac{K_c\ f_M\ K_a}{f_a\ K_c} - q\ \frac{0.0591}{n} \log C_x\ f_x \qquad \ldots\ldots (10)$$

Since the concentration of the metal ion complex does not enter into the expression for its half wave potential, $E_{\frac{1}{2}}$ should be constant and independent of the concentration of complex metal ion.

<u>Relation between $E_{\frac{1}{2}}$ and dissociation constant</u>

From Equation (10) we find that the $E_{\frac{1}{2}}$ depends on the logarithm of the dissociation constant of the complex metal ion, and it is more negative, the smaller the value of K_c, i.e. the more stable the complex ion.

Although K_c can be evaluated from the observed value of the $E_{\frac{1}{2}}$ itself, it is usually more accurate to determine it from the difference between the $E_{\frac{1}{2}}$ value of the complex metal ion and that of the corresponding simple metal ion. Lingane[21] and Stackelberg[22] have shown that $E_{\frac{1}{2}}$ of a simple metal ion is expressible by

$$(E_{\frac{1}{2}})_s = \epsilon - \frac{0.0591}{n} \log \frac{f_a K_s}{f_s K_a} \quad \ldots\ldots (11)$$

where K_s is proportional to the square root of the diffusion coefficient of the simple metal ions. From equations (10) and (11) it follows that the shift of $E_{\frac{1}{2}}$ by complex formation should obey the relation

$$(E_{\frac{1}{2}})_c - (E_{\frac{1}{2}})_s = \frac{0.0591}{n} \log \frac{K_c f_c f_s}{f_s K_c} - q \frac{0.0591}{n} \log C_x f_x \quad \ldots\ldots (12)$$

in which the subscripts "c" and "s" refer to the complex and simple metal ions respectively, $(E_{\frac{1}{2}})_c$ and $(E_{\frac{1}{2}})_s$ are to be given their proper signs with respect to particular reference electrode against which they are measured. The ratio K_s/K_c which is equal to $(\frac{D_s}{D_c})^{\frac{1}{2}}$ is usually close to 1 and so it may also be neglected. Hence for approximate purpose equation (12) can be simplified to

$$(E_{\frac{1}{2}})_c - (E_{\frac{1}{2}})_s \cong \frac{0.0591}{n} \log K_c - q \frac{0.0591}{n} \log Cx \quad \ldots\ldots (13)$$

Also ratio K_s/K_c can be determined experimentally from the ratio of the observed diffusion currents of the simple and complex metal ions at the same concentration and with all other conditions constant.

Determination of the coordination number

From equation (10), the $E_{\frac{1}{2}}$ of the complex metal ion should shift with changing activity of the complex forming substance according to

$$\frac{\Delta E_{\frac{1}{2}}}{\Delta \log Cxfx} = - q \frac{0.0591}{n} \quad \ldots\ldots (14)$$

This relation is important because it enables the determination of the coordination number, q, of the complex metal ion to be made and hence its formula determined. For this purpose it is usually sufficiently accurate to employ the concentration of X^{-b} in place of activity.

(b) Diffusion current or kinetic current

In polarographic measurement, it is essential

that the current should be diffusion controlled. It has been pointed out that polarographic limiting current, under certain conditions do not depend upon the height of the mercury reservoir, whereas the true diffusion currents are always proportional to the square root of the height of the reservoir[23]. Maas[24] demonstrated earlier, that i_d is directly proportional to the quantity $m^{2/3} t^{1/6}$. From the Poiseuille relation,

$$m = \frac{Vd}{t} = \frac{r^4 c d P}{8 l} \qquad \ldots\ldots (15)$$

(t is the drop time, V the volume and Vd is the weight of a single drop), it is evident that the following relation should exist

$$m = A h_{corr} \qquad \ldots\ldots (16)$$

and $$t = \frac{B}{h_{corr}} \qquad \ldots\ldots (17)$$

where h_{corr} is the effective or corrected pressure on the dropping mercury in cm. of mercury and A and B are constants which depend on the geometrical properties of the capillary.

Then $$m^{2/3} t^{1/6} = (A h_{corr})^{2/3} \left(\frac{B}{h_{corr}}\right)^{1/6}$$

$$= C h^{\frac{1}{2}} \qquad \ldots\ldots (18)$$

and hence $i_d = K\, m^{2/3}\, t^{1/6}$ = Constant x $h_{corr}^{\frac{1}{2}}$.
That is with all other factors constant the diffusion current should be proportional to the square root of the effective pressure on the dropping mercury.

As per condition of the position of equilibrium, the observed current may represent the properties in between that of the true diffusion current at one extreme or a pure kinetic current at the other. This means that the wave height may be proportional to $h_{corr}^{\frac{1}{2}}$, independent of h or may increase with increasing h. This was tested by measuring the diffusion currents of the solutions of 0.2 mM. Cu^{+2}, 0.1 mM. nucleosides, in 0.01 M KNO_3 at six different heights of the mercury columns. The results as obtained are given in Table I from which it is observed that the ratio $h_{corr}^{\frac{1}{2}}/i_d$ is a constant which proves that the currents in such cases are diffusion controlled.

TABLE I

h_{corr} cm.	h_{corr} cm.	i_d μA	$i_d/h_{corr}^{\frac{1}{2}}$
62.0	7.874	1.687	0.214
56.0	7.483	1.597	0.213
52.0	7.211	1.512	0.209
46.5	6.819	1.437	0.210
41.5	6.442	1.350	0.209
36.5	6.041	1.300	0.215

(c) Effect of copper concentration on diffusion current depression at constant nucleoside concentrations

Polarograms of various concentrations of copper solutions in the range 0.1 mM. - 0.4 mM. in 0.01 M solutions of KNO_3 in the absence and presence of nucleosides (0.1 mM.) were obtained and from these the values of diffusion currents were obtained which are given in Tables II and III. The effect of nucleosides on the diffusion currents of copper show that the reduction of the diffusion currents $(i_d/(i_d)_o)$ ratio of the diffusion current in the presence of nucleosides (i_d) to the diffusion current in the absence of nucleosides $(i_d)_o$ in all these cases, except with thymidine where this is not significant, is due mainly to copper-nucleoside interaction and is found to be in the order guanosine > cytidine > adenosine.

(d) Relation between $E_{\frac{1}{2}}$ and nucleoside concentrations

A series of polarograms were obtained with 0.2 mM. Cu^{+2} and varying concentrations of guanosine, cytidine, adenosine and thymidine. From these polarograms, the half wave potentials $(E_{\frac{1}{2}})$ were found by plotting the logarithm of $i/(i_d - i)$ versus the applied

TABLE II

Cu^{++} mM.	Guanosine, 0.1 mM.			Cytidine, 0.1 mM.		
	$(i_d)_o$	i_d	$i_d/(i_d)_o$	$(i_d)_o$	i_d	$i_d/(i_d)_o$
0.400	2.412	2.175	0.902	2.412	2.212	0.8917
0.333	2.212	1.975	0.892	2.212	2.012	0.909
0.266	2.025	1.7875	0.882	2.025	1.825	0.901
0.200	1.850	1.625	0.878	1.850	1.650	0.891
0.133	1.650	1.412	0.856	1.650	1.450	0.879
0.100	1.588	1.312	0.826	1.588	1.338	0.848

TABLE III

Cu^{++} mM.	Adenosine, 0.1 mM.			Thymidine, 0.1 mM.		
	$(i_d)_o$	i_d	$i_d/(i_d)_o$	$(i_d)_o$	i_d	$i_d/(i_d)_o$
0.400	2.412	2.275	0.943	2.412	2.397	0.994
0.333	2.212	2.062	0.932	2.212	2.198	0.993
0.266	2.025	1.875	0.926	2.025	2.000	0.987
0.200	1.850	1.700	0.919	1.850	1.822	0.984
0.133	1.650	1.500	0.909	1.650	1.621	0.982
0.100	1.588	1.375	0.866	1.588	1.554	0.979

potential. At least ten such points were used along the mid portion of the curve. It is evident from equation (9) that these plots should produce a straight line with a slope equal to $\frac{0.0591}{n}$ at 25°C and the potential where the log term becomes zero should measure the half wave potential.

The values of half wave potentials so obtained are given in Table IV from which it is evident that the shift in $E_{\frac{1}{2}}$ values towards negative direction is quite insignificant with thymidine as compared to other cases, and is in the order guanosine > cytidine > adenosine.

Unfortunately, the equations (14) and (13) for the calculation of the coordination numbers and the dissociation constants, could not be applied in the present investigation because the necessary condition for the applicability of Lingane's equation[20] could not be satisfied due to the low solubility of purine nucleosides.

3. Spectrophotometric studies

It has been reported in Chapters II and III that cupric ions interact with the nitrogen atoms of the nucleoside bases of DNA at higher temperatures and produce bathochromic and hypsochromic shifts of 3 mμ and 60 mμ

TABLE IV

Cu^{++} (0.2 mM.); $E_{\frac{1}{2}} = 0.010$

Guanosine		Cytidine		Adenosine		Thymidine	
Conc. mM.	$E_{\frac{1}{2}}$	Conc. mM.	$E_{\frac{1}{2}}$	Conc. mM.	$E_{\frac{1}{2}}$	Conc. mM.	$E_{\frac{1}{2}}$
0.657	-0.008	0.440	+0.002	0.518	+0.005	0.518	+0.006
1.312	-0.014	0.880	-0.006	1.030	-0.002	1.036	-0.001
2.626	-0.026	1.760	-0.014	2.070	-0.005	2.072	-0.004
6.567	-0.037	4.400	-0.025	5.180	-0.012	5.180	-0.008
9.193	-0.043	6.160	-0.034	7.250	-0.021	7.252	-0.010
11.820	-0.046	10.160	-0.038	9.32	-0.029	9.334	-0.014
15.760	-0.050	15.560	-0.042	14.43	-0.034	18.443	-0.016

in the U-V and visible regions respectively. Since such spectra result from the transition of the nucleoside bases, it is expected that they should be affected by the presence of copper. It is only with this aim that the present spectrophotometric studies were made.

Both the ultra-violet and visible absorption spectra of nucleosides were recorded in the presence and absence of copper. Where-ever necessary solutions of dilute HNO_3 and KOH prepared in 0.01 M solution of KNO_3 were added to maintain the pH values to the value which has been described in the text.

(a) U-V absorption spectra

The U-V absorption spectra of these compounds is mainly due to the presence of the purine and pyrimidine components. During past years many attempts have been made to correlate the spectrum of DNA with its constituent bases or nucleotides[25]. Hotchkiss[26] has given data for the ribosides of cytosine and adenine and has found that their molar extinction do not differ from those of free bases.

The general shapes of the U-V absorption curves of all the nucleosides and nucleotides studied herein, were found to be the same as reported earlier by

Doty et al[27]. Addition of copper in different molar ratios does not change the shape of the curves but increase in the molar absorptivities.

The results with adenosine are shown in Fig. 1 from which it is observed that the addition of copper in high molar ratio (1:100) does only increase the absorption at the maximum. A series of spectra, with varying molar ratios of guanosine and copper were recorded at pH 4.30, a few of them are given in Fig. 2. In this case the increase in the absorption intensity is more than adenosine and also distinct shoulder formation is observed towards longer wavelength. The increase in the absorption intensity with cytidine was more than adenosine but less than guanosine and no such effects were observed with thymidine (Figs. 3 and 4).

To observe further the effects and role played by phosphate groups on the absorption intensity of these nucleosides, the spectra of deoxyadenylic, deoxyguanylic, deoxycytidylic and thymidylic acids were recorded at pH 4.30, 3.60, 4.50 and 4.50 respectively. The spectra so obtained are given in Figs. 5-8. It is evident from these graphs that there is an increase in the absorption at the maximum both with deoxyguanylic and deoxyadenylic acids but there is no difference in the spectra of

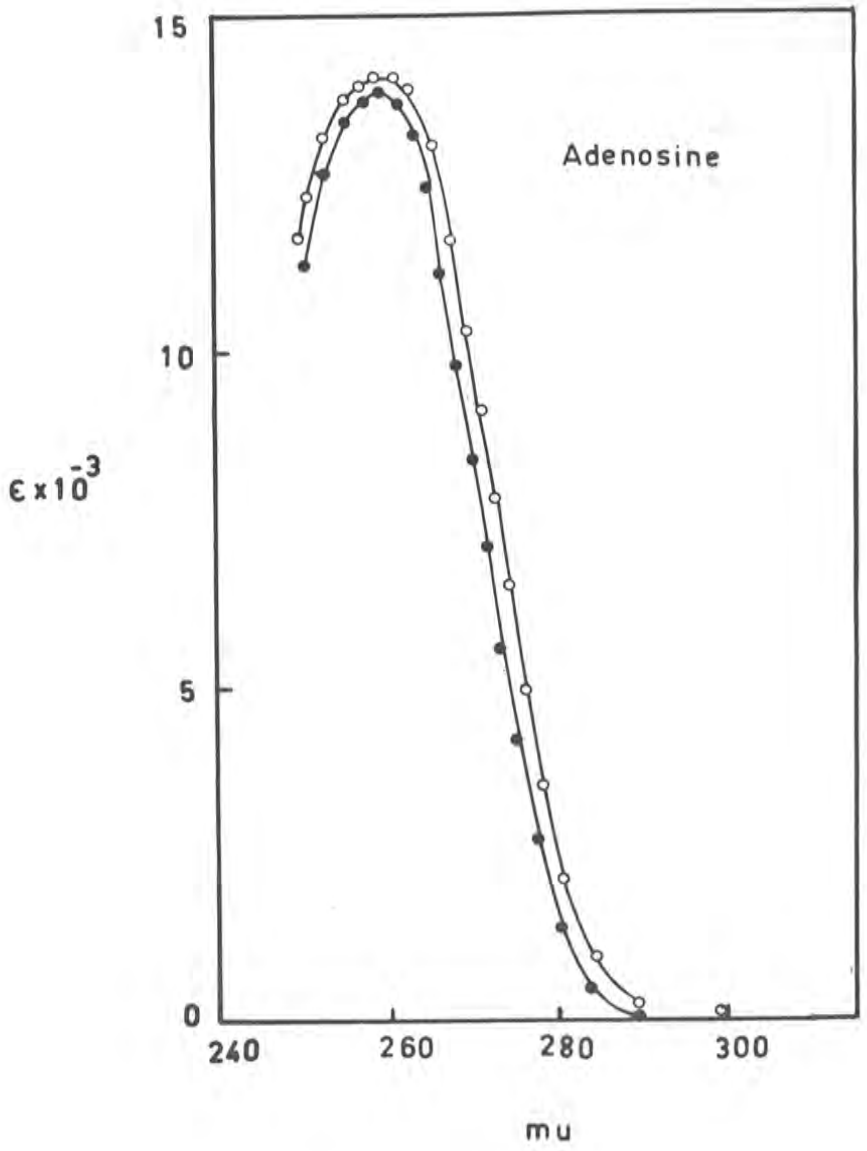

Fig. IV-1. Effect of cupric ions on the U-V. spectrum of adenosine, pH 4.60; ● ● adenosine, 8×10^{-4} M; ○ ○ copper, 8×10^{-2} M and adenosine 8×10^{-4} M.

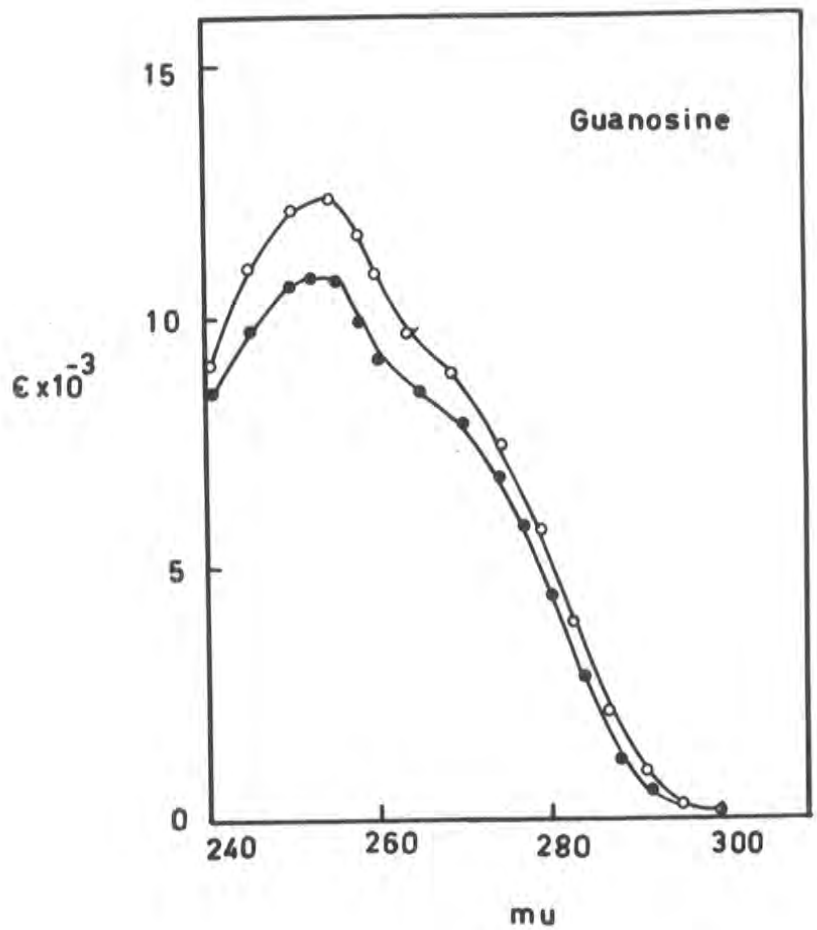

Fig. IV-2. Effect of cupric ions on the U-V. spectrum of guanosine, pH 4.30; ● ● 2.80×10^{-4} M guanosine; ○ ○ copper, 2.80×10^{-2} M and guanosine, 2.80×10^{-4} M.

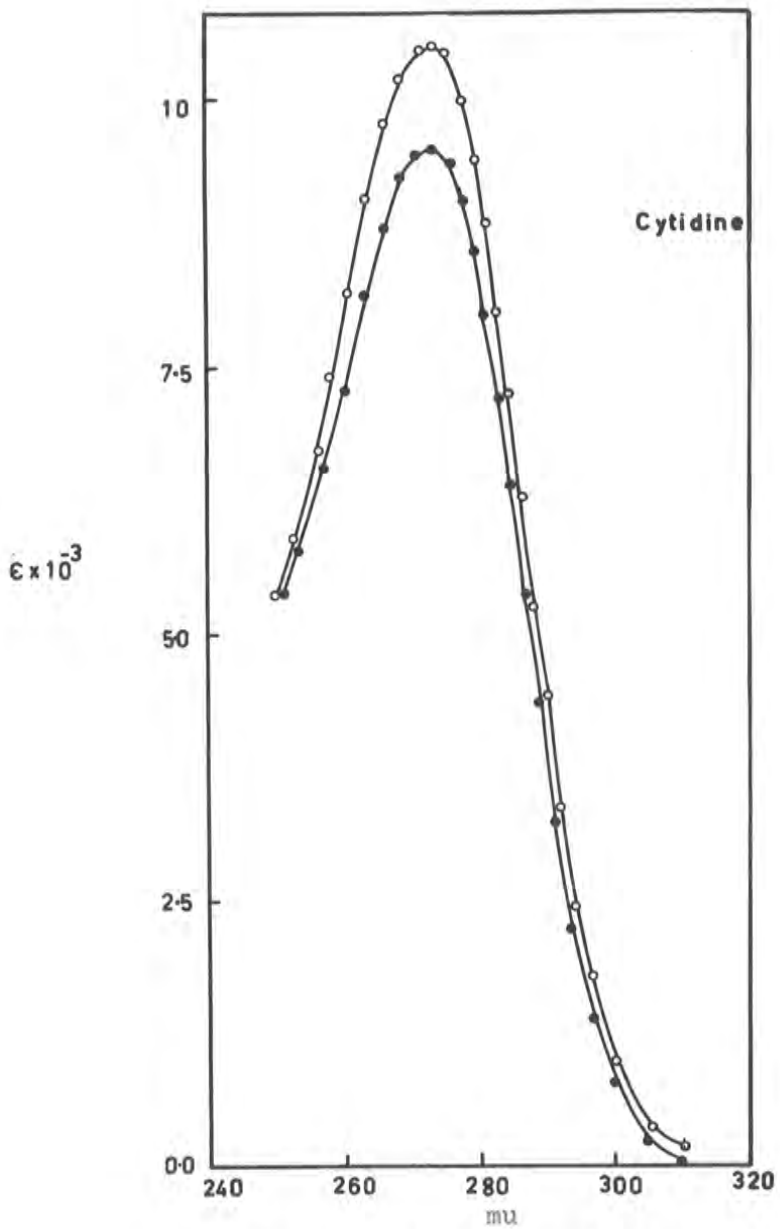

Fig. IV-3. Effect of cupric ions on the U-V. spectrum of cytidine, pH 4.50; ● ● cytidine, 8.0×10^{-4} M; ○ ○ copper, 8.0×10^{-2} M and cytidine, 8.0×10^{-4} M.

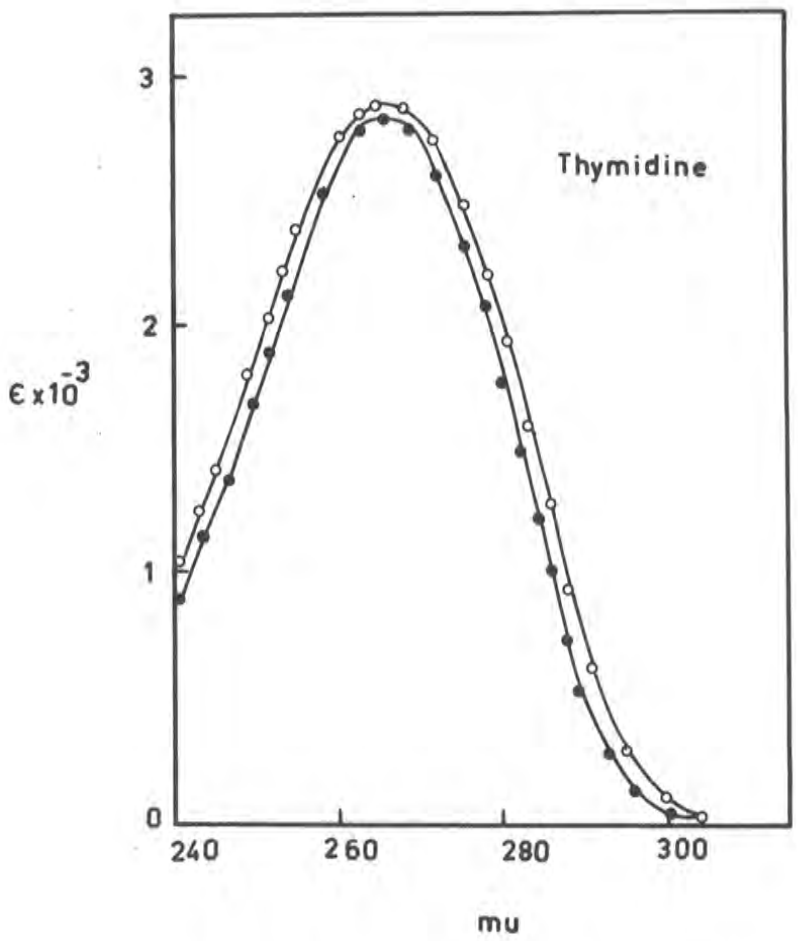

Fig. IV-4. Effect of cupric ions on the U-V. spectrum of thymidine, pH 3.0; ● ● thymidine, 8.0×10^{-4} M; ○ ○ copper, 8.0×10^{-2} M and thymidine, 8.0×10^{-4} M.

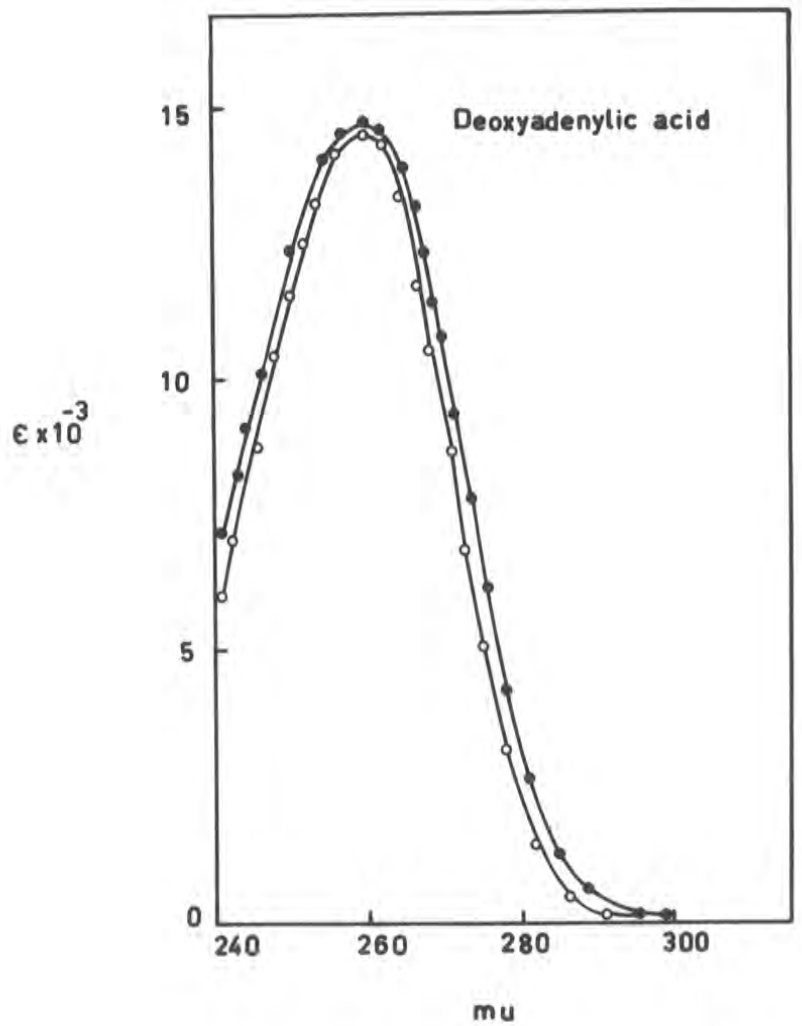

Fig. IV-5. Effect of cupric ions on the U-V. spectrum of deoxyadenylic acid, pH 4.30; ○ ○ deoxyadenylic acid 8.0×10^{-4} M; ● ● copper, 8.0×10^{-2} M and deoxyadenylic acid, 8.0×10^{-4} M.

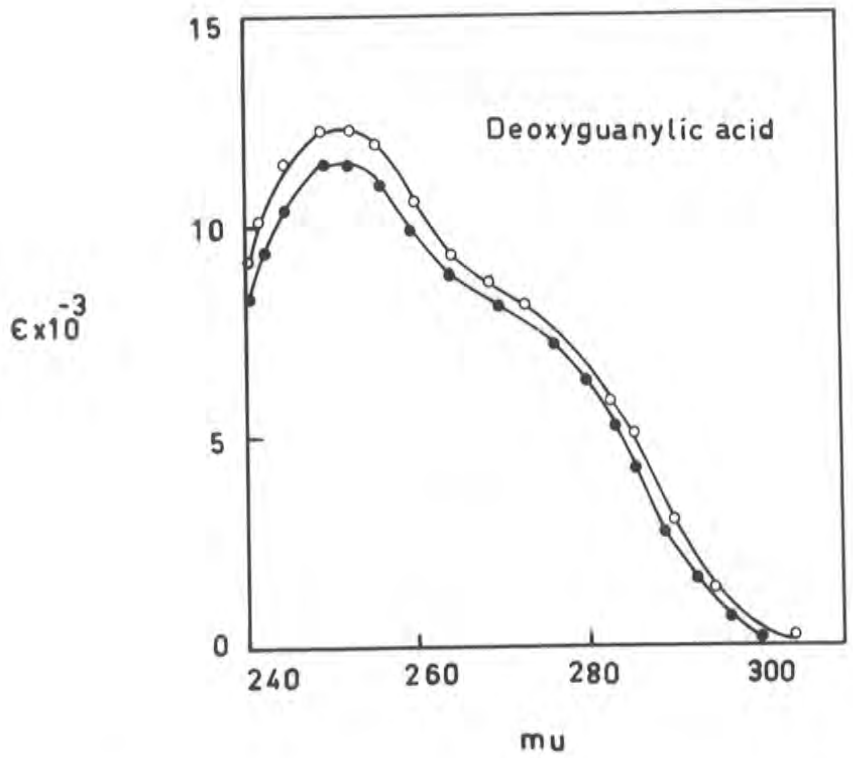

Fig. IV-6. Effect of cupric ions on the U- V spectrum of deoxyguanylic acid, pH 3.60; ● ● deoxyguanylic acid, 8.0×10^{-4} M; ○ ○ copper, 8.0×10^{-2} M and deoxyguanylic acid, 8.0×10^{-4} M.

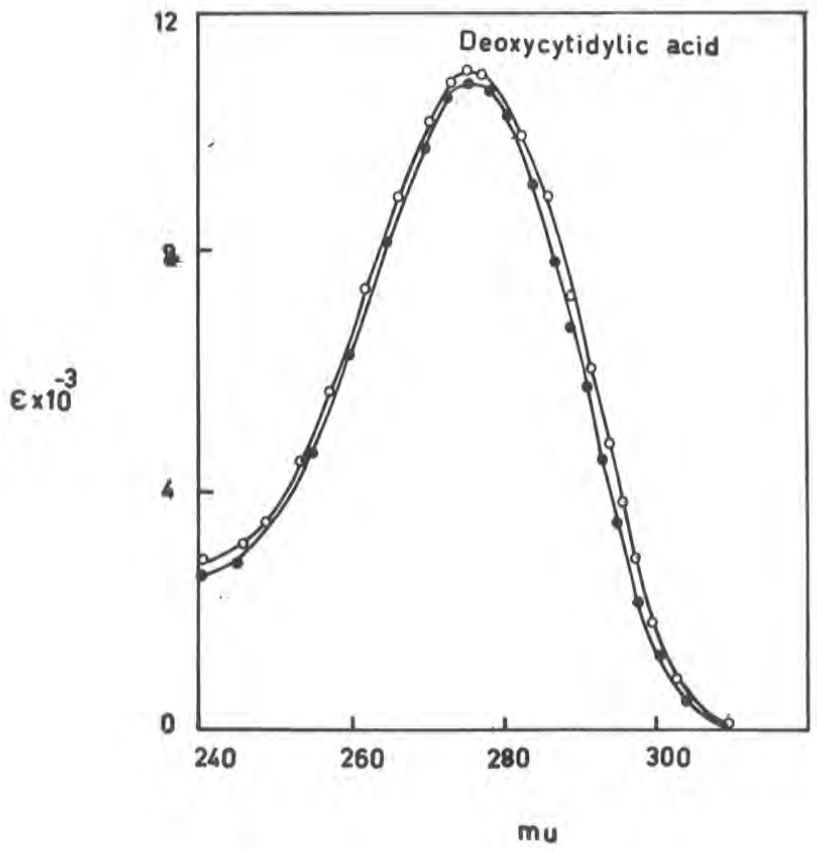

Fig. IV-7. Effect of cupric ions on the U-V. spectrum of deoxycytidylic acid, pH 4.50; ● ● deoxycytidylic acid, 8.0×10^{-4} M; ○ ○ copper, 8.0×10^{-2} M and deoxycytidylic acid, 8.0×10^{-4} M.

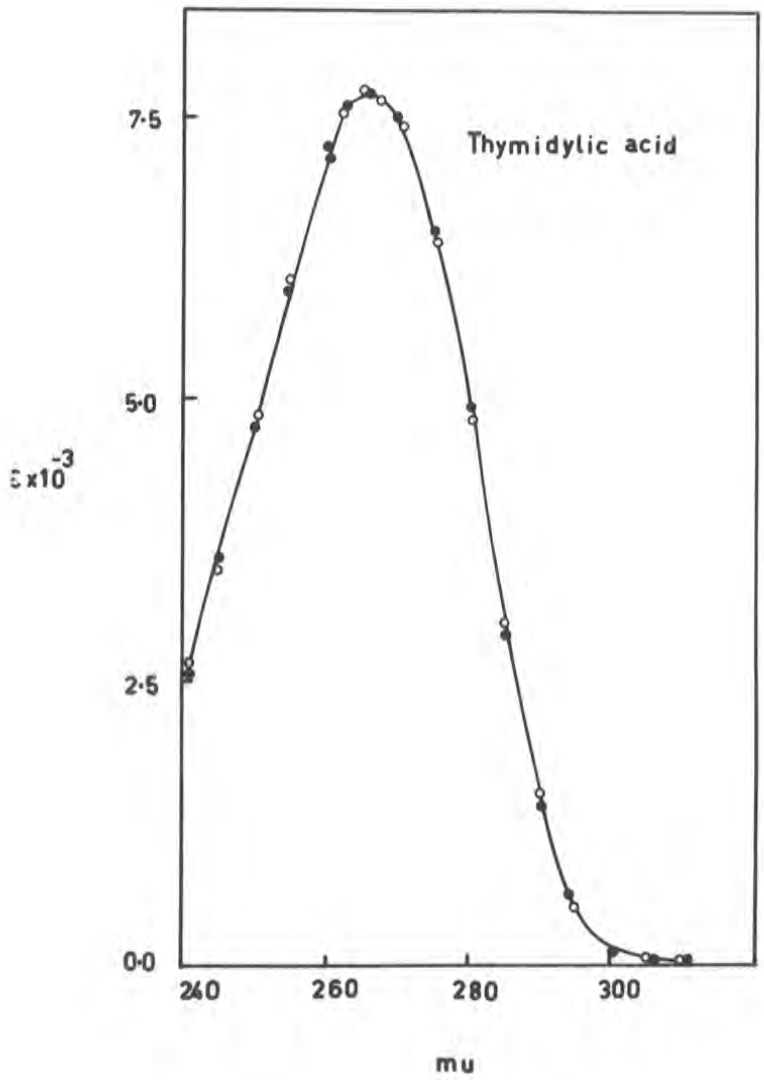

Fig. IV-8. Effect of cupric ions on the U-V. spectrum of thymidylic acid, pH 4.50; ○ ○ thymidylic acid 8.0×10^{-4} M; ● ● copper, 8.0×10^{-2} M and thymidylic acid 8.0×10^{-4} M.

deoxycytidylic and thymidylic acids obtained in the presence and absence of cupric ions.

(b) Visible absorption spectra

Visible absorption spectra of copper and guanosine in different molar ratios were obtained at pH 4.30 which are shown in Fig. 9. These curves give a maximum at 780 mμ which is 20 mμ less than the maximum of copper alone (800 mμ). No such shift in the maximum towards shorter wavelength was observed with adenosine (Fig. 10). Higher concentrations of guanosine and adenosine could not be used because of their low solubility.

The spectra of cytidine and copper as high as 4:1 in molar ratios and at pH 4.50 were recorded which gave the maximum at 760 mμ (Fig. 11). Thymidine did not give any such shift (Fig. 12).

Since there was no such solubility problem with deoxyguanylic, deoxyadenylic, deoxycytidylic and thymidylic acids, efforst were made to use them in higher concentrations as compared to the nucleosides. But it was observed that about 0.03 M solution of all of them except deoxycytidylic acid, gave precipitate in 1:1 molar ratio with copper. The spectra of deoxycytidylic

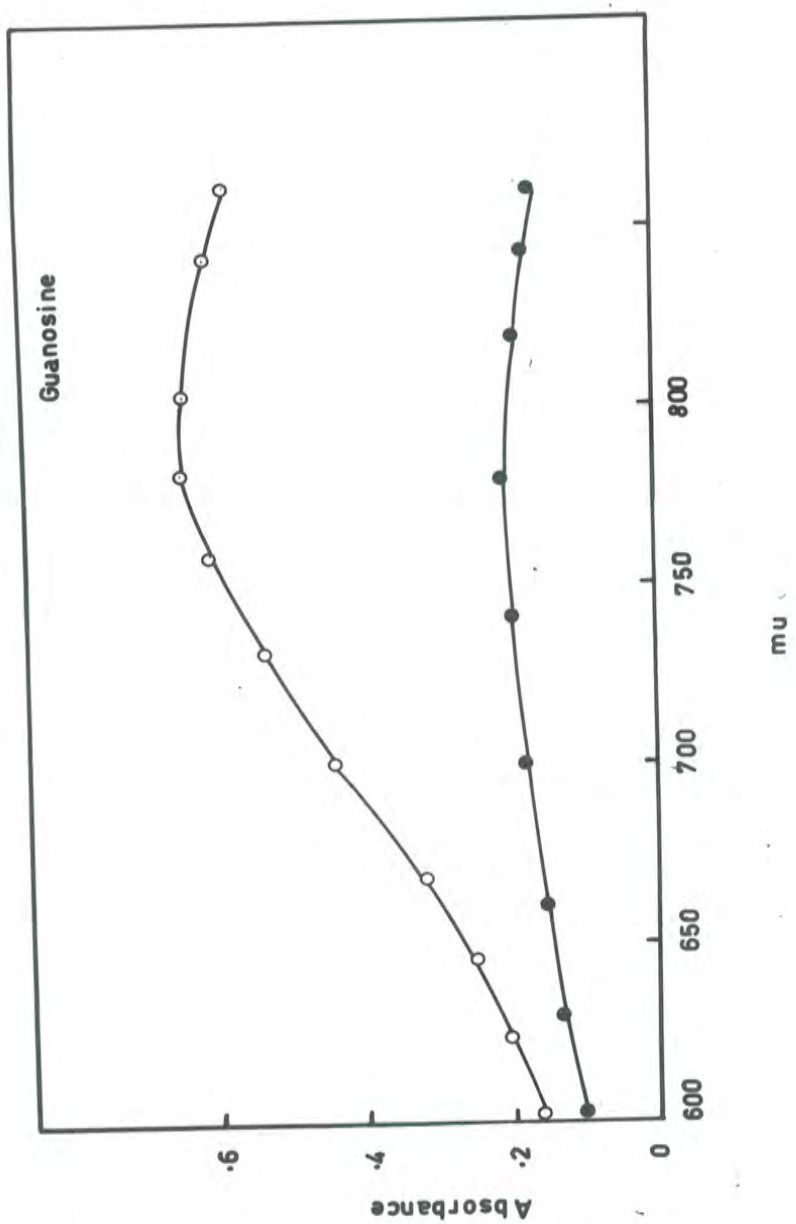

Fig. IV-9. Visible absorption spectra of copper and guanosine in ● ● 1:1 and ○ ○ 1:2 molar mixtures at pH 4,30.

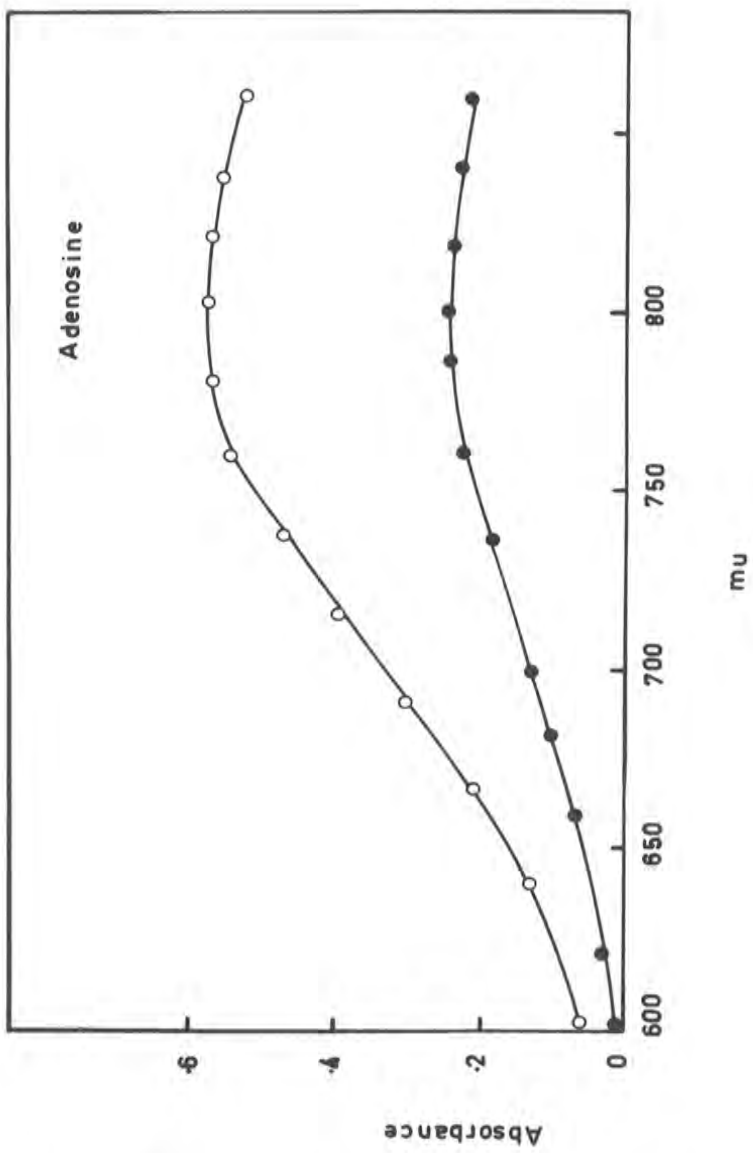

Fig. IV-10. Visible absorption spectra of copper and adenosine in ● ● 1:1 and ○ ○ 1:2 molar mixtures at pH 4.60.

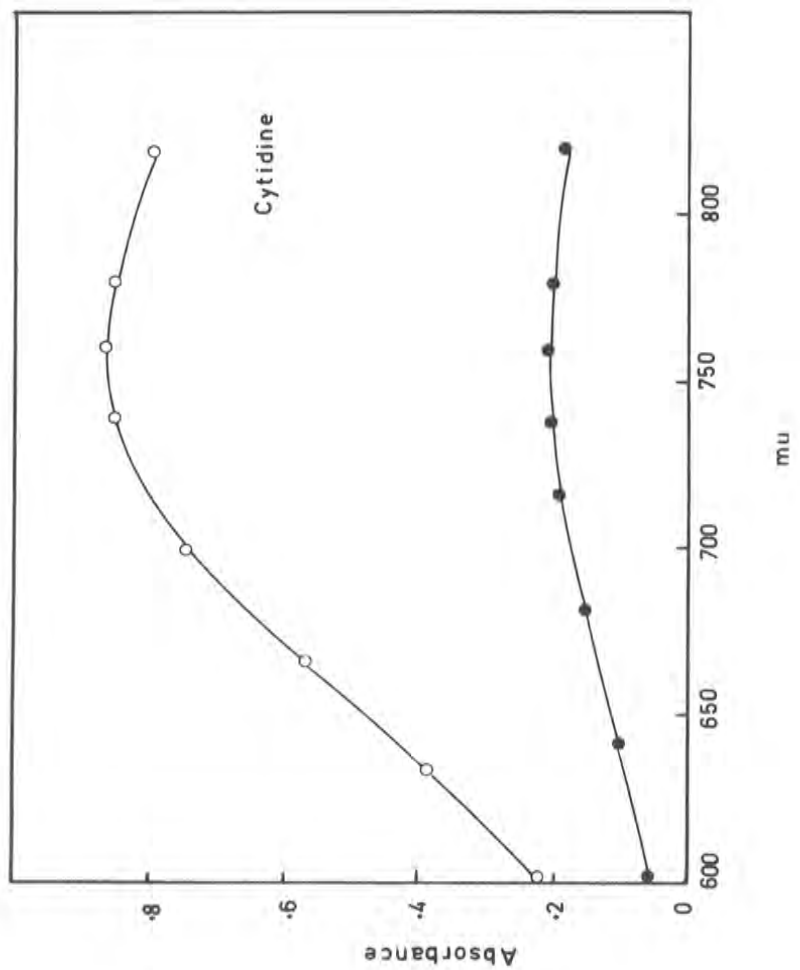

Fig. IV-11. Visible absorption spectra of copper and cytidine in ● ● 1:1 and ○ ○ 1:4 molar mixtures at pH 4.50.

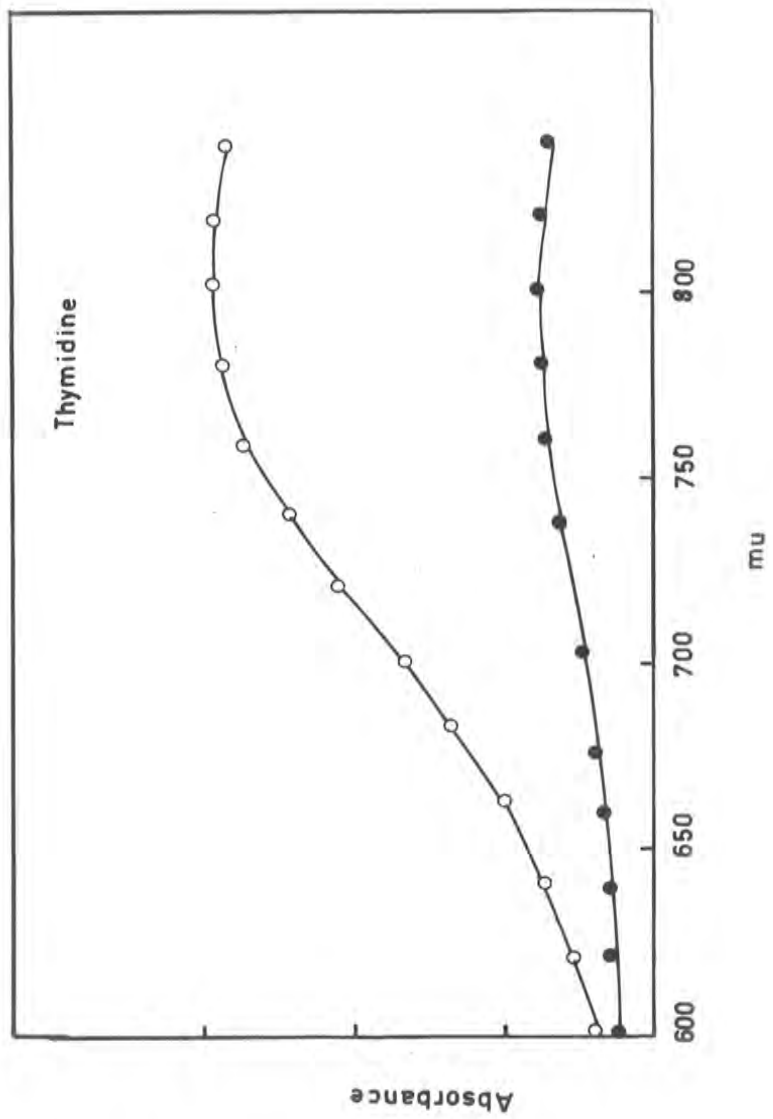

Fig. IV-12. Visible absorption spectra of copper and thymidine in ● ● 1:1 and ○ ○ 1:2 molar mixtures at pH 3.55.

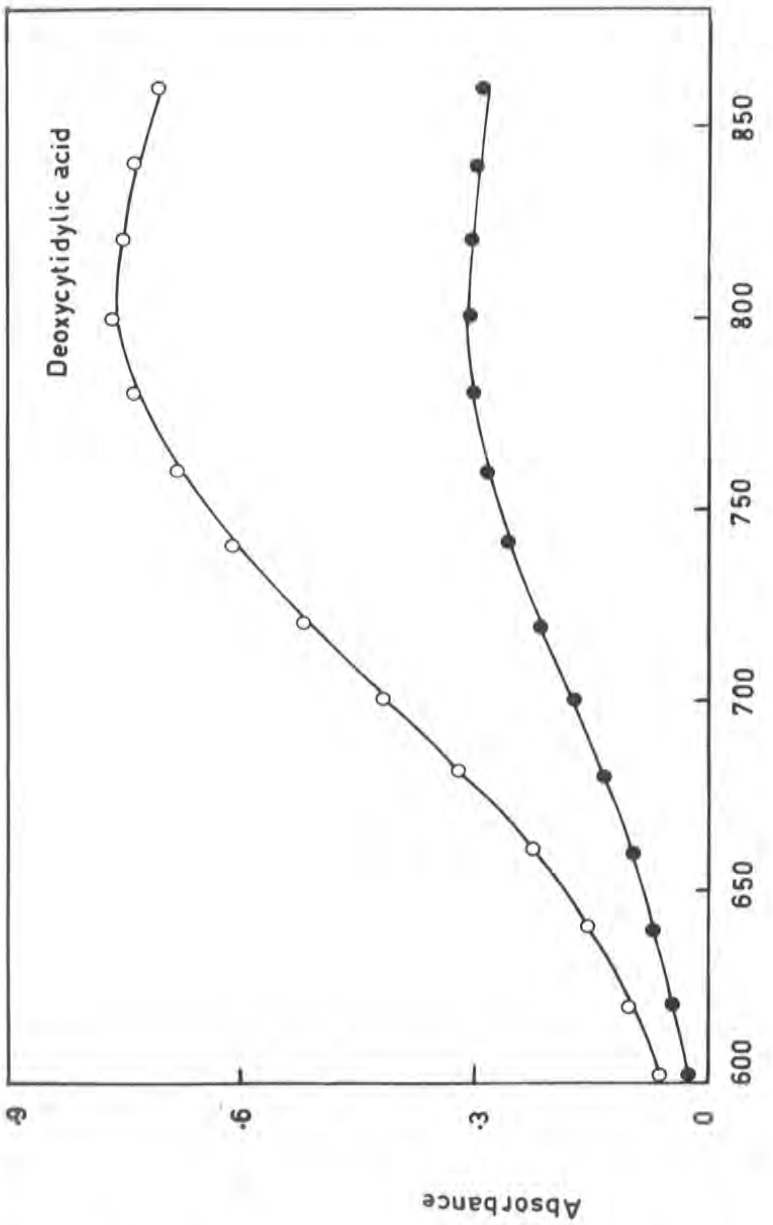

Fig. IV-13. Visible absorption spectra of copper and deoxycytidylic acid in ● ● 1:1 and ○ ○ 1:2 molar mixtures at pH 4.50.

in different molar ratios with copper were only recorded a few of them are shown in Fig. 13. From the figure it is evident that no change in the absorbance and shift in the maximum occurs in this case.

4. Conclusions

Since phosphate and sugar are common to all nucleosides and nucleotides, the specificity resides in the variation of purine and pyrimidine bases. In most cases nucleosides are preferred because of the phosphate moieties of the nucleotides which cause a slight modification of the physical properties such as U-V absorption and the dissociation constant. The little variation which has been observed in the U-V absorption of nucleotides with copper ions as compared to the nucleosides is primarily due to the charge effects carried by the phosphate groups.

There is observed a slight discrepancy in the results which have been obtained in the U-V and visible regions. In the U-V region the increase in the molar absorptivities at the maximum is in the order guanosine cytidine adenosine, whereas in the visible region the shift of the maximum towards shorter wavelength appears

to be more with cytidine (760 mμ) than with guanosine (780 mμ). Definite conclusions, however, cannot be drawn from these observations as the same molar ratio of cytidine to copper could not be used in case of guanosine because of its low solubility.

Binding of copper to the nucleosides is greatly dependent on pH and their relative concentrations as was noticed by Fiskin and Beer[7] who observed that at pH 3.0 about 90% guanosine was complexed with copper and that they bind in the order guanosine > cytidine > adenosine. At pH 4.0, the percentage of binding increases in all the cases while the order remains the same.

Under the limited conditions in the polarographic studies, qualitative approach has been made and from the shift in the half wave potentials towards negative direction and also from the depression in the diffusion currents it is concluded that with thymidine these changes are quite insignificant and they vary in the order guanosine > cytidine > adenosine.

It can be concluded that thymidine, the only nucleoside with no NH_2 group, fails to bind copper and since adenosine is the last in the binding order, the most effective nucleoside pair in binding copper is guanosine-cytidine.

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CHAPTER V

GENERAL DISCUSSION

1. Effect of copper on Tm
   - (a) Variation with copper concentration
   - (b) Variation with DNA composition
2. Measurements on overall binding parameters.
3. Direct evidence for the binding of copper to the bases of DNA.
4. Reversibility of denaturation
   - (a) U-V absorption and sedimentation velocity values
5. Direct evidence of complexing of copper to the nucleoside bases
   - (a) U-V absorption spectra
   - (b) Visible absorption spectra
   - (c) Polarographic studies
6. The way in which binding of copper to DNA is different to binding of copper to nucleosides.
7. Mechanism of reaction
8. Possible binding sites
9. References

The interaction of metal ions with DNA has been a subject of much importance specially regarding the controversy of the sites to which the metal ions are bound. There are many bivalent metal ions which bind to the bases as well as to the phosphates. Cupric ions, besides such type of bindings, have a specific effect in destabilising the DNA helix and produce other diverse effects which are discussed as below.

1. Effect of copper on Tm

(a) Variation with copper concentration

From the thermal denaturation of DNA from Calf Thymus and E. Coli in the presence of copper, as has been described in Chapter II, it is observed that in the presence of small amounts of copper, the Tm approaches the value obtained in the absence of copper. It is also noted that the lowering in Tm is dependent on the copper concentration. As is evident from Fig. 3, the Tm is lowered significantly with increasing R values both for Calf Thymus and E. Coli DNA up to a value of R = 1.6 after which the decrease in Tm with increasing value of R is less. This denaturation of DNA thus

appears to be a cooperative process.

(b) Variation with DNA composition

The presence of copper seems to modify the base pairing by association with them. It has been reported earlier by Marmur and Doty that there is a linear relationship between Tm and G-C content of DNA[1] and also DNA's with high G-C content have high Tm values. In the present study, it was found that the Tm of E. Coli DNA (73.5°C) was more than Calf Thymus DNA (63.5°C) because of its high G-C content. But in the presence of 0.1 mM. copper, both of them recorded the same Tm values of 45.0°C. This type of behaviour was also noted by Hiai[3] who found that in the presence of 0.1 mM. copper, six different DNA's recorded the Tm values of 45.0°C. Hiai[3] also observed that copper brings elevation in the Tm of dAT copolymer and also the hyperchromicity was reversible upon cooling. This suggests that the interaction of copper with A-T content of DNA is very weak or not at all. It can thus be concluded that the denaturation of DNA with copper is related directly to its G-C content. Lowering of Tm value is larger with increasing G-C composition. Earlier, Venner and Zimmer[4] also reported that in the

presence of 0.1 mM. copper, bacterial DNA's showed decreasing stability with increasing G-C content. But this observation is marked with the fact that the experiments were done at a much lower concentration of electrolyte (0.002 M) where G-C binding becomes more unstable.

From the observations as described above, it is concluded that the G-C bases of DNA are primarily involved in such a process.

2. Measurements on overall binding parameters

In the earlier publication, Eichhorn and Clark[5] reported a break at R = 1.5 in the plot of Tm against R and from this, they concluded that one copper is bound to phosphate and the rest to the nucleoside bases. No such break was observed in the present study and the behaviour was similar to the one which has been reported by Hiai[3]. Hiai[3] noted the maximum hyperchromicity at R = 0.8 at 55°C, and from this he proposed that one copper may be bound per 1-2 nucleotides. From these observations, it is difficult to formulate any such conclusions, as there is also the possibility that some of the copper may not be bound at all. To work

out the possible stoichiometry of the reaction, binding studies were done by the equilibrium dialysis method which is described in Chapter III. From these studies it is noted that,

(a) the binding increases with concentration and that there is only one type of binding site at $5^{\circ}C$, the same is true for DNA denatured in the absence of copper and then placed in an environment containing these ions. However, at $55^{\circ}C$, the number of binding sites per phosphate increases to 2.3 approximately.

(b) The binding constant at $55^{\circ}C$ is more than at $5^{\circ}C$. But the binding constant with denatured DNA is less as compared to native DNA.

Thus it can be suggested that one copper is bound per phosphate and the rest to the nucleoside bases in view of the increase in the type of the binding sites as noted above.

3. Direct evidence for the binding of copper to the bases of DNA

As has been described in Chapters II and III,

the U-V spectrum of DNA remains unchanged at the room temperature. But at the denaturation temperature, the bathochromic shift of 3 mμ in the U-V region is observed. Similar behaviour is also observed in the visible region where a hypsochromic shift of 60 mμ was noted. Also, formation of shoulders was observed in both the cases. These observations prove conclusively that at the temperature above Tm the bonding of copper to the nitrogen atoms of the bases does take place.

4. Reversibility of denaturation

The copper induced hyperchromicity was found to be irreversible upon cooling. It is suggested that the bonding of copper to the nitrogen atoms of the bases blocks the hydrogen bonding and also the longitudinal interaction among the bases with the result that the hyperchromicity is maintained upon cooling. All the U-V and visible spectra, as described above, behaved in the same fashion.

But this hyperchromicity disappeared completely at 25°C by increasing the concentration of KNO_3 to 0.1 M. The reversal of this hyperchromicity was not instantaneous and it was observed that it went to completion

in about 15 hours time. Renaturation can generally be achieved with bacterial DNA's by slow cooling but not with Calf Thymus DNA. The renaturation of Calf Thymus DNA after the denaturation in the presence of copper is specific. This suggests that the strands are not separated in the apparently denatured state and copper binds to the bases by the displacement of the hydrogen bonds. Thus the partial separation of the strands takes place on denaturation and reformation occurs on renaturation. The fact that the hyperchromicity is accompanied by the cleavage of most of the hydrogen bonds was also observed by Hiai[3].

(a) <u>U-V absorption and sedimentation velocity values</u>

That almost complete renaturation of the denatured solution takes place is evident by the return of the sedimentation velocity and absorption values as described in Chapter II. The slight deviation in the sedimentation velocity values can be well remarked by the fact that the complete renaturation of Calf Thymus DNA is difficult because its solution is not completely homogeneous, a property which has been pointed out by previous workers[6,7]. This reversibility of copper

denatured DNA can serve as a method of estimating the extent of denaturation of the DNA solution.

5. Direct evidence of complexing of copper to nucleoside bases

Since the U-V and visible spectra of DNA in the presence of cupric ions at higher temperatures, result from the electronic transition of the nucleoside bases, it is expected that individually these nucleoside bases should be complexed by cupric ions. This has been found to be so as was observed by spectrophotometric and polarographic methods which are described in Chapter IV.

The absence of any shift in the potentiometric titration of copper and the nucleosides as reported by Harkins and Frieser[8] and Eichhorn et al[9] shows that such complex formation proceeds without the removal of proton from the nucleosides. If this is so, the binding should not be affected by the variation in pH values, because such conclusion eliminates the competition of copper ions with protons in the reaction with the nucleosides. But Fiskin and Beer[10] made calculations of the stabilities of these complexes which was based

on the proton displacement in the acid solution.

(a) U-V absorption spectra

In case of the spectrum of DNA and copper, a bathochromic shift of 3 mμ was observed but no such shift was observed when the spectra of the nucleosides and copper were recorded individually and separately, although they were worked out in a reasonably high molar ratio. In all such cases the increase in the molar absorptivities at the maximum was observed which was good enough to show the complexing of these nucleosides with copper. No shift in the maxima was also observed with copper and nucleotides.

(b) Visible absorption spectra

Eichhorn et al[9] reported that at pH 5, the maximum of 1:2 mixture of copper and guanosine was at 800 mμ but the same solution, at pH 11, gave the maximum at 680 mμ. At such a high pH value, the situation is complicated because of the hydrolysis of cupric ions. However, in the present study, it was found that even 1:1 mixture of copper and guanosine at pH 4.30, recorded the maximum at 780 mμ. Such shift in the maxima was also observed with cytidine, but adenosine and thymidine did

not give shift in the maximum. This observation again gives direct evidence of complexing of copper with nucleosides and from the overall spectrophotometric observations, it is concluded that the complexing order is guanosine cytidine adenosine.

(c) Polarographic studies

The evidence for the complexing ability of nucleosides with copper was also shown by means of polarography which is described in Chapter IV. The shift of the half wave potential towards negative direction and the depression in the diffusion current of copper in the presence of nucleosides is a direct consequence of the complex formation between copper and the nucleosides. It is also shown that the adsorption of nucleosides on the mercury drop and other non specific factors do not have a significant role.

6. The way in which binding of copper to DNA is different to binding of copper to nucleosides

The few differences which were observed in case of binding of copper to DNA and nucleosides are

listed as below.

(a) The binding of copper to DNA is characterised by a bathochromic shift of 3 mμ in the U-V region but no such shift was observed in case of copper binding to nucleosides.

(b) DNA gives a distinct shoulder formation towards longer wave length in the region 265 to 280 mμ. In case of guanosine and cytidine the formation of shoulders exists in the same region, but not so distinct, whereas no such shoulder formation is observed with adenosine and thymidine.

7. Mechanism of reaction

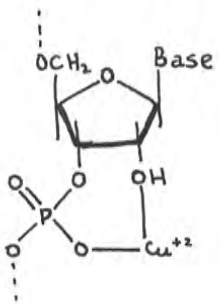
The mechanism by which copper significantly decreases the thermal stability of DNA is obscure. Eichhorn and Clark[5] have interpreted their data by assuming that copper is interposed between the complementary strands of DNA by coordination to the bases in such a manner that the hydrogen bonds are broken and the secondary structure of each strand destroyed. However, in view of the renaturation of the denatured DNA on increasing the concentration of the simple electrolyte,

it is assumed that the copper must somehow keep the bases in register even in the denatured state. This view is also supported by the fact that the bases in DNA helix only become available for interaction with copper at elevated temperature although the interaction can readily take place at lower temperatures after the helix has been disrupted. This fact has also been shown to be so by Hiai[3] and Eichhorn et al[9].

8. Possible binding sites

Basically there are only two possible types of sites where copper could interact with DNA and they are the phosphates and the bases. At room temperature, however, copper binds to only one site as is evident from the binding experiments and that could be due to the phosphate binding, which is easily accessible to these ions. This fact is also supported by the spectral measurements both in the U-V and visible regions where no change in the spectrum of DNA on the addition of copper was observed at room temperature. That the cupric ions bind to the phosphates is also supported by the experiments of Butzow and Eichhorn[11] who noticed the formation of small oligo-nucleotides by heating copper and

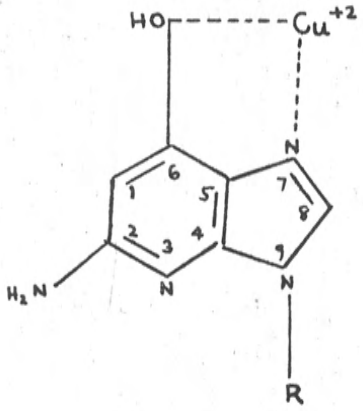
polyribonucleotides. This degradation took place by the cleavage of the phosphate bonds as is given by the following structure



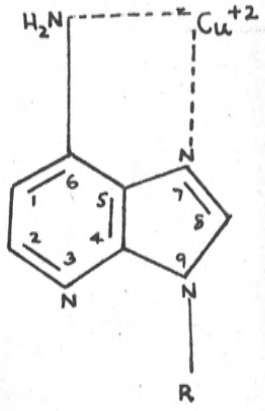
where the seven membered chelate ring is not a stable structure. Binding of copper to the phosphate groups was also confirmed by Eichhorn et al[9] who noted the broadening of the phosphate resonance in deoxyadenosine monophosphate and deoxythymidine monophosphate in the presence of copper. Katz[12], Shack et al[13] have earlier reported that bivalent metal ions combine with DNA in a maximum ratio of one metal atom per two phosphorus atom which was supported later by the findings of Wieberg and Neuman[14].

The situation is entirely different when solutions of DNA and copper are heated together. Instead of degradation, as described above, lowering in Tm values are observed. This is simply because the degradation requires the chelation of phosphate and 2'OH group and since the latter is not present in DNA, the degradation does not occur. Thus the obvious choice for copper is to interact with the nucleoside bases under such conditions.

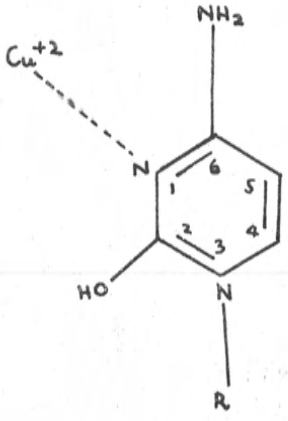
In the nucleosides, the sugar residues do not contribute towards the binding of copper which has been described in Chapter IV. The bases are, therefore, entirely responsible for such effects. The differences in the spectral behaviour of the nucleosides with and without copper is probably due to bonding of copper to the nitrogen atoms of the bases. Since thymine does not have any amino group attached to it, no such effects have been observed. Guanine and adenine supply the steric configuration suitable for metal chelation, which represents the most stable configuration[15] as is shown in Fig. 1. In guanine and adenine, the position N_7 is important for the formation of these chelates. This view is supplemented by the fact that guanine and



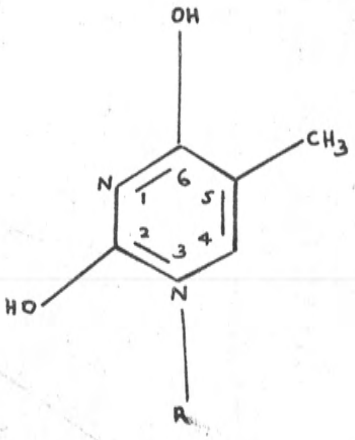
guanine



adenine



cytosine



thymine

Fig. V-1.

adenine when methylated at N_7, i.e. caffeine and theobromine, failed to form complexes with copper[16]. Apart from the experimental result that copper binds more to guanine than adenine, theoretically also the superiority of guanine over that of adenine resides in the fact that the basicity difference of guanine (-1.67) and adenine (-1.42) provides an advantage to the former to form stronger coordinate bonds than the latter[17]. Also more binding with the O atoms of guanine is favoured by the absence of steric complications involved with the presence of two protons on the $-NH_2$ group of adenine. Spectroscopic, polarographic, potentiometric and E.P.R. measurements have shown that cytosine binds copper ions rather better than adenine but less than guanine. The N.M.R. measurements of Eichhorn[9] indicate N_1 as the coordination site. None of the physical methods mentioned have revealed any evidence for the slightest binding of copper to thymine (Fig. 1). A theoretical explanation of this is not at present available.

It is thus concluded that copper binds to the phosphate sites only at the room temperature, but at higher temperatures when some relative motion of the two strands in the DNA helix is possible, such as occurs at the "annealing temperature"[18], penetration of the helix by the copper can occur which results in

binding of the copper to nitrogen atoms of the bases such as to bring about marked distortion or partial disruption of the helix to produce denaturation. Of the bases, the most effective base pair for copper binding is guanine - cytosine.

The state of DNA molecules in the "Denatured form" produced by heating in the presence of copper is characterised thus:

the copper ions

(1) cause collapse of the DNA double helix

(2) bind to many phosphate groups and also to many bases

(3) prevent hypochromicity

(4) hold the bases in register in such a way that increase in ionic strength results in the complete reversal of hyperchromicity even in the case of Calf Thymus DNA. This reversibility of the hyperchromicity is similar to some extent to the observation made by Colvill and Jordan[19] when they found that with Calf Thymus DNA, denaturation by dilution was apparently reversible, although there was a marked difference between the behaviour of the

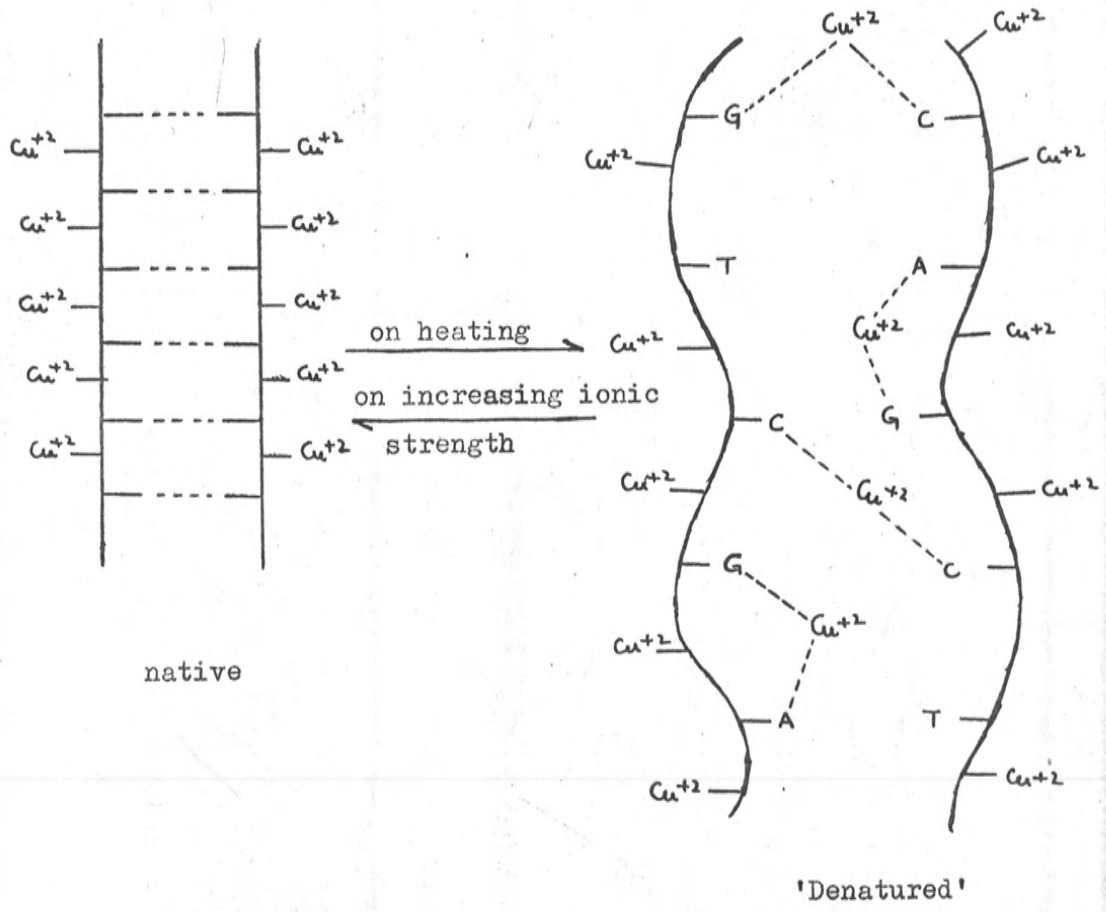


Fig. V-2.

material prepared by slowly or rapidly increasing the ionic strength. But this did not occur in case of heat denaturation of Calf Thymus DNA as reported earlier by Doty et al[20].

A suitable model for copper containing denatured DNA should account for all these features. It is known that native DNA has a high density of negative charge on the outside and has the non polar bases on the inside of the double helix, which is held together by hydrogen bonds and other secondary forces. In this native DNA, copper ions bind to the phosphate groups at room temperature as is shown in Fig. 2. But on heating partial separation of the strands takes place and the copper ions are interposed between the complementary strands or possibly cross bridged as is shown in Fig. 2. This occurs in such a manner that all of the hydrogen bonds are broken. As is shown in the figure, the only base which is not involved in such a process is thymine.

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CHAPTER VI

MATERIALS AND METHODS

1. Preparation of samples
2. Thermal denaturation
3. U-V absorption spectra
4. Visible absorption spectra
5. Kinetics of denaturation
6. Sedimentation coefficients
7. Equilibrium dialysis
8. Polarography
    - (a) apparatus
    - (b) capillary
    - (c) maximum suppressor
    - (d) agar plug
    - (e) removal of oxygen
    - (f) measurement of diffusion current
9. References

1. Preparation of samples

Calf Thymus DNA was prepared by the method of Kay, Simmons and Dounce[1] whereas E. Coli was prepared by the method as described by Marmur[2]. The solutions were always made in 0.01 M potassium nitrate. Stock solutions of approximately 0.15% by weight of these DNA samples were prepared which were then diluted with 0.01 M solution of potassium nitrate.

$Cu(NO_3)_2, 3H_2O$ (Analytical Reagent, Hopkin and Williams Ltd., England) was recrystallised twice from water and dried in vacuo to a constant weight. Solution of cupric nitrate was always made by weight measurement in 0.01 M solution of potassium nitrate.

Commercial samples of the nucleosides and nucleotides were obtained from Schwarz Bioresearch Inc., New York. Solutions of these were also prepared by weight measurement (in 0.01 M solution of potassium nitrate) and the concentrations were checked by optical density measurements.

2. Thermal denaturation

The melting curves were determined by keeping

the solution of DNA and copper in 1 cm. silica cell with 0.01 M KNO_3 solution in a matched silica cell as reference. A layer of paraffin on the top of the solution in the silica cells prevented any loss due to evaporation. These cells were placed in an electrically heated and thermostatically controlled cell holder. For the solutions to achieve equilibrium after each increase in temperature by a definite amount, time interval of 5-6 minutes was always maintained during which period constancy in the optical density readings was obtained. All optical density measurements were made using a Unicam SP-500 spectrophotometer and were corrected for increase in volume with temperature.

3. U-V absorption spectra

The U-V absorption spectra, as described in Chapters II and IV, were determined using Gilford model 2000 Absorbance recorder set up with Beckman DU monochromator. 2 mm. and 10 mm. silica cells were commonly used. All measurements were made at 25°C and the temperature was maintained constant by regular circulation of water through an annular space in the cell compartment.

4. Visible absorption spectra

All the visible absorption spectra were obtained with a Shimadzu spectrophotometer QR 50 using 10 cm. cells, with a matched cell of 10 cm. containing 10^{-2} M solution of potassium nitrate except in the case of DNA (Chapter III) where identical concentration of DNA solution was used.

5. Kinetics of denaturation

DNA solution of known volume (0.01 - 0.03 mls.) was placed in the cell and conc. copper solution in a small teflon plunger which was fitted into the cell. At zero time, the plunger was used to mix copper solution with DNA. The absorbance values were then recorded at definite intervals of time. The same operation was repeated at all the temperatures. It is observed that the absorbance begins to form plateau after about 3 minutes time. The heating time of 4 minutes for other experiments was therefore selected which was sufficient to complete denaturation.

6. Sedimentation Coefficients

A spinco model E Ultracentrifuge equipped with ultraviolet optics was used to measure the sedimentation coefficients. The measurements were made of the solutions of copper and DNA (heated and cooled), at 44, 770 r.p.m. for 25.0°, 38.6°C; at 35, 600 r.p.m. for 41.0°C and at 24, 630 r.p.m. for 45.4°, 48.0° and 50.6°C. But in all those cases where KNO_3 (0.1 M) was added to the above heated solutions, speed of 44, 770 r.p.m. was used. This measurement was made using a 12 mm. aluminium centrepiece cell in a D rotor, thermostated at 20.0 ± 0.1°C. All the solutions were made up in 0.01 M potassium nitrate and the photographs were taken at intervals of 4 minutes with an exposure time of 30 seconds. These photographs were then converted to plots of concentration versus distance with a Spinco Analytrol photodensitometer attached with a microanalyser equipment. The position in the cell corresponding to the 50% concentration is obtained from the tracing done by the photodensitometer, and which is used for the calculation of the sedimentation coefficient values.

7. Equilibrium dialysis

The 18/32 Visking tubings which were used for

the dialysis purpose were first heated at 80.0°C approximately with a 50% solution of sodium bicarbonate for about 40 minutes. These tubes were then washed several times with twice distilled water and stored in a cold place at 4°C.

5 ml. solutions of DNA and copper in 10^{-2} M KNO_3 of varying copper concentrations were taken inside the dialysis bags, which were then placed in a pyrex tube with 15 ml. of identical copper concentrations in 10^{-2} M KNO_3. These tubes were fitted with stoppers. Special care was taken in selecting the size of the tubes, the dialysis bag and the amount of the solution in the tubes, so that all the dialysis bags were covered with solution and also the movement of the solution was free. These tubes were then placed in holders and shaken gently for 72 hours at 5°C.

For the dialysis at 55°C, the tubes with solutions as above, were placed in the water bath thermostated at 55°C and the equilibration was done for 48 hours.

8. Polarography

(a) Apparatus

The instrument used for this work was constructed in this laboratory and is the type generally referred to as a "manual instrument". It was designed on the basis of the manual instrument described by Vogel[3] with some modifications which is shown in Fig. 1.

The instrument consists mainly of three parts

(i) The cell and the dropping mercury electrode (d.m.e.)

(ii) The current measuring circuit

(iii) The potential circuit.

(i) The cell used was of the type shown in Fig. 1. The bottom of this cell was filled with mercury till the beginning of the side arm which is used for bubbling nitrogen gas through the solution. Near the top of the cell, there is another inlet for passing nitrogen over the surface of the solution at the time of recording polarograms. Electrical connection through the pool of the mercury was made by inserting platinum wire inside it. The e.m.f. of saturated calomel electrode (S.C.E.) used was checked against standard normal calomel half cells.

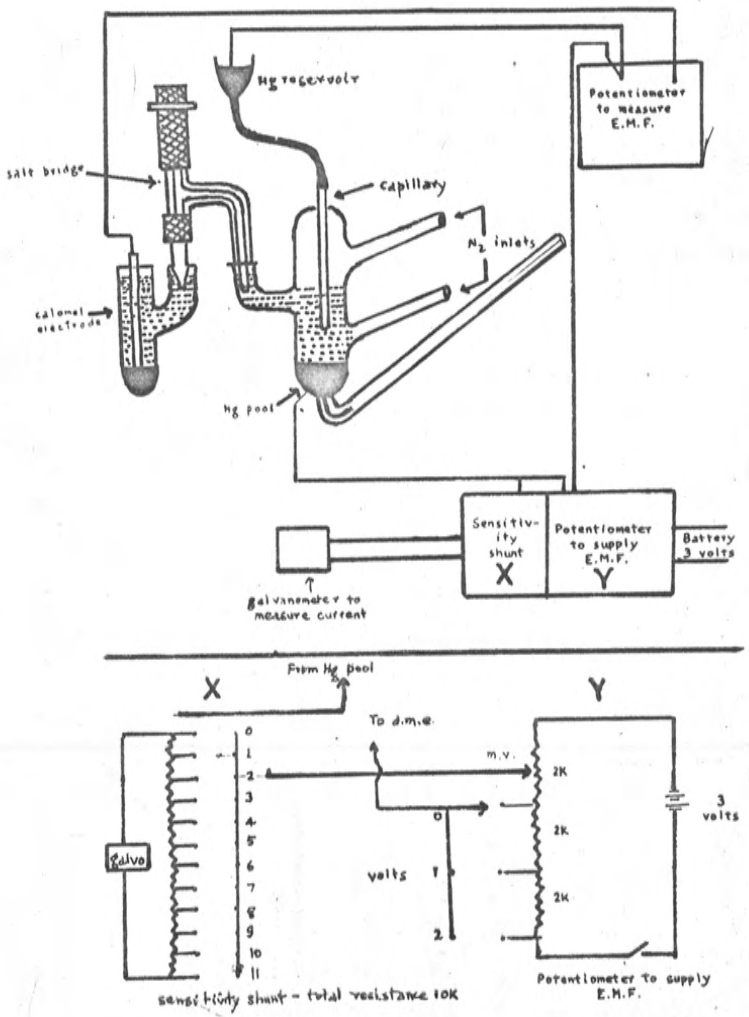

Fig. VI-1. Schematic of Polarograph.

switch position	μA per 10 cm. deflection
0	out of circuit
1	63.6
2	47.7
3	31.8
4	15.9
5	6.4
6	3.2
7	1.6
8	0.64
9	0.32
10	0.13
11	0.065

It has generally been observed that the liquid junction produces a considerable error in the determination of e.m.f. Care was taken, therefore, to use a more reproducible type of junction as is shown in the figure. The agar plug is held by a piece of rubber tubing and is renewed after each polarogram. The other end of the tube is connected through a ground joint to a capillary which dips inside the solution of the cell. In order to make the connection, the solution is sucked through the tube and then clipped. This type of junction has been found to give a reproducibility of at least 0.4 mV[4] and a constant potential over a period of several hours[5].

(ii) The current measuring circuit consists of a sensitive galvanometer (Cambridge D'arsonval, 41148/2) with an internal resistance of 450 ohms. A suitable shunt in the circuit makes it possible to alter the sensitivity and was so adjusted that it gave 0.64 μA per 10 cm. deflection.

(iii) The e.m.f. circuit consists of a source of e.m.f. from a 6 volt accumulator. A voltage of 3 volts is placed across the resistance and by the means of

helipot potentiometer, small increments of 10-50 mV from 0-1.8 volts can be supplied to the cell, taking into the fact that the e.m.f. of S.C.E. is zero. Thus all measurements refer to the S.C.E. These e.m.f. values were then measured by a portable D.C. potentiometer Croydon type P.3.

(b) Capillary

The capillary used in these measurements has an internal diameter of 0.05 mm. with the following characteristics

$$m = 1.523 \text{ mg. sec.}^{-1},$$

$$t = 4.12 \text{ sec./drop}$$

measured at -1.0 volt against S.C.E.

(c) Maximum suppressor

Non-ionic detergent, Lissapol N450, of about 0.002% in the solution was used as a maximum suppressor.

(d) Agar plug

Small capillary tubes (as shown in Fig. 1) were filled with the hot solution of agar gel which was

prepared by dissolving 2 gm. of agar (B.D.H. London) in 45 ml. of water in a beaker kept in a large boiling water bath and then 15 gm. of KCl (A.R. quality B.D.H.) was added and stirred properly. These tubes were cooled and then used for making connection to the solution.

(e) Removal of Oxygen

Because of the fact that oxygen is very readily reduced at the dropping mercury electrode, it was necessary to remove dissolved oxygen from the solution. It was achieved by passing oxygen free nitrogen gas through a pair of bottles containing vanadous chloride. Vanadous chloride was prepared by boiling 2.0 gm. of ammonium metavanadate with 25.0 ml. of concentrated HCl, diluting it to 200 ml. and then shaking with a few grains of heavily amalgamated zinc. To this some more concentrated HCl was added to secure a clear violet solution. The gas emerging from these two bottles was passed through a third similar bottle containing doubly distilled water to remove any acid or impurities. The gas, after passing through these bottles, was allowed to pass through the cell from the

top and bottom of the solution for about 15 minutes. The inlet at the bottom of the cell was closed and nitrogen was allowed to pass over the top of the solution while recording the polarograms.

(f) Measurement of the diffusion current

The average oscillation of the galvanometer was used for measuring the diffusion current instead of using maximum or minimum throw. This was found to be essential because the oscillations which are due to the periodic growth and fall of the mercury drops, depends entirely on the ratio of the period of the galvanometer to the drop time. Generally the type of galvanometer which is used does not respond rapidly as to follow the true change in the current during the life of each mercury drop and therefore the maximum or minimum throw does not correspond to the true maximum or minimum current. And when the oscillations are not too large, their average always refers to the true average current to which Ilkovic equation refers.

There is also observed a small residual current produced from traces of impurities in the solution or due to the supporting electrolyte. This

is observed before the decomposition potential is reached and thus to know the value of the true diffusion current of the solution, it is necessary to make correction due to the residual current.

The polarograms of all the solutions, in the present study, show only a single wave, the evaluation of the true diffusion current was, therefore, made by determining the residual current in a separate experiment by obtaining the polarogram of the supporting electrolyte i.e. 10^{-2} M KNO_3 alone. The value so obtained was subtracted from the total diffusion current of the solution.

After making this correction, the diffusion current was found to be practically constant in all the cases.

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APPENDIX

Publication

Binding of Cu(II) ions to DNA

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THE BINDING OF COPPER(II) IONS TO DNA

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Eichhorn (1962), Eichhorn and Clark (1965) and Hiai (1965) have shown that Cu(II) ions have a specific effect in decreasing the stability of the DNA helix to thermal denaturation. The decrease of T_m with increase of Cu^{++} concentration at constant ionic strength is markedly different from the effect observed with other ions and the denaturation at relatively low temperatures induced by the presence of Cu(II) ions has been shown by Eichhorn and Clark (1965) and by Hiai (1965) to be reversible, addition of electrolyte (KCl, KNO_3) to produce a high ionic strength renaturing the denatured DNA. The assumption that the loss of hypochromicity at relatively low temperatures in the presence of Cu(II) ions is consequential on a denaturation process is supported by the evidence [Hiai (1965)] that under the same conditions there is observed a marked decrease in viscosity, a loss of biological activity and a decrease in molecular asymmetry.

The mechanism whereby Cu(II) ions specifically decrease the thermal stability of the DNA helix is obscure. Eichhorn and Clark (1965) interpret their data by assuming that the Cu(II) ions are interposed between the complementary strands of DNA by co-ordination to the bases in such a manner that the hydrogen bonds are broken and the secondary structure of each strand destroyed. However, in view of the ready renaturation

of the denatured DNA on increasing the concentration of simple electrolyte, it is assumed by Eichhorn and Clark that the Cu(II) ions must somehow keep the bases in register even in the denatured state. This view is also held by Hiai (1965) who further considers that the bases in the DNA helix only become available for interaction with Cu(II) ions at elevated temperatures although the interaction can readily take place at lower temperatures after the helix has been disrupted. No direct evidence for the interaction of Cu(II) ions and the base-pairs of DNA was given by either of these groups of workers.

Our experiments on the thermal stability of DNA in the presence of Cu(II) ions in general confirm the experimental results of Eichhorn and Clark (1965) and of Hiai (1965). However, we wish to present direct evidence that there is interaction between Cu(II) ions and nitrogen atoms of the DNA bases after denaturation in the presence of Cu(II) ions at elevated temperatures, but not at room temperature. Cu(II) ions in the presence of non-complexing anions (e.g. NO_3^-) exhibit an absorption spectrum in the region 600 to 900 mμ with a

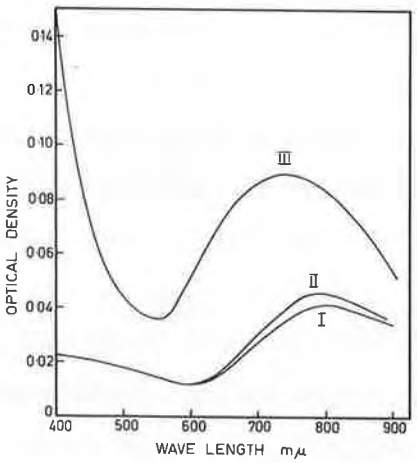

Fig. 1. Absorption spectra of Cu(II) ions in the presence of DNA. Concentrations: Cu^{++} (as nitrate), 4×10^{-4} M; DNA, 7.2×10^{-4} M; KNO_3, 2×10^{-3} M. I, Cu^{++} at 25°C; II, Cu^{++} and DNA at 25°C; III, Cu^{++} and DNA after heating at 55°C, measured at 25°C.

maximum at 800 mμ (curve I, Fig. 1) which is characteristic of the $[Cu(H_2O)_6]^{++}$ ion. This spectrum is little changed on the addition of DNA at 25°C (curve II, Fig. 1). After heating DNA at 55°C in the presence of Cu(II) ions for as little as one minute, a procedure sufficient to produce denaturation, there is a marked increase in absorption and a shift of the maximum to 740 mμ (curve III, Fig. 1). This increased absorption and shift of the maximum to shorter wavelengths is similar to the effect observed on co-ordination of Cu(II) with ammonia and ethylene diamine, the stronger ligand field due to the nitrogen derivative causing the shift and the increase in intensity of the absorption band. The maximum for the ion $[Cu(NH_3)(H_2O)_5]^{++}$ occurs at 740 mμ and for $[Cu(NH_3)_2(H_2O)_4]^{++}$ at 680 mμ. These spectral data thus afford direct evidence for the bonding of the Cu(II) ions to the nitrogen atoms in the heterocyclic bases after denaturation.

The U.V. spectrum of DNA, as is well known, shows a maximum at 258 mμ. The position of this maximum remains unchanged by the addition of Cu(II) ions at 25°C but on increasing the temperature there is a small shift in the maximum towards longer wavelengths and an increase in absorption due to the decrease in hypochromicity as shown in Table 1. Accompanying the shift in the position of the absorption

Table 1. Shift of U.V. absorption maximum of calf thymus DNA in presence of Cu(II) ions after heating at various temperatures.
(Concentrations: DNA, 2.99×10^{-4} M; Cu^{++}, 0.95×10^{-4} M)

Temperature °C	Position of maximum absorbance mμ	$O.D._{max.}\ T/O.D._{max.\ 25}$
25	258	1.00
35	259	1.04
45	260	1.45
55	261	1.55

maximum is a change in the absorption in the region 260 to 520 mμ towards longer wavelengths (this may be seen in Fig. 1 in the region 400 to 520 mμ) and which may best be described as the formation of a shoulder in the absorption curve in the region 265 to 280 mμ. The appearance of this shoulder may be attributed to the perturbation of the allowed energy levels of the bases by the co-ordinating Cu(II) ions. This behaviour is similar to that observed by Yamane and Davidson (1961) for the spectral shift associated with the interaction of DNA with mercury(II) ions, which was also interpreted as an interaction between the Hg^{++} ions and the ring nitrogen atoms of the heterocyclic bases.

It is noteworthy that no bonding of Cu(II) ions to heterocyclic nitrogen atoms occurs until the thermal denaturation process occurs. At 25°C, determination of the extent of binding of Cu(II) ions to DNA shows that binding increases with concentration and that there is only one type of binding site, the same is true for DNA denatured in the absence of Cu(II) ions and subsequently placed in an environment containing these ions; however, at 55°C there is a marked increase in the absorption compared with the values at 25°C and the number of binding sites is greater (Coates, Jordan and Srivastava, to be published). It is thus concluded that the Cu(II) ions at room temperature bind to the phosphate sites only, but at higher temperatures when some relative motion of the two strands in the DNA helix is possible, such as occurs at the "annealing temperature" [Marmur, Rownd and Schildkraut (1963)], penetration of the helix by the Cu(II) ions can occur which results in binding of the Cu(II) ions to nitrogen atoms of the bases such as to bring about marked distortion or partial disruption of the helix to produce denaturation.

EXPERIMENTAL

Calf thymus DNA was prepared by the method of Kay, Simmons and

Dounce (1952) and E. coli DNA by the method described by Marmur (1961). U.V. spectra were obtained using a Gilford model 2000 absorbance recorder used in association with a Beckman DU monochromator and visible spectra were determined with a Shimadzu spectrophotometer model QR.50 using 10 cm. cells.

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