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THE KINETICS OF THE RENATURATION OF DEOXYRIBONUCLEIC ACID DENATURED IN THE PRESENCE OF COPPER(II) IONS

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CONTENTS

I INTRODUCTION 1

II THE INTERACTION OF COPPER(II) IONS WITH DNA

1. Introduction 17

2. Interaction of Cu^{++} ions with native DNA 17

   a. Effect of Cu^{++} on the UV spectrum of native DNA 19

   b. Other techniques used to determine the site of Cu^{++} interaction with native DNA 27

   c. Proposed sites 30

3. The formation of the denatured DNA-Cu^{++} complex 33

   a. The effect of Cu^{++} concentration 34

   b. The mechanism of formation of the denatured DNA-Cu^{++} complex 37

4. The structure of the denatured DNA-Cu^{++} complex 39

   a. The site of interaction: Mononucleotides 40

   b. The site of interaction: Polynucleotides 45

   c. The site of interaction: DNA 49

   d. The conformation of the strands 50

5. The renaturation of the denatured DNA-Cu^{++} complex 53

   a. Comparison with renaturation of DNA in the absence of Cu^{++} 55

   b. Hypotheses to explain the renaturability 57

References 60

III THE RENATURATION OF THE DENATURED DNA-Cu^{++} COMPLEX BY INCREASING THE IONIC STRENGTH: SPECTROPHOTOMETRIC RESULTS

1. Introduction 65

2. Experimental procedure 65

   a. Solutions 65

   b. Denaturation 66

   c. Renaturation 66

3. Renaturation in 0.15 M KNO_3 at 25°C 67

   a. Effect of slow cooling the denatured solution 68

   b. Kinetic analysis 68

   c. Accuracy and reproducibility 69

   d. Effect of DNA and Cu^{++} concentrations 76

4. Renaturation in 0.10 M KNO_3 at 25°C 76

5. Effect of the time of standing on the denatured DNA-Cu^{++} solution 77

   a. Viscosity measurements on the denatured DNA-Cu^{++} solution 78

6. Effect of temperature on the renaturation rate 79

   a. 35°C 79

   b. 15°C 80

   c. 5°C 80

   d. Arrhenius plot 82

7. Effect of solution viscosity on the renaturation rate 83

   a. Calculation of viscosity of sucrose solutions 84

   b. Preparation of solutions 84

   c. Results 85

References 86

IV THE RENATURATION OF THE DENATURED DNA-Cu^{++} COMPLEX BY INCREASING THE IONIC STRENGTH: CHANGE IN FREE Cu^{++} ION CONCENTRATION

1. Introduction 87
2. The specific cupric ion activity electrode 88
   a. The Cu^{++} ion activity 89
   b. Calibration of the Cu^{++} ion electrode 90
3. Experimental procedure 92
   a. Measurement of potential 93
   b. Denaturation and renaturation 94
4. The renaturation as followed by the Cu^{++} ion electrode 95
   a. Relative positions of the renaturation curves 96
   b. Reproducibility 97
   c. The spectrophotometrically observed reaction under the same conditions 98
   d. Calculation of the total EMF change on renaturation 99
   e. Comparison between the renaturation data from the Cu^{++} ion electrode and the spectrophotometer 103
5. The renaturation in high viscosity solution as followed by the Cu^{++} ion electrode 104

References 106

V THE RENATURATION OF THE DENATURED DNA-Cu^{++} COMPLEX BY ETHYLENE DIAMINE

1. Introduction 107
2. Experimental procedure 109

3. Renaturation at an ethylene diamine/Cu^{++} ratio of 1:1 — 110

a. Kinetic analysis — 111

b. First order rate constants — 112

c. Correction for the absorbance of the Cu^{++}-EDA complex — 113

4. Effect of the EDA/Cu^{++} ratio on the renaturation rate — 114

5. Reactions followed by the stopped-flow rapid reaction apparatus — 116

a. Results — 116

b. Analysis of the data — 118

6. Effect of solution viscosity on the renaturation rate — 122

Reference — 123

VI GENERAL DISCUSSION AND CONCLUSIONS — 124

1. Reaction scheme for the renaturation brought about by increasing the ionic strength — 124

2. Reaction scheme for the renaturation brought about by ethylene diamine — 129

3. The rate-determining step — 130

4. The viscosity dependence — 135

5. The time dependence — 136

6. The renaturation brought about by ethylene diamine — 138

VII MATERIALS AND METHODS

1. Cleaning of apparatus 142

2. Preparation of solutions 142

a. DNA solutions 142

b. $Cu(NO_3)_2$ solutions 143

c. KNO_3 solutions 144

d. Other solutions 144

3. Spectrophotometer 144

a. Description 144

b. Calibration 146

c. Use of the instrument for renaturation reactions 148

4. Procedure for renaturation by increasing the ionic strength 149

a. Denaturation 150

b. Renaturation 151

c. Experiments at 5°C and 15°C 151

5. The Cu^{++} ion electrode measurements 152

6. The stopped-flow rapid reaction apparatus 153

a. Optical and recording systems 153

b. Operating procedure 154

References 155

SUMMARY

The transition between the double helix and the random coil configuration of deoxyribonucleic acid (DNA) is of great biological significance and in spite of considerable study over recent years, the physical chemistry of this transition is not well understood. The helix to coil transition, termed denaturation and its reverse process, renaturation, can be observed in aqueous solutions of DNA under a number of experimental conditions, but in this study, the particular case of the presence of Cu(II) ions was employed. It has been previously shown that Cu^{++} ions cause the helix-coil transition of DNA to occur at a much lower temperature than in their absence. DNA denatured under these conditions can be renatured on dissociation of the Cu^{++}-DNA complex by increasing the ionic strength of the solution or by adding a Cu^{++}-complexing agent. This renaturation reaction, proceeding rapidly to completion under conditions unfavourable for the renaturation observed in the absence of Cu^{++} ions, appeared likely to give valuable information concerning the helix-coil transition of DNA.

The rate of the renaturation brought about by increasing the ionic strength and followed by the change in UV absorption was found to depend on the ionic strength, the Cu^{++} concentration, the temperature, and on the time elapsing between denaturation and the commencement of renaturation. The data could be fitted to first order kinetics for a considerable extent of the reaction. It was found that the rate constant decreased markedly as the bulk viscosity of the solution was increased.

The renaturation was also followed by measuring the rate of increase in free Cu^{++} ion concentration using a specific cupric ion activity electrode. The rate determined by this method agreed well with that obtained by spectrophotometry.

Ethylene diamine, a complexing agent for Cu^{++} ions, was also used to bring about the renaturation. In this case, the reaction was observed to obey first order kinetics, to be time dependent, but to be independent of the solution viscosity.

The interpretation of these results in terms of the interaction between Cu^{++} and DNA and the possible mechanism for the renaturation reaction is discussed.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, nor any material previously submitted for a degree or diploma in any University, except when due reference is made in the text.

L.R. Holman

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CHAPTER I

INTRODUCTION

The central role of deoxyribonucleic acid (DNA) in molecular biology is well established. As the carrier of the genetic information of the living cell, this macromolecule transmits to each generation the vital instructions for the synthesis of proteins, including the enzymes which direct and control every process occurring in the cell. Clearly, an understanding of the structure and properties of DNA is essential to the detailed description of its biological function.

The primary structure of DNA has been established[1] to be a highly polymerised linear chain of alternating deoxyribose sugar and phosphate units, each sugar having attached to it one of four heterocyclic bases which, in general, are guanine, adenine, cytosine, and thymine. The secondary structure was elucidated by Watson and Crick[2] by model building based on the X-ray study of DNA fibres performed by Wilkins et al.[3] They proposed that each molecule consists of two polynucleotide chains wound in right hand helices about a common axis, with each base of one chain paired to its complementary base on the other chain by hydrogen bonding, and with the plane of the bases perpendicular to the helix axis. With few modifications, the Watson-Crick model has been accepted as the basic structure for DNA molecules in vivo and in vitro as well as in the fibrous state. Techniques such as spectroscopy, low angle X-ray scattering, light scattering and hydrodynamic measurements,[4] when applied to DNA in aqueous solution, have given data which are in

conformity with the Watson-Crick model for DNA structure, and have led to a greater understanding of the solution properties of this unusual molecule. Also useful in this respect have been studies on the interaction of DNA with other species such as various organic molecules[5] and metal ions.

Interest in the possible interaction of metal ions with nucleic acids was aroused, even before the Watson-Crick model was proposed, by the demonstration of the frequent involvement of metal ions in enzyme function and in the organization of protein molecules, and by several reports of the presence of metal ions in isolated nucleic acids.[6] It has subsequently been found that metal ions have varied and pronounced effects on the primary and secondary structure of nucleic acids.

Thomas[7,8] was the first to point out that the presence of a certain concentration of metal ions is required for the stability of the native secondary structure of DNA in aqueous solution. If DNA is placed in a solution with less than a certain critical concentration of univalent electrolyte, such as NaCl, there is a loss of the ordered secondary structure which can only be partially reversed by the subsequent addition of salt.[7-9] This critical ionic strength depends on the DNA concentration, for it has been shown[10,11] that DNA can be maintained in the native form in the absence of added electrolyte, provided the solution is sufficiently concentrated, in which case the Na^+ counterions present in the solid DNA sample are sufficient to stabilise the molecules in the solution.

The transition from the double helical structure (native form) to the disordered structure (denatured form) can also be brought about by heating the solution above a critical temperature.[12] This thermal denaturation is a cooperative transition usually followed by observing the increase in UV absorbance which accompanies the process. The position of the transition, as indicated by its mid-point, the melting temperature (T_m), may be taken as a measure of the stability of the native structure. Thus, to extend the earlier work of Thomas[7] concerning the effect of univalent cations on DNA stability, a number of determinations have been made of the dependence of the melting temperature on the ionic strength of the solution.[13-17] Over a range of univalent electrolyte concentration from 0.003 M to 0.5 M, a linear relationship between T_m and the logarithm of the ionic strength has been established.[14-17]

The explanation of these observations becomes evident when it is realised that in the neutral pH range employed by the above workers, the phosphate groups of the DNA backbone were ionised, each bearing a single negative charge. The double helical conformation of such a molecule in the absence of any counterions would be expected to be highly unstable for the forces holding the strands together[18] would not overcome the electrostatic repulsion between the two highly negative strands. The cations when present reduce the negative potential of the chains, thus stabilising the double helical structure. Application of polyelectrolyte theory to describe the properties of this system, in particular, the dependence of T_m on ionic strength, has been attempted

with some success.[17,19,20]

In his investigation of the effect of electrolyte concentration on DNA, Thomas[7] noted that the critical concentration of Mg^{++} salt required for stability of the native structure was approximately one hundred times lower than that of the Na^{+} salt. Subsequent work has amply confirmed the enhanced stabilising effect of Mg^{++} ions on DNA and RNA from various sources,[14,21-27] both in solutions in which Mg^{++} is the only counter ion present[24] and when a small amount of Mg^{++} salt is added to a solution with excess univalent electrolyte present.[14,23,25] The other alkaline earth metals behave similarly[23,25,28,29] but the Mg^{++}-DNA system has stimulated most interest because of the involvement of Mg^{++} ions in many of the enzyme systems associated with DNA. At first there was controversy over the site of interaction of Mg^{++} with DNA, some workers[30,31] postulating binding to purine bases, but it now seems evident that, at least with native DNA, Mg^{++} is involved in a strong stoichiometric interaction with the phosphate groups.[32-35]

In 1962, Eichhorn[23] undertook a systematic study of the effect of divalent metal ions, including those of the alkaline earths and the first transition series elements, on the melting temperature of DNA in low ionic strength solutions. He found that, at a ratio of two metal ions per nucleotide residue, most metal ions (Mg^{++}, Ca^{++}, Ba^{++}, Mn^{++}, Co^{++}, Ni^{++}, Zn^{++}) were effective in stabilising the native structure, as shown by the increase in melting temperature; in contrast, Cu^{++}, Cd^{++} and Pb^{++} were found to decrease T_m, the latter two to just

a small extent, but Cu^{++} ions to the extent of at least 20°C. The difference between these three and the other divalent ions became even more apparent when the denatured solution was cooled. In solutions of the former group of ions, the UV absorbance decreased considerably on cooling, but with the latter group, no decrease was observed. This effect of Cu^{++} on the thermal denaturation of DNA has since been studied in greater detail.[25,36-38]

To explain the above phenomena it is necessary to consider briefly the relation between the helix-coil transition and the UV absorption of nucleic acids. The absorption band centred at 259 nm arises from electronic transitions of the bases, but the extinction coefficient of this band in native DNA is approximately 40% less than would be expected from the sum of the contributions of the constituent chromophores (bases). This decrease in extinction coefficient, called hypochromicity, is a consequence of the interaction between the bases when they are in an ordered, stacked conformation, as in the double helical model. On denaturation, the ordered conformation is destroyed, resulting in a reduction in the interaction between the bases which causes an increase in the UV absorption (hyperchromicity). When DNA is cooled after thermal denaturation, interaction between the bases again becomes possible. Thus the decrease in hyperchromicity on cooling, observed in the presence of monovalent ions or divalent ions of the Mg^{++}-like group, is due to the arrangement of the bases in some degree of order, the extent of which depends on conditions such as the rate of cooling and the ionic strength. The failure to observe any

decrease in hyperchromicity on cooling the denatured solution in the presence of Cu^{++} ions indicates that the Cu^{++} ions prevent any interaction between the bases, presumably by complexing with them.

It was also found[36,37] that the hyperchromicity of the denatured DNA-Cu^{++} solution which was unaffected by cooling, could be completely quenched if the supporting electrolyte concentration was raised to 0.1 M - 0.2 M. The product of this reaction was unequivocally shown by many techniques[36,37] to be 100% native DNA. Thus it has been established that DNA denatured in the presence of Cu^{++} ions can be completely and rapidly renatured by increasing the ionic strength, even in the case of the very heterogeneous Calf Thymus DNA. The uniqueness of this reaction can be appreciated when it is realised that DNA thermally denatured in the absence of Cu^{++} undergoes a very slow renaturation at elevated temperatures in a high ionic strength solution.[39-41] For mammalian DNA the renaturation proceeds only to a small extent (25% in 24 hours for Calf Thymus DNA[41]). As a result of this property, the Cu^{++}-DNA system appears to be significant in the study of the helix-coil transition which is fundamental to the understanding of the structure and stability of DNA. The transformation of the molecules from the double helix to the random coil form has been studied by a number of methods,[42-45] but the reverse process, the formation of the helix from disordered strands, has proved less accessible to study. Since the renaturation in the absence of Cu^{++} is complex from the point of view of both experimentation and mechanism, the complete and

rapid renaturation observed in the Cu^{++}-DNA system may give valuable information on the mechanism of helix formation.

Several other metal ions have been shown to have a marked effect on the secondary structure of DNA. Mercuric and silver ions interact with the bases at room temperature causing substantial spectral changes,[46-49] an increase in T_m in the case of Ag^{+},[14,50] and, in the case of Hg^{++}, a pronounced decrease in viscosity.[51] The effects are completely reversible for on addition of complexing agents for these ions, native DNA is reformed. It has been proposed that the Hg^{++} ions form bridges between the two strands with one Hg^{++} per base pair.[47,52] Important evidence for this type of complex has come from experiments[53] using the methylmercuric ion ($HgCH_3^{+}$) where only one site is available for complexing instead of two. In this case, the interaction causes the DNA to become denatured, and native DNA cannot be formed when the mercury-base complex is dissociated. Silver ions form a number of different complexes with the bases depending on the Ag^{+}/nucleotide ratio, but bridging of the metal ions between complementary bases has also been postulated for one type of complex.[49,50]

Very little work has been carried out thus far on the interaction of Fe^{++} and Fe^{+++} ions with nucleic acids, possibly because of experimental difficulties, Fe^{++} being easily oxidised and Fe^{+++} being easily hydrolysed. Eichhorn[23] observed that, at $2Fe^{++}$ per phosphate, the T_m of DNA was raised, but on cooling, instead of a decrease in hyperchromicity, there was a small increase. Ivanov and Minchenkova[54] found that T_m was lowered by small amounts of Fe^{+++} ions (Fe^{+++}/

Phosphate < 0.5) and on reduction of the ions to Fe^{++}, T_m returned approximately to its original value. Using an NMR proton relaxation enhancement technique, Eisinger et al.[55] showed that Fe^{+++} was bound to an "internal" site while Fe^{++} was bound "externally". Apart from these observations, very little is known of iron-DNA interaction in spite of suggestions that such an interaction may be of biological significance.[56]

Metal ions can also affect the primary structure of nucleic acids. Pb^{++}, La^{+++} and other rare earth ions as well as being precipitants for nucleic acids,[57,58] bring about the depolymerisation of RNA and polyribonucleotides by catalysing the hydrolytic cleavage of the phosphodiester bond.[59,60] Zn^{++} also causes degradation of RNA and polyribonucleotides at temperatures around 60°C and a similar but slower reaction has been observed with Mn^{++}, Co^{++}, Ni^{++} and Cu^{++}.[61] In no case could DNA be degraded by metal ions, which suggests that the mechanism for cleavage of ribose containing nucleic acids involves the metal ions chelating between the phosphate and the 2'-OH of the ribose.

In view of the quantity and diversity of the effects of metal ions on nucleic acids in vitro, it has often been suggested that some may have a biological function. Indeed, metal ions have often been found closely associated with cell organelles which contain nucleic acid, such as ribosomes and chromosomes,[62,63] and Mg^{++} ions have been proven to be essential in the structure of microsomal nucleoprotein.[64,65] There have been a number of reports of various metals isolated in

preparations of viruses, DNA and RNA. Some workers dismissed the presence of metals as being due to contamination or as being in too small a quantity to be significant.[66,67] However, there have been fairly consistent reports of iron,[68-73] copper,[70,73] calcium and magnesium[68,70] present in viruses, and a number of these reports stated that the metal persisted in the nucleic acid isolated from the virus.[68,70-72] Altmann[74] detected Cu, Zn and Mn in isolated rat liver RNA, but the most comprehensive work was undertaken by Wacker and his coworkers.[68,75] Spectroscopic analysis of RNA from different mammalian sources and from Tobacco Mosaic virus showed small but significant amounts of a variety of metals, the most prevalent being Mg, Ca, Fe, and Zn. Mammalian DNA also contained metals but at a lower concentration. Small amounts of Cu were generally detected. These investigators meticulously avoided any contamination with metal ions during precipitation and handling of the nucleic acid samples, and yet Blois, Maling and Taskovich,[76] studying the EPR signal due to the presence of Fe^{+++} in DNA samples, found that the signal disappeared when all sources of contamination were removed. When they isolated Calf Thymus DNA under strict metal-free conditions, even avoiding stainless steel instruments, the EPR signal was much reduced, and after washing the gland homogenate five times with EDTA solution prior to DNA isolation, the signal disappeared completely. They concluded that the metal ions in DNA samples arose from contamination during isolation by ions present in the tissue.

Thus, whether metal ions are intimately associated with nucleic

acids in vivo is still an unsettled question, and what biological function such an association would have is even more elusive. There have been several observations[77,78] of pronounced changes in bacterial strains grown in the presence of low concentrations of Cu^{++}, including a change in the GC content of DNA isolated from them,[77] which certainly suggests that Cu^{++} ions have some biological function. The most detailed suggestion concerning this has come from Ivanov and Minchenkova[54,56,79] who have proposed that Cu^{++} and/or Fe^{+++} ions control the winding and unwinding of DNA in vivo. Their in vitro results showed that Cu^{++} and Fe^{+++} lowered the T_m of DNA and on reduction by ascorbic acid, the Cu^{+} and Fe^{++} ions so formed raised T_m. (The cuprous ion appears to be similar to Ag^{+} in its effect on DNA.) They proposed that such a stabilising-destabilising system could be involved in the "priming" of DNA for replication or transcription. While the in vitro evidence is clear, there seems to be little evidence to indicate that such a process could control the complex DNA mechanisms in vivo.

The purpose of the research described in this thesis was to study the helix-coil transition of DNA under the unique set of conditions afforded by the reaction in which DNA is renatured after denaturation in the presence of Cu^{++}. The aim was not to clarify any biological significance of the Cu^{++}-DNA system, nor even to elucidate more fully the interaction of Cu^{++} ions with DNA, although there are many unanswered questions concerning this system, as will be discussed in Chapter II. As discussed above, it was considered that the study

of this reaction could lead to a better understanding of the process by which the double helix of DNA is formed from the disordered conformation.

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CHAPTER II

THE INTERACTION OF COPPER(II) IONS WITH DNA

1. Introduction

2. Interaction of Cu^{++} ions with native DNA

   a. Effect of Cu^{++} on the UV spectrum of native DNA

   b. Other techniques used to determine the site of Cu^{++} interaction with native DNA

   c. Proposed sites

3. The formation of the denatured DNA-Cu^{++} complex

   a. The effect of Cu^{++} concentration

   b. The mechanism of formation of the denatured DNA-Cu^{++} complex

4. The structure of the denatured DNA-Cu^{++} complex

   a. The site of interaction: Mononucleotides

   b. The site of interaction: Polynucleotides

   c. The site of interaction: DNA

   d. The conformation of the strands

5. The renaturation of the denatured DNA-Cu^{++} complex

   a. Comparison with renaturation of DNA in the absence of Cu^{++}

   b. Hypotheses to explain the renaturability

References

1. Introduction

The first suggestion of a strong interaction between Cu^{++} ions and nucleic acids came from Frieden and Alles[1] in 1958. Studying the Cu^{++} catalysis of ascorbic acid oxidation, they found that DNA, RNA and purine base derivatives strongly inhibited the reaction which they considered to be due to the chelation of Cu^{++} ions by these compounds. The more recent investigations of the effect of Cu^{++} ions on the secondary structure of DNA carried out by Eichhorn,[2] Eichhorn and Clark,[3] Hiai,[4] Venner and Zimmer[5] and Srivastava[6] have led to certain tentative ideas concerning the interaction. The picture which has emerged from these studies is that at room temperature, Cu^{++} interacts only with the phosphate groups of native DNA; when the temperature is raised, interaction with the bases becomes possible reducing the stability of the helix and producing a lowering of the T_m; the Cu^{++} ions complexed to the bases prevent interaction between them on cooling, but at the same time, the Cu^{++} ions hold the strands in register so that on removal of the Cu^{++} ions, native DNA is reformed.

In this chapter, the sections of this hypothesis will be examined in detail in the light of the available experimental evidence.

2. Interaction of Cu^{++} ions with native DNA

Since in neutral solution at low ionic strength, the native DNA helix carries a high negative charge, only partially shielded by counterions, it would be expected that the principal site of interaction

of Cu^{++} ions is the negatively charged phosphate group. In view of the known interaction of other divalent ions such as Mg^{++}, Ca^{++} and Mn^{++} with the phosphates,[7-11] such behaviour in the case of Cu^{++} could be reasonably expected. The bases in the interior of the helix do not appear to be readily available for interaction with metal ions. However, Hg^{++} [12,13] and Ag^{+} [14,15] ions interact with the bases of DNA at room temperature, and so such a possibility should not be discounted for Cu^{++} ions.

One method of distinguishing between interaction at these two possible sites is to examine the effect of Cu^{++} ions on the UV absorption band of DNA which is centred at 259 nm.* Since this band arises from electronic transitions within the bases, it is unlikely that an interaction of Cu^{++} ions with the bases would leave their electronic structure so undisturbed as to cause no change in the absorption spectrum. Thus interaction with the bases would in all probability result in a shift in the 259 nm band. On the other hand, interaction with only the phosphate groups would not cause any spectral change unless the interaction had a marked effect on the conformation of the bases, which is unlikely since no UV shift is observed when ions such as Mg^{++} interact strongly with the phosphates. Hence, a number of workers have examined the effect of Cu^{++} on the native DNA spectrum.

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* Some workers use 260 nm as the wavelength of the maximum of this band, but the majority employ the accepted value of 259 nm.

a. Effect of Cu^{++} on the UV spectrum of native DNA

In the extensive work of Hiai in 1965 on the effects of Cu^{++} on the denaturation of DNA,[4] it was stated that the presence of Cu^{++} did not change the absorption at 260 nm at room temperature. This was supported by Coates et al.[16] and by Eichhorn et al.[17] who found that Cu^{++} had no effect on the UV spectrum of native DNA. Minchenkova and Ivanov[18] in 1967 reported that the spectrum of DNA in the presence of Cu^{++} was modified by a very small decrease in absorbance at the peak and a slight broadening to longer wavelengths. They attributed this to a small amount of reduction of the Cu^{++} ions by a reducing contaminant because they found that the slight spectral changes were enhanced when a reductant was added. In 1968 Eichhorn's research group again stated that Cu^{++} did not significantly shift the absorption maximum of DNA.[19] All these workers supported an interaction with solely the phosphates at room temperature.

However, there have been five reports of changes in the UV spectrum of native DNA caused by Cu^{++} ions at room temperature. The earliest of such reports were made by a number of Russian investigators in 1966. Yatsimirskii et al.[20,21] found that when the ratio of the Cu^{++} concentration to the DNA concentration, expressed in terms of the nucleotide or phosphate group concentration, was less than 0.5, there was a small general decrease in absorbance. The difference spectrum between the Cu^{++}-DNA solution and DNA alone (Cu^{++} does not absorb in this region) had a minimum around 250 nm. For Cu^{++}/Phosphate ratios of greater than 0.5, there was an increase in O.D. throughout

the band, the difference spectrum exhibiting a minimum at 250 nm and a maximum at 275 nm, with an increase of approximately 20% in the absorption at this wavelength. Another study was made by Zakharenko and Moshkovskii[22] who observed that for $Cu^{++}/P \leq 1.0$ there was a decrease in the absorbancy throughout the whole band, the decrease being a maximum when Cu/P = 0.5. Only in the case of a ratio of Cu/P = 10 was any increase in absorbance seen.

In 1967 Bryan and Frieden[23] reported a study of the binding of Cu^{++} to native DNA below 30°C in 5×10^{-3} M $NaNO_3$. They stated that difference spectra in the presence of Cu^{++} were observed with a maximum at 270 nm, but no diagrams of such difference spectra were presented nor any indication of the observed changes in O.D. given. The results were represented as a fraction of the maximum absorbance change at 270 nm but the value of this maximum was not stated. The increase at 270 nm appeared when Cu/P was approximately 0.5, and their plot of ΔO.D.(270) against log(Cu^{++} concentration) had a maximum slope at approximately Cu/P = 2. A time effect was also reported in the form of a gradually increasing absorbance, sometimes not reaching an equilibrium value for several hours after the addition of Cu^{++} to the DNA solution. In another section of the paper a value of 1.15 was given for the relative increase in absorbance at 260 nm for Cu/P = 3. It appears that this value was obtained with a more concentrated DNA solution than used for the other results.

A recent publication of Zimmer and Venner,[24] as well as reporting difference spectra of Cu^{++}-DNA complexes formed on heating

above T_m measured against native DNA, also included spectrophotometric titrations of native DNA with Cu^{++} at room temperature, followed at 260 nm. When DNA of high GC content was used, the absorbance increased by 20%-30% for $Cu/P \geqslant 1.0$, but for DNA samples with GC content less than 50% the corresponding increase was only 10%.

The most detailed and systematic study of the binding of Cu^{++} to native DNA was presented by Schreiber and Daune[25] in 1969. The difference spectra observed in 10^{-1} M $NaClO_4$ with different Cu/P ratios are shown in fig. II-1. It should be noted that, in contrast to the difference spectra discussed above, a negative minimum at 245 nm and a negligibly small change at 260 nm were observed. The increase in O.D. at 280 nm was only small, particularly when it was compared with the total O.D. of the DNA solution (DNA concentration = 3×10^{-4} M_P, O.D.(260) $\sim$ 2). In fact, for Cu/P = 1, the change in O.D. at 280 nm observed by these workers (calculated from figure 2 of their paper) was of the order of 10%, 5% and 2% in 0.005 M, 0.1 M and 1.0 M $NaClO_4$ respectively. Schreiber and Daune explicitly stated that because the difference spectra observed were so small, it was necessary to use DNA solutions with concentrations as high as could be allowed within the limitations of the experimental technique in order to measure the changes satisfactorily.

Thus, although five different research groups have reported spectral changes for this system at room temperature, there is little agreement concerning the nature or magnitude of such changes. There are a number of complicating factors capable of causing serious anom-

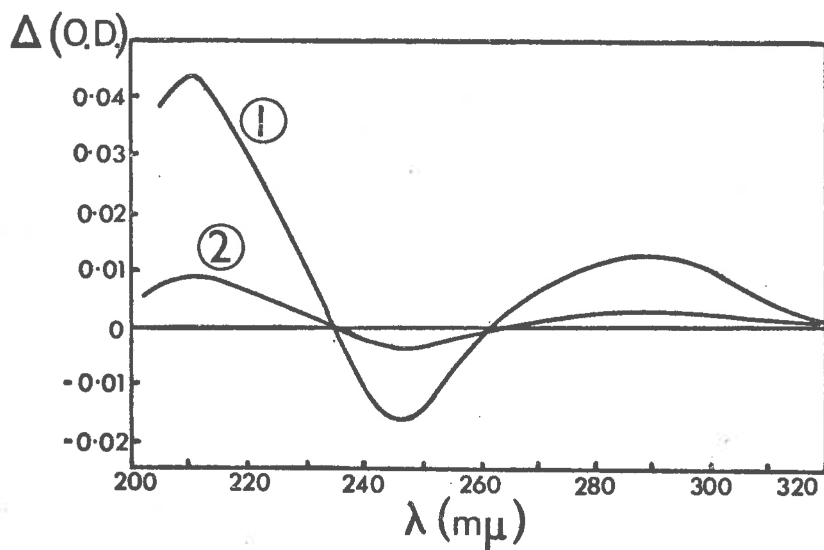


Fig. II-1. Difference spectra of Cu^{++}-native DNA complex against native DNA as observed by Schreiber and Daune (ref. 25)

(1) Cu/P = 0.025
(2) Cu/P = 0.005

$NaClO_4$ concentration = 0.1 M
DNA concentration $\sim 3 \times 10^{-4}$ M_P ($O.D._{259} \sim 2$).

alies in the difference spectra which were generally not considered by the above workers.

Firstly, consider the case of a small amount of the DNA being present in the denatured form. It is known that Cu^{++} ions interact with denatured DNA causing spectral changes.[17] Hiai[4] showed that with partially denatured DNA, prepared by brief exposure to a temperature above T_m, there was a considerable increase in hyperchromicity when Cu^{++} ions were added at room temperature. In fact the hyperchromicity at 260 nm of a partially denatured DNA sample with a hyperchromicity of 15% increased in the presence of Cu^{++} to 27%. Since Hiai did not observe any hyperchromicity with native DNA at room temperature, it appears that such an effect is due to the Cu^{++} ions causing an increase in the amount of disordered secondary structure. This could be interpreted as the interaction of the Cu^{++} ions with the bases which are readily available in the denatured regions of the molecule, resulting in destabilisation of the base stacking in adjacent regions which leads to further denaturation. In this way, a small number of denatured sites in native DNA could lead, in the presence of Cu^{++}, to an increased extent of denaturation. With this in mind, it is interesting to note that the difference spectrum of the Cu^{++}-denatured DNA complex[24] is very similar to the difference spectrum reported by Yatsimirskii, Kriss and Akhrameeva[20] for native DNA and Cu^{++}; a maximum between 270 and 280 nm, a minimum at 250 nm, still with a positive change in O.D., and a very steep rise less than 240 nm. In fact, the explanation put forward by these workers to account for

their spectral changes is that for $Cu/P > 0.5$, Cu^{++} ions cause a breakdown of the double helix followed by chain separation. Also, the general decrease in absorbancy observed when $Cu/P < 0.5$ is remarkably similar to the effect of stabilising divalent cations such as Mg^{++} on denatured DNA,[26,27] taking into account that it has been suggested that when $Cu/P < 0.4$ the Cu^{++} ions exert a stabilising effect on DNA secondary structure.[3,5]

As well as the effect of a small amount of denaturation, the possible effect of other irregularities in the native structure on the interaction of Cu^{++} with DNA should be considered. How the presence of irregularities such as single strand scissions or disturbances in the ordered base stacking due to thymine dimers caused by UV radiation would influence the interaction has not been studied, but it is reasonable to propose that if Cu^{++} ions cannot interact with the bases of normal native DNA, they may be able to do so at such points of irregular structure. This would lead to denatured regions with more Cu^{++} bound, in a similar way to that discussed above for partially denatured DNA. Such a scheme has been suggested by Sutherland and Sutherland[28] to explain their results concerning the effect of Cu^{++} ions on the UV-induced dimer formation in DNA. From these considerations it can be seen that it is possible that the observed spectral changes arise not from the interaction of Cu^{++} ions with completely native DNA but from the interaction with denatured regions caused or enhanced by the presence of the Cu^{++} ions, and the conclusion that the difference spectra indicate Cu^{++} binding to the bases of native DNA may not

necessarily be a correct one.

There have been a number of reports of aggregation in Cu^{++}-DNA solutions, which has been explained by the tendency of Cu^{++} ions to form salt bridges between DNA molecules. For example, with native DNA and Cu^{++} ions in the millimolar concentration range viscous solutions[29] and precipitation[29,30] have been observed, while at lower concentrations, precipitation or extremely high sedimentation values have resulted from heating the solution.[3,4,6,31] Aggregation present in the solution even before precipitation is observed by the eye, complicates the observation of difference spectra due to the excess light scattering by the aggregates. For example, when a comparison is made between the two studies of the effect of native DNA on the visible spectrum of Cu^{++}, it is noted that one study[16] using solutions in the 10^{-4} M concentration range observed no change, while Zimmer and Venner,[24,32] working in the 10^{-3} M range, found that the Cu^{++} spectrum was markedly changed by the addition of DNA; the absorbance increased by 150% throughout the band, and the maximum shifted to shorter wavelengths. Extensive aggregation has been reported for native DNA and Cu^{++} in this concentration range[29,30] and so it seems likely that this spectral change was due to light scattering, the wavelength dependence of the scattering explaining the blue shift of the peak.

On considering the reports discussed above, it appears that, in several cases, light scattering may have contributed to the difference spectra observed. Yatsimirskii et al.[20] stated that when the

Cu^{++} concentration exceeded a critical value, they observed precipitation. Thus the aggregation present in solutions with less than this critical concentration would most likely cause a light scattering contribution to their difference spectra. The DNA concentrations employed by Bryan and Frieden[23] in their spectrophotometric titration with Cu^{++} at 270 nm were too low for significant aggregation to occur. However, in the later section of their paper where they reported a 15% increase in 260 nm absorption, they used a solution of higher DNA and Cu^{++} content which showed an increased sedimentation coefficient, indicating the possibility of a light scattering factor in their absorbance results. Zimmer and Venner[24] gave no indication of the DNA concentrations present in the solutions they studied, and so it is not possible to draw any conclusions in this case.

In contrast to the above investigators, Schreiber and Daune[25] were very careful to recognise and, if possible, avoid the effects of aggregation. Their binding curve, reduced absorption at 280 nm against the number of Cu^{++} ions bound per phosphate group (r), exhibited a straight line portion, independent of DNA concentration, up to about $r = 0.04$ at which point a very sharp increase in slope occurred. In 10^{-1} M $NaClO_4$, and with DNA concentrations $(3 \text{ to } 6) \times 10^{-4}$ M_p, the deviation occurred when between 3 and 4 Cu^{++} ions were bound per 100 phosphates. They attributed this deviation to aggregation brought about by Cu^{++} and also to small conformational changes in the DNA

molecules in the presence of Cu^{++}, which they demonstrated by light scattering measurements on solutions from which the aggregates had been removed by centrifugation. They stated that the intensity of the scattered light during their spectral measurements was only small compared to the total optical density but it had a significant effect on the difference spectra. No details of light scattering in their solutions of low ionic strength (0.005) were given, but it could be expected that because of the stronger Cu^{++}-phosphate association at the lower ionic strength aggregation may occur to an even greater extent.

To summarise the discussion of this section, the changes in the UV spectrum of native DNA caused by Cu^{++} as observed by Schreiber and Daune[25] are sufficiently small to explain why the previous workers[16,17,19] failed to observe them, particularly as the technique of difference spectra used by Schreiber and Daune was not employed by them. Also, these workers were more concerned with the much larger changes which appeared on denaturation. In Schreiber and Daune's results the change at 259-260 nm was minimal and would most likely be within the experimental uncertainty of other investigators who, using either of these wavelengths to follow the melting transition, observed no hyperchromicity in the presence of Cu^{++} at 25°C.[3,4,5,6]

The results of Zakharenko and Moshkovskii[22] are totally incompatible with all the others, and those of Yatsimirskii et al.[20,21] are uncertain because of possible denaturation and aggregation. There is insufficient data presented by Bryan and Frieden[23] for significant

conclusions concerning their work to be made, but their report of the effect of time on the difference spectrum is not supported by any others and may indicate some anomaly in their DNA solutions. The changes reported by Zimmer and Venner[24] at 260 nm are too large to be compatible with Schreiber and Daune's data. The changes observed by Schreiber and Daune have been verified in this laboratory[33] for solutions of 0.005 ionic strength. With DNA of 50% GC content, no change in the 259 nm absorption was observed for Cu/P ratios up to 2.0 in contrast to the 10% hyperchromicity reported by Zimmer and Venner. Small O.D. increases seen in the range 275-285 nm agree with Schreiber and Daune's difference spectra. With DNA of 72% GC content the increase at 259 nm was only 10% for Cu/P = 2.0 instead of 25% as observed by Zimmer and Venner. The reason for such large discrepancies in the experimental results obtained under supposedly identical conditions is not at all clear.

b. Other techniques used to determine the site of Cu^{++} interaction with native DNA

From the previous section it can be seen that the results of the UV absorption studies are inconclusive, and thus other experimental techniques which have been applied to this system are now considered.

The effect of DNA on the visible absorption spectrum of Cu^{++} has been studied, but no conclusion can be drawn because, as was mentioned in the previous section, Coates et al.[16] reported no change in the Cu^{++} band while Zimmer and Venner's results indicated a change but

were uncertain because of the possibility of light scattering.[24,32] In any case, because of the low concentration of Cu^{++} ions which must be employed due to the problem of aggregation and precipitation, it may be experimentally difficult to detect significant changes in the visible spectrum brought about by DNA.

From their IR studies, Fritzsche and Zimmer[34] concluded that Cu^{++} interacted with guanine and cytosine of DNA but because of the manipulations performed on the solution to prepare a solid sample for IR measurements, the state of the DNA in the complex was a little uncertain.

There has only been one preliminary study on the effect of metal ions on the UV rotary dispersion of native DNA.[35] This showed that Ca^{++}, Zn^{++}, Mg^{++} and Mn^{++} caused only minor changes in the dispersion curve, Cu^{++} ions caused a marked change in each peak, and Hg^{++} ions, which are known to interact with the bases, brought about such an extensive change that the dispersion spectrum bore no resemblance to the original DNA curve. No conclusions were drawn from the study, but it would appear that Cu^{++} either interacts with the bases or indirectly causes a marked conformational change of the bases.

Techniques which would be expected to be very sensitive to the environment of copper are electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR). The application of NMR to this system is complicated by the paramagnetism of the Cu^{++} ion. However, Eisinger and his coworkers[29,36] made use of this property and studied the effect of paramagnetic ions on the proton relaxation times of

water molecules using an NMR spin-echo technique. The changes in the longitudinal relaxation time of the protons brought about by interaction between water molecules and a paramagnetic ion such as Cu^{++} are dependent on the characteristics of the hydration sphere of the ion, and thus are sensitive to interactions between the ion and a third species such as a nucleic acid molecule present in the solution. Their results indicated that Cu^{++} interacted with DNA at an "external" site where the ions retained most of their hydration sphere. (The nature of this "external" site, unspecified here, will be discussed in the following section.)

Ropars and Viovy[37,38,39] studied the interaction between Cu^{++} and native DNA by EPR. They analysed the complex EPR signal of the system into three bands with different g factors. The first was identified as being due to free Cu^{++} ions, the second, by comparison with the signal from Cu^{++}-phosphate and Cu^{++}-polyphosphate solutions, as being due to a covalent interaction between the Cu^{++} ions and the phosphate group. The third peak, with the lowest g value, was assigned to the interaction of Cu^{++} with an internal site. When they studied the interaction between Cu^{++} and the nucleosides and nucleotides, the strongest interaction was with guanine, and thus they proposed the guanine base as the internal site. The interaction of Cu^{++} ions with heat denatured DNA was also studied, and the authors stated that, under these conditions, the EPR spectrum could still be reduced to three bands, and the proportion of Cu^{++} ions giving rise to the second and third bands was very much increased. Since Cu^{++} is known to interact with

the bases of denatured DNA (see section II.4), these results to some extent support the assignment of the third band to a Cu^{++}-base complex.

Direct measurements of binding parameters such as the association constant and number and type of sites for this system have been attempted using the techniques of gel filtration,[23] UV absorption spectroscopy,[20,23] Cu^{++} catalysis of peroxide oxidation of hydroquinone,[20] and polarography.[40,41,6] The results vary considerably, and in general such studies concluded either implicitly or explicitly that there was only one type of binding site, which is in contrast to the conclusion of Ropars and Viovy.[39] The approach which yielded the most significant information concerning the site of binding of Cu^{++} to native DNA was that of Schreiber and Daune[41] who used DNA samples of different GC content in their polarographic measurements. They demonstrated clearly that the number of binding sites at high ionic strength (0.1 to 0.3) increased with increasing GC content, again suggesting base binding as phosphate binding would be independent of base content. Further binding studies expected to give more information concerning this system are currently in progress in this laboratory.[33]

c. Proposed sites

In their paper on the UV difference spectra, Schreiber and Daune[25] have proposed three sites for the interaction of Cu^{++} with native DNA.

(i) An electrostatic interaction with the phosphate groups.

(ii) Cu^{++} chelation between the N_7 of a purine base and an adjacent

phosphate group.

(iii) Cu^{++} chelation between two guanine residues adjacent on the same chain, involving the N_7 and O_6 of each base. This "sandwich" type complex is illustrated in fig. II-2, and is similar to a complex which has previously been proposed for the interaction of Ag^+ ions with native DNA.[42,43]

The first two types of interaction, since they involve the phosphate groups, are expected to depend on the ionic strength of the solution, and the third type of site is stated by Schreiber and Daune to be independent of ionic strength. Since their polarographic measurements[41] showed that the number of binding sites was practically constant in 0.1M-0.3M $NaClO_4$, they concluded that at these ionic strengths there is no phosphate interaction and binding is exclusively of type (iii). The number of type (iii) sites per nucleotide was 0.04 to 0.12 depending on the GC content of the DNA which agreed closely with the values obtained by Ropars and Viovy[39] for the base-binding sites detected by EPR.

The other techniques which have been applied to this system, as discussed in the previous section, generally indicate a base interaction and thus support Schreiber and Daune's proposals. The possible exception is the NMR study of Eisinger et al.[29] which showed that Cu^{++} was bound to an external site on native DNA and led these workers to conclude that this external site was the phosphate group. However this conclusion is not necessarily the only one which can be drawn from their experimental results. Since they used a low ionic strength solution, binding to the phosphates would certainly be predominant, and it is unlikely that their NMR technique would be sensitive to a small amount of

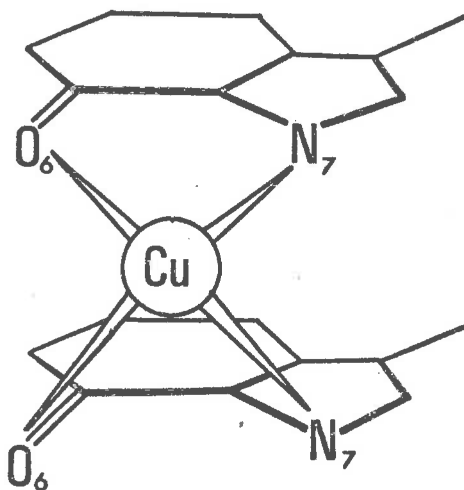


Fig. II-2. Type (iii) "sandwich" complex as proposed by Schreiber and Daune (Ref. 25) with Cu^{++} ion chelated between two guanine residues adjacent on the same DNA chain.

base interaction occurring simultaneously. It can also be proposed that, since the sites for type (ii) and type (iii) binding are accessible on native DNA at room temperature, interaction of the Cu^{++} ion at these sites may not affect the hydration sphere of the ion to a significantly greater extent than interaction at the phosphate site. Because the criterion for an external site, in the work of Eisinger et al., was the effect of the binding on the protons in the hydration sphere of the Cu^{++} ion, from this consideration also it appears that the NMR technique may not be able to distinguish between phosphate and base interaction. In the discussion which follows in later sections of this thesis, the term "external site" is used to denote any site on native DNA that is available for Cu^{++} interaction without prior distortion of the secondary structure, and thus this term applies to the three sites proposed by Schreiber and Daune. An "internal site" is one which requires partial unwinding or distortion of the double helix before it is available for interaction.

Examination of a model of the DNA molecule, shows that the purine N_7 is reasonably well exposed in the grooves of the double helix and should be available for Cu^{++} interaction without prior distortion of the structure. Schreiber and Daune have stated that binding of type (ii) distorts the helix geometry and is a destabilising interaction and that on raising the temperature in a low ionic strength solution, more Cu^{++} becomes bound to this type of site causing the helix to melt. However, the positions of the purine N_7 and the phosphate group appear in the DNA model to be ideally suited for a Cu^{++} bridge between them, and

it is difficult to see why Schreiber and Daune have said that this is a destabilising interaction. They have also proposed that the "sandwich" complex stabilises the helix both through the reduction of the negative potential of the chains by the positive charges and through each Cu^{++} ion holding the two guanine moieties involved in the complex more strongly in the stacked conformation. Once again, from the DNA model it is difficult to support these claims. The guanine O_6 involved in the type (iii) complex appears rather inaccessible in the interior of the helix and it would be thought that interaction here may distort the geometry of the structure, and may weaken the hydrogen bonding since the O_6 is involved in a hydrogen bond to cytosine. Also, the vertically adjacent guanine are displaced at an angle to each other and the complex may tend to twist them more into vertical alignment, weakening the structure once again.

Thus, although Schreiber and Daune's binding mechanism is attractive, its main merit lies in its use as a working hypothesis for the design of further experiments to solve some of the difficulties inherent in it and to elucidate more fully the details of the interaction between Cu^{++} ions and native DNA.

3. The formation of the denatured DNA-Cu^{++} complex

Investigators studying the denatured DNA-Cu^{++} complex have used one of two methods for forming the complex. Some have first denatured the DNA solution by heating at 90°-100°C and then added Cu^{++} ions, either at the elevated temperature or after rapid cooling to room temperature. The other method employed has been to heat a DNA

solution containing Cu^{++} ions to a temperature above T_m. Identical results are obtained by adding Cu^{++} ions to a DNA solution preheated to a temperature between the T_m of the DNA in the absence of Cu^{++} and the T_m in the presence of Cu^{++} at that concentration, (55°C for example), whereupon denaturation occurs.

It has often been assumed that the complexes formed by these two methods are identical, but in fact this is incorrect. It is true that in certain aspects they are similar, as indicated, for example, by their very similar UV absorption spectrum[4,16,17] but the difference between them becomes clear when the complex is made to dissociate. When the Cu^{++} ions are removed from the complex formed by denaturing a DNA solution containing Cu^{++} ions, native DNA is reformed.[3,4,6] However no such process occurs for the complex formed by the addition of Cu^{++} to denatured DNA.

The complexes formed by these two methods both contain a much greater amount of Cu^{++} bound than in the native DNA-Cu^{++} complex. This has been established by EPR[39] and polarography[40] for complexes formed by the first method, and by specific Cu^{++} ion activity electrode measurements on complexes formed by the second method.[44]

As it is the renaturable complex formed by the second method which is of particular significance in this study, all discussion following will be concerned with this complex only.

a. The effect of Cu^{++} concentration

The temperature of formation of the complex, that is, the melting temperature of the DNA-Cu^{++} solution, has been extensively

studied as a function of the Cu/P ratio. Eichhorn and Clark,[3] and Venner and Zimmer[5] observed in low ionic strength solution (0.005 to 0.006) that there was a small increase in T_m for Cu/P < 0.4 but this was followed by a very rapid decrease, the T_m value being lowered by approximately 20°C at Cu/P = 2.0. Higher ratios produced only a further small decrease. However, there have been a number of other workers who do not report an increase in T_m at low Cu/P ratios. Hiai[4] using 5×10^{-3} M NaCl solutions, Srivastava[6] in 10^{-2} M KNO_3, and Ivanov and Minchenkova[45] in 10^{-2} M NaCl, each observed destabilisation throughout the whole Cu/P range.

The explanation generally proposed for the stabilisation at low Cu/P ratio is that when only a few Cu^{++} ions are present they interact preferentially with the phosphate groups causing stabilisation, and it is only at higher concentrations of Cu^{++} that interaction with the bases and the destabilisation associated therewith predominates. The decrease in the phosphate interaction with increasing ionic strength may explain why the graph of the variation of T_m with Cu/P ratio in 10^{-2} M electrolyte[6,45] showed only an initial flat portion instead of an increase. However, this does not account for the discrepancy between Hiai's results[4] and those of Eichhorn and Clark,[3] and Venner and Zimmer[5] who all worked at an ionic strength of 0.005 to 0.006. As the increase observed when Cu/P < 0.4 only amounts to several degrees, the discrepancies between these studies may reflect either the experimental uncertainty in measuring T_m, or slight differences in the characteristics of the DNA solutions. Certainly Hiai observed very

little change in T_m for Cu/P between 0 and 0.4 which may well be interpreted as a balance between two types of interaction with opposite effect on the helix stability. Thus it appears that there is a stabilising interaction between Cu^{++} ion and DNA but it only exerts a significant effect at low Cu/P ratios. This stabilising interaction has generally been attributed to phosphate binding, but Daune and his collaborators[25,46] have reached a different conclusion. They have analysed the hyperchromic spectra through the melting region using the method of Felsenfeld et al.,[47,48] whereby the melting curve is transformed into two curves showing the fraction of AT and GC pairs melted at any temperature. Using a high ionic strength solution (0.1) to minimise Cu^{++}-phosphate interactions, they found that when 3 Cu^{++} per 100 phosphate were present, the AT melting curve was identical to that of DNA without Cu^{++} present but the GC curve was stabilised by several degrees with respect to the GC curve for DNA alone. When the concentration of Cu^{++} ions was increased to 30 Cu^{++} per 100 phosphate, both the AT and the GC melting curves were destabilised. Since phosphate interaction would stabilise GC and AT pairs alike, they attributed the exclusive GC stabilisation to their type (iii) complex as discussed in section II.2.c with the Cu^{++} chelating between two vertically stacked guanine bases. As this complex is independent of ionic strength it would still be present under the conditions in which the previous workers[3,5] observed destabilisation. Thus it is likely that it is not Cu^{++}-phosphate interaction alone but also this particular type of Cu^{++}-base complex which determines the characteristics of the Cu^{++}-DNA

complexes at low Cu/P ratios.

Another property besides the melting temperature which depends on the Cu/P ratio is the hyperchromicity on cooling the solution from above the melting temperature. Venner and Zimmer[5] observed a considerable decrease in hyperchromicity on slowly cooling a solution with Cu/P = 0.25 from above T_m, but with Cu/P = 2.5 no decrease occurred. The lowest value of the ratio which was necessary to prevent any interaction between the bases after forming the complex, as indicated by the maintenance of full hyperchromicity on cooling, was found by others[4,45] to be approximately Cu/P = 1.0. Thus, it is concluded that the typical properties of the denatured DNA-Cu^{++} complex are only fully developed when the Cu^{++} ions are present in at least 1:1 ratio with DNA nucleotides.

b. The mechanism of formation of the denatured DNA-Cu^{++} complex

Any reaction mechanism naturally depends on the initial and final states involved. In this case, the initial state, the Cu^{++}-native DNA system, is not fully understood, as was indicated in section II.2. The final state, the denatured DNA-Cu^{++} complex, has also not been determined in detail, as will be discussed in section II.4. In spite of this, by considering a description of the properties of the initial and final states in general terms only, a mechanism for the formation of the denatured DNA-Cu^{++} complex can be postulated which, although not greatly detailed, is able to account for the marked destabilisation of the DNA helix by Cu^{++} ions.

Consider the initial state involving Cu^{++} ions bound to external

sites which can be either phosphate groups or sites on the bases which are accessible at low temperatures. The final state consists of Cu^{++} ions bound to internal base sites, an interaction which at room temperature is thermodynamically favoured but sterically hindered. It has been shown by proton exchange methods[49-52] that, even at low temperatures, DNA is subject to local structural fluctuations which result in frequent opening and closing ("breathing") of small regions of the molecule, of the order of several, or perhaps even single, nucleotide pairs. Such small distortions may enable a Cu^{++} ion to enter the helix and interact with the internal base site, thus interfering with the hydrogen bonding and/or ordered stacking in this region. The "breathing" fluctuations will become more frequent as the temperature is increased and so on raising the temperature, more Cu^{++} ions can pass from the exterior to the interior of the helix until at a certain temperature, well below the normal melting temperature, the helix is so weakened that it begins to collapse, exposing even more internal sites for complexing with Cu^{++}. By this means a cooperative transition is observed and it will be a more cooperative, sharper transition than the normal thermal transition, as has been experimentally observed.[2,4-6]

An initial state in which the external site of Cu^{++} interaction is a base permits another mechanism to be postulated. The interaction of Cu^{++} ions with the bases will disturb the electronic structure of the bases and most probably affect the forces between the bases which are responsible for maintaining the double helical structure of the molecule. If the destabilising effect resulting from the perturb-

ation of these forces outweighs the stabilisation through the decreased negative potential on the chains due to the presence of the positive ions, then the helix melts at a lower temperature. The excess Cu^{++} ions present are then able to interact with the base sites, exposed during the transition.

4. The structure of the denatured DNA-Cu^{++} complex

A description of the denatured DNA-Cu^{++} complex formed under the conditions outlined in the previous section must specify two basic aspects.

(i) The site or sites at which the Cu^{++} is complexed.

(ii) The conformation adopted by the DNA strands.

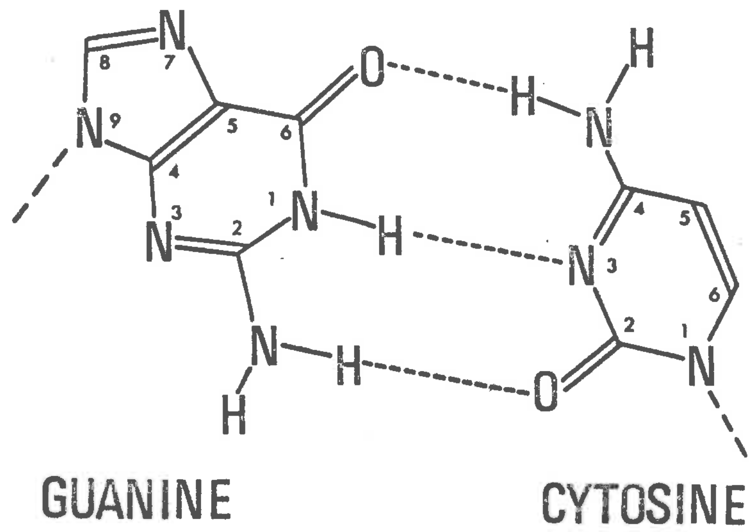
Since the first report of the effect of Cu^{++} ions on the DNA secondary structure, there has been little question that the bases are the site of Cu^{++} binding in this complex. Such an interaction explains, in general terms, the destabilisation of the helix, the lack of hypochromicity on cooling and the hyperchromicity of RNA and partially denatured DNA with Cu^{++} at 25°C.[4] Direct evidence for the Cu^{++}-base complex is the red shift in the UV absorption band of DNA[4,6,16,17,19] and the blue shift in the visible spectrum of Cu^{++}.[6,16] However, there has been considerable controversy over which of the bases and which particular sites on the bases are involved in the complex. In order to solve this problem, many techniques have been applied to determine the effect of Cu^{++} ions on the individual bases, the nucleosides and nucleotides, and also the synthetic polynucleotides.

a. The site of interaction: Mononucleotides

The structure and numbering of the four bases in the tautomeric form in which they occur in DNA are shown in fig. II-3. Uracil, the base which replaces thymine in RNA, has the same structure as thymine but without the C_5 methyl group. Also inosine is often used as a substitute molecule for guanine, as it differs from it only in the absence of the C_4 amino group.

It has been clearly demonstrated[3,17] that there is no proton release accompanying the interaction of Cu^{++} with the bases in DNA, which is in contrast to the behaviour of Hg^{++} and Ag^{+}.[12-15] Therefore, complexes which can be demonstrated between Cu^{++} and mononucleotides, if they are to be applicable to the interaction with DNA, must not involve the release of protons, or if they do, there must be some mechanism whereby the released proton is transferred to some other part of the DNA molecule. It is also known[1,53] that the sugar ring is not directly involved in the DNA-Cu^{++} complex and thus either the ribose or the deoxyribose form of the nucleosides or nucleotides can be used in studies of the Cu^{++} interaction with the monomer compounds.

When Cu^{++} ions were added to the nucleosides or nucleotides, there were only very slight changes in the UV spectra. Hiai[4] reported no change at all but Srivastava[6] found small increases in the extinction coefficients of G, C and A at a copper to base ratio of 100:1. There was no shift in the position of the maximum nor was there any suggestion of a long wavelength shoulder forming on the bands. Also, no changes were observed for the thymine compounds. Small shifts in the maximum



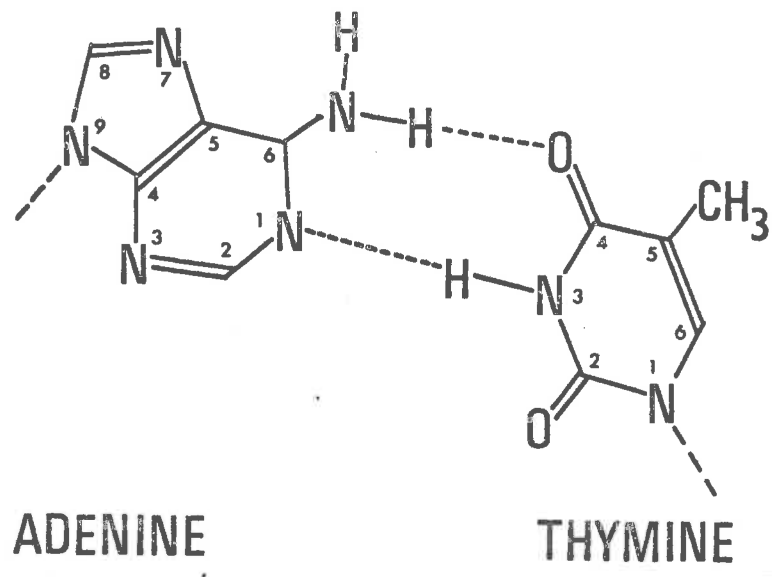


Fig.II-3. The four bases of DNA.

of the 800 nm band of Cu^{++} were observed on addition of G, C and A nucleosides or nucleotides.[6,32] Polarography[6] and EPR[39] also indicated an interaction between Cu^{++} and these species, with the exception, once again, of the thymine compounds. The magnitude of the change in the EPR signal of Cu^{++} varied G > C > A for deoxynucleosides and G > A > C for deoxynucleotides.

Eichhorn et al.[17] performed potentiometric titrations on all the ribonucleosides and concluded that each, including uridine, complexed with Cu^{++}, while Fiskin and Beer,[54] also using a potentiometric method, determined association constants of Cu^{++} with G, C, and A nucleosides. An NMR study[17] in both D_2O and DMSO solvents showed certain lines of the spectra of G, C and A deoxynucleotides broadened in the presence of Cu^{++}, but no change was seen with thymine. However, the infra-red spectra of both A and T deoxynucleosides in D_2O solutions were unaffected by a 20-fold excess of Cu^{++} ions while G and C compounds exhibited marked changes.[34] On the other hand, the early work of Frieden and Alles[1] on the effect of the nucleic acid components on the copper catalysis of ascorbic acid oxidation reported strong inhibition by adenine and guanine compounds but much higher concentrations of C, U and T compounds were required for any inhibition to occur. No complexes between Cu^{++} and cytidine or uridine could be detected by conductometric, potentiometric or spectrophotometric titrations according to Tu and Friederich.[53]

Thus it appears from these studies using different techniques and different conditions that a strong Cu^{++}-guanine interaction exists,

there is no significant interaction with thymine or uracil, and whether or not adenine or cytosine form complexes with Cu^{++} is still uncertain.

By examining the structures for guanine, cytosine and adenine and taking into account the tendency of Cu^{++} ions to complex with a lone pair of electrons of a nitrogen or oxygen atom, the possible sites of interaction can be listed as follows.

(i) The heterocyclic ring nitrogens:

G	N_3, N_7
A	N_1, N_3, N_7
C	N_3

(ii) The amino groups on C_2 of G, on C_6 of A and on C_4 of C.

(iii) The imido site, N_1, of G.

(iv) The keto groups at C_6 of G, and C_2 of C.

Pullman and Pullman[55] who performed molecular orbital calculations on the bases in DNA showed theoretically that the N_7 of guanine is the most basic site of all the bases, and they predicted that this would be the most favoured site of metal chelation, which has been confirmed experimentally.[17,53,54] A clear demonstration[53] of N_7 binding was the absence of any Cu^{++} complex formation with caffeine, a guanine derivative with methylation at N_1, N_3, and N_7, whereas theophylline with only N_1 and N_3 methylation, formed an equimolar complex with Cu^{++}. N_1 or N_3 as possible sites were eliminated by the NMR experiments of Eichhorn, Clark and Becker,[17] as was the involvement of the amino group. The question which remains concerns the participation of the C_6 keto group. The five-membered ring resulting from Cu^{++}

chelation to N_7 and C_6-O, a structure which is commonly observed in coordination chemistry, has caused this complex to be frequently postulated.[1,39,43,56] The only direct evidence for it is the change brought about by Cu^{++} in the IR band associated with the stretching mode of the C_6 keto group.[53,57] Drozdov-Tikhomirov and Kikoin[57] interpreted the change as a shift in the tautomeric equilibrium to more enol form, so that, although the attachment of the proton to N_1 is favoured in the free nucleotide, on complexing with Cu^{++} it is shifted to O_6 (the enol form) enabling the oxygen lone pair of electrons to be coordinated with the Cu^{++}.

Another source of information concerning the nature of the Cu^{++}-guanine interaction is the X-ray diffraction study of cyrstals of a complex formed between guanine and cupric chloride in hot aqueous hydrochloric acid.[58] A binuclear complex was found with the two copper atoms, bridged by chlorine atoms, each bonded to the N_9 of guanine. The C_6-O and the amino group were definitely not involved in the complex. Because the complex was formed in acid solution, N_7 and N_9 were both protonated before addition of Cu^{++} but for some reason the N_9 site was much preferred for Cu^{++} binding. However, when N_9 is blocked, as in the nucleoside and nucleotide, N_7 may become the site of binding, and the C_6-O group may then be in a favourable position to participate in the complex.

The interaction of Cu^{++} and other metal ions with adenine has received considerable attention due to the biological importance of the mono-, di-, and triphosphates of adenosine.[59] For ATP and ADP

a number of studies have presented evidence of a simultaneous interaction of Cu^{++} with one of the phosphate groups and the adenine ring.[60-62] For the free base, Harkins and Frieser[63] reported complex formation with proton release and suggested a chelate between N_7 and the C_6 amino group. The N_7 site was supported by the NMR experiments of Eichhorn, Clark and Becker[17] but no interaction with the amino group could be detected. They also observed a smaller perturbation of the H_2 peak in the NMR spectrum which they thought could be due to a weaker interaction at N_1 or N_3. Weiss and Venner,[64] and later Reinert and Weiss[65,66] suggested N_3 and N_9 (or possibly N_7) as the sites on adenine, but stated that no complex formation could be observed by a potentiometric technique with adenosine or deoxyadenosine.

Thus the situation seems quite confused. N_1 is the most basic site of adenine and Fiskin and Beer[54] have measured a formation constant for a Cu^{++} complex at this site. However it was only a low association constant and little other evidence suggests N_1 as the site for Cu^{++}. N_7 is also a likely site by analogy with guanosine and also by comparison with the Zn^{++}-adenosine complex, which was shown by NMR to involve a chelate to N_7 and C_6 amino group.[67] Interaction at N_7 makes involvement of the amino group in the complex sterically likely, although a chelate involving N_7 and the phosphate group may be more favoured.

The only indications of the site for Cu^{++} binding to cytidine have been from NMR,[17] which supported N_3 and eliminated the amino group, and from Fiskin and Beer's potentiometric titration[54] which again

suggested N_3. Zn^{++} has also been shown to interact at N_3 of cytidine.[67] However it should be recalled that other studies have been unable to detect binding to cytidine.[53]

The most reasonable conclusion to be drawn from the results discussed above for A and C compounds is that Cu^{++} complexes with these ligands are weaker than with guanine and its derivatives, and the site of the interaction and even the detection of the interaction by a particular technique depends on the conditions employed, particularly pH and concentration. Therefore, evidence for the involvement of these bases in the interaction of Cu^{++} with DNA may perhaps be more reliably deduced from studies involving the polynucleotides or DNA itself.

b. Site of interaction: Polynucleotides

There have been only two detailed studies of the interaction of Cu^{++} ions with any of the synthetic polynucleotides. Eichhorn and Tarien[68] have studied the effect of Cu^{++} on the double helices, poly(A + U) and poly(I + C), and Zimmer and Szer[69] have observed the interaction of Cu^{++} ions with poly C and substituted derivatives in both the single-stranded and double-stranded forms.

Prior to these investigations, Hiai[4] had noticed that the melting temperature of the alternating copolymer poly dAT was increased by 10°C in the presence of Cu^{++} ions under the same conditions which were used to observe the marked decrease in T_m of DNA from various sources. Due to the regular alternating sequence of purine and pyrimidine bases in this polynucleotide the hyperchromicity-temperature

profile is completely reversible on cooling, but in the presence of Cu^{++} this behaviour was not observed. The absorbance decreased gradually to a hyperchromicity of about 12% at room temperature. Thus the behaviour of dAT melted in the presence of Cu^{++} was neither typical of DNA denatured with Cu^{++} which displays no decrease in hyperchromicity on cooling, nor typical of dAT in the absence of Cu^{++} in which case the cooling curve is exactly superimposable on the heating curve. The explanation for these results is elusive, but it is most probably related to the lack of interaction of Cu^{++} with thymine. Some interaction with the adenine moieties must have occurred to prevent the full amount of double helix from forming on cooling, but this interaction evidently did not cause a decrease in T_m.

Eichhorn and Tarien,[68] having demonstrated an interaction of Cu^{++} with poly A but not with poly U, proceeded to show that the presence of Cu^{++} in a low ionic strength solution of poly A and poly U prevented the formation of the poly(A + U) double helix, provided that the ratio of Cu^{++} to total polymer phosphate was at least equimolar. Also, addition of Cu^{++} to a preformed poly(A + U) helix caused it to unwind. These workers then observed the interaction between Cu^{++} ions and poly C and poly I, and verified a destabilising effect of Cu^{++} on the poly(I + C) helix similar to that observed for poly(A + U). When the ionic strength of a solution of Cu^{++}, poly I and poly C ($Cu/P \geq 1.0$) which showed maximum hyperchromicity, was increased from 0.005 to 0.1 or to 1.0, there was a decrease in hyperchromicity indicating helix formation. The reaction was not complete however, the

final absorbance being between that expected for separate poly I and poly C at the high ionic strength and that of double-stranded poly(I + C).

Once again the interpretation of these phenomena is not obvious. The case of poly(I + C) bears close resemblance to that of DNA, since the unwinding of the poly(I + C) helix in the presence of Cu^{++} at low ionic strength is equivalent to the lowering of the T_m of DNA by Cu^{++} in low ionic strength. Similarly, the hypochromicity on increasing the ionic strength parallels the renaturation of DNA in high ionic strength. However, it is difficult to explain why the poly(I + C) helix formation does not go to completion under these conditions which cause 100% renaturation of DNA. The destabilisation of the poly(A + U) helix by Cu^{++} does not seem to be in agreement with the previously observed stabilisation of poly dAT by Cu^{++}.[4] In both polynucleotides, Cu^{++} can only interact with the adenine moiety but on the other hand, it must be remembered that the distribution of the two bases in the molecule is entirely different. These observations on poly dAT and poly(A + U) have not at present been confirmed by any other studies, but if they prove to be substantially correct, then it appears that the relative position of the different bases may be a very important factor in the action of Cu^{++}.

Zimmer and Szer[69] studied the effect of Cu^{++} ions on another polynucleotide conformation, the highly ordered single strand with stacked bases, as taken up by poly C in neutral solution. The addition of Cu^{++} to such a solution caused a hyperchromic change with the

difference spectrum exhibiting a maximum at 279 nm. The hyperchromic effect depended on the ionic strength of the solution, decreasing as the ionic strength increased. When the cytosine bases were methylated at the C_5-position, similar results were obtained, but methylation at both C_5 and the C_4 amino group prevented the Cu^{++} ions from exerting any effect on the spectral properties. Unusual results were obtained when the hyperchromicity at the wavelength of maximum extinction was presented as a function of the Cu/P ratio. For poly C, no hyperchromicity was observed until a ratio of Cu/P = 3.5 was obtained, and the maximum change was reached at Cu/P = 10. However, for poly-5MeC hyperchromicity was observed at very low Cu^{++} concentrations and the maximum was reached at approximately Cu/P = 1. An explanation of the different behaviour of the three polynucleotides was not attempted by the authors, nor was any suggestion made concerning the high Cu/P ratio required for any effect of Cu^{++} on the secondary structure of poly C to be observed.

Poly C is known to form a double helical structure in acid solution with a maximum stability at pH 4.5 where half the cytosine moieties are protonated. Zimmer and Szer showed that lowering the pH of a poly C solution caused a decrease in the effect of Cu^{++} as indicated by the difference spectrum. That is, as the double-helical form of poly C was formed, the extent of Cu^{++} interaction was decreased. In fact a direct competition between Cu^{++} ions and protons was demonstrated indicating that the site of protonation, N_3, was also involved in the Cu^{++} complex. As expected, the T_m of the acid form of poly C

was decreased by Cu^{++} ions.

Thus the studies using polynucleotides have strengthened the case for the involvement of A and C in the interaction of Cu^{++} ions with DNA, but have contributed little to the detailed description of the site or sites of binding in the denatured DNA-Cu^{++} complex. However, further research using these model compounds will most probably yield more valuable results.

c. The site of interaction: DNA

As would be expected from the size and complexity of the molecule, studies of Cu^{++} binding using DNA as the substrate do not usually give much information concerning the site of binding. The most profitable approach is to observe some property as a function of the GC content for a number of DNA samples.

The variation of the melting temperature in the presence of Cu^{++} with the GC content has been determined.[4,5] In the absence of Cu^{++}, T_m is a linear function of GC content with a positive slope of the order of 0.5 in 0.005 ionic strength solution.[4,5,70,71] When Cu^{++} is added, there is a marked change in slope to a very small negative slope. Hence there is very little dependence of T_m on GC content, the GC-rich DNAs being slightly less stable than AT-rich. When the decrease in T_m on adding Cu^{++} is calculated, it is clearly seen that the destabilisation is much greater the higher the GC content. This observation has led to the conclusion that the GC pair are involved in the interaction with Cu^{++} ions. It has also been observed

that the spectral changes on denaturing DNA in the presence of Cu^{++} are more marked for GC rich DNAs.[24]

A study by Zimmer and Venner[24] using a DNA of high GC content with 80% of the guanine residues methylated at the N_7 position, indicated that such a DNA sample was much less sensitive to the presence of Cu^{++}. Approximately twice the concentration of Cu^{++} was required to produce the same decrease in T_m, and the spectral changes were decreased.

Thus the prediction from the studies on the individual nucleosides that the N_7 position of guanine is a favourable site for Cu^{++} interaction appears to be fulfilled in the case of DNA itself. However the role of the adenine and cytosine bases remains uncertain.

d. The conformation of the strands

The UV spectrum is sensitive to changes in the conformation of the DNA strands to the extent to which such changes affect the interaction of the bases and hence their hypochromicity, but care must be taken in interpreting the spectral changes brought about by metal ions. For example, when mercuric ions are added to native DNA, pronounced spectral changes occur which could be taken to indicate marked disorientation of the bases.[12] However, Hg(II) causes considerable modification of the spectra of the individual bases,[72] and so two possible sources of the spectral change in the case of DNA must be considered; the effect of Hg(II) on each base, and the change in the relative positions of the bases. Since Hg(II) is believed to form a

bridge between each complementary base pair, the change in base conformation may not be very great and may make only a small contribution to the spectral change.

For Cu^{++} ions, however, only slight changes in the UV spectra of the bases were observed in the form of a small increase in extinction throughout the band.[6] Such changes alone could not account for the difference between the spectrum of the Cu^{++}-denatured DNA complex and the spectrum of native DNA. It seems that in this case the disordering of the bases is the major source of the spectral change. In fact, if a comparison is made between the spectrum of thermally denatured DNA, in which the bases are completely disordered, and the spectrum of the Cu^{++}-denatured DNA complex, only a small difference is observed,[4,6] small enough to be accounted for by the effect of Cu^{++} on the individual bases. Thus in the absence of any evidence to the contrary it appears that the bases of the Cu^{++}-denatured DNA complex are so disordered as to have lost all hypochromic interaction between them, as is the case for thermally denatured DNA.

The specific viscosity of the denatured DNA-Cu^{++} solution is very low compared to that of native DNA[4] indicating that the extended rigid structure of native DNA is collapsed and the molecule is in a much more flexible form. Similarly, light scattering measurements[4] have shown that the complex has a much reduced radius of gyration and is therefore more collapsed. From these considerations it seems that the conformation of the strands in the complex is typical of DNA denatured in the absence of Cu^{++}. However measurement of the

sedimentation coefficient has indicated the presence of a complicating factor, that of aggregation.

Srivastava[6] showed that at the temperature at which the optical density started to increase, the sedimentation coefficient also began to rise, and at a temperature of 50°C where the hyperchromicity is a maximum, the sedimentation coefficient had increased by a factor of four. Another report[3] also stated that the sedimentation coefficient was increased 5- to 6-fold on heating to 55°C in the presence of Cu^{++}. This phenomenon was not studied in further detail in either investigation because the aim was to show the return of the sedimentation coefficient to the native value on dissociation of the complex. The effect of the relative and absolute concentrations of Cu^{++} and DNA on the sedimentation behaviour would be of considerable interest because the suggested explanation for such a pronounced increase in the sedimentation coefficient is the occurrence of aggregation. Whether the aggregation occurs through Cu^{++} bridging between phosphate groups or between base groups of different DNA molecules, and why it is observed to such an extent at these low DNA concentrations are questions that remain to be answered.

In a number of Eichhorn's papers[3,17,19] he has referred to the DNA strands as being unwound in the presence of Cu^{++} and has stated[19] that this does not necessarily imply strand separation. However, when the unwinding of a double helix is pictured, it is most usual to think of it proceeding to separated strands. The description "unwound without strand separation" seems to necessitate a mechanical joining of the complementary strands. Whether the complementary strands are

completely separated, or joined at a single point or at many points along their length, are considerations which will have great bearing on the explanation of the renaturability of this complex. Thus, it seems that the local conformation of the DNA chains in the Cu^{++}-denatured DNA complex is similar to that of thermally denatured DNA, but the conformation of the whole macromolecule, particularly as it relates to the aggregation and renaturation phenomena, remains to be determined.

5. The renaturation of the denatured DNA-Cu^{++} complex

Hiai,[4] and Eichhorn and Clark[3] were the first to notice that the denatured DNA-Cu^{++} complex formed under the conditions discussed in section II.3 appeared to renature on increasing the ionic strength ten- or twenty-fold. Hiai stated that the hyperchromicity was completely quenched within one minute in 0.2 M NaCl, but required about ten minutes for completion in 0.15 M NaCl. If the excess electrolyte was added at 55°C the same behaviour was observed even if the period of heating the complex at 55°C was eight hours. Similarly, Eichhorn and Clark observed that changing the ionic strength from 0.005 to 0.1 resulted in complete loss of hyperchromicity in five hours. Both groups have established that an ionic strength of 0.04 is required for partial reversal to be observed, and 0.1 is sufficient to bring about total reversal. Other methods which have been found[4] to bring about the same decrease in hyperchromicity are addition of the metal ion chelating agent, EDTA, and removal of the Cu^{++} ions from the solution by dialysis.

The solution which, after treatment as above, had the same absorbance as native DNA was shown to contain native DNA by a number of methods. The reduced viscosity[4] and the sedimentation coefficient,[3] both markedly changed in the denatured DNA-Cu^{++} complex, returned to their native values. For this supposedly renatured material, the elution pattern from a methylated albumin kieselguhr column,[4] and the buoyant density in a CsCl gradient[3] were identical to the corresponding observations on native DNA. When the renatured DNA was mixed with native DNA they sedimented at a single peak in the CsCl density gradient.[3] The optical rotation at 436 nm returned to the value of native DNA[3] and when the Cu^{++} ions and excess electrolyte were removed by dialysis, the resulting solution exhibited a melting transition profile superimposable on that obtained with native DNA at the low ionic strength without Cu^{++} ions.[4] Conclusive evidence was the retention of the original bacterial transforming activity after the processes of denaturation and renaturation.[4]

Thus it appears that when the denatured DNA-Cu^{++} complex is made to dissociate by either increasing the ionic strength, or removing the Cu^{++} ions by dialysis or chelation, rapid renaturation follows giving DNA which cannot be distinguished from native DNA by any of the techniques discussed above. It should be noted that these observations were made on Calf Thymus DNA[4] and on a commercial sample of DNA from an unspecified source.[3]

a. Comparison with renaturation of DNA in the absence of Cu^{++} ions

It was observed by Doty et al.[73] that when bacterial DNA was cooled after denaturation, the secondary structure that resulted was dependent on the conditions employed. Rapid cooling produced single strands with some random base interactions and short imperfect helical regions giving a decrease in hyperchromicity. However slow cooling in high ionic strength solution (> 0.1) produced a considerable amount of pairing between complementary strands in the form of long stretches of double helix. The presence of substantial amounts of native structure were shown by the sharp melting profile on reheating,[73] by the retention of a large degree of the original transforming activity,[74] by immunological assay,[75] and by electron microscopy.[73]

This renaturation on slow cooling can be more systematically studied by maintaining the denatured solution at a single temperature, the annealing temperature, between T_m and room temperature, and this method has been used by a number to determine the optimum conditions for the reaction and to elucidate its mechanism.

The dependence of the rate of renaturation upon the annealing temperature was found to be a bell-shaped curve with a broad maximum at 25°C below the melting temperature of the DNA sample at the particular ionic strength used.[76,77,78] The rate was very dependent on ionic strength below 0.4 but above this it showed only a gradual increase with increasing ionic strength.[77,78,79] It was recognised from the earliest report of this phenomenon[73] that the renaturation would depend strongly on the heterogeneity of the DNA sample as this would affect

the rate at which complementary strands separated on denaturation could recombine. In accordance with this, some observed that the very heterogeneous DNA from mammalian sources could not be renatured at all,[73,76,80] but Subirana and Doty[81] reported a very slow renaturation of Calf Thymus DNA resulting in 25% native DNA after twenty-four hours annealing. More recently, other workers[82] have followed this reaction for periods up to 4,000 minutes and have observed considerable renaturation in 1.0 M $NaClO_4$ at 70°C. The renaturation of the less heterogeneous DNAs derived from various bacterial sources has been well studied[76-81] as has the very rapid reaction for the homogeneous bacteriophage DNAs.[77,78,79,81] Investigators[79] who followed the renaturation of E. Coli DNA and T_7 bacteriophage DNA under comparable conditions observed that the rate was 100 times faster for the bacteriophage DNA than for the bacterial DNA, while others[78] who compared T_4 DNA with E. Coli DNA noted a 20- to 25-fold rate increase. The extent of the renaturation of bacterial or viral DNA was generally observed to be 100% within experimental error if optimum conditions were employed,[77,79,83] although long periods of annealing were required for complete reaction. The renaturation rate was also found to decrease when the average chain length of a DNA sample was decreased.

Cavalieri et al.[80] in 1962 determined the kinetic order of the renaturation reaction to be first order, and consequently concluded that there was no strand separation in denatured DNA. However since then there has been ample evidence that the reaction follows second order kinetics. With DNA from different sources, including mammalian,[82]

and under different solution conditions, workers have used various methods of denaturation and of bringing the solution to the annealing temperature and have employed different analyses of the data, but second order kinetics have been consistently observed for a considerable extent of the reaction.[77,78,79,81,82] As the reaction rate was found to decrease when the solution viscosity was increased by sucrose, glycerol, or ethylene glycol,[77,78,79,81] it was proposed that the reaction mechanism involved the diffusion together of separated strands to form a nucleus of base pairs between them as the rate-determining step, followed by a rapid "zippering up" process to form the hydrogen-bonded double helix. Wetmur and Davidson[78] placed a different interpretation on the experimental data, and suggested that the bimolecular nucleation step was rate limiting but that the "zippering up" reaction was the hydrodynamically limited process. Whichever mechanism is correct, it is well established that the renaturation by annealing involves the recombination of separated strands.

b. Hypotheses to explain renaturability

As the previous section indicates, the renaturation by annealing at elevated temperatures in high ionic strength solution is much slower than the renaturation of DNA denatured in the presence of Cu^{++} ions which is brought about at low temperatures in moderate ionic strength solution. This is true for bacterial and viral DNA and an even greater difference in both rate and extent is apparent for mammalian DNA. Thus those who observed the renaturation of the denatured DNA-Cu^{++} complex have proposed that in this case, the diffusion together

of separated strands is not involved, but that in the complex the Cu^{++} ions by some means hold the complementary strands in register.[3,4,6] When the Cu^{++} is removed from the complex, "zippering up" of the strands can proceed rapidly.

Suggestions concerning how the Cu^{++} ions hold the strands in register have been made, but such proposals are only speculative as the available experimental evidence gives little insight concerning this. The most obvious suggestion is that the Cu^{++} ions form bridges by chelating between two bases, one on either strand. This may involve each base pair, although this is unlikely because of the lack of any demonstration of a Cu^{++} complex with thymine. All the GC pairs may be involved or just a few Cu^{++} bridges along the length of the molecule may be present, either between GC pairs or between two non-complementary bases. Eichhorn and Clark[3] have proposed a Cu^{++} bridge between N_7 and O_6 of guanine and N_3 of cytosine. Such a complex could not form between the two bases in their relative orientation in the native state as the guanine N_7 is situated in the groove pointing away from the cytosine. The hydrogen bonds between the bases would have to be broken so that they could twist around into a suitable conformation to form such a complex. The difficulty with the base-bridging hypothesis is that there has been no experimental evidence of a complex of this type. Zn^{++} ions have been shown[67] to bridge between adenosine and cytidine, and between adenosine and guanosine, but similar complexes for the case of Cu^{++} have not been observed thus far. Another suggested explanation for the ready renaturation is that the Cu^{++} ions, instead

of bridging, interact with each strand in such a way as to place some kind of topological restraint on the strands so that they are unable to twist around each other and completely separate.

Thus it is evident that a detailed study of the renaturation reaction is required, with a view to ascertaining its mechanism and, if possible, the structure of the Cu^{++} complex.

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CHAPTER III

THE RENATURATION OF THE DENATURED DNA-Cu^{++} COMPLEX BY INCREASING THE IONIC STRENGTH: SPECTROPHOTOMETRIC RESULTS

1. Introduction

2. Experimental Procedure

   a. Solutions
   b. Denaturation
   c. Renaturation

3. Renaturation in 0.15 M KNO_3 at 25°C

   a. Effect of slow cooling the denatured solution
   b. Kinetic Analysis
   c. Accuracy and reproducibility
   d. Effect of DNA and Cu^{++} concentrations

4. Renaturation in 0.10 M KNO_3 at 25°C

5. Effect of the time of standing on the denatured DNA-Cu^{++} solution

   a. Viscosity measurements on the denatured DNA-Cu^{++} solution

6. Effect of temperature on the renaturation rate

   a. 35°C
   b. 15°C
   c. 5°C
   d. Arrhenius Plot

7. Effect of solution viscosity on the renaturation rate

   a. Calculation of viscosity of sucrose solutions
   b. Preparation of solutions
   c. Results

References

1. Introduction

Preliminary experiments on the renaturation of DNA denatured in the presence of Cu^{++} ions brought about by increasing the ionic strength were carried out prior to this investigation.[1] The most pertinent information from that initial work was that the rate of renaturation decreased on increasing the time the denatured solution was allowed to stand before commencing the renaturation. This finding has since been confirmed by Richard and Pacault,[2] who, in a report on the effect of ionic strength on this reaction, stated that the renaturation rate depended on the time interval between denaturation and renaturation. However no details of their observations were presented. This time effect is quite unrelated to that reported by Bryan and Frieden[3] who found a dependence of the native DNA spectrum on the time in the presence of Cu^{++}.

In this chapter, the results of the kinetic studies of this renaturation reaction under various conditions, followed by the change in absorbance at 259 nm are presented.

2. Experimental Procedure

The detailed description of the materials, apparatus, and procedures used is presented in Chapter VII. However, the basic procedure for the denaturation and renaturation reactions is outlined here to facilitate the discussion of the results which follows.

a. Solutions

All DNA and $Cu(NO_3)_2$ solutions were prepared in 10^{-2} M KNO_3.

The concentrations of the solutions and the volumes involved in the mixing procedures were calculated to give final DNA and Cu^{++} concentrations of 5.00×10^{-5} M_p and 1.00×10^{-4} M, respectively. In all reactions, unless otherwise stated in the text, the ratio of Cu^{++} to DNA phosphate was 2:1, and the ionic strength at which the renaturation was performed was 0.15.

The DNA solution in 10^{-2} M KNO_3 had a melting temperature of 76°C, while at a Cu/P ratio of 2.0, T_m decreased to 41.6°C.

b. Denaturation

A sample of DNA solution was weighed into a small stoppered flask, which was thermostatted at 55°C for ten minutes. A small quantity of concentrated $Cu(NO_3)_2$ solution was then delivered using a 250 µl Beckman polythene micropipette. This solution was maintained at 55°C for four minutes, the maximum hyperchromicity being reached within the first minute of this period.[1] The solution was shock cooled in ice-water until the temperature was within ½°C of that at which the renaturation was to be performed, at which point the flask was placed in a water bath at the renaturation temperature. The cooling of the solution from 55°C was accomplished in 30 seconds for a final temperature of 35°C and in approximately 100 seconds for a final temperature of 5°C. The time at which the denatured solution first reached the renaturation temperature was taken as zero time, and all subsequent time intervals were measured relative to this point.

c. Renaturation

From the thermostatted denatured DNA-Cu^{++} solution, samples

were withdrawn for renaturation at various time intervals. Generally four to six samples were renatured from each denatured solution prepared. When a renaturation reaction was scheduled to commence at a certain time, τ, twenty minutes before this time 2.3 to 2.4 mls of solution were withdrawn from the flask into a clean dry cuvette and weighed. The cuvette was placed in the spectrophotometer and the absorbance of the solution at 259 nm determined. At (τ - 1) minutes, the cuvette was removed from the cell compartment, and a small quantity of 1.0 M KNO_3 was layered at the bottom of the cuvette using an Agla micrometer syringe. At time τ, a stopwatch was activated and the contents of the cuvette rapidly stirred for 2-3 seconds. The cuvette was stoppered, replaced in the cell compartment, and the absorbance change with time at 259 nm followed by a Gilford Model 2000 automatic absorbance recorder. The first absorbance reading was obtained 9-15 seconds after commencement of the reaction, the correspondence between the time parameter of the recorder output and the actual zero time for the reaction being indicated by the stopwatch. The absorbance was followed until no further change could be detected. The temperature during the reaction was maintained to ± 0.2°C by water circulating around the cell compartment.

3. Renaturation in 0.15 M KNO_3 at 25°C

A typical set of renaturation curves obtained from a denatured solution after various times of standing at 25°C prior to commencing the renaturation by increasing the ionic strength to 0.15 is shown in

Fig. III-1. Renaturation at 25°C in 0.15 M KNO_3.

Time of standing at 25°C between denaturation and renaturation:

(1) 30 mins.
(2) 500 mins.
(3) 1000 mins.
(4) 2000 mins.
(5) 2750 mins.

DNA concentration = 5.00×10^{-5} M_P;
Cu/P = 2.

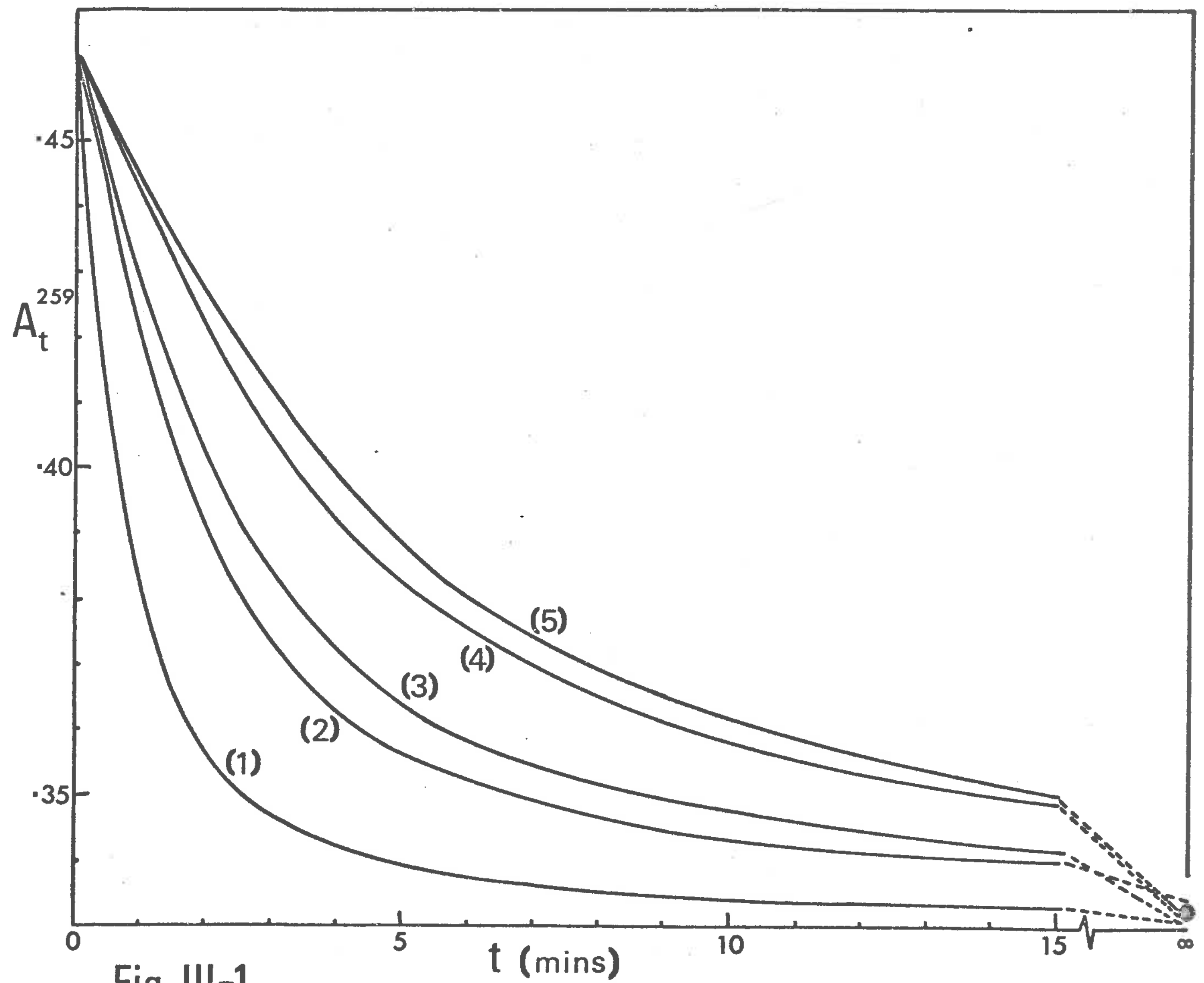

Fig. III-1.

fig. III-1. It is noted that the curves all have the same initial point, A_0, which indicates that the absorbance of the denatured solution was constant during the interval between denaturation and renaturation. Also, the final state of the reaction, A_∞, is the same for all curves. Hence, the extent of renaturation was not affected by the time of standing. For all reactions studied the total change, $(A_0 - A_\infty)$, was 0.132 ± 0.003 O.D. units, which is equivalent to the loss of $40 \pm 1\%$ hyperchromicity, indicating that complete renaturation was occurring.

a. Effect of slow cooling the denatured solution

Several experiments were performed in which the Cu^{++}-DNA solution at 55°C was slowly cooled to 25°C at a uniform rate of 1°C per 25 minutes instead of shock cooling in ice. Fig. III-2 presents four renaturation curves obtained in this way. Curves B, C, and D were obtained on samples of a solution which had been slow cooled over 750 minutes and then renatured immediately (B), or thermostatted further at 25°C for a total time after denaturation of 1500 minutes (C), or 3000 minutes (D). The results did not show good reproducibility, for when another solution was slow cooled in an identical manner, curve A was obtained under the conditions which correspond to curve B. However, it is quite clear that slow cooling gives rise to a much reduced renaturation rate.

b. Kinetic Analysis

The normal first and second order plots of the data in fig. III-1 are given in fig. III-3 and fig. III-4. The second order plots

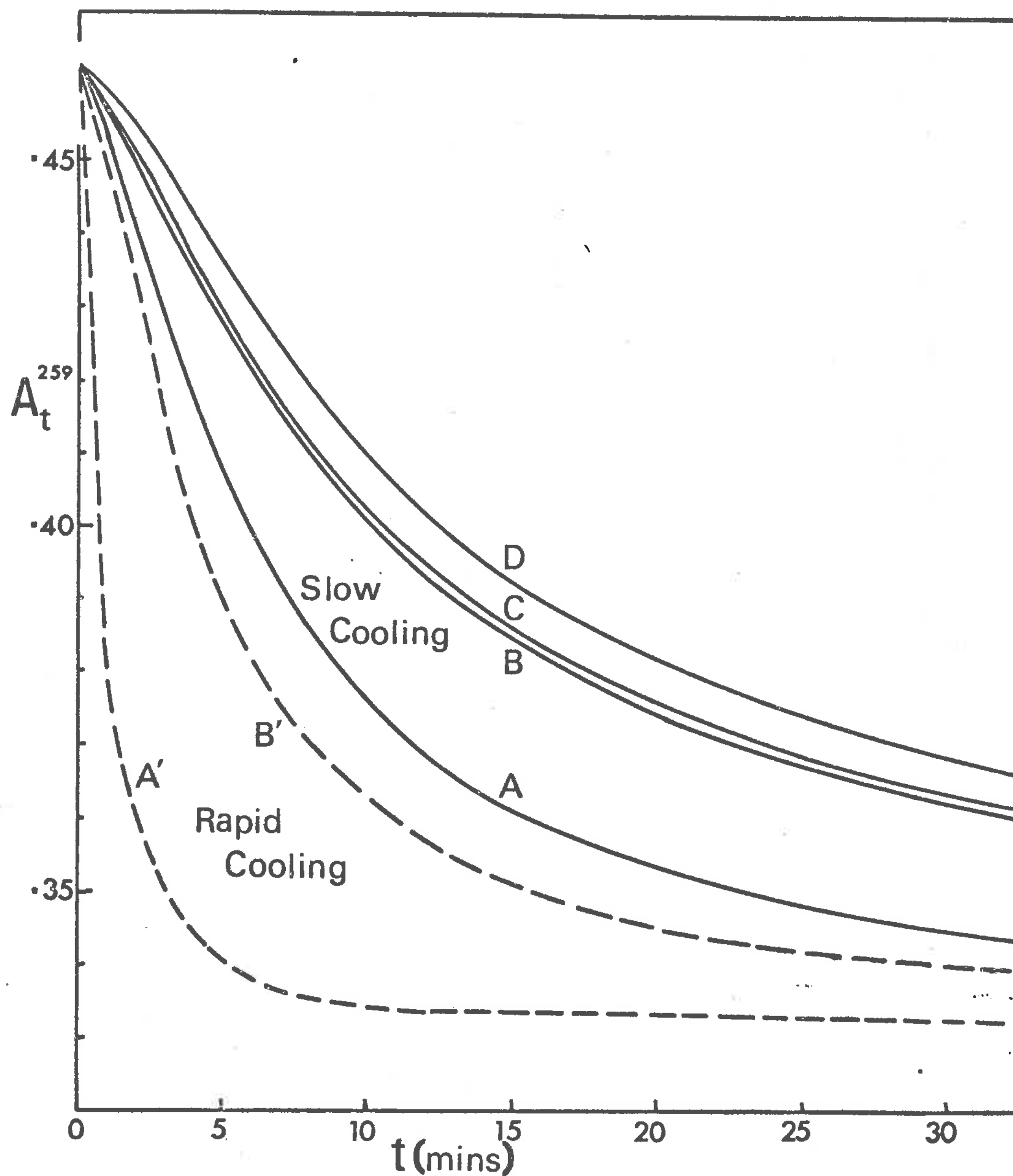

Fig. III-2. The effect of slow cooling to 25°C.

A',B': Correspond to (1) and (5) of fig. III-1.

A,B: Slow cooling for 750 minutes.

C,D: Maintained at 25°C after 750 minutes of slow cooling. Total time after denaturation = 1500 minutes and 3000 minutes, respectively.

See text, section III.3.a, for details.

KNO_3 concentration = 0.15 M;

DNA concentration = 5.00×10^{-5} M_p; Cu/P = 2.

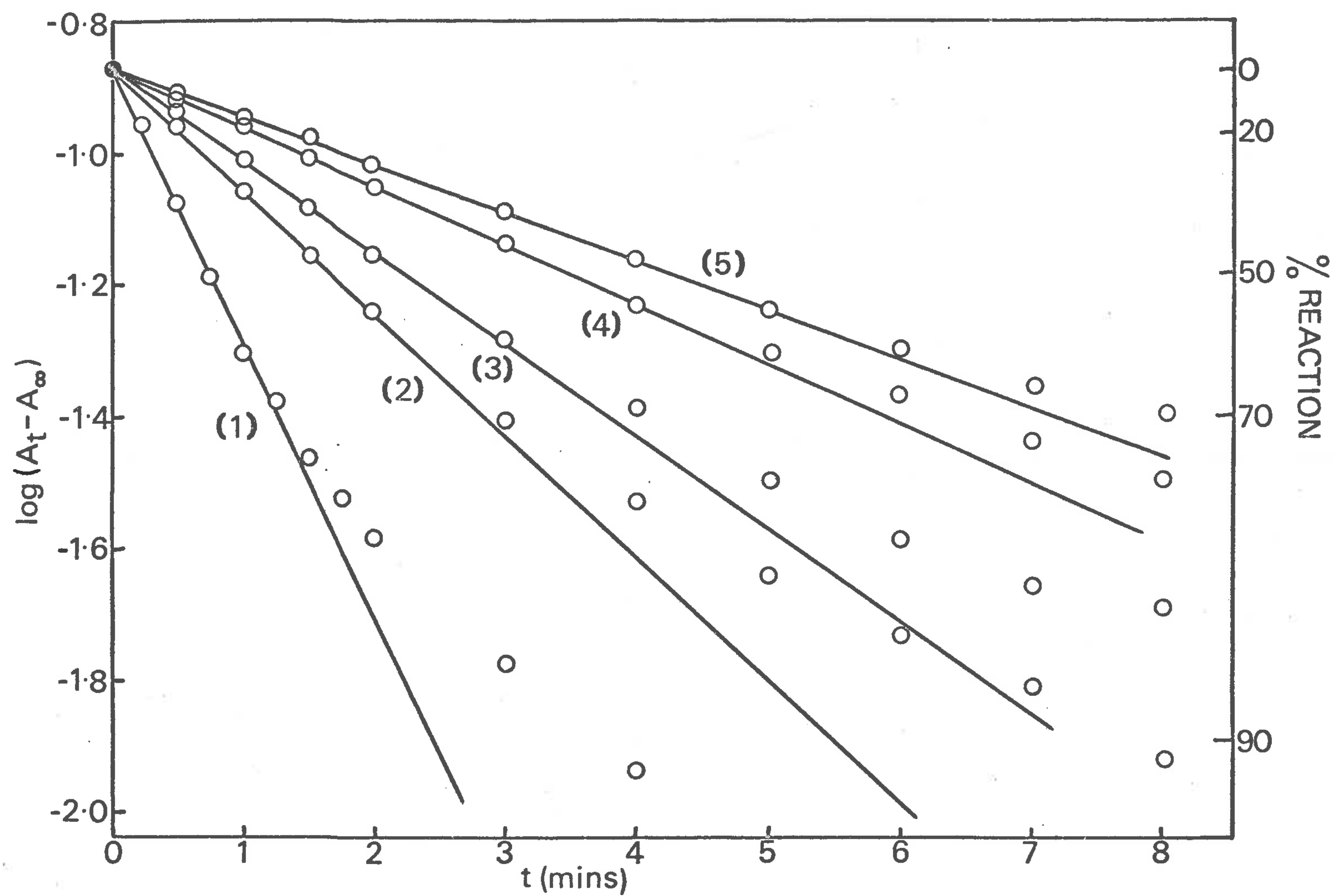

Fig. III-3. First order analysis of data of fig. III-1, 25°C, 0.15 M KNO_3.

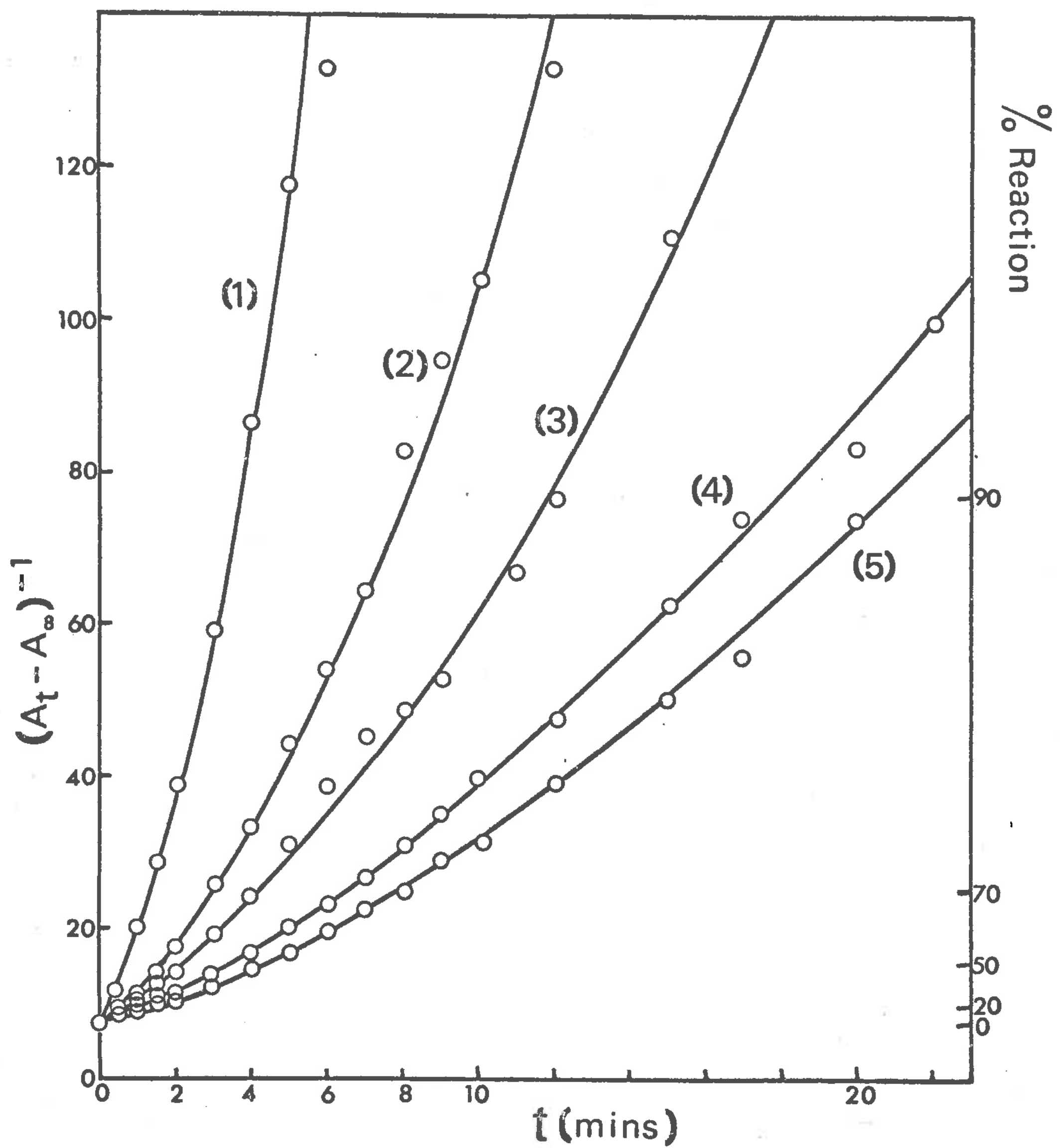

Fig. III-4. Second order analysis of data of fig. III-1, 25°C, 0.15 M KNO_3.

are obviously curved, but the first order plots show an initial straight line portion. Standard least squares analysis applied to the linear region gave a value for the slope and hence the rate constant, k, which shall be referred to as an apparent first order rate constant. The least squares method was not used in every case as it was found that the graphical line of best fit had a slope which rarely differed from the least squares value by more than 1%. The standard deviation of the points from the straight line was very small, of the order of 0.006.

The extent to which the reaction followed first order kinetics can be seen in fig. III-3 to be 60%-70%. This was generally the case for all the reactions observed under these conditions, the only exceptions being the reactions after slow cooling where the extent to which the reaction followed first order kinetics was less.

The variation of the apparent first order rate constant, k, with the time of standing at 25°C between denaturation and renaturation (τ) is shown in fig. III-5.

c. Accuracy and reproducibility

Fig. III-5 indicates that the apparent first order rate constants obtained at a particular time of standing showed a considerable scatter. For example, the seven values at $\tau = 30$ ranged from $15.85 \times 10^{-3}\ sec^{-1}$ to $20.65 \times 10^{-3}\ sec^{-1}$, a variation of approximately 25%. Fig. III-6 illustrates the difference between the experimental renaturation curves which gave rise to these varied values of k. Thus the factors affecting the accuracy and reproducibility of the results will now be considered.

(k x10^3) sec^-1

20

10

0

0 1000 2000 3000

T (mins) = TIME OF STANDING

○ Dilution 1
● Dilution 2
◐ Dilution 3
⊖ Dilution 4

Fig. III-5. Apparent first order rate constant, k, against time of standing at 25°C. KNO_3 concentration = 0.15 M; DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

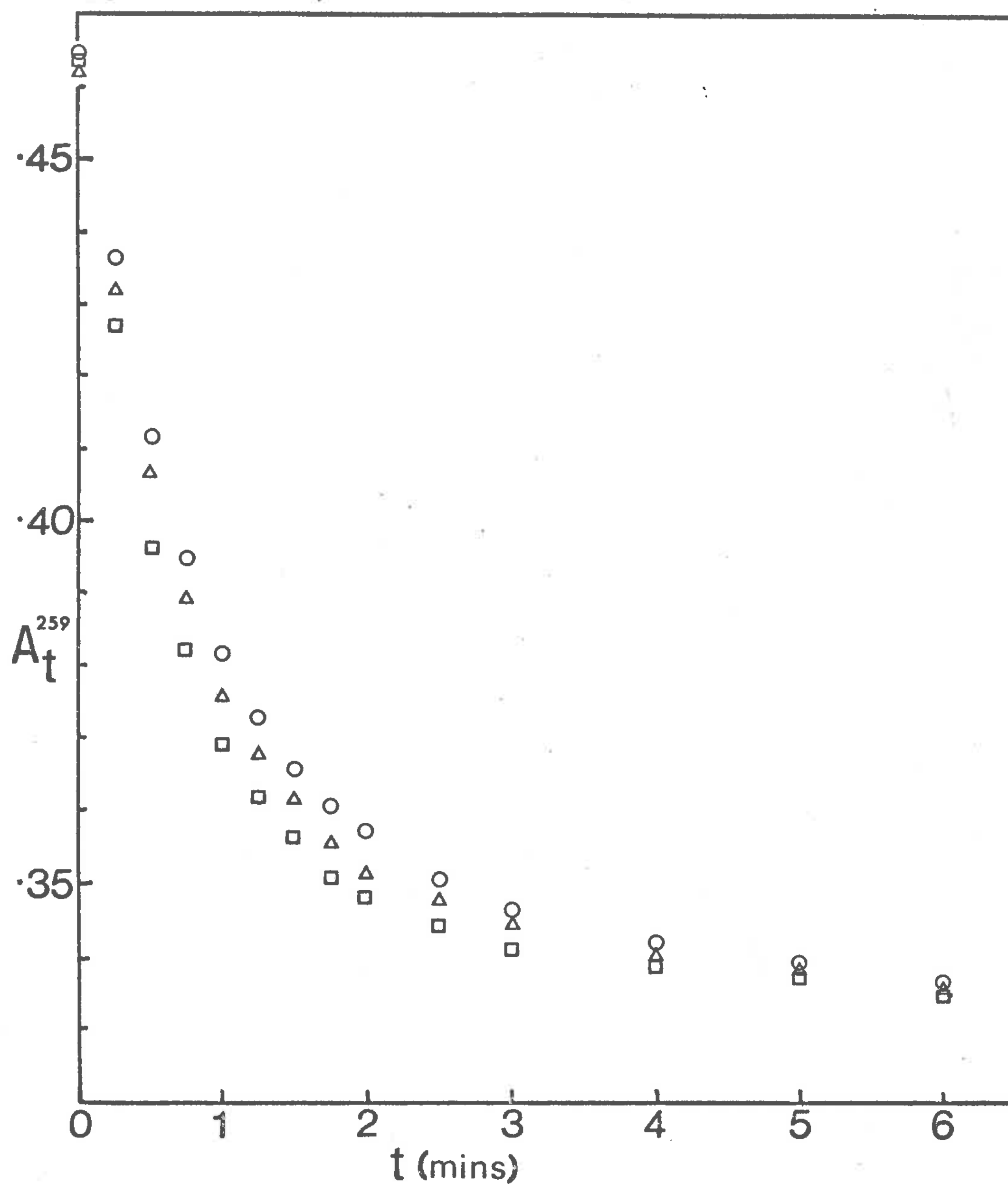

Fig. III-6. Comparison of three renaturation curves from different denatured solutions, each obtained after 30 minutes of standing at 25°C.
Corresponding k values are 15.8×10^{-3} sec^{-1} (○),
17.89×10^{-3} sec^{-1} (△),
and 20.65×10^{-3} sec^{-1} (□).
Conditions as in fig. III-1.

Within a renaturation curve the uncertainty in the relative values of A_t was very low, always within the limits of $\pm$ 0.001, because the instrumental output was very stable and any occasional instrumental noise was easily recognised and a smooth curve drawn through such regions. The value of A_0, obtained by extrapolating back from the first reading at t = 9 to 15 seconds, was subject to a similar uncertainty in the case of the slower reactions but the extrapolation was much more inaccurate for the rapid reactions. In such instances, the value of A_0 could be estimated by two possible methods. Firstly, it could be calculated from the absorbance of the denatured DNA-Cu^{++} solution measured before renaturation, by adding to this the increase in O.D. on going from 0.01 M KNO_3 to 0.15 M KNO_3 and reducing by the dilution factor for the 1.0 M KNO_3 addition. The A_0 value thus calculated had a possible error of 0.005. Secondly, when slower reactions were recorded for the same denatured solution, the value of A_0 obtained by the small extrapolation in these cases could be applied directly to the fast reactions, since it was known that the time of standing did not affect the initial absorbance. In cases where both these methods of calculating A_0 could be applied, the agreement between the values was satisfactory within the error limits. The value of A_0 was not essential to the kinetic analysis of the data and errors in it would not significantly affect the value of k.

On the other hand, the value of A_∞ was critical to the analysis of the data. For the rapid reactions where the final value was realised quickly, the error was only $\pm$ 0.001, but for the slower reactions which

had to be followed for long periods of time, the difficulty of distinguishing between the reaction still proceeding and slow base line drift increased the uncertainty to $\pm$ 0.002. However, while a change of 0.004 in A_∞ markedly changed the shape of the first order plot once the reaction had exceeded 70%, it had only a very slight effect on $\log(A_t - A_\infty)$ for 0%-50% reaction, and thus affected the slope of the first order plot in only the third decimal place. This is clearly insignificant compared to the variation of k in fig. III-5.

In support of the above discussion, it was found when least squares analysis was applied to the first order plot, $\log(A_t - A_\infty)$ against t, the standard deviation of the points from the straight line and the standard error in the slope were very low. Taking one particular set of data for an example, the standard deviation of the experimental points was 0.006, which is equivalent to an error in $(A_t - A_\infty)$ of 0.001 or 0.002. This is of the order of the instrumental uncertainty in A_t and A_∞ as mentioned above. The standard error of the slope was 0.003, giving rise to an uncertainty in k of 1.7% for this particular reaction. Thus, there was only a small error in the value of k derived from each renaturation reaction, but when the reproducibility of k over a number of reactions was tested, the accuracy of the concentrations of the components in the reaction mixture became an important factor.

On comparing a family of curves obtained using the same denatured DNA-Cu^{++} solution, the source of concentration inaccuracy is the addition of 1.0 M KNO_3. The calibration of the micrometer syringe used showed that it delivered the quantity of 1.0 M KNO_3 required

(approx. 0.4 gms) to an accuracy of ± 0.0005 gms (0.25%). The weight of DNA-Cu^{++} solution to which it was added was correct to better than ± 0.0005 gms (0.04%). The final weight of solution, of the order of 2.7 gms, was accurate to ± 0.001 gms (0.07%). Hence the uncertainty in DNA and Cu^{++} concentrations due to dilution by the addition of 1.0 M KNO_3 was 0.1%, and the uncertainty in the final KNO_3 concentration was 0.3%.

The reproducibility of reactions performed on different denatured solutions is dependent on the accuracy of the denaturation technique. To add the concentrated $Cu(NO_3)_2$ solution to the hot DNA solution, a Beckman polythene 250 µl micropipette was used, the calibration of which showed an accuracy of ± 0.0001 gms. However, there was reason to believe that the accuracy decreased from this value because on repeated use, small droplets were seen to adhere to the polythene capillary during delivery. Hence an error of ± 0.001 gms was assigned to this apparatus (0.8%). Since the volume of DNA into which the Cu^{++} solution was delivered was determined by weighing, it would be expected that negligible error was associated with this volume, but in fact there were two sources of error. Firstly, there was evaporation from the DNA solution when the stopper was removed from the flask at 55°C to permit the addition of the Cu^{++} solution. Although the flask was only open for 30-40 seconds, there was a considerable surface area to facilitate evaporation, for on heating the contents of the flask from room temperature to 55°C, droplets of solution condensate formed on the upper surface of the flask and on the stopper. Also, the chloroform which

saturated the stored DNA solution at 4°C was completely driven out of the solution at 55°C, and, on removing the stopper, escaped from the flask. Some of the chloroform present at 4°C was lost from the solution during the room temperature weighing, but some remained contributing to the weight of DNA solution at 25°C but absent when the Cu^{++} was added at 55°C. Thus the volume of DNA to which the small amount of Cu^{++} solution was added was less than the volume required to give the correct DNA and Cu^{++} concentrations. The error involved is difficult to estimate but since the total volume was of the order of 12 mls, 1% would be the upper limit to the error, and perhaps 0.5% would be a more realistic estimate. The 0.8% error in the delivery of the required volume of Cu^{++} solution as discussed above, was also in the direction of decreasing the volume, and so these two errors would tend to compensate each other.

The different stock solutions used to prepare the reaction mixture for each run also contributed to the reproducibility of the results. For all the experiments performed to obtain the points of fig. III-5, the same stock $Cu(NO_3)_2$ solution was used, and so no uncertainty was introduced here. However, four different DNA solutions were employed. These DNA solutions were each prepared by diluting a concentrated stock solution of DNA at various times during the seven month period spanned by the results of fig. III-5. Each diluted DNA solution was required to have an absorbance at 259 nm of 0.407 so that after addition of Cu^{++} and KNO_3 its final concentration would be 5.00×10^{-5} M_P. However each individual O.D. reading was subject to an error of $\pm$ 0.002 which introduced a possible error of 1% in the DNA concentration.

That such a lack of reproducibility between DNA solutions was not significant can be seen in fig. III-5 where the maximum and minimum k values at $\tau = 30$ were both obtained on the same DNA solution. A fresh 1.0 M KNO_3 solution was prepared for each series of renaturations on a denatured solution. The uncertainty of 0.4% in concentration contributed to the error in the final KNO_3 concentration.

To recapitulate the discussion of this section, the possible errors in the concentrations of the three components of the reaction mixture are summarised.

(i) DNA: Error in the initial concentration of the different solutions used was 1%. Error on adding Cu^{++} solution to denature was approximately 1%. No significant error occurred on adding 1.0 M KNO_3.

(ii) Cu^{++}: The only significant source of error was the process of addition of concentrated $Cu(NO_3)_2$ solution to bring about denaturation. This was approximately 1%.

(iii) KNO_3: Error in the different 1.0 M KNO_3 solutions used was 0.4%. Error in the final KNO_3 concentration when 1.0 M KNO_3 was added to 0.01 M KNO_3 was 0.3%.

It is not possible to estimate the effect of such concentration errors on the value of k because, although the dependence of the renaturation rate on the Cu^{++} concentration and the ionic strength has been examined briefly (sections II.3.d and II.4), there is not sufficient data for a quantitative relationship between k and these parameters to be derived. It may be that, of the errors listed above, the one which

most significantly affects k is the uncertainty in KNO_3 concentration. However it seems unlikely that these concentration errors alone can account for the scatter of the values observed in fig. III-5. It is considered that this scatter arises instead from the past history of each solution sample from the point at which the Cu^{++} is added to the DNA to the point at which the concentrated KNO_3 is added to commence the renaturation.

It is known[2] that the temperature of denaturation and the time the solution is at this temperature affect the renaturation rate. It has been established that the rate of cooling from this temperature to the renaturation temperature influences the renaturation rate (section III.3.a), as does the time interval between denaturation and renaturation and also the temperature of the denatured solution in this interval (section III.6). The effect of the time of standing is of course accounted for by representing k as a function of τ as in fig. III-5, and although an attempt was made to keep all the other factors constant by rigidly following the same experimental procedure for each reaction, it is possible that small differences between experiments occurred. For example, the time the DNA-Cu^{++} solution was at 55°C was always 4 minutes, but due to the finite time required for addition of the Cu^{++}, this time was only accurate to 10 or 15 seconds. Small fluctuations in the temperature of the 55°C water bath or minor differences in the rate of cooling from 55°C to 25°C might have occurred. Also temperature fluctuations during the weighing of the sample into the cuvette or during the addition of 1.0 M KNO_3 to

the cuvette might have contributed to the experimental error. The effect of such factors on k cannot be quantitatively estimated, but it is considered that they contribute significantly to the 20-25% scatter shown in fig. III-5.

Although all DNA solutions were stored at 4°C in the presence of chloroform, on occasions a deterioration in such solutions was observed. Solutions which had given satisfactory results for several months suddenly produced slow irreproducible renaturation reactions, the rates of which were less than half those previously observed. Therefore, frequent checks were made to test that the DNA solution used in any experiment had not deteriorated in any way by verifying that the renaturation rate at 25°C, in 0.15 M KNO_3, at Cu/P = 2 and at τ = 30 minutes and τ = 500 minutes was in agreement with the data shown in fig. III-5.

d. The effect of DNA and Cu^{++} concentrations

The effect of DNA and Cu^{++} concentrations on the renaturation rate was not systematically studied. The only observation made was that when the Cu/P ratio was decreased to 1:1 at a constant DNA concentration, the reaction rate significantly increased.

4. Renaturation in 0.10 M KNO_3 at 25°C

To study the effect of ionic strength on the reaction rate, the renaturation was carried out in 0.10 M KNO_3 as well as 0.15 M KNO_3. The ionic strength of the denatured solution was raised to 0.10 using a solution of 0.667 M KNO_3 instead of the 1.0 M KNO_3 solution employed when a final ionic strength of 0.15 was required. In this way, the

volume of concentrated KNO_3 solution added to the DNA-Cu^{++} solution in the cuvette, and hence the dilution factor, was the same for both final ionic strengths. A typical set of results is presented in fig. III-7, and the kinetic analysis in fig. III-8. The rate constants for the various times of standing at 25°C are shown in fig. III-9 in comparison with the rate constants in 0.15 ionic strength.

5. Effect of the time of standing on the denatured DNA-Cu^{++} solution

The relation between the first order rate constant and the time of standing as illustrated in fig. III-5 does not appear to be able to be represented by a simple mathematical relationship. Neither log k nor k^{-1} against τ are linear plots, and the log k versus log τ plot is only approximately linear with a slope of the order of -0.4.

In order to suggest an explanation for the dependence of k on τ, a search was made for some property of the solution which changed during this period prior to renaturation. It had previously been shown[1] that there was no change in the UV spectrum of the denatured DNA-Cu^{++} solution in the 24 hour period following denaturation. An attempt was also made[1] to see whether the environment of the Cu^{++} ion altered sufficiently during this period to cause a change in the visible absorption band. However, to observe the Cu^{++} visible spectrum, even in a cell of 10 cm path length, required an increase of Cu^{++} and DNA concentrations, and on denaturing such a solution a precipitate was formed precluding observation of the spectrum.

Measurement of the hydrodynamic properties of the solution

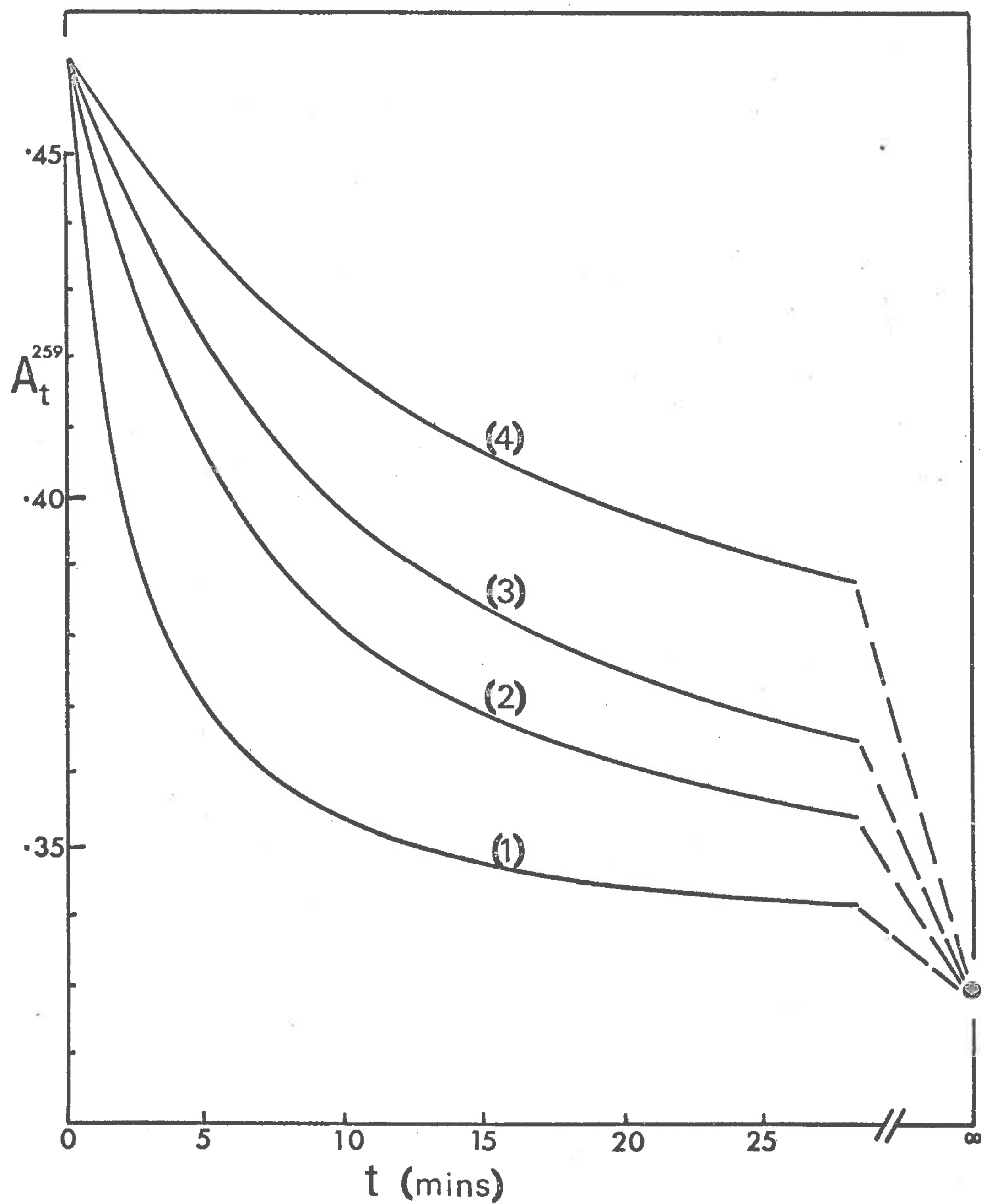

Fig. III-7. Renaturation in 0.10 M KNO_3 at 25°C.
Time of standing after denaturation:
(1) 30 mins.
(2) 500 mins.
(3) 1000 mins.
(4) 1500 mins.
DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

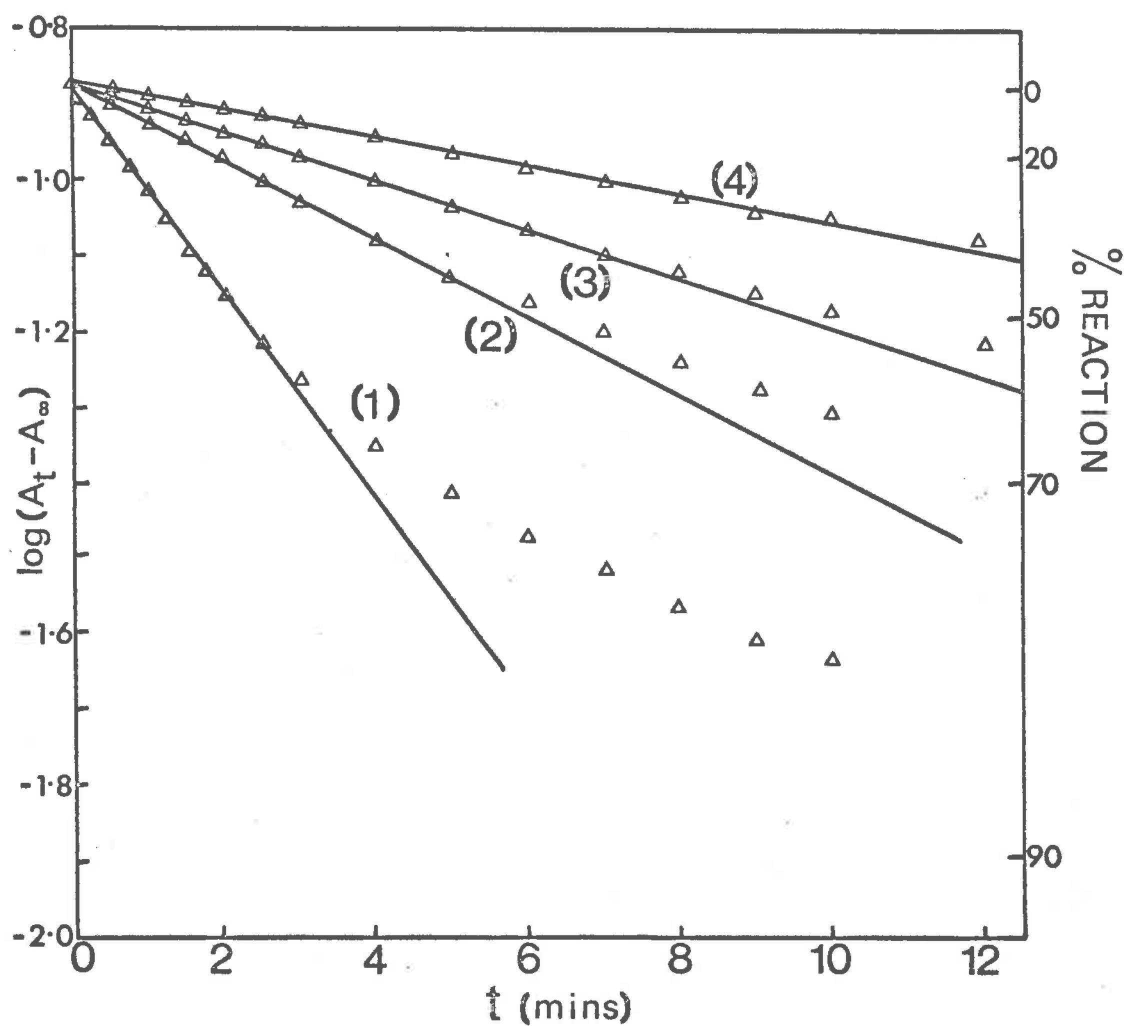

Fig. III-8. First order analysis of data of fig. III-7, in 0.10 M KNO_3, at 25°C.

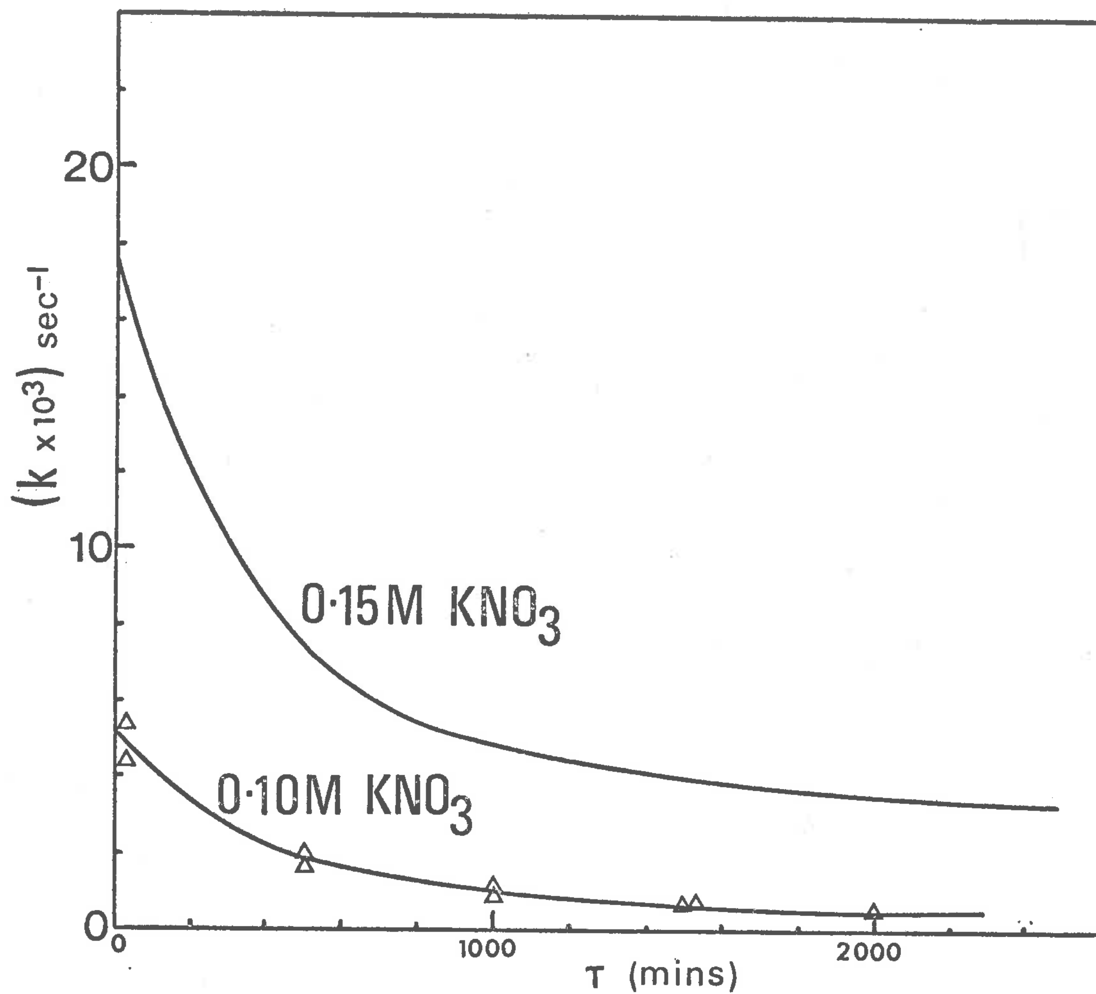

Fig. III-9. Apparent first order rate constant, k, in 0.10 M KNO_3 at 25°C, against time of standing. Curve for k against τ at 0.15 M KNO_3 from fig. III-5 included for comparison. DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

was considered valuable as this may detect some conformational change of the DNA during this period. Previous workers had reported[4,5] that the sedimentation coefficient of the denatured DNA-Cu^{++} solution was very high and somewhat variable, and so it was considered very unlikely that this technique would be sensitive to small conformational changes. Hence, it was decided to use viscosity measurements as the probe for such changes.

a. Viscosity measurements on the denatured DNA-Cu^{++} solution

The denatured DNA-Cu^{++} solution was prepared in the normal way and 2 mls placed in an Ubbelohde low shear viscometer in a 25°C water bath. The flow time through the capillary was then determined at intervals of approximately 10 minutes over a period of 8 to 12 hours. It can be seen from fig. III-5 that the renaturation rate of this solution changed markedly during this time period. Although the stopwatch used measured the flow time to 0.01 second, the variation among a series of flow times of distilled water determined over several hours was often as high as 0.3 seconds (0.15%). Factors which affected the reproducibility of the measurements were the personal reaction time involved in starting and stopping the stopwatch as the meniscus passed the marks on the capillary tube, the attaining of temperature equilibrium of the solution which was slow because of the thick glass walls of the viscometer, the clarity of the solutions and the cleanliness of the apparatus which affected markedly the draining of the liquid from the capillary and bulb walls.

No significant change in the viscosity of the denatured DNA-

Cu^{++} solution could be detected during the 12 hours after denaturation, for the flow times showed only a random scatter of the same order as that observed in the case of distilled water. The viscosity of the denatured DNA-Cu^{++} solution was very low, close to that of solvent alone. In fact, the average flow time for the solution was slightly higher than the average solvent flow time, but the difference was within the 0.3 second uncertainty of the measurements and so not significant. When the DNA concentration was doubled, the expected marked increase in the native DNA viscosity was observed, but the denatured solution exhibited such a small increase that its flow time was still within the 0.3 second uncertainty. Even at this higher DNA concentration, no significant change in viscosity with time could be found.

Hence, if conformational changes of the DNA occur during the time of standing after denaturation, they are not sufficiently large to be detected by viscosity measurements.

6. Effect of temperature on the renaturation rate

a. 35°C

A typical set of renaturation curves obtained for the reaction at 35°C is shown in fig. III-10, and the first order plots of this data are illustrated in fig. III-11. The shape of the plots is very similar to that observed at 25°C, although the extent to which the reaction followed first order kinetics appears on the average to be slightly less, namely 50-60% instead of 60-70%. The values of the rate constants obtained are compared in fig. III-12 with those at 25°C.

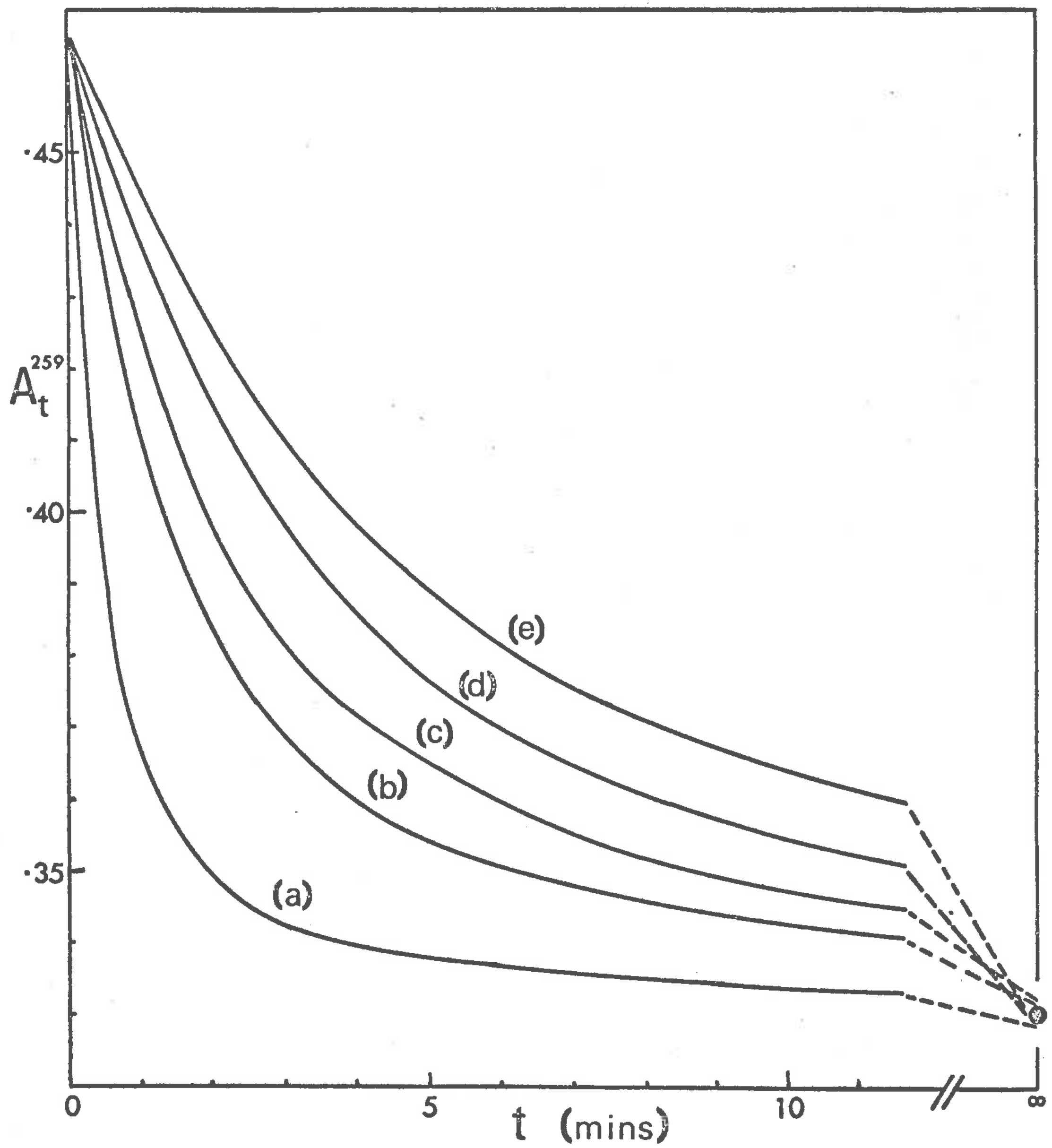

Fig. III-10. Renaturation at 35°C in 0.15 M KNO_3.

Time of standing: (a) 30 mins.
(b) 500 mins.
(c) 1100 mins.
(d) 1850 mins.
(e) 2520 mins.

DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

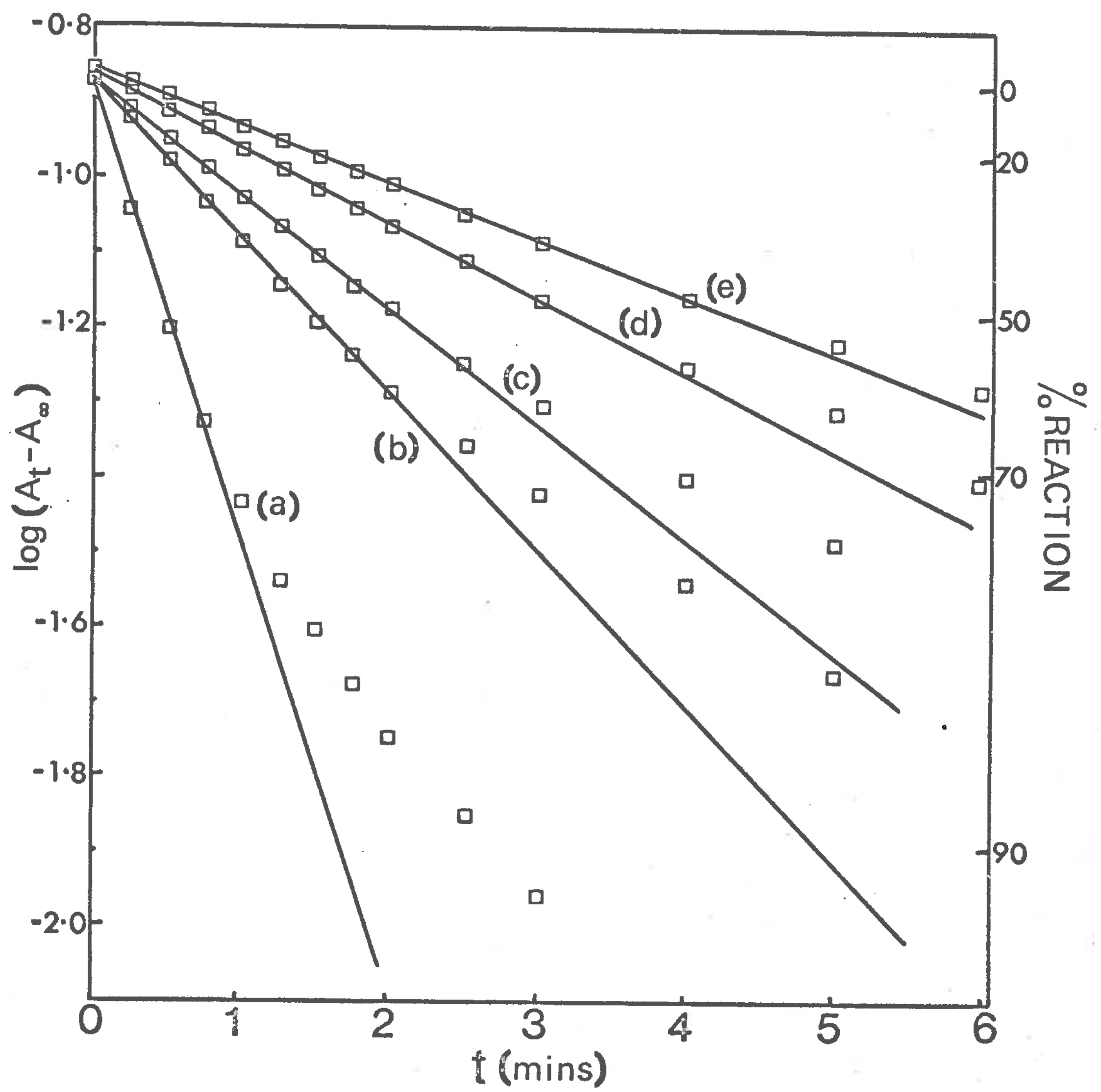

Fig. III-11. First order analysis of data of fig. III-10, at 35°C, 0.15 M KNO_3.

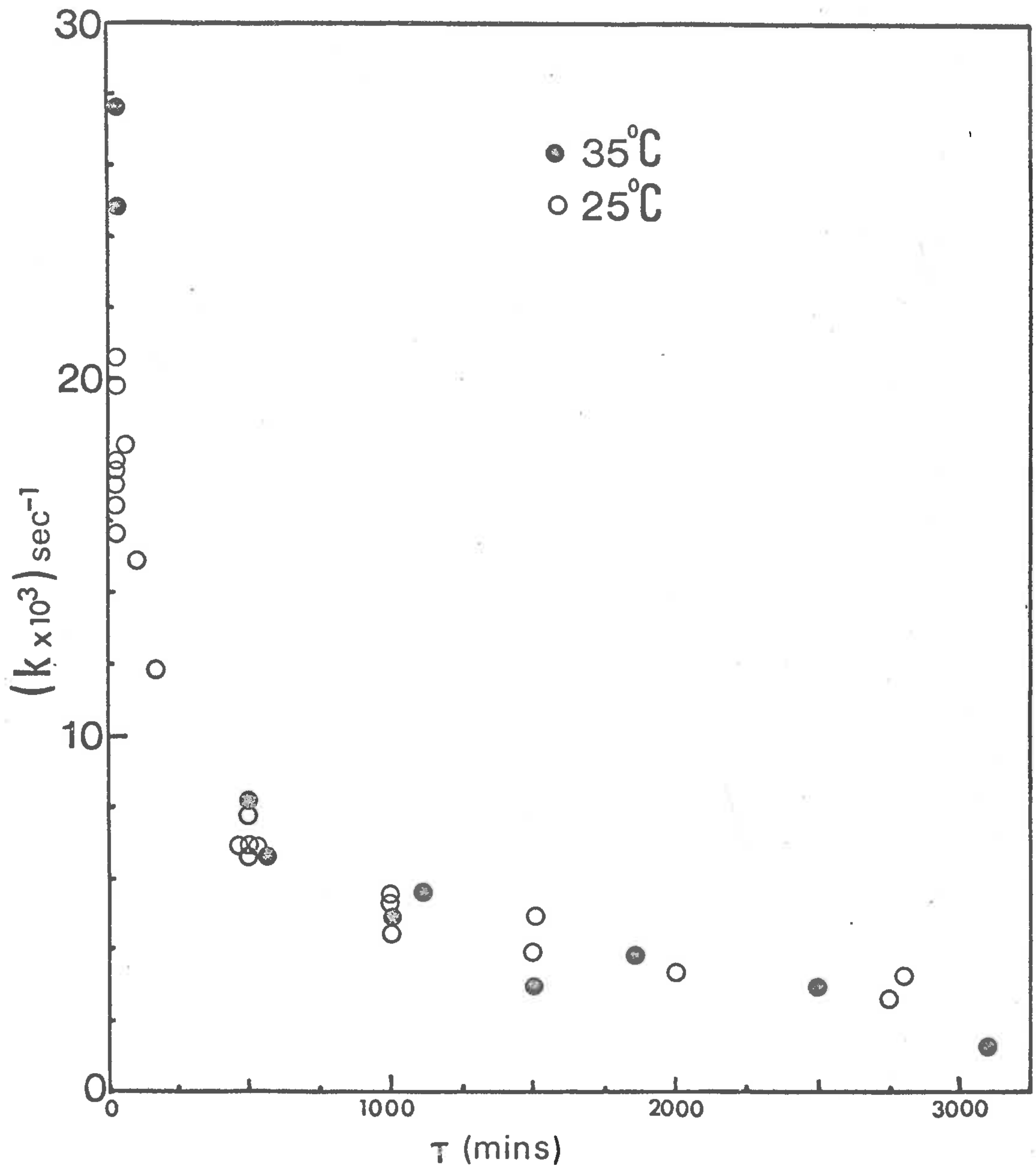

Fig. III-12. Comparison between apparent first order rate constants, k, obtained at 35°C and 25°C.
KNO_3 concentration = 0.15 M;
DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

It can be seen that after short times of standing (30 minutes) the reaction at 35°C was much faster, but after 500 minutes, the rate constants at the two temperatures are not significantly different.

b. 15°C

Fig. III-13 and fig. III-14 show the data obtained at 15°C and the first order analysis, respectively. In this case the renaturation followed first order kinetics for 70-75% reaction which is slightly longer than at 25°C.

c. 5°C

To carry out the renaturation at 5°C some modifications in the experimental procedure were required. The method of maintaining the cell compartment at 5°C and of keeping the cells free from condensation is described in Chapter VII. The denaturation was performed at 55°C as previously, the solution cooled rapidly in ice to 5°C and then placed in the bath supplying the water at 2°C-3°C for circulation around the spectrophotometer cell compartment. To place an aliquot of the solution into a cuvette, the normal procedure of weighing could not be used as this was too slow, allowing the solution to warm, and also the condensation forming on the cell during the weighing introduced errors. Instead, it was necessary to use the less accurate method of delivering 2.5 mls with a 5 ml graduated pipette. Although this pipette was calibrated to an accuracy of 0.3%, its reproducibility under actual conditions was somewhat less than this due to the speed at which the operation had to be carried out and the difficulty of satisfactorily

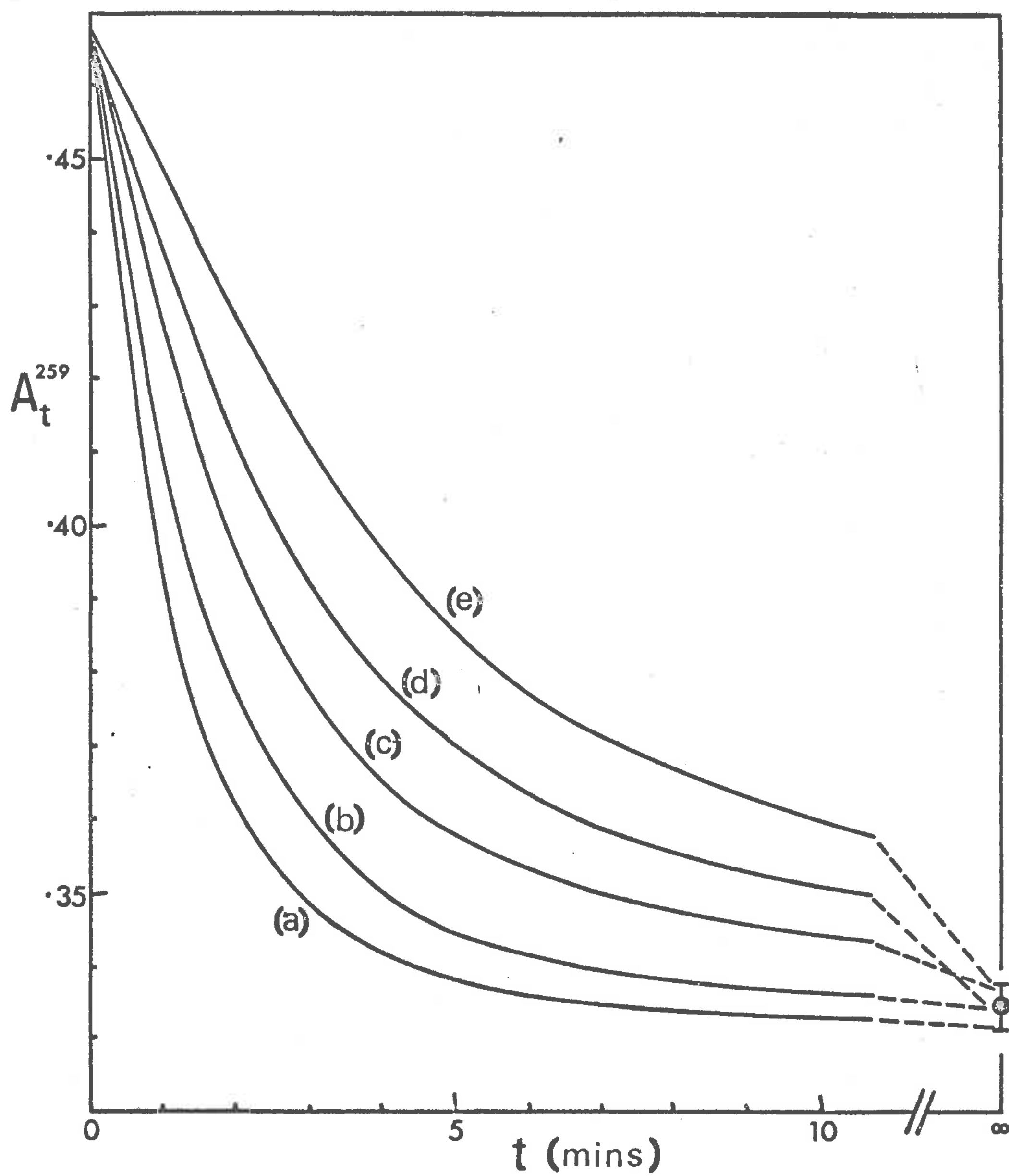

Fig. III-13. Renaturation at 15°C in 0.15 M KNO_3.

Time of standing: (a) 30 mins.
(b) 500 mins.
(c) 1070 mins.
(d) 1840 mins.
(e) 2500 mins.

DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

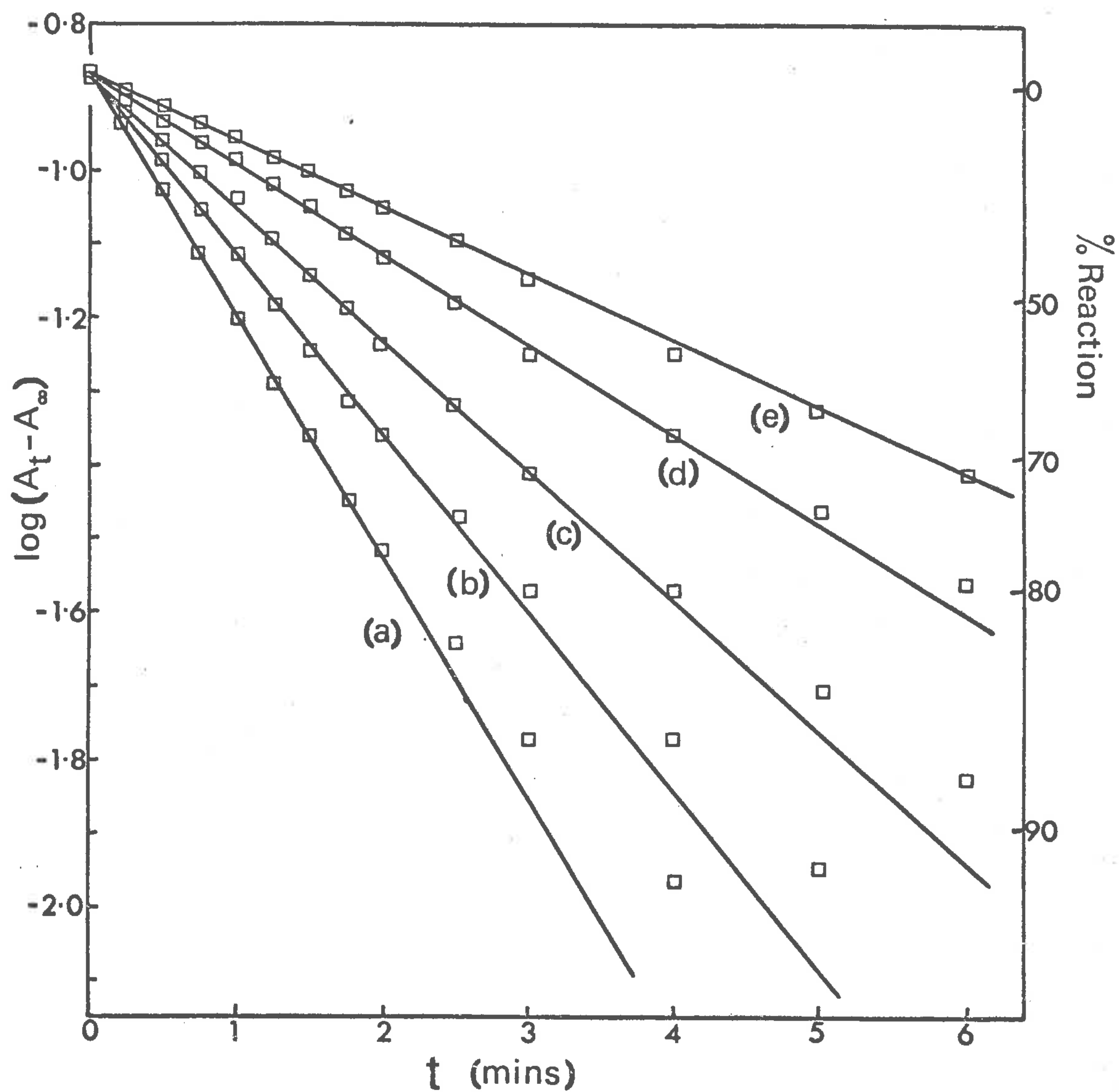

Fig. III-14. First order analysis of data of fig. III-13, at 15°C, 0.15 M KNO_3.

draining the pipette into the cuvette.

The addition of the 1.0 M KNO_3 solution to commence the renaturation was carried out without removing the cuvette from the cell compartment as done previously to avoid an increase in the temperature of the cuvette or any condensation forming on the optical surfaces. The 1.0 M KNO_3 was at room temperature for the addition as it was considered that the small volume of warmer solution would not cause a significant temperature change. The amount of the 1.0 M KNO_3 required was calculated taking into account the differences in solution densities at 5°C and 20°C (room temperature). The accuracy of the final ionic strength brought about by this modified technique was tested on several blank runs by measuring the O.D. (259) increase which accompanies the 0.01 M KNO_3 to 0.15 M KNO_3 change; the reproducibility was only 3%.

A set of renaturation curves at 5°C is shown in fig. III-15. It can be seen that the variation between the curves bears no relation to the time of standing, and is due only to the experimental error which was greater in this situation than in the higher temperature experiments due to three factors. The temperature of the cell compartment was less constant (5.0 ± 0.5°C) than during the higher temperature runs; the temperature fluctuations between denaturation and renaturation during the necessary manipulations of the solution were greater at 5°C than at higher temperatures; the concentration of the components was less accurate, as discussed above and as clearly shown by the range of A_0 values in fig. III-15.

Thus it can be concluded that at 5°C the rate of renaturation

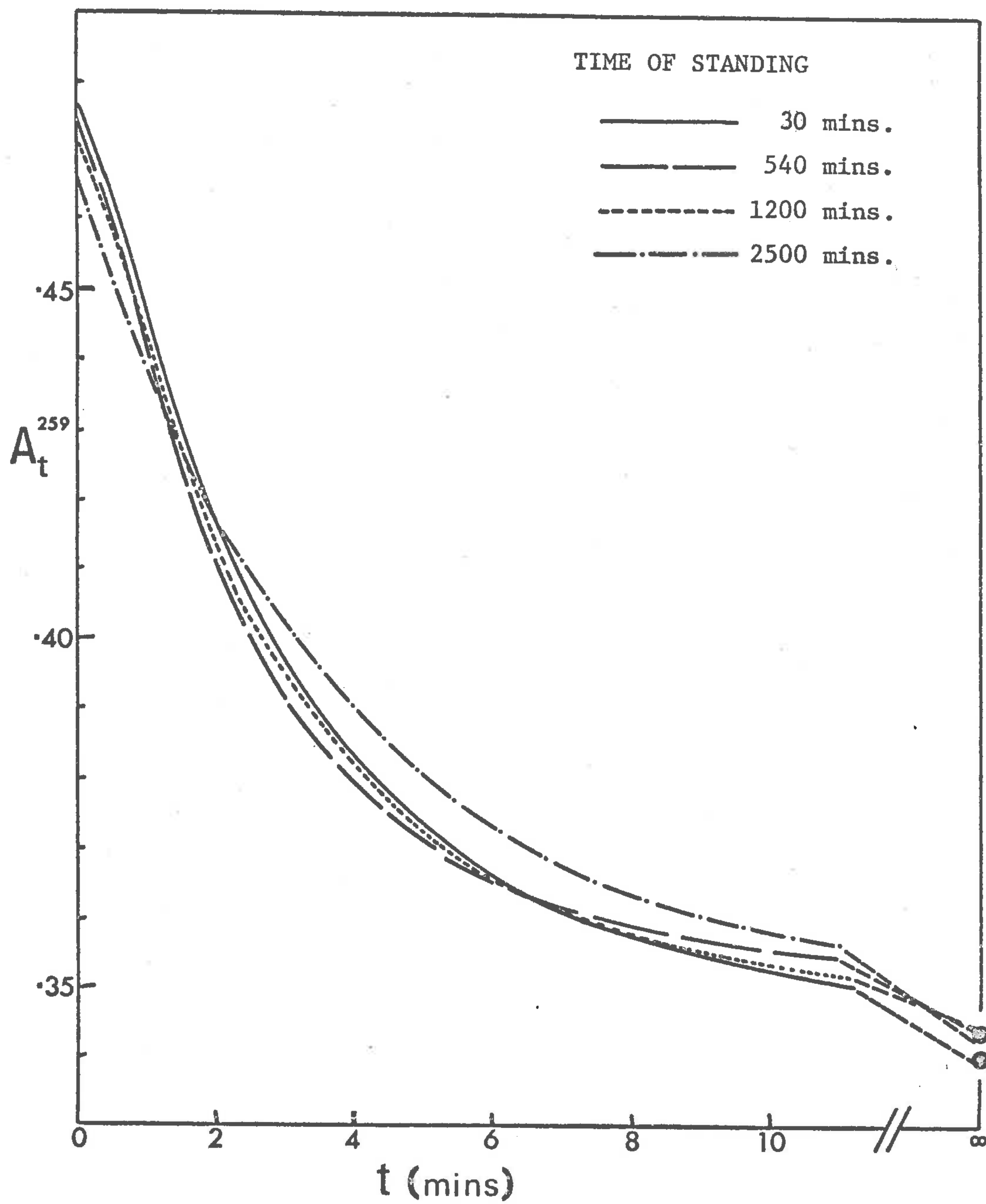

Fig. III-15. Renaturation at 5°C in 0.15 M KNO_3. Time of standing at T < 5°C as indicated.
DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

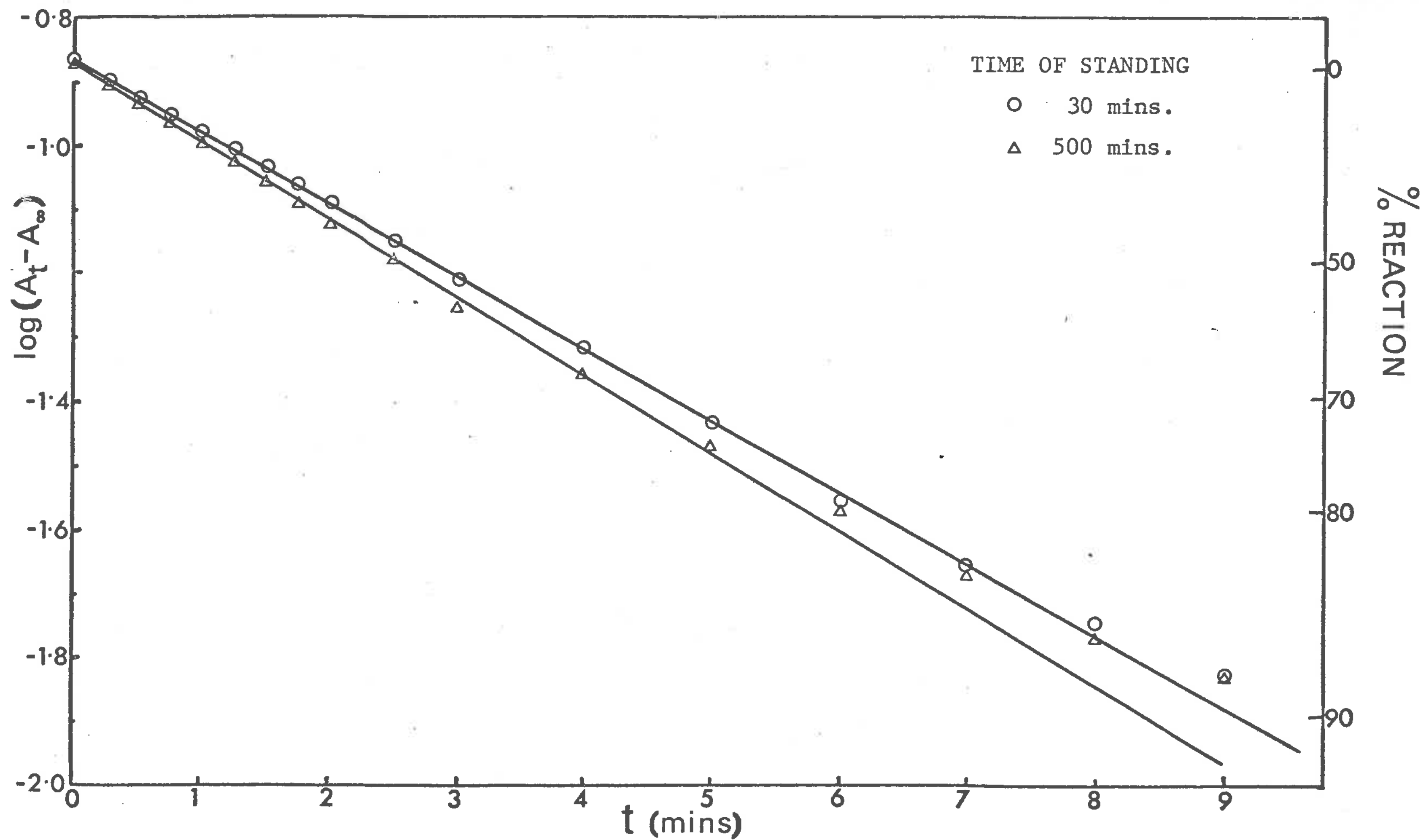

Fig. III-16. First order analysis of renaturation curves at 5°C. KNO_3 concentration = 0.15 M; DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

is independent of the time of standing between denaturation and renaturation. Actually the more correct interpretation of the results is that when the solution is maintained at 2°C–3°C there is no dependence of the subsequent renaturation rate at 5°C on the time of standing. Since the bath in which the solution was thermostatted had to be at 2°C–3°C to maintain the temperature inside the cell compartment at 5°C, this discrepancy could not be avoided. These results are supported by the previous observation[1] that the time the denatured solution was allowed to stand in ice had no effect on the renaturation rate at 25°C. Hence, whatever process is responsible for the time dependence of the renaturation rate, it does not occur below 5°C.

Fig. III-16 shows two typical first order plots of reactions at 5°C. The renaturation follows first order kinetics for 75%–85% of the reaction, which agrees with the trend of an increasing first order extent with decreasing renaturation temperature.

The apparent first order rate constants at 5°C, 15°C, and 25°C are compared in fig. III-17.

d. Arrhenius Plot

The rate constants obtained at a time of standing, $\tau = 30$ minutes for the four temperatures between 5°C and 35°C are presented as an Arrhenius plot in fig. III-18. The points are approximately linear, the slope being close to -2×10^3 which indicates an activation energy of the order of 10 kcals/mole. The significance of such a plot is somewhat doubtful because of the dependence of k on the time of

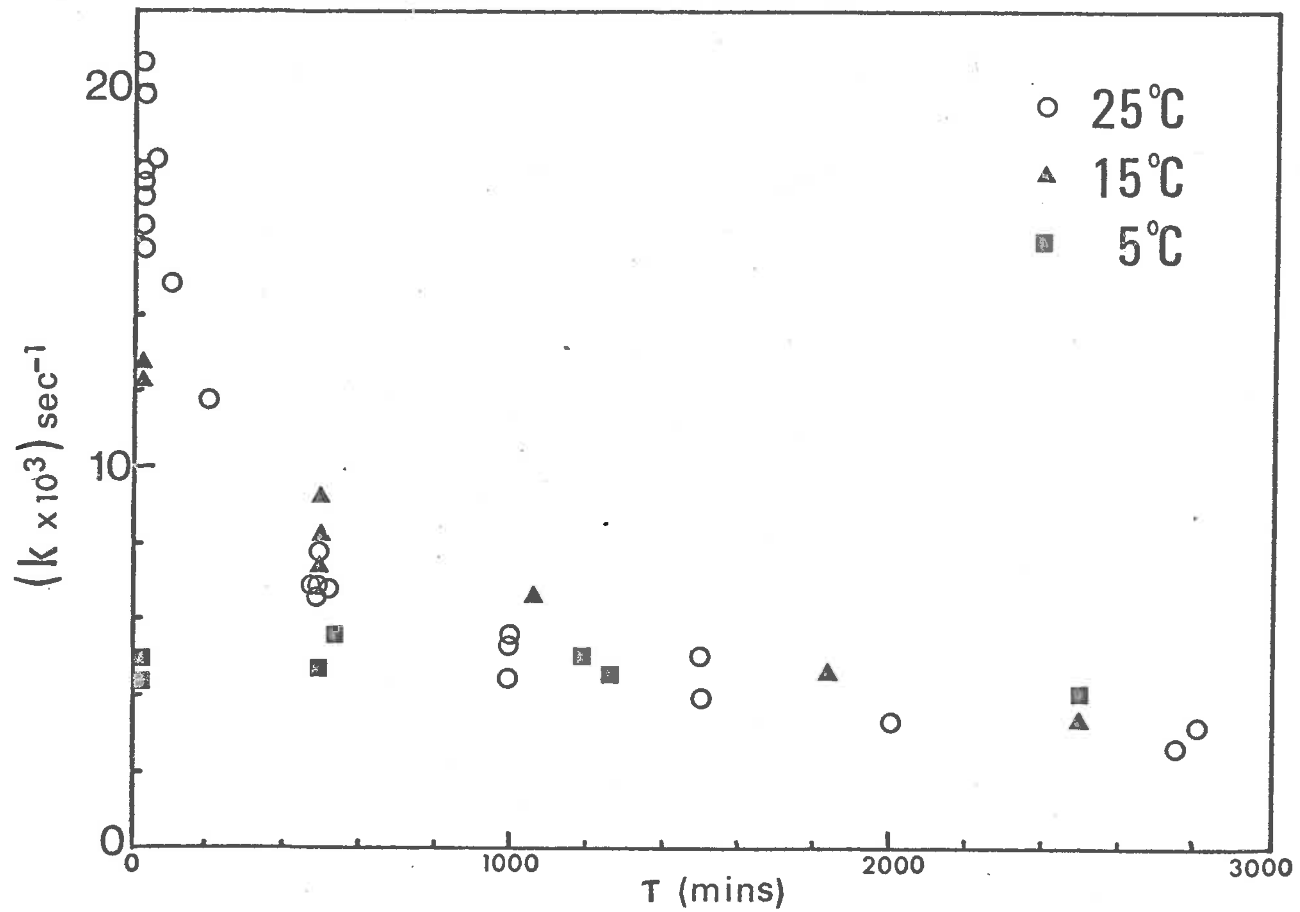

Fig. III-17. Comparison between apparent first order rate constants, k, obtained at 5°C, 15°C, and 25°C. KNO_3 concentration = 0.15 M; DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

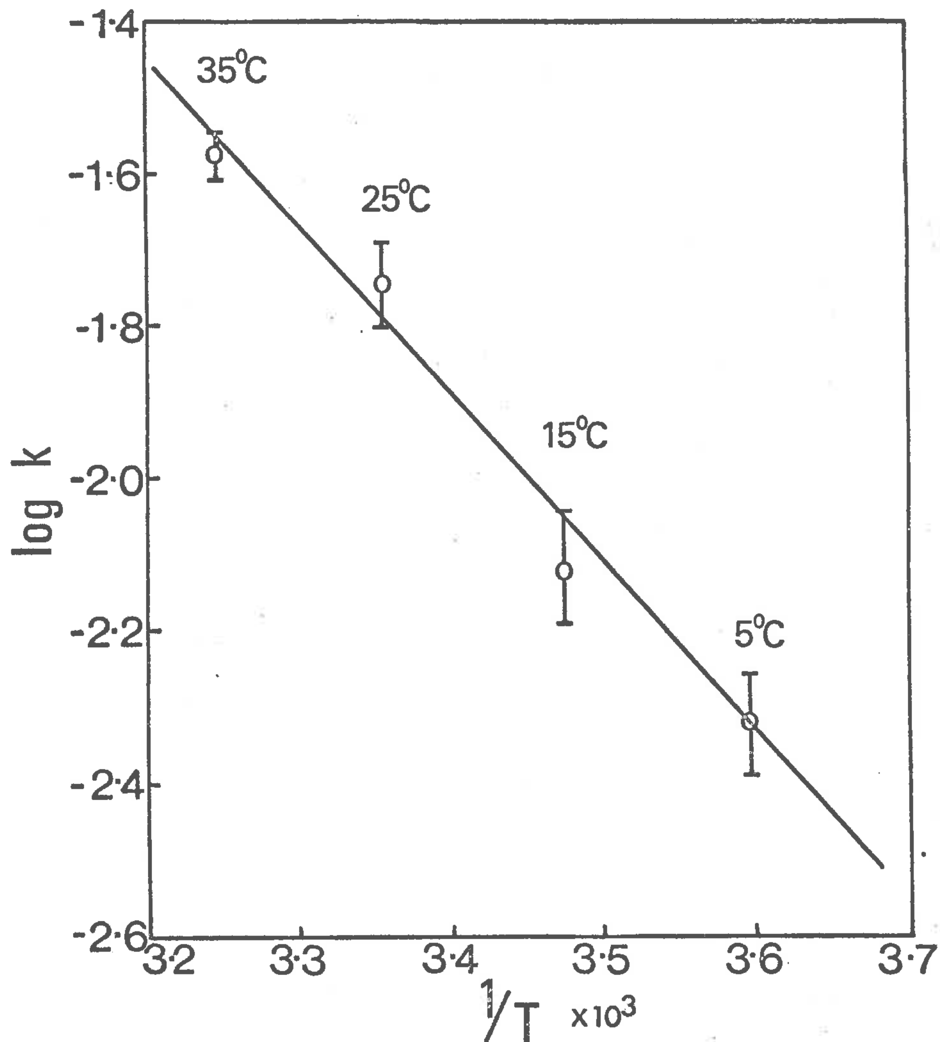

Fig. III-18. Arrhenius plot for reactions after 30 minutes of standing. (T = Absolute Temperature). Conditions as in fig. III-12 and fig. III-17. Slope ∿ -2×10^3. Energy of activation ∿ 10 kcals.

standing. However, it is considered that the values of k at τ = 30 represent renaturation reactions which have not been significantly affected by the process responsible for the decrease in reaction rate at longer times of standing. (An experiment where k was obtained at τ = 10, 20 and 30 minutes showed no significant change of k with τ.) Once the reaction rate has been modified by the time of standing, an Arrhenius plot of the rate constants at the different temperatures is no longer valid. However it is of interest to note from fig. III-12 and fig. III-17 that after periods of standing for greater than 1500 minutes, the temperature dependence of k seems to disappear. There is even some indication that around 2500 minutes, the higher temperature rates are slower than the lower temperature rates, but the difference is within the experimental error and no reliable statement can be made concerning this. Why most of the rate constants obtained after long periods of standing at different temperatures should fall within the range 2.5×10^{-3} to 5.0×10^{-3} sec^{-1} is a puzzling question, the answer to which must be related to the explanation for the time dependence.

7. Effect of solution viscosity on the renaturation rate

To study the effect of bulk viscosity on the renaturation, the solution viscosity was increased by the addition of sucrose. As previous workers[6,7] had used sucrose in other DNA systems for this purpose, it was considered that no spurious chemical effects would be introduced. This assumption appears to be reasonable in the Cu^{++}-

DNA system because full denaturation was accomplished within 4 minutes at 35°C, and on increasing the ionic strength, complete renaturation was observed.

a. Calculation of the viscosity of sucrose solutions

The viscosity of solutions containing various quantities of sucrose were calculated with the aid of an equation derived from the diffusion theory of viscosity as given by Robinson and Stokes.[8] The equation which is valid for high concentrations of large solute particles is

$$\log \eta_{rel} = \frac{A_3 c}{1 - Q'c}$$

where η_{rel} is the relative solution viscosity, c is the concentration in moles/litre, A_3 is a constant related to the molar volume of the solute, and Q' is an arbitrary constant, specific for a particular solute. The values of A_3 and Q' for sucrose are 0.380 litre/mole and 0.230 litre/mole, respectively.

b. Preparation of solutions

To prepare a DNA solution containing the desired concentration of sucrose, a sample of DNA stock solution was diluted with 10^{-2} M KNO_3 in the presence of the appropriate weight of sucrose, and the DNA concentration checked by measuring the O.D. (259). Since the sucrose contributed a small amount to the absorbance at 259 nm (0.09 O.D. units for 40% sucrose), the solvent reference cells all contained the appropriate sucrose concentration. Because of the difficulties involved in preparing accurate $Cu(NO_3)_2$ stock solutions and because

the accuracy of the Cu^{++} concentration in the renaturation is very important, the $Cu(NO_3)_2$ stock solution containing no sucrose which had been used in the previous experiments was employed throughout the high viscosity studies. It was considered that the inaccuracy in the sucrose concentration caused by adding a small volume of this Cu^{++} solution to the DNA-sucrose solution (2% for the case of 40% sucrose) was preferable to the large uncertainty in Cu^{++} concentration which would have been present if a fresh $Cu(NO_3)_2$-sucrose solution had been prepared for each sucrose concentration studied.

The 1.0 M KNO_3 solution used to increase the ionic strength was prepared containing the appropriate sucrose concentration. The density of each sucrose solution was measured so that when a mixing procedure involved the weight of a solution, the conversion to volume could be made.

Using these solutions, the denaturation and renaturation were carried out in the usual manner.

c. Results

The renaturation in 0.15 M KNO_3 at 25°C was studied at sucrose concentrations of 40% (η_{rel} = 4.06), 23% (η_{rel} = 2.00) and 10.5% (η_{rel} = 1.33). A typical set of renaturation curves in 40% sucrose is shown in fig. III-19, and the first order plots of these curves in fig. III-20. Clearly in the high viscosity medium the renaturation is markedly slower than in fig. III-1. Similar families of curves were obtained in 23% sucrose and in 10.5% sucrose, and the relationship between the first order rate constants at corresponding

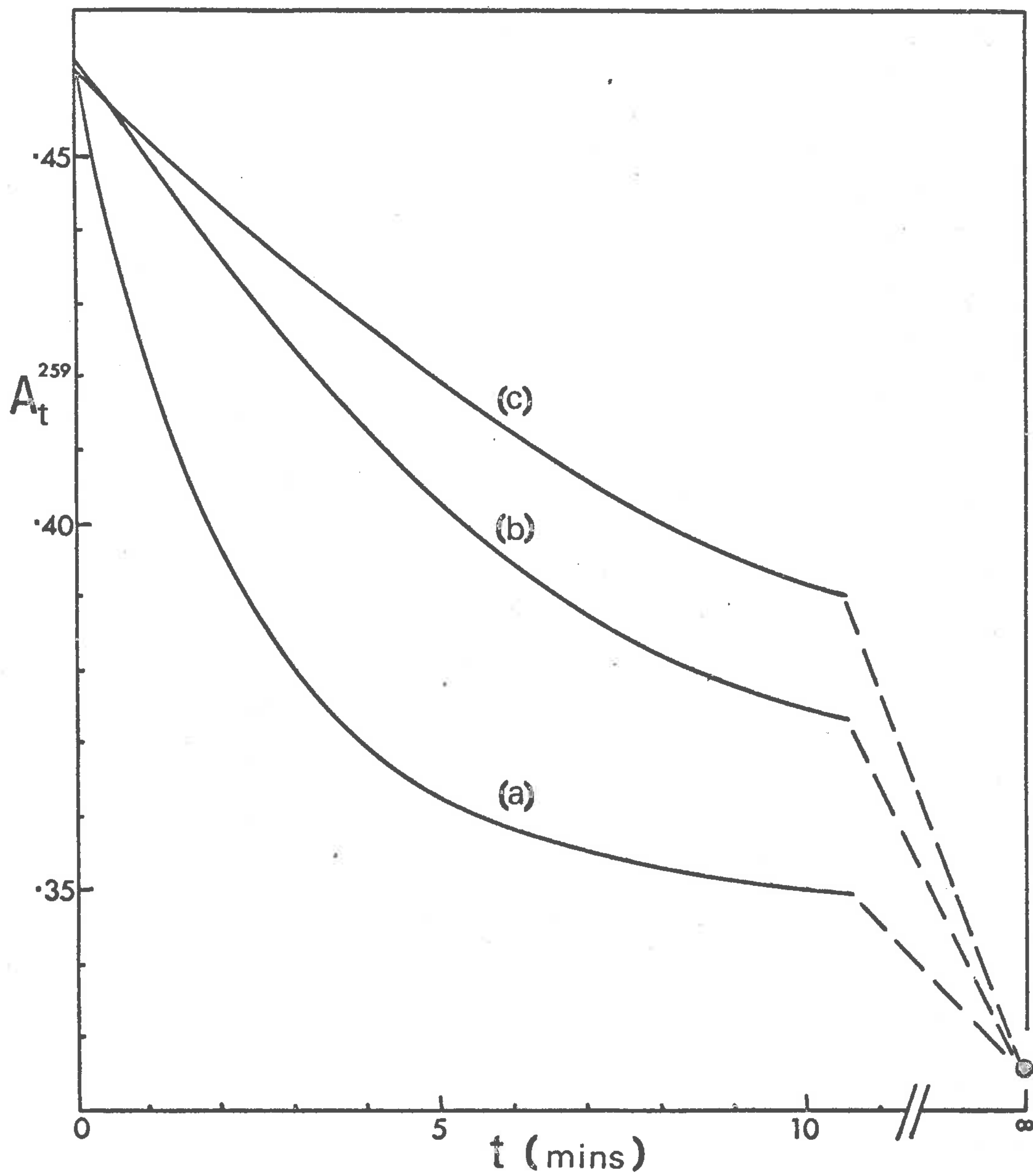

Fig. III-19. Renaturation in 40% sucrose solution, at 25°C, in 0.15 M KNO_3.

Time of standing: (a) 30 mins.
(b) 485 mins.
(c) 1140 mins.

DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2.

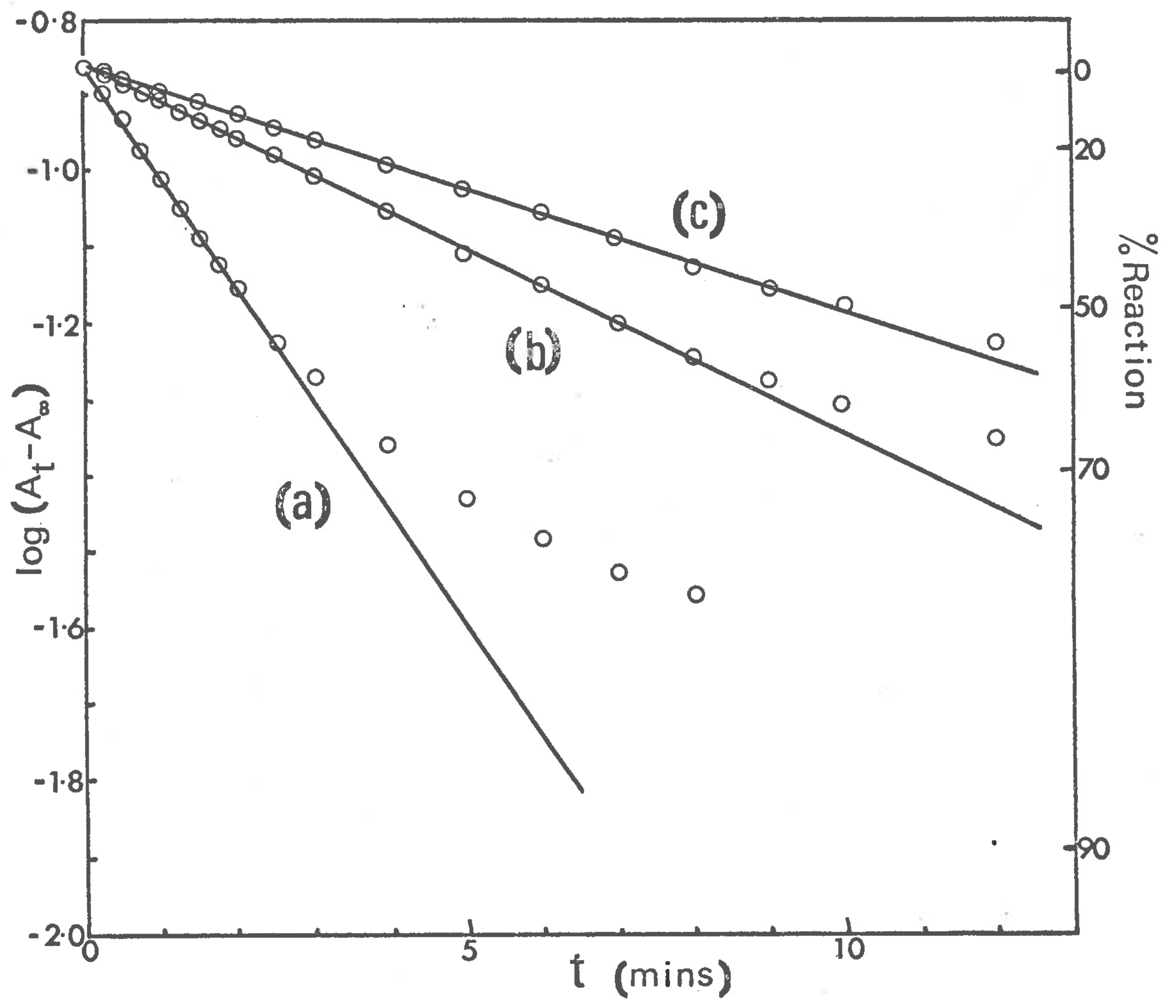

Fig. III-20. First order analysis of data of fig. III-19, in 40% sucrose solution.

times of standing and the relative viscosity is shown in fig. III-21. It can be concluded that at any particular time of standing the rate is inversely proportional to the relative viscosity of the solution.

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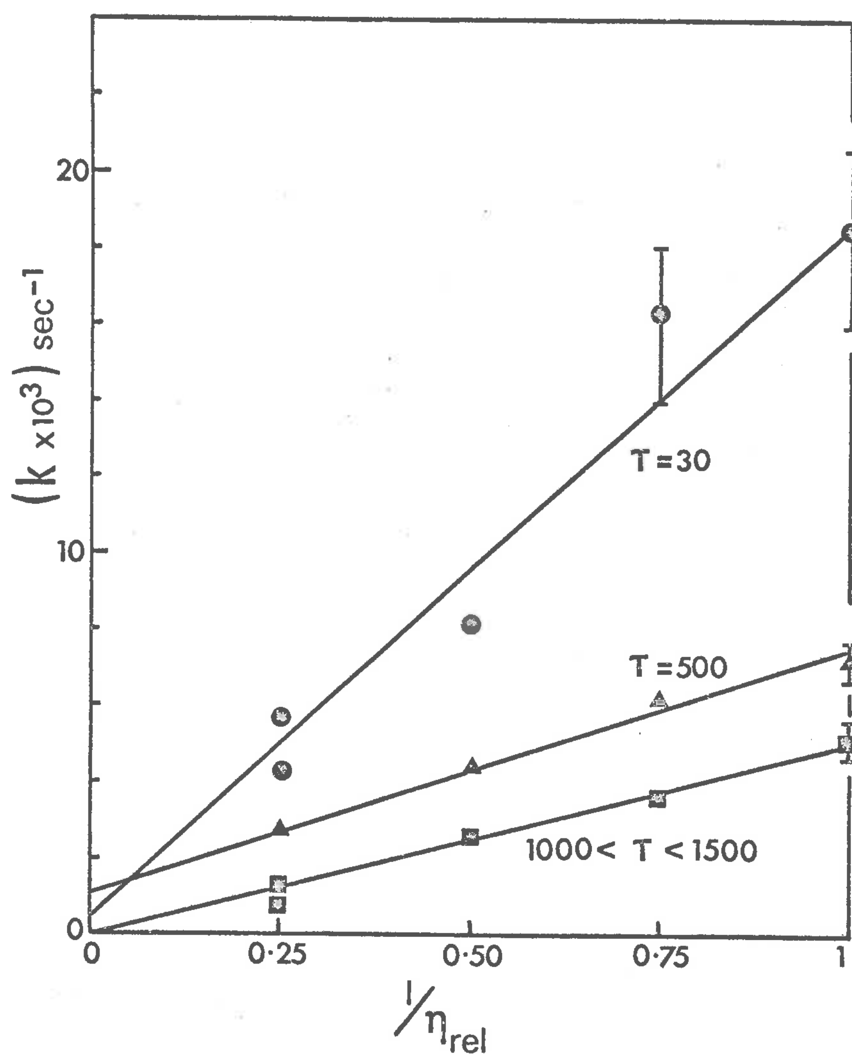

Fig. III-21. The dependence of k on the relative viscosity of the solution. The rate constants obtained in solutions of varying sucrose concentrations plotted for corresponding times of standing against $1/\eta_{rel}$.

CHAPTER IV

THE RENATURATION OF THE DENATURED DNA-Cu^{++} COMPLEX BY INCREASING THE IONIC STRENGTH: CHANGE IN FREE Cu^{++} ION CONCENTRATION

1. Introduction

2. The specific cupric ion activity electrode
   a. The Cu^{++} ion activity
   b. Calibration of the Cu^{++} ion electrode

3. Experimental procedure
   a. Measurement of potential
   b. Denaturation and renaturation

4. The renaturation as followed by the Cu^{++} ion electrode
   a. Relative positions of the renaturation curves
   b. Reproducibility
   c. The spectrophotometrically observed reaction under the same conditions
   d. Calculation of the total EMF change on renaturation
   e. Comparison between the renaturation data from the Cu^{++} ion electrode and the spectrophotometer

5. The renaturation in high viscosity solution as followed by the Cu^{++} ion electrode

References

1. Introduction

From the previous chapter it is evident that the renaturation reaction is complex. Even though first order kinetics were consistently observed for a certain extent of the reaction, the deviation from first order kinetics as the reaction proceeded, the variation with the time between denaturation and renaturation, and the viscosity dependence showed that the reaction does not proceed by a simple one-step mechanism. It was recognised that following the course of the reaction by spectrophotometric means gave information only on the reaction step or steps which involve a change in the conformation of the bases affecting the hyperchromicity of the DNA. No information concerning the dissociation of the Cu^{++}-base complex could be given by this technique.

Two proposals can be put forward for the relationship between the dissociation step and the overall reaction. On increasing the ionic strength, the Cu^{++}-base complex may dissociate rapidly followed by the return of the bases to the helical conformation at a slower rate, the spectrophotometrically observed reaction rate. On the other hand, the dissociation may proceed much more slowly, at a rate comparable to the overall reaction rate, in which case it will have some effect on the renaturation rate and may even be rate determining. In the first instance, the increase in free Cu^{++} ion concentration would occur practically instantaneously while in the second case, there would be a gradual increase accompanying the renaturation. Thus, by following the increase in free Cu^{++} ion concen-

tration on renaturation using an electrode, the potential of which is dependent on the Cu^{++} concentration, it should be possible to distinguish between these two situations. The results of such an investigation are presented in this chapter.

2. The specific cupric ion activity electrode

The Orion specific cupric ion activity electrode, by means of a solid state sensing element, develops a potential which is a function of the Cu^{++} ion activity of the solution in which it is placed. To measure the electrode potential, the Cu^{++} ion electrode is used in conjunction with a saturated calomel reference electrode and the EMF of the electrochemical cell thus formed is measured on a potentiometer.

The theoretical relationship between the potential of the cell and the Cu^{++} ion activity is given by the Nernst equation:

$$E = E_a + \frac{2.3RT}{2F} \log (a_{Cu^{++}})$$

where E is the measured potential of the system,

E_a is a constant incorporating the standard electrode potential of Cu^{++}, the potential of the reference electrode and any liquid junction or asymmetry potentials,

$a_{Cu^{++}}$ is the cupric ion activity,

and $\frac{2.3RT}{2F}$ is the Nernst factor which equals 29.58 mV at 25°C.

To account for the unknown constant, E_a, the electrode must first be calibrated in a solution of known Cu^{++} activity.

a. The Cu^{++} ion activity

The relation between the activity and the concentration of a copper solution, as given by the activity coefficient (γ), is a function of the total ionic strength of the solution. Since, in using the electrode to follow the renaturation reaction, measurements were made in solutions of two different KNO_3 concentrations, it was necessary to know the activity coefficient at each ionic strength in order to relate the observed changes in potential to actual changes in Cu^{++} concentration.

The activity coefficient of Cu^{++} ions as a function of total ionic strength for pure $Cu(NO_3)_2$ solutions has been calculated from the Debye-Huckel theory by Keilland.[1] It is necessary to assume that the Cu^{++} ion activity coefficient in a pure $Cu(NO_3)_2$ solution is the same as in a solution containing only a low $Cu(NO_3)_2$ concentration in the presence of excess KNO_3 to the same total ionic strength. This assumption is believed to be satisfactory at low ionic strength where the activity coefficient is independent of the ionic composition but is doubtful at an ionic strength of 0.1 or higher due to the influence of the other ions in the solution. In the solutions containing 0.01 M KNO_3 prior to renaturation, the literature value[1] of the Cu^{++} ion activity coefficient is 0.675. The renaturation, for reasons to be discussed later, was carried out in 0.10 M KNO_3, and the activity coefficient under these conditions is given as 0.405. Due to the doubtful validity of these theoretical values in this system, an experimental test of the relative activity coefficients was made by

preparing solutions of identical $Cu(NO_3)_2$ concentration in both 0.01 M and 0.10 M KNO_3 and measuring the Cu^{++} electrode potential. A series of measurements on two solutions both containing 1.05×10^{-4} M $Cu(NO_3)_2$ indicated that the decrease in potential on going from 0.01 M KNO_3 to 0.10 M KNO_3 was 4.7 ± 0.2 mV from which the difference in the Cu^{++} activity coefficients in the two ionic strengths can be calculated. Therefore, by assigning the literature value of 0.675 as the value of γ in the 0.01 ionic strength solution, the calculated value of γ in the 0.10 ionic strength solution was 0.47 ± 0.01. A similar determination using Cu^{++} solutions of 2.09×10^{-4} M gave γ in 0.10 M KNO_3 as 0.45. Both of these values are significantly different from the literature value of 0.405 for γ in 0.10 ionic strength. Because of this uncertainty in the activity coefficients, an experimental calibration curve of potential against log (Cu^{++} concentration) was plotted for each ionic strength which avoided the problem of the assignment of activity coefficients, and the accuracy of the calibration curves gave some indication of the accuracy to which the Cu^{++} concentration could be determined.

b. <u>Calibration of Cu^{++} ion electrode</u>

Three $Cu(NO_3)_2$ solutions with concentrations between 2×10^{-5} M and 2×10^{-3} M were prepared in both 0.01 M KNO_3 and 0.10 M KNO_3. The Cu^{++} concentration of each solution was determined by titration against standard EDTA solutions containing the appropriate KNO_3 concentration, using the Cu^{++} ion electrode to detect the equivalence point. For the solutions containing Cu^{++}

at 2×10^{-3} M or 1×10^{-4} M, the concentrations were determined to an accuracy of 0.2% but the more dilute solutions (2×10^{-5} M) gave less reproducible titrations, and the concentration was only accurate to 1%.

On some occasions when the potential of a standard Cu^{++} solution was measured over a period of 20 minutes, the reading remained constant to within 0.1 mV, but on other occasions a drift of up to 1 mV was observed. Generally, the value taken was the average of the readings in the first 5 minutes after 5 minutes of stirring, and an error range of $\pm$ 0.1 mV or $\pm$ 0.2 mV was assigned depending on the constancy over the 5 minute interval.

The data for the three standard Cu^{++} solutions in 0.01 M KNO_3 showed that within experimental error the electrode response obeyed the Nernst equation with the theoretical factor of 29.58 mV. However, the calibration using the standard Cu^{++} solutions in 0.10 M KNO_3 was less satisfactory, for the correct linear relationship between E and log (Cu^{++} concentration) could only be obtained if the error range of the potential readings was increased to $\pm$ 0.5 mV for two of the solutions and to $\pm$ 1.0 mV for the most dilute solution. These errors were larger than the fluctuations observed over 5-10 minutes, and the reason for the decreased accuracy in the higher ionic strength solutions is not readily apparent.

All the measurements for the above calibration were performed on the one day but when they were repeated later, the potentials of the standard solutions had changed, but as each had shifted by the

same factor it was concluded that only the constant E_a had changed and the slope of 29.58 mV still applied. For example, repeated readings on the standard Cu^{++} solutions containing 1.05×10^{-4} M in 0.01 M and 0.10 M KNO_3 were made over a period of two months, and a sample of the observations is set out in Table IV-1. This drift in the characteristic E_a of the electrode system could not be attributed to changes in any external conditions such as the light intensity, solution temperature or room temperature and it appears that such long term instability could be an inherent weakness of the Cu^{++} ion electrode since the instrument manual suggests that calibration should be carried out several times a day.

3. Experimental procedure

For the most accurate observation of the potential change accompanying the renaturation reaction, it was advantageous to make the change in free Cu^{++} ion concentration as large as possible. To accomplish this, the Cu/P ratio was decreased from 2:1, as used in the previous spectrophotometric experiments, to 1:1 which had the effect of increasing the fraction of the total Cu^{++} concentration bound in the denatured DNA-Cu^{++} solution. When the renaturation of the solution in which Cu/P = 1 was observed spectrophotometrically in 0.15 M KNO_3 after 30 minutes standing, 65% of the reaction was complete in the first 15 seconds. To lose this much of the reaction in the mixing period would certainly not be desirable when following the renaturation with the Cu^{++} ion electrode, and thus it was decided to decrease the KNO_3 concentration used to bring about the renaturation

Date	E (mV), Solution A	Δ	E (mV), Solution B	Δ
7/10	-158.9	0	-154.3	0
28/10	-158.0	+.9	-153.4	+.9
29/10	-159.5	-.6	-154.6	-.3
7/11	-151.7	+7.2	-146.5	+7.8
24/11	-141.0	+17.9	-136.1	+18.2

Table IV-1

Drift in the potential of standard Cu^{++} solutions.

Solution A: Cu^{++} concentration = 1.053×10^{-4} M

KNO_3 concentration = 0.01 M

Solution B: Cu^{++} concentration = 1.051×10^{-4} M

KNO_3 concentration = 0.10 M.

from 0.15 M to 0.10 M. This made the renaturation sufficiently slow to be followed conveniently with the Cu^{++} ion electrode, and it also avoided the problem of the activity coefficient of Cu^{++} which is uncertain in an ionic strength of 0.15.

a. Measurement of potential

Potential measurements were made in a specially constructed glass electrode vessel with three ground glass access joints. The Cu^{++} ion electrode and the calomel reference electrode were each fitted into an access joint by means of a teflon sleeve. The remaining access joint was used for addition of solutions to the vessel and as this was generally stoppered, the vessel was effectively sealed to prevent evaporation.

A volume of at least 10 mls of the solution to be measured was placed in the electrode vessel which was thermostatted in a water bath at 25.0 ± 0.1°C. By means of a small teflon coated magnet, approximately 1 cm long, placed in the electrode vessel and a magnetic stirrer placed in the waterbath beneath the vessel, the solution was stirred for at least 10 minutes to obtain thermal equilibrium before the electrodes, prepared as described in section VII.5, were inserted into the vessel. It was found that due to the small volume of solution used, the Cu^{++} electrode was close to the stirring magnet, causing the reading to depend on the rate of stirring. As it was not possible to keep the rate of stirring constant, it was decided to record potential readings on unstirred solutions only, in spite of the electrode instruction manual recommending continuous stirring to

improve the electrode response time. The difference between the reading with and without stirring often exceeded 1 mV, depending on the relative positions of the electrode and the stirrer, the rate of stirring, and also the Cu^{++} concentration, the effect being much greater at low Cu^{++} concentrations. The difference on stirring also seemed to increase as the electrodes remained longer in a solution, possibly due to some effect on the calomel electrode liquid junction. Thus, to determine E for solutions of constant free Cu^{++} concentration, the solution was stirred for 5 minutes after placing the electrodes in the vessel, the reading taken every minute for the next 5 minutes, the stirring recommenced for a further 2 minutes and then readings taken for another 5 minutes. This procedure was continued until satisfactorily steady readings were obtained, but attention was always given to the two opposing considerations, that taking readings over a longer period of time ensures good equilibrium in the system, but it also increases the likelihood of contamination of the solution with K^+ and Cl^- ions from the calomel electrode.

b. Denaturation and renaturation

The denaturation was carried out as previously, the only differences being that the relative amounts of the Cu^{++} and DNA solutions used were adjusted to give a final Cu/P ratio of 1:1, and that the volume of denatured solution prepared was much greater ($\sim$ 40 mls) because of the larger samples required for renaturation in the electrode vessel. At various time intervals after denaturation, a sample of approximately 12 gms of the denatured DNA-Cu^{++} solution

was weighed into an electrode vessel which was then thermostatted for 10 minutes at 25°C before inserting the electrodes and recording the potential of the solution by the procedure outlined in the previous section. While the solution was stirred, 1 ml of 1.20 M KNO_3 was added by means of a graduated pipette to bring the KNO_3 concentration to 0.10 M. The time at which the addition of the 1.2 M KNO_3 was complete was taken as zero time for the reaction and the stirring was continued for 30 seconds after this time. The stirring was then stopped and the change of E with time, as indicated by the movement of the needle across the potentiometer scale on its most expanded setting, was recorded manually. By this means, values of E to 0.1 mV could be recorded as frequently as every 5 seconds if necessary. It was not possible to use a chart recorder to follow the potentiometer output during the reaction because the most sensitive setting on the recorder of 1 cm = 10 mV was not sufficiently accurate for the small potential change accompanying the renaturation. The reaction was followed for 15-20 minutes before the solution was stirred for 2 minutes and then more measurements taken until no further change was observed.

4. <u>The renaturation as followed by the Cu^{++} ion electrode</u>

Fifteen renaturation reactions were performed and followed by the Cu^{++} ion electrode as described above, under the conditions of DNA concentration = 5.00×10^{-5} M_p, Cu/P = 1, KNO_3 concentration = 0.10 M, temperature = 25°C. Samples of five denatured solutions were renatured after standing at 25°C for 30-40 minutes, 350-500

minutes, and 1200-1500 minutes. A typical set of data is shown in fig. IV-1.

a. Relative positions of the renaturation curves

It can be seen that there is a marked error in the relative positions of the renaturation curves, for the 350 minute curve appears to be displaced by approximately 2 mV from the other two, a discrepancy which is far too large to be due to an error in the Cu^{++} concentration of this solution. However it should be reported that the results in fig. IV-1 were the most extreme in this respect, and in other cases, there was much less scatter in the relative positions of the three renaturation curves obtained from a denatured solution. This scatter is a consequence of the instability in the value of E_a of the electrode system over such time intervals as discussed in section IV.2.b. A method of correcting this error is to measure the potential of one or two standard Cu^{++} solutions before each renaturation, and by comparing these readings to arbitrarily chosen reference values of the standard solutions, a correction factor can be assigned which must be applied to all the subsequent readings. However, this procedure was found to be unsatisfactory because the correction factor could not be obtained with sufficient accuracy. When two standard solutions were measured prior to a renaturation reaction, the correction factor derived from each sometimes differed by more than 0.5 mV (see Table IV-1, for example), or when a standard solution was measured immediately before and immediately after a reaction, the two correction factors calculated often differed by 0.5 mV. Hence an error of $\pm$ 0.5 mV was associated

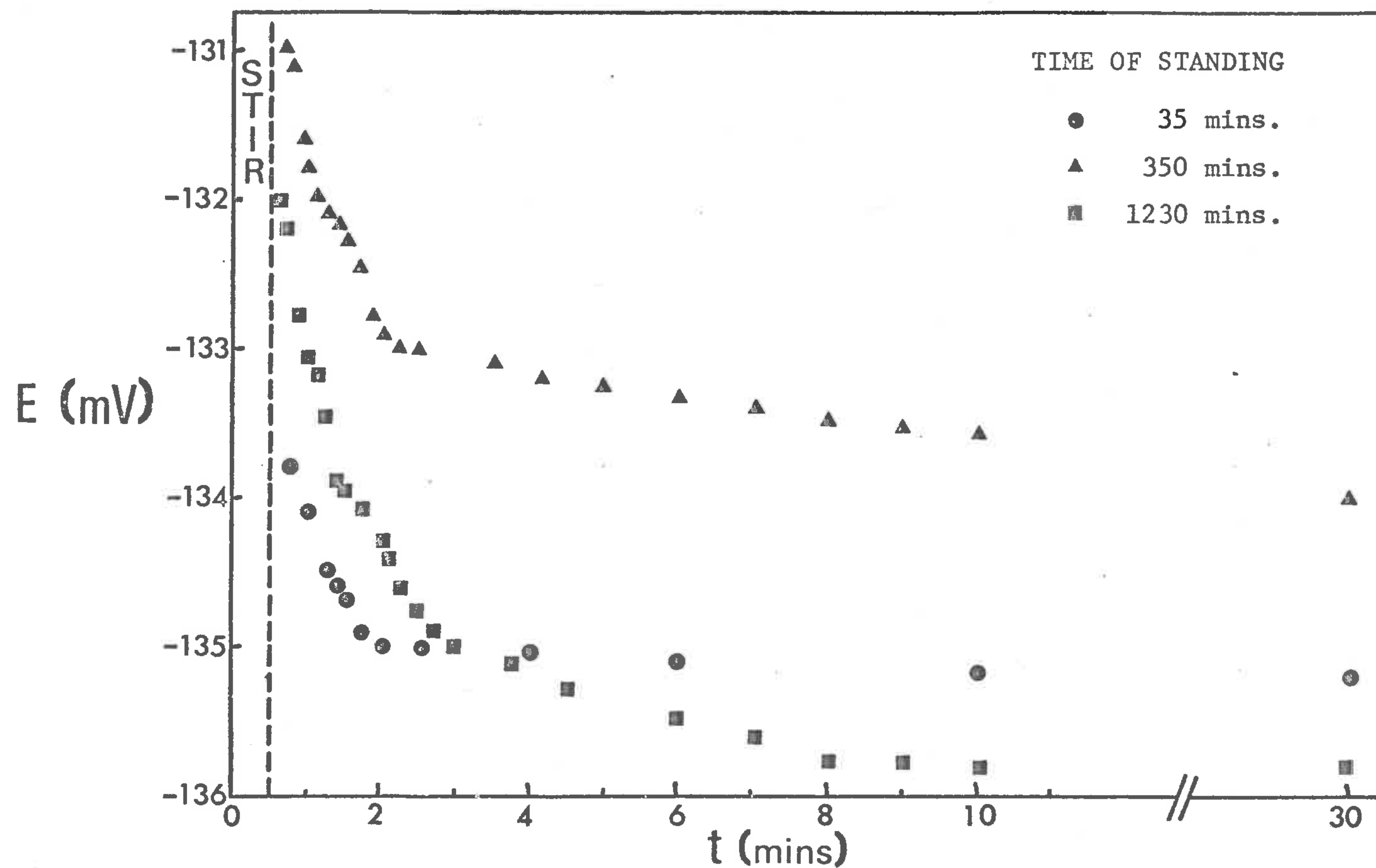

Fig. IV-1. The measured EMF of the Cu^{++} ion electrode with time during the renaturation reaction. DNA concentration = 5.00×10^{-5} M_P; Cu/P = 1; KNO_3 concentration = 0.10 M; Temperature = 25°C. Time of standing as indicated.

with each correction factor, resulting in an uncertainty of 1 mV in the relative positions of the renaturation curves. In comparison with the total change accompanying the renaturation, such a large uncertainty would make interpretation of the results very difficult.

To overcome this problem, it was decided to present the renaturation curves in terms of ΔE, the difference in the potential at time t and at completed reaction. That is, the infinity potential value was assigned to zero and the renaturation curve shown relative to this. A set of renaturation curves presented in this way is shown in fig. IV-2.

The potential readings of the samples of denatured DNA-Cu^{++} solution prior to addition of 1.2 M KNO_3 are indicated on the extreme left of fig. IV-2, relative to E_∞ but in fact the relationship between these points and the rest of the graph is not directly related to the free Cu^{++} ion concentration because these first three points are at the lower ionic strength and have not been corrected for the change in potential due to the change in ionic strength. The lines in fig. IV-2 between these "PRE-initial" points and the renaturation curves represent approximately the course of the potential during the addition of the 1.2 M KNO_3 and the initial stirring period before the accurate potential readings were commenced.

b. Reproducibility

When the renaturation curves are represented in terms of ΔE, the only source of uncertainty in their relative positions is the determination of E_∞ with respect to the values E_t. In most cases

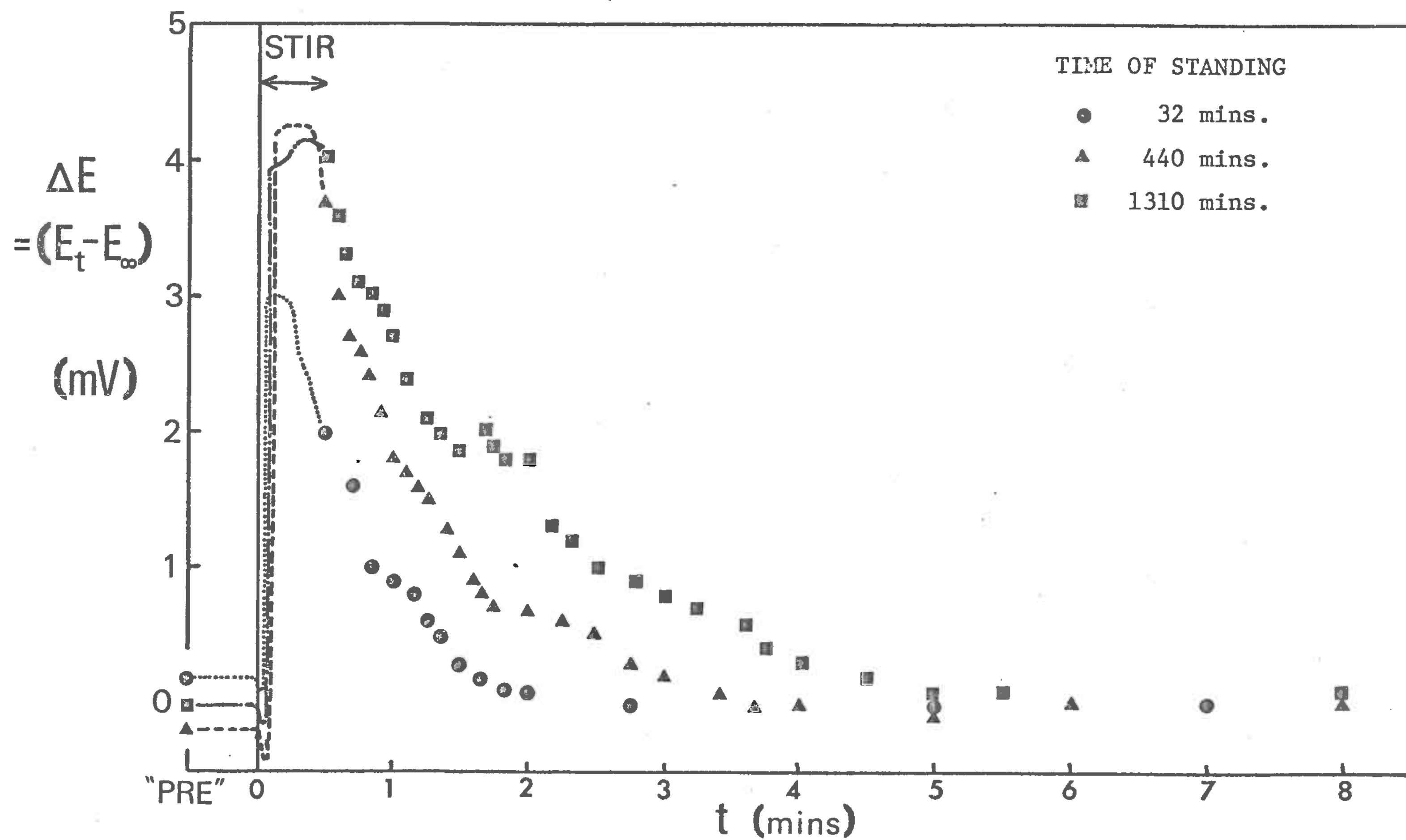

Fig. IV-2. The renaturation curves as determined by the Cu^{++} ion electrode presented relative to the EMF value at complete renaturation. Conditions as in fig. IV-1. The "PRE-initial" points (labelled "PRE" at the left of the figure) are the EMF readings on the denatured DNA-Cu^{++} solution prior to each renaturation.

the value of E remained constant to within $\pm$ 0.1 mV between t = 10 and t = 15 minutes and if measurements were taken for an extended period of time following this (30-60 minutes) a random fluctuation around this value was observed. Therefore the average value of E from t = 10 to t = 15 minutes was taken as E_∞ and an uncertainty of $\pm$ 0.1 mV associated with it, although in several instances where the stability of the electrode was not as satisfactory, E_∞ could only be given to $\pm$ 0.2 mV.

Fig. IV-3 demonstrates the reproducibility of the renaturation curves obtained at times of standing of 30-40 minutes, and 1200-1400 minutes. A similar reproducibility was observed for curves obtained after 350-450 minutes standing. With an error in E_∞ of $\pm$ 0.1 mV and in E_t of at least $\pm$ 0.1 mV, the ΔE values would be expected to have an uncertainty of $\pm$ 0.2 mV and it can be seen that for the renaturation curves after any particular time of standing, the experimental points generally lie within $\pm$ 0.2 mV of an average curve.

c. The spectrophotometrically observed reaction under the same conditions

In order to compare the renaturation reaction as observed by the Cu^{++} ion electrode with the reaction followed spectrophotometrically, the spectrophotometric data were obtained under the conditions of Cu/P = 1 and KNO_3 concentration = 0.10 M, and are shown in fig. IV-4. On comparing fig. IV-3 and fig. IV-4, it is clear that the different techniques of following the reaction give very similar results. The reactions are all 90% complete within 6 minutes, the

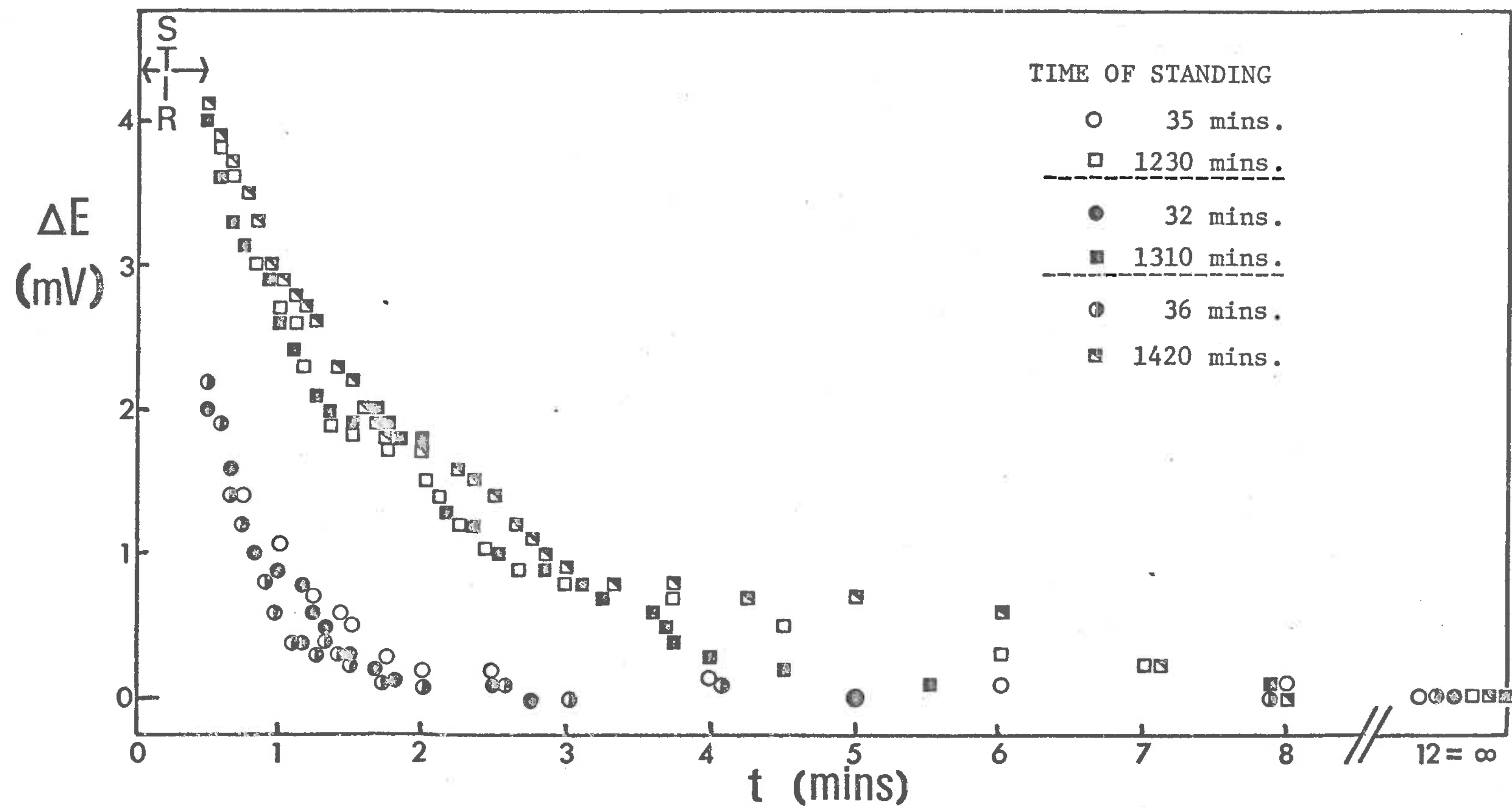

Fig. IV-3. Reproducibility of the renaturation curves as determined by the Cu^{++} ion electrode. Representative data from renaturation reactions carried out under the conditions defined in fig. IV-1.

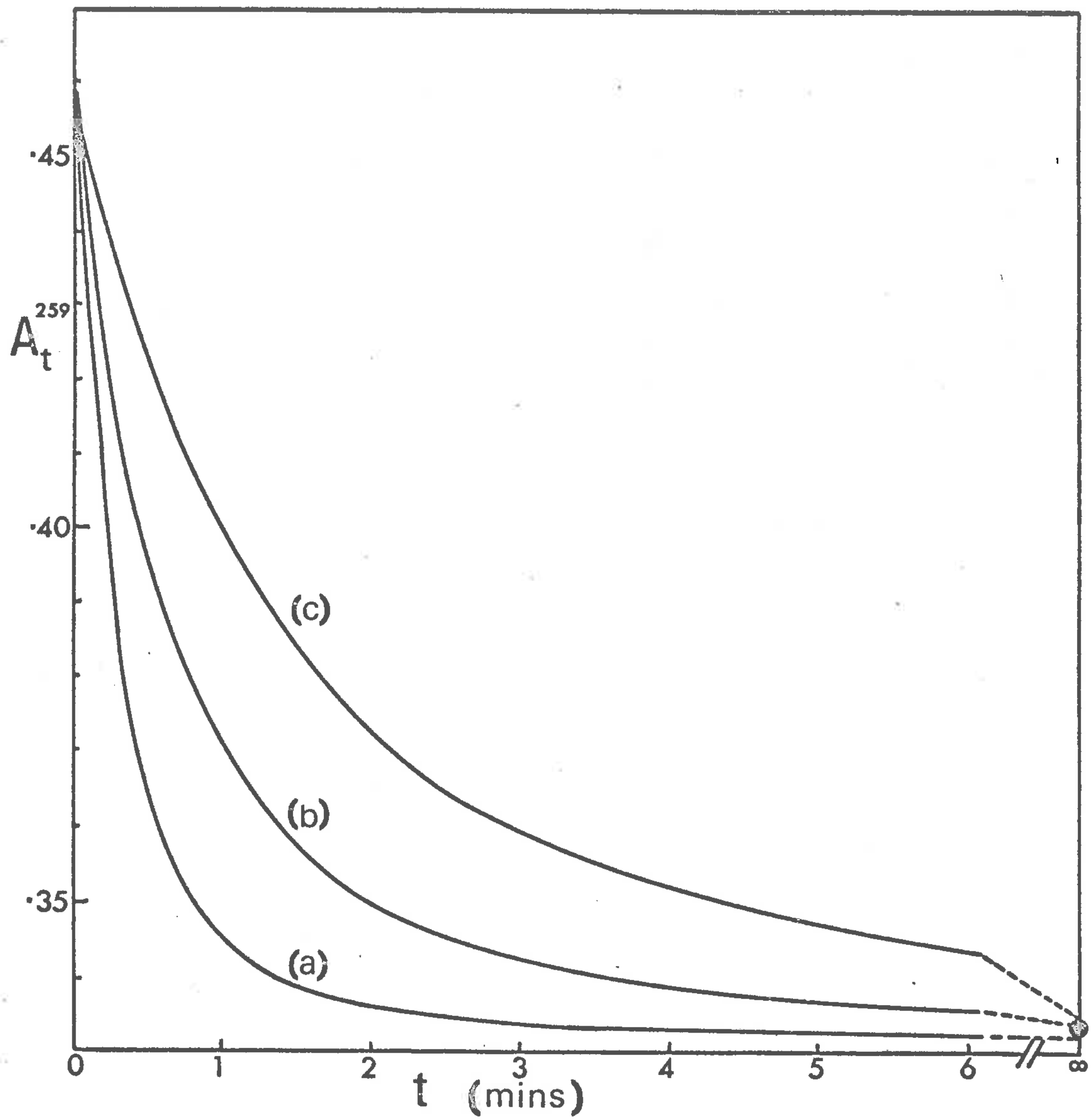

Fig. IV-4. Renaturation observed spectrophotometrically at DNA concentration = 5.00×10^{-5} M_P; Cu/P = 1; KNO_3 concentration = 0.10 M; temperature = 25°C.
Time of standing: (a) 30 minutes
(b) 410 minutes
(c) 1360 minutes.

infinity value was reached within 10-20 minutes, and the dependence on the time of standing was similar in both cases. However, for a more precise comparison between the sets of results, the renaturation curves must be presented as % reaction against time. For the spectrophotometric data, A_0 can be readily obtained by extrapolation and calculation, and so all A_t values can be represented as percentages of the total change, $A_0 - A_\infty$. On the other hand, it can be seen from fig. IV-2 or fig. IV-3 that it is most difficult to obtain the value of E_0 or of ΔE $(t = 0)$ by extrapolation and so an attempt must be made to calculate the total potential change associated with the renaturation.

d. Calculation of the total EMF change on renaturation

The calculation of this value required potential measurements on several solutions, all subject to experimental error. These values had to be converted to Cu^{++} concentrations, a procedure which not only required the theoretical Nernst factor but also a knowledge of the activity coefficients as the measurements were made at different ionic strengths and, as discussed in section IV.2.a, the activity coefficients are somewhat uncertain. The calculation then involved taking differences between these Cu^{++} concentrations which unavoidably enhanced the previous errors in measurement and calculation. Because of these considerations, it was only possible to estimate the probable range of values for the total EMF change.

(i) Total Cu^{++} concentration

The total Cu^{++} concentration in the solution before the

addition of the concentrated KNO_3 was 5.409×10^{-5} M. When this was tested a number of times by potential measurements on Cu^{++} solutions prepared from the stock solution by the normal procedure with 10^{-2} M KNO_3 substituted for the DNA solution, the experimental values lay within the range $(5.4 \pm \cdot 3) \times 10^{-5}$ M. This large error range, which corresponds to $\pm$ 0.7 mV, is not due to real errors in Cu^{++} concentrations as the volumetric error would certainly be less than 1%, but is a consequence of the electrode irreproducibility. Therefore, the total Cu^{++} concentration for all calculations was taken as the theoretical value, 5.41×10^{-5} M.

On dilution of the solution by addition of 1.2 M KNO_3, the experimental measurements of the Cu^{++} concentration showed a similar large uncertainty and the theoretical concentration of 5.00×10^{-5} M was used in calculations.

(ii) Cu^{++} bound to native DNA in 0.01 M KNO_3

Potential measurements on solutions containing native DNA and Cu^{++} at Cu/P = 1 and in 10^{-2} M KNO_3 gave a range of values for the free Cu^{++} concentration which, when related to the total Cu^{++} concentration of 5.41×10^{-5} M, indicated that the fraction of Cu^{++} bound to native DNA was between 0.1 and 0.2. The majority of the values lay between 0.15 and 0.2 and so this more restricted range was taken to define the amount of Cu^{++} bound to DNA under these conditions, which is considered to be Cu^{++} bound to external sites on the DNA.

(iii) Cu^{++} bound to native DNA in 0.10 M KNO_3

Two solutions of identical Cu^{++} concentration were prepared,

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one with and one without native DNA present, and 1 ml of 1.2 M KNO_3 was added to a sample of each solution in an electrode vessel to bring the KNO_3 concentration to 0.10 M. A comparison of the potentials of the resulting solutions, measured within several hours of each other, always showed an agreement to within 1 mV, often better. This indicated that, within an experimental error of 8%, the free Cu^{++} concentration in each solution was the same, and thus effectively no Cu^{++} is bound to native DNA in 0.10 M KNO_3. (This conclusion is not in disagreement with Schreiber and Daune's value for the amount of Cu^{++} bound to native E. Coli DNA in 0.10 ionic strength[2] because their value is within the experimental uncertainty of the Cu^{++} electrode measurements.)

(iv) Cu^{++} bound in the denatured DNA-Cu^{++} solution in 0.01 M KNO_3

The potential measurements on the denatured DNA-Cu^{++} solution prior to each renaturation reaction gave the free Cu^{++} concentration in the denatured solution in 0.01 M KNO_3. These measurements indicated that the fraction of Cu^{++} bound under these conditions was between 0.40 and 0.45. It is proposed that the Cu^{++} ions are bound on two types of sites, internal sites and external sites, and furthermore the assumption is made that the fraction of the total Cu^{++} concentration bound to the external sites in this denatured solution is the same as that bound to the external sites of native DNA in 0.01 M KNO_3, which has been determined. To illustrate the calculation, a particular example is given using the lower bound of each range of

values, (fraction of Cu^{++} on all sites = 0.40, fraction of Cu^{++} on external sites = 0.15). It can be deduced that for a total Cu^{++} concentration of 5.41×10^{-5} M, the concentration of Cu^{++} bound to external and internal sites is 2.16×10^{-5} M, to external sites is 0.81×10^{-5} M, and thus to internal sites is 1.35×10^{-5} M.

(v) Cu^{++} bound in the denatured DNA-Cu^{++} solution in 0.10 M KNO_3

Since it has been shown that no Cu^{++} is bound to native DNA in 0.10 M KNO_3, it is proposed that when the KNO_3 concentration of the denatured DNA-Cu^{++} solution is increased to 0.10 M, the Cu^{++} ions which were bound to the external sites at the lower ionic strength, are immediately released. The Cu^{++} ions bound to the internal sites are released gradually as the DNA is renatured, and the potential change with time during the renaturation is due to the release of Cu^{++} from the internal sites. Thus the initial state (t = 0) of the renaturation is defined by a total Cu^{++} concentration of 5.00×10^{-5} M, the concentration of Cu^{++} bound to the external sites = 0, the concentration of Cu^{++} bound to the internal sites = 1.35×10^{-5} M from the above calculation, and hence the free Cu^{++} concentration = 3.65×10^{-5} M.

(vi) The total EMF change accompanying the renaturation

By the procedure discussed above, the concentration of free Cu^{++} at t = 0 is calculated, and as the free Cu^{++} concentration present at complete renaturation is known to be 5.00×10^{-5} M, the potential change from t = 0 to t = ∞ is readily obtained. The deriv-

ation outlined above, starting from values of 0.15 and 0.40 for the fractions of Cu^{++} bound to native DNA and denatured DNA respectively, leads to a total ΔE of 4.1 mV. When other values for these fractions are selected from within the experimental ranges, the calculation gives values which define the possible range for the total ΔE as 4.1 mV to 5.0 mV. The assumptions concerning the various states of the system which had to be made at various points through the calculation are the most reasonable postulates in the light of the available evidence but they are by no means proven fact. However, the plausibility of the final answer is encouraging concerning the validity of the assumptions in the general form in which they were stated.

e. Comparison between the renaturation data from the Cu^{++} ion electrode and the spectrophotometer

The renaturation curves of fig. IV-2 were converted to % Reaction by dividing by the total ΔE of either 4.1 mV or 5.0 mV and a comparison between the resulting data and the spectrophotometric data of fig. IV-4 is shown in fig. IV-5. It can be seen that the biggest discrepancy between the two sets of data occurs in the early part of the reaction. In fact, if the time scale of the Cu^{++} ion electrode data is shifted by ¼ minute, the agreement becomes much better, as is shown in fig. IV-6, in which the Cu^{++} ion electrode data are plotted against $t^* = (t - ¼)$. Whether this apparent 15 second induction period is real or an artefact is not known, and neither is the explanation for it if it is a real phenomenon. The reaction zero time was certainly less accurately defined in the Cu^{++} ion electrode

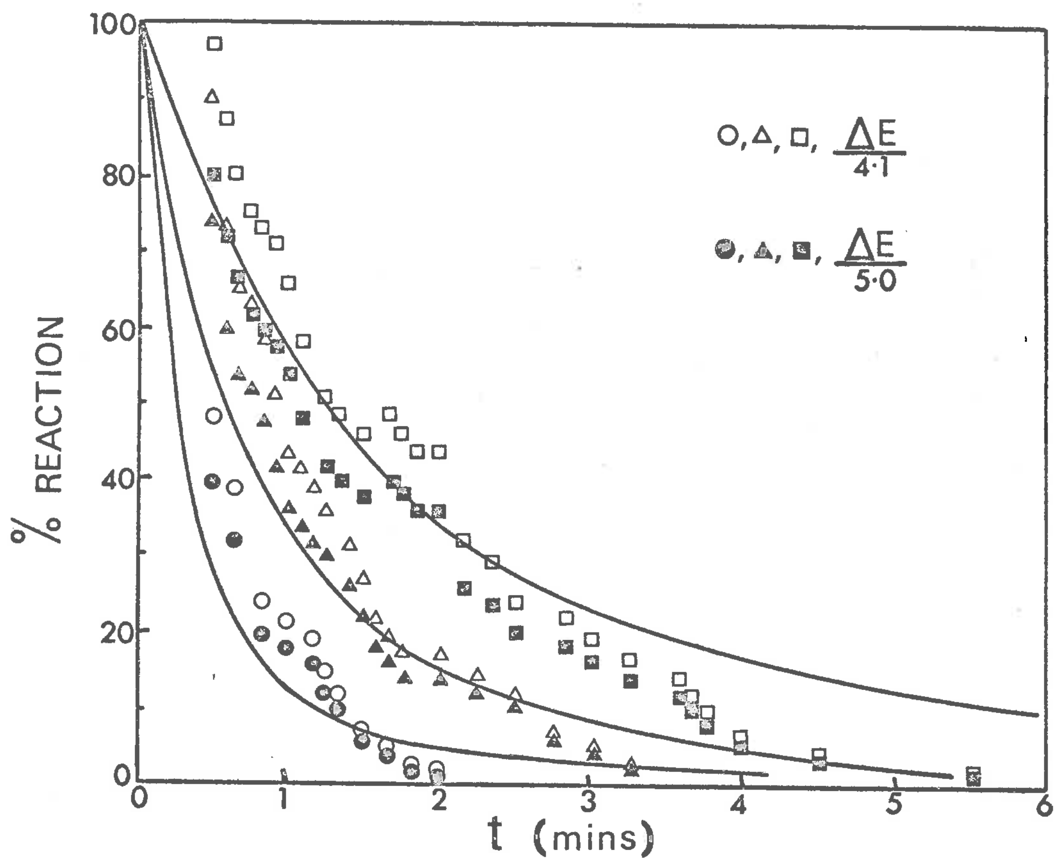

Fig. IV-5. Comparison between renaturation curves observed by the absorbance change (————), and by the Cu^{++} ion electrode (points). Open symbols represent Cu^{++} ion electrode data from fig. IV-2 converted to % reaction using the value of 4.1 mV as the total EMF change on renaturation; closed symbols correspond to 5.0 mV as the total EMF change. See text for details. Concentrations and times of standing as in fig. IV-2 and fig. IV-4.

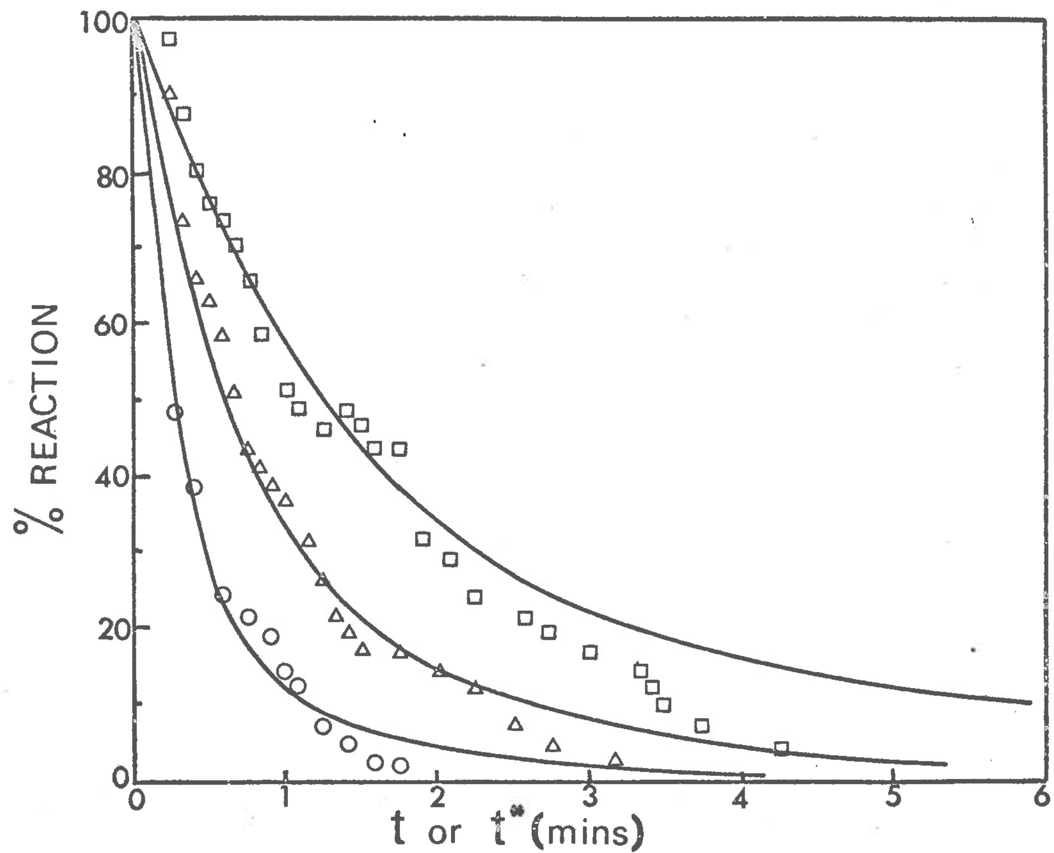

Fig. IV-6. Comparison between renaturation curves observed by the absorbance change and by the Cu^{++} ion electrode, with the Cu^{++} ion electrode data plotted against $t^* = (t - \frac{1}{4})$ mins, that is, shifted to left by ¼ minute compared to fig. IV-5. All conditions as in fig. IV-5.

experiments than in the spectrophotometric ones, for the addition of 1 ml of 1.2 M KNO_3 required approximately 30 seconds. However, the end of this addition period was taken as t = 0, and as the solution was rapidly stirred during the addition, it would be expected that at t = 0 the KNO_3 concentration would be 0.10 M throughout the solution. It can be noticed from fig. IV-2 that in the 30 second stirring period after t = 0, the value of E continued to rise for at least the first 10 seconds, and then it remained fairly steady before starting to decrease between t = 20 and t = 25 seconds. This behaviour may be related to the response time of the electrode, or the readings may be a true indication of the course of the free Cu^{++} ion concentration.

Whatever may be the interpretation of this apparent induction period, it can be concluded that there is sufficient agreement between the data obtained by the two techniques to indicate that during the renaturation reaction the rate of release of Cu^{++} ions from the complex is identical to the rate of decrease in hyperchromicity.

5. The renaturation in high viscosity solution as observed by the Cu^{++} ion electrode

In order to test whether the renaturation as followed by the Cu^{++} ion electrode has the same viscosity dependence as the spectrophotometrically observed reaction, the experiments of the previous section were repeated in 23% sucrose solution. This particular concentration of sucrose, corresponding to $\eta_{rel} = 2.0$, was

chosen in preference to the 10.5% or 40% solutions used previously (section III.7) because if there is a viscosity dependence, the reaction rate in 23% sucrose should be readily detected to be slower than in the absence of sucrose and yet not so slow as to make the observation of the reaction by the Cu^{++} electrode difficult.

Thus a denatured DNA-Cu^{++} solution containing 23% sucrose and with Cu/P = 1 was renatured by the addition of 1.1 M KNO_3 in 23% sucrose to a final KNO_3 concentration of 0.10 M. A typical set of renaturation results is shown in fig. IV-7. It is apparent that the reaction in the viscous solution is slower than that observed without sucrose under the same conditions (fig. IV-2).

There were two main difficulties associated with these experiments. Firstly, the electrodes appeared to be less stable in the solutions containing sucrose; for example, measurements on the denatured DNA-Cu^{++} solutions prior to each renaturation within a space of 24 hours showed a variation as high as 5 mV. However, the stability of the potential reading on a single solution over a period of an hour or more was quite reasonable apart from an occasional large fluctuation such as can be seen between t = 5 and t = 6 in the 30 minute curve of fig. IV-7. The second difficulty concerns the determination of the value of E_∞ due to the much slower reactions observed under these conditions. It was much more difficult to decide when a constant EMF value was reached, for superimposed on the slow approach of E to its equilibrium value from approximately t = 5 minutes onwards are the small electrode fluctuations of the order of $\pm$ 0.2 mV and any

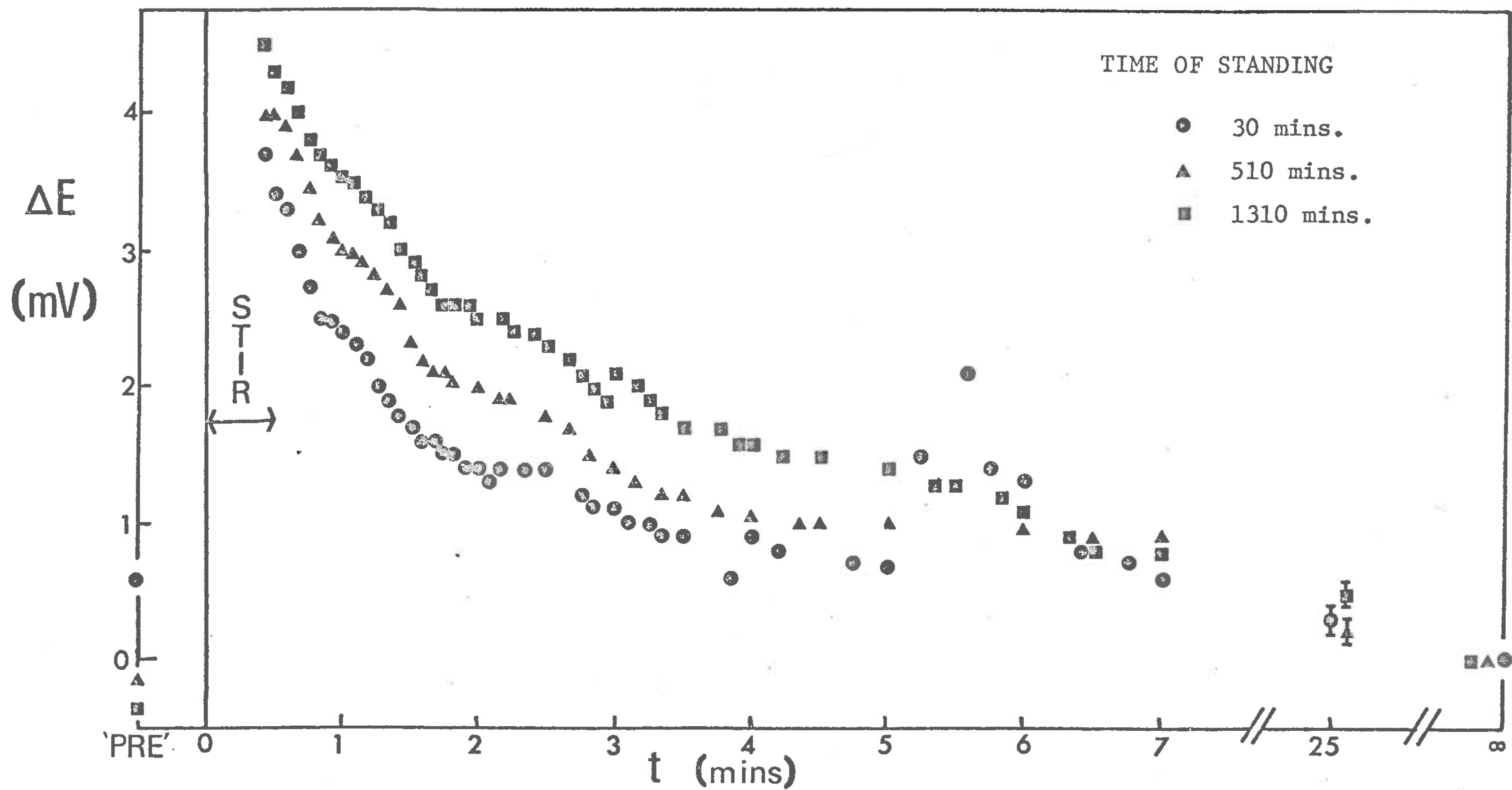

Fig. IV-7. Renaturation in 23% sucrose solution (η_{rel} = 2.0) as observed by Cu^{++} ion electrode. "PRE-initial" points represent EMF readings on the denatured DNA-Cu^{++} solution prior to each renaturation.
DNA concentration = 5.00 × 10^{-5} M_P; Cu/P = 1; KNO_3 concentration = 0.10 M; temperature = 25°C.

drift in the electrode output which may occur. Generally, a value of E, steady to within ± 0.2 mV, was observed between t = 50 and t = 70 minutes, although if the electrodes were left in the solution for a further hour, the reading after this time had decreased quite considerably but as the reading was very unstable, it was discounted. Thus the average value between t = 50 and t = 70 minutes was taken as E_∞ with an accuracy of ± 0.2 mV.

Because of these difficulties associated with the sucrose solution measurements, no attempt was made to calculate the total EMF change accompanying the renaturation, and so no formal comparison could be made between these results and the renaturation followed spectrophotometrically under the same conditions. However, since the renaturation rate is significantly reduced by the presence of 23% sucrose, it is concluded that the rate of release of Cu^{++} ions from the denatured DNA-Cu^{++} complex is dependent on the viscosity of the solution.

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CHAPTER V

THE RENATURATION OF THE DENATURED DNA-Cu^{++} COMPLEX BY ETHYLENE DIAMINE

1. Introduction

2. Experimental procedure

3. Renaturation at an ethylene diamine/Cu^{++} ratio of 1:1

   a. Kinetic analysis

   b. First order rate constants

   c. Correction for the absorbance of the Cu^{++}-EDA complex

4. Effect of the EDA/Cu^{++} ratio on the renaturation rate

5. Reactions followed by the stopped-flow rapid reaction apparatus

   a. Results

   b. Analysis of the data

6. Effect of solution viscosity on the renaturation rate

Reference

1. Introduction

An approach which could possibly assist in elucidating the mechanism of the renaturation reaction was considered to be the addition of a Cu^{++} complexing agent to bring about the renaturation in place of an increase in ionic strength.

Hiai[1] observed that when 3×10^{-4} M disodium diaminoethane tetraacetate (EDTA) was added to the denatured DNA-Cu^{++} solution, complete and rapid renaturation of the DNA followed. It was thought that by using a low concentration of complexing agent, such as a 1:1 ratio between ligand and Cu^{++} ions, the renaturation may proceed at a sufficiently slow rate for the course of the reaction to be followed by a spectrophotometric method. However, when preliminary experiments were conducted it was found that EDTA was not a suitable reagent to bring about the renaturation because although EDTA alone had no absorbance in the 200-300 nm range, the Cu^{++}-EDTA complex exhibited an absorbance band in this region with a broad maximum between 240 and 270 nm. With Cu^{++} and EDTA both present at a concentration of 1×10^{-4} M, the absorbance at 259 nm was 0.28, and thus the decrease in hyperchromicity during renaturation was masked by the increase in absorbance as the Cu^{++}-EDTA complex was formed. These experimental observations are in conformity with a brief footnote to Hiai's paper[1] reporting a UV absorption band of the Cu^{++}-EDTA complex. Ethylene diamine (EDA), another common complexing agent for Cu^{++} ions, was examined to see if it too gave an interfering UV

absorbance but it was found to be more satisfactory. The spectra between 240 nm and 300 nm of the compounds of interest are shown in fig. V-1. Ethylene diamine at a concentration of 1.00×10^{-4} M has no absorbance in this region (curve A), nor does it have any effect on the spectrum of native DNA (5.00×10^{-5} M_p) as shown in curve C. The ethylene diamine-Cu^{++} complex, concentration = 1.00×10^{-4} M, has a spectrum given by curve B. It can be seen that there is a significant absorbance at 259 nm but at wavelengths greater than 275 nm the absorbance is very small. When a solution of native DNA, Cu^{++}, and ethylene diamine was prepared, the spectrum of this solution followed curve E, which is the sum of curves B and C. Curve D is the spectrum of a denatured DNA-Cu^{++} solution, DNA concentration = 5.00×10^{-5} M_p, Cu^{++} concentration = 1.00×10^{-4} M. Thus, prior to the renaturation reaction, the spectrum of the solution is curve D; when ethylene diamine has been added and the renaturation has come to completion the spectrum of the solution is curve E. As fig. V-1 shows, at wavelengths between 270 nm and 280 nm, an O.D. change of the order of 0.1 O.D. units accompanies the renaturation by ethylene diamine which is sufficiently large to enable the course of the renaturation reaction to be followed accurately. The wavelength selected was 280 nm because the absorbance of the Cu^{++}-EDA complex was less at this wavelength and when the experimental absorbance values were corrected for the Cu^{++}-EDA contribution, it was found that the correction was not significant at this wavelength (section V.3.c).

Fig. V-1. UV spectra of various DNA, Cu^{++} and ethylene diamine solutions.

A: Ethylene diamine, 1.00×10^{-4} M.

B: Ethylene diamine-Cu^{++} complex, 1.00×10^{-4} M.

C: Native DNA, 5.00×10^{-5} M_p or native DNA + ethylene diamine, 1×10^{-4} M.

D: Denatured DNA-Cu^{++} solution;
DNA concentration = 5.00×10^{-5} M_p,
Cu^{++} concentration = 1.00×10^{-4} M.

E: Native DNA (5.00×10^{-5} M_p) + Cu^{++} (1.00×10^{-4} M) + Ethylene diamine (1.00×10^{-4} M).

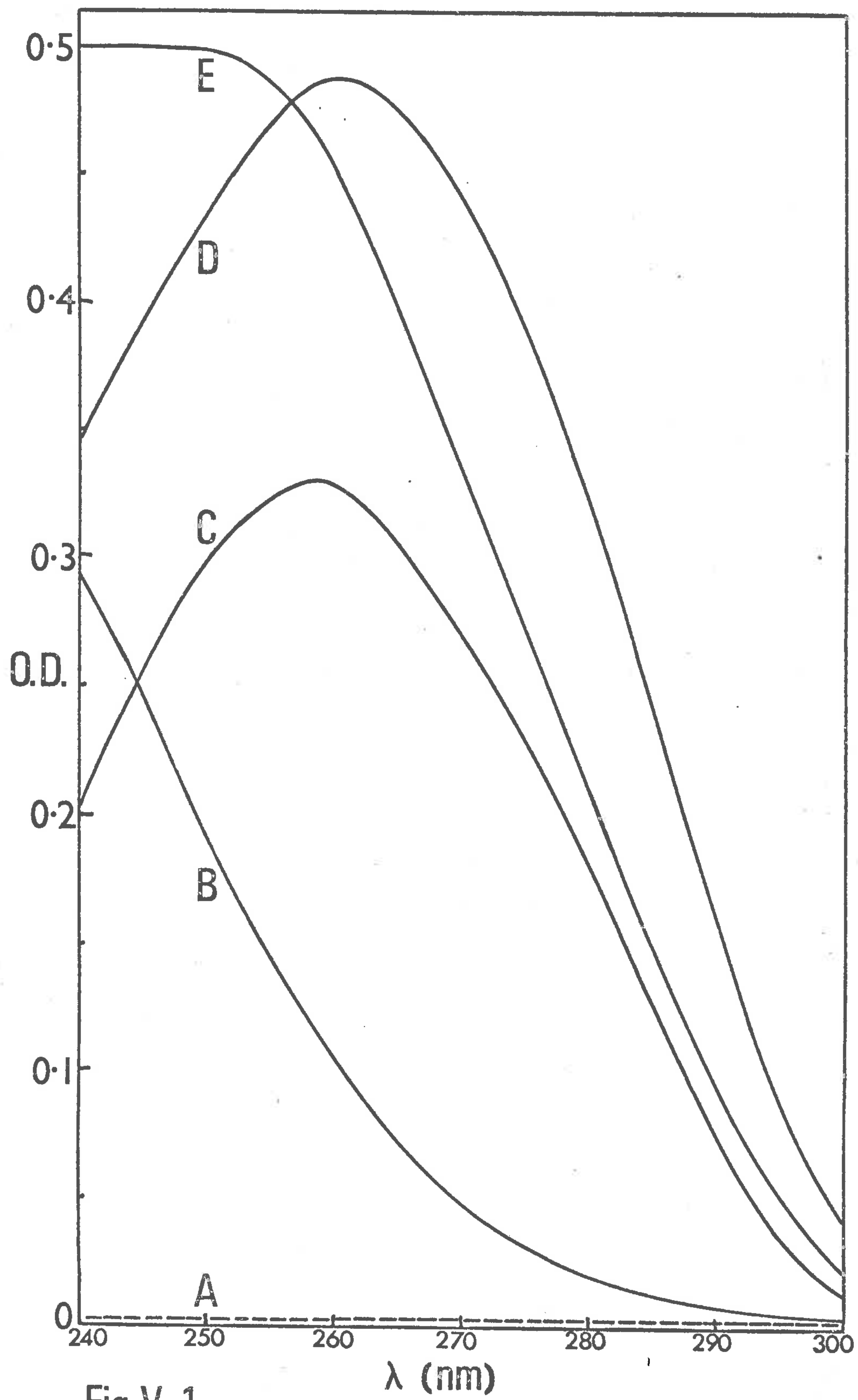

Fig.V-1

Measurements of the pH of various solutions were made to ensure that the use of ethylene diamine to bring about the renaturation did not cause any undesirable pH changes. An EDA solution, 1×10^{-4} M, had a pH of 9.0, but when the Cu^{++}-EDA complex was formed (1×10^{-4} M) the solution had a pH of 5.9. As this was an acceptable pH at which to carry out the renaturation, all solutions were used unbuffered or unadjusted in pH.

2. Experimental procedure

The denatured DNA-Cu^{++} solution was prepared by the same procedure as previously described (section III.2.b). The renaturation was commenced by adding a volume of 1.943×10^{-3} M ethylene diamine solution by means of a micrometer syring to a weighed sample of denatured solution in a cuvette. The layering technique used previously for the addition of concentrated KNO_3 solution could not be used in this case as there was not sufficient difference in the densities of the solutions. To bring about rapid mixing, a small teflon coated magnet, approximately 1 cm long was placed in the cuvette, and by means of a magnetic stirrer the contents were stirred rapidly while the EDA solution was added, the process requiring approximately 5 seconds. This uncertainty in the point taken as zero time is relatively insignificant when the total reaction time exceeds three minutes. The cuvette was stoppered, replaced in the spectrophotometer and the absorbance change at 280 nm recorded.

The final DNA and Cu^{++} concentrations were always

5.00×10^{-5} M_p and 1.00×10^{-4} M, respectively and the ethylene diamine concentration depended on the ratio of EDA/Cu^{++} required in each particular reaction. All solutions contained 10^{-2} M KNO_3, and all reactions were performed at 25°C.

To obtain reproducible results, care had to be taken to prevent evaporation of the volatile ethylene diamine from the stock solution. In order to fill the micrometer syringe prior to each renaturation reaction, a few mls of the EDA stock solution (stored in a sealed flask) were placed in a small beaker. The syringe was quickly rinsed several times with this solution and then filled, and the excess EDA solution discarded. The time the sample of EDA solution was exposed to the air was approximately 5 minutes.

3. Renaturation at an ethylene diamine/Cu^{++} ratio of 1:1

A typical set of renaturation curves observed at 280 nm at an EDA/Cu^{++} ratio of 1:1 is shown in fig. V-2. It can be seen that a dependence on the time of standing between denaturation and renaturation is observed as in the case of the renaturation brought about by increasing the ionic strength. It can also be seen that the renaturation curves are not very reproducible; for example, curves (e) and (f) which were observed after approximately the same time of standing are quite dissimilar. This lack of reproducibility is more evident when the rate constants calculated from these curves are examined (section V.3.b).

Fig. V-2. Renaturation brought about by ethylene diamine.

$EDA/Cu^{++} = 1.0$; DNA concentration = 5.00×10^{-5} M_p; $Cu/P = 2$; temperature = 25°C.

Time of standing:
(a) 33 mins.
(b) 80 mins.
(c) 150 mins.
(d) 555 mins.
(e) 1660 mins.
(f) 1700 mins.

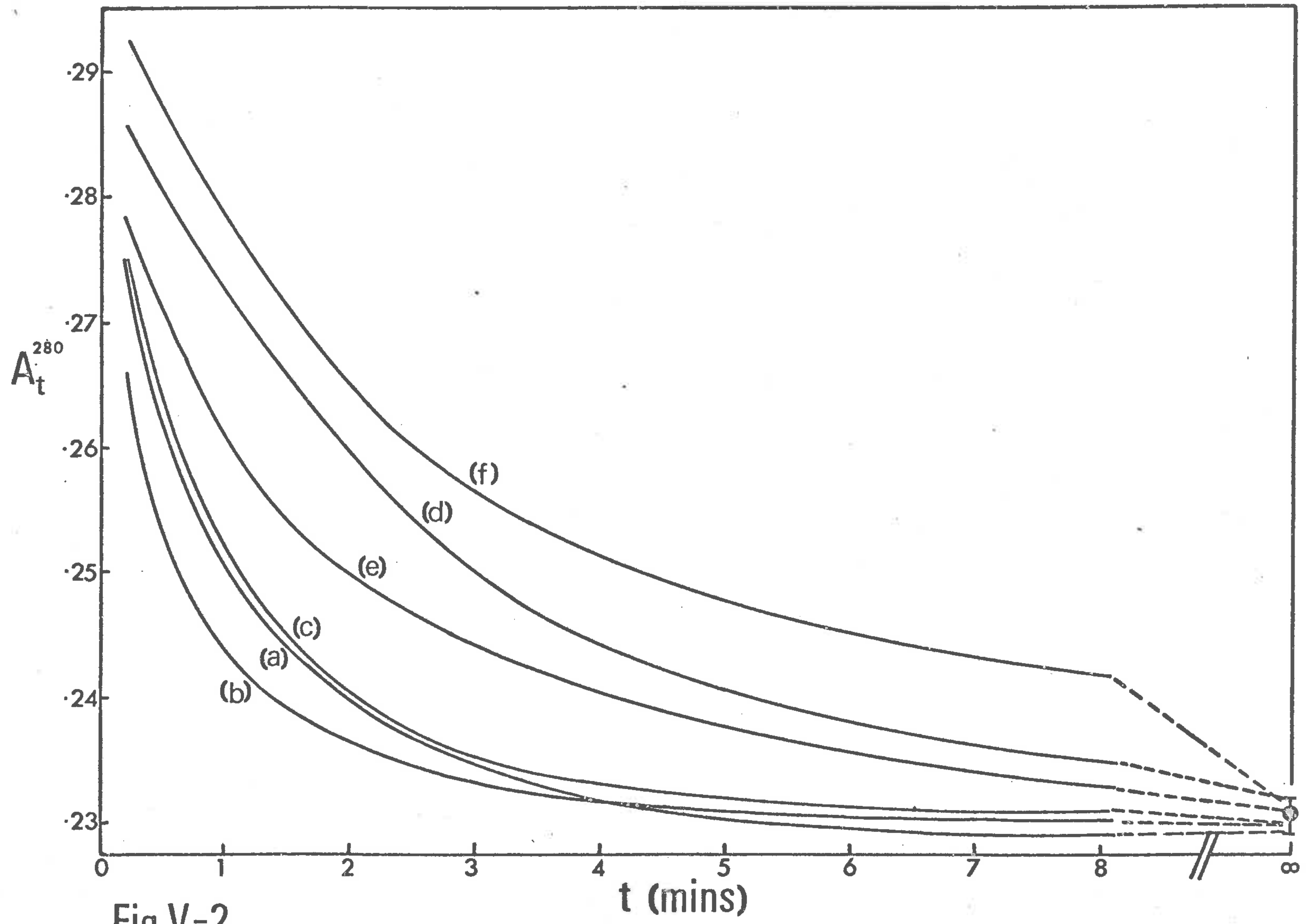

Fig.V-2.

a. Kinetic analysis

First order analyses of all renaturation curves were carried out and in all cases straight lines were obtained. The first order plots of three of the renaturation curves of fig. V-2 are shown in fig. V-3. The slope of the line and its intercept with the y-axis was calculated in each case by least squares analysis of the data.

Fig. V-3 illustrates two important characteristics which were observed for all reactions at EDA/Cu^{++} = 1.0. Firstly, the extent to which the reaction follows first order kinetics is 80-90% which is significantly higher than the extent to which the renaturation at 25°C in 0.15 M KNO_3 adhered to first order kinetics (60-70%). Secondly, the intercepts of the straight lines with the y-axis (t = 0) do not correspond to log $(A_0 - A_\infty)$ = 0% reaction as would be expected. The value of A_0 is obtained by reducing the O.D. (280 nm) of the denatured DNA-Cu^{++} solution measured before each renaturation reaction by the dilution factor for the addition of the ethylene diamine solution which was known from the mixing procedure and from an experimental check to be 0.950. The value of A_∞ was observed experimentally to an accuracy of ± 0.001 and for all reactions the value of $(A_0 - A_\infty)$ lay in the range 0.095 - 0.100 O.D. units, which predicts that the intercept for 0% reaction should be -1.022 to -1.000. The observed values lay in the range -1.30 to -1.11.

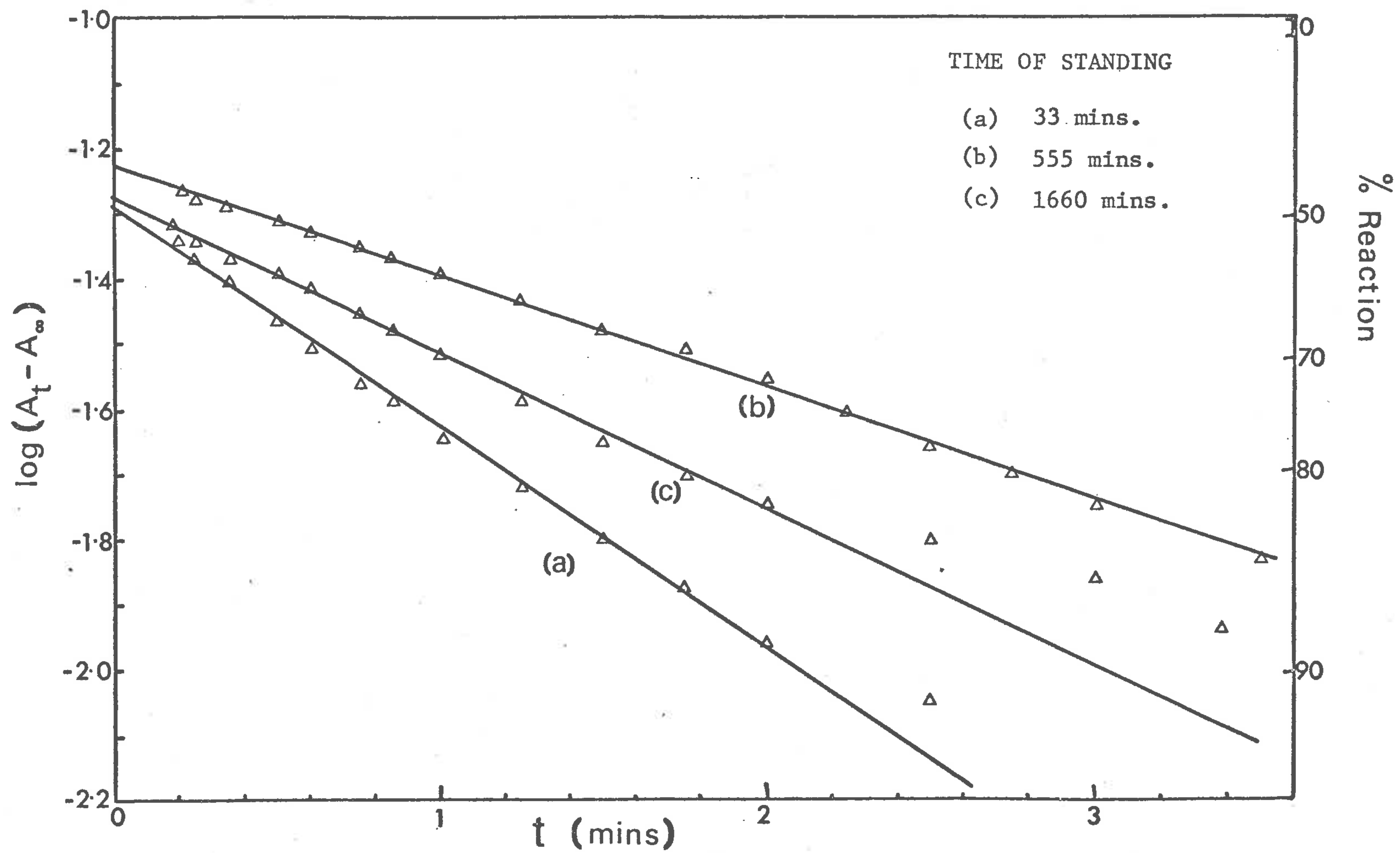

Fig. V-3. First order analysis of data taken from fig. V-2. $EDA/Cu^{++} = 1 \cdot 0$.

b. The first order rate constants

From the slope of the first order plot calculated by the least squares method, the rate constant of each renaturation reaction was obtained and in fig. V-4 these are plotted against the time of standing for the seventeen reactions observed under these conditions.

The lack of reproducibility of the renaturation rate noticed in fig. V-2 is more clearly demonstrated by the marked scatter of the points in fig. V-4. Most of the sources of error discussed in section III.3.c with respect to the renaturation by increased ionic strength are still relevant to this system, but the reason for the further decrease in accuracy in this case is believed to be the uncertainty in the ethylene diamine concentration. Since a very small volume ($\sim$0.1 ml) of concentrated EDA solution was added to the denatured solution, the final EDA concentration may be subject to a considerable error. The calibration of the micrometer syringe used to perform the addition showed that the delivery of 0.1 ml was accurate to 0.4%. In actual usage where a rapid addition and mixing procedure was desired, the uncertainty in the volume may have been larger than this. Another source of inaccuracy is evaporation of ethylene diamine from the stock solution with which the micrometer syringe was filled. It was attempted to minimise this error by carrying out the required procedure as quickly as possible, but some irreproducibility in conditions for different reactions was inevitable. From these considerations it is considered that the final EDA concentration may be subject to an error approaching 1%. Although this

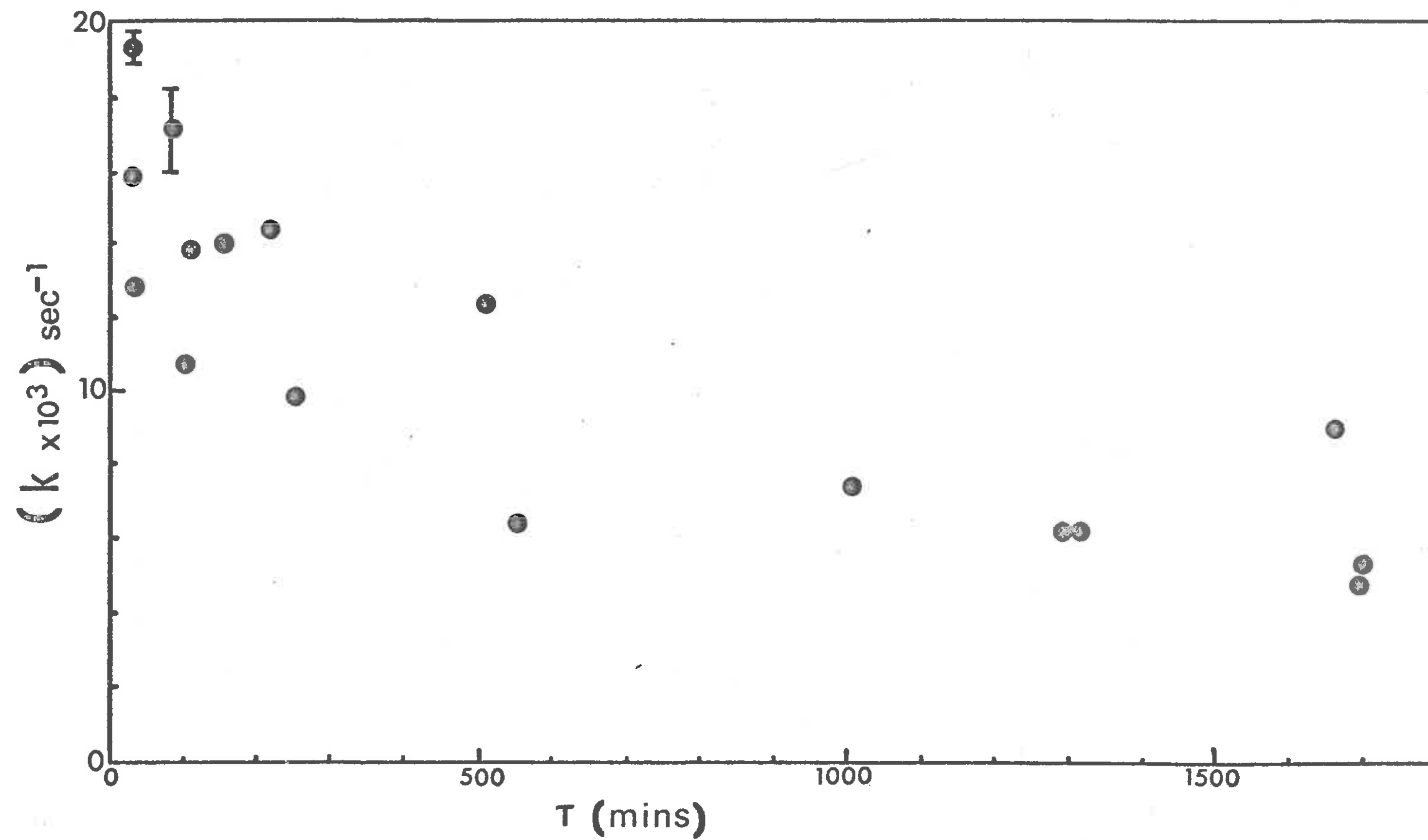

Fig. V-4. Dependence of the first order rate constants at $EDA/Cu^{++} = 1.0$ on the time of standing between denaturation and renaturation.

DNA concentration = 5.00×10^{-5} M_P; Cu/P = 2; temperature = 25°C.

may not appear very large, it is demonstrated in section V.4 that the renaturation rate is extremely sensitive to ethylene diamine concentration and so an uncertainty of 1% may account for the considerable scatter of the rate constants seen in fig. V-4.

c. Correction for the absorbance of the Cu^{++}-EDA complex

The absorbance of the Cu^{++}-EDA complex at a concentration of 1×10^{-4} M at 280 nm is 0.017 ± 0.001. Since the total O.D. change on renaturation is only of the order of 0.1, correcting for the Cu^{++}-EDA absorbance may significantly change the analysis of the data. At each stage of the reaction, the concentration of the Cu^{++}-EDA complex must be determined to enable the correction to be made. Consider the situation at $t = 0$ when the EDA has been added to the solution but no renaturation has taken place. The concentration of the Cu^{++}-EDA complex at this instant depends on which of three possible mechanisms has occurred; the EDA may have complexed with all the free Cu^{++} ions present in the denatured solution, or with the free Cu^{++} ions and those bound on external sites of the DNA, or with all the Cu^{++} ions present. In the last case, the concentration of the Cu^{++}-EDA complex remains constant throughout the renaturation and the same correction of 0.017 is applied to all A_t values, which has no effect on the first order analysis. In the first two cases, a knowledge of the free Cu^{++} concentration and the concentration of Cu^{++} bound to the external sites on the DNA is necessary to make the corrections. Cu^{++} ion electrode measurements (section IV.4.d) showed that at $Cu/P = 1$

the fraction of Cu^{++} ions free in the denatured DNA-Cu^{++} solution was 0.55-0.6. Since at Cu/P = 2 the fraction of free Cu^{++} ions is increased, a value of 0.7 would appear reasonable in this case, and this value is assumed in order to estimate the magnitude of the corrections. At t = 0 the absorbance due to the complex between EDA and the free Cu^{++} ions (0.7×10^{-4} M) is 0.011. When the renaturation is complete, all the Cu^{++} ions are complexed with EDA and the absorbance due to the complex is 0.017. Thus, during the reaction, the correction which must be applied varies from 0.011 to 0.017, and so the change in the values of $(A_t - A_\infty)$ required for the first order analysis decreases from 0.006 at $t = 0$ to zero at $t = \infty$. On examining fig. V-3, it can be realised that the effect of corrections of this magnitude on the slope of the first order plot is insignificant, and the same conclusion would be reached if any value greater than 0.5 was taken for the fraction of Cu^{++} ions free in the solution or if the Cu^{++} ions bound to external sites on the DNA were also assumed to react with the EDA at $t = 0$.

4. Effect of the EDA/Cu^{++} ratio on the renaturation rate

The renaturation was carried out at a number of EDA/Cu^{++} ratios from 0.75 to 1.50, the Cu^{++} concentration being kept constant at 1.00×10^{-4} M; examples of the renaturation curves thus obtained are shown in fig. V-5. For each EDA/Cu^{++} ratio, a number of reactions were obtained at various time intervals after denaturation but only the one corresponding to an interval of 30-40 minutes is presented

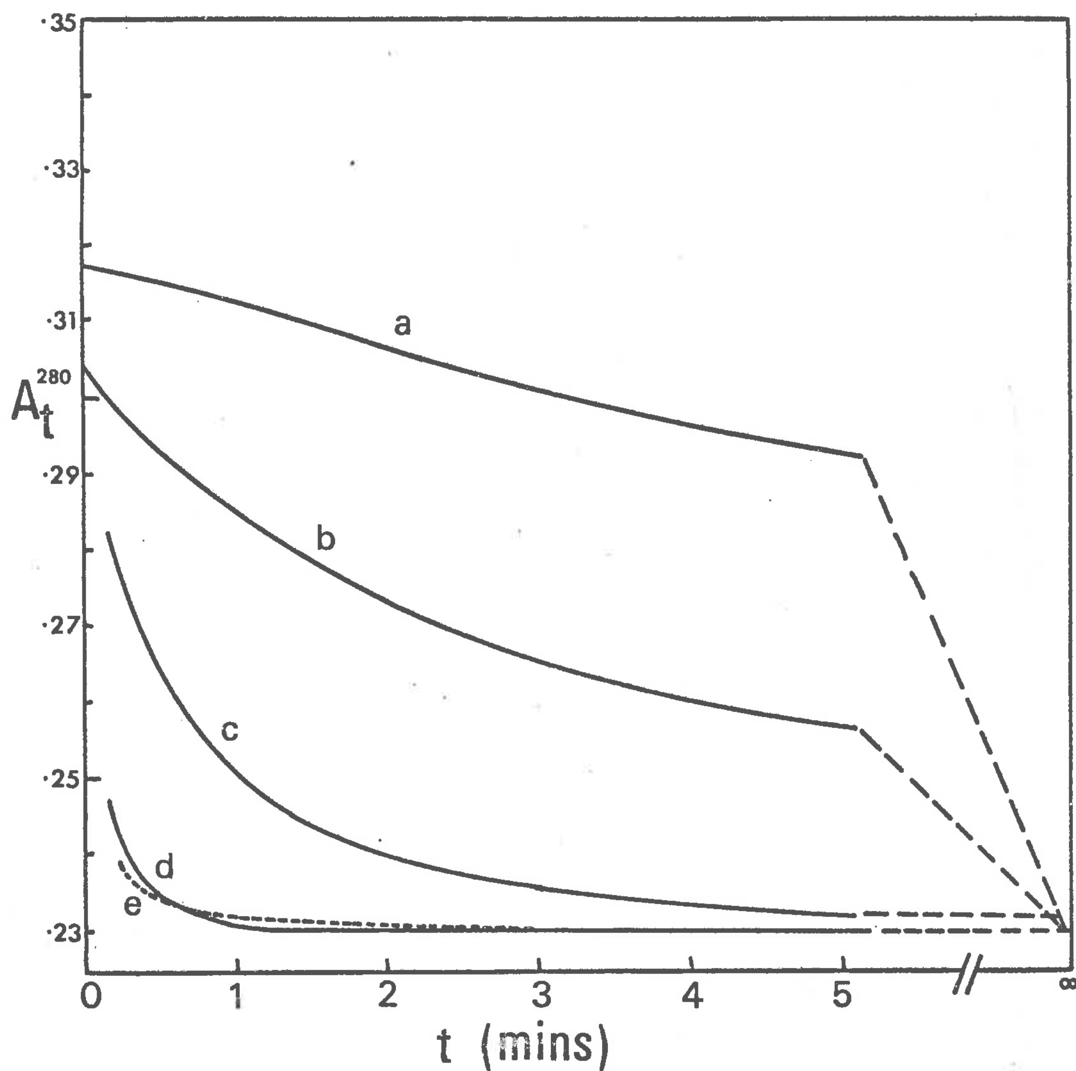

Fig. V-5. Renaturation curves at varying concentrations of ethylene diamine.

a) EDA/Cu^{++} = 0.75
b) " = 0.85
c) " = 1.00
d) " = 1.25
e) " = 1.50

Time of standing = 30-36 minutes for all curves.

here for the sake of clarity. The expected dependence of the renaturation rate on the time of standing was evident for the reactions in which EDA/Cu^{++} = 0.75, 0.85 or 1.0. At EDA/Cu^{++} = 1.25 or 1.50, only the final 10-15% of the reaction was experimentally observed and thus no conclusions can be drawn from this data.

The first order analysis of the renaturation at EDA/Cu^{++} = 0.85 showed that the reactions followed first order kinetics for the initial 60-75% reaction. The rate constants varied from 4.34×10^{-3} sec^{-1} at a time of standing of 30 minutes to 1.14×10^{-3} sec^{-1} at a time of standing of 1800 minutes.

When EDA/Cu^{++} = 0.75, only the initial 40-45% reaction could be fitted to a first order plot and the rate constants for times of standing from 30 minutes to 1270 minutes lay in the range 1.07×10^{-3} sec^{-1} to 0.43×10^{-3} sec^{-1}.

The intercept of each first order straight line with the axis at t = 0 was generally less than the expected value of -1.022 to -1.000, as was also observed for the case of EDA/Cu^{++} = 1.0 (section V.3.a). The range of values for the intercepts at each EDA/Cu^{++} ratio was found to be -1.05 to -1.03 for EDA/Cu^{++} = 0.75, -1.15 to -1.10 for EDA/Cu^{++} = 0.85, and -1.30 to -1.11 for EDA/Cu^{++} = 1.00. These values suggest that there may be a dependence of the apparent extent of the reaction as indicated by the first order intercept on the EDA/Cu^{++} ratio, but this would have to be tested over a wider range of EDA/Cu^{++} ratios before a conclusion is drawn.

5. Reactions followed by the stopped-flow rapid reaction apparatus

As the renaturation reactions when EDA/Cu^{++} ratios greater than 1.0 were used proceeded too rapidly to be observed using a conventional mixing technique in a spectrophotometer cell, the reactions at such ratios were followed using a stopped-flow rapid reaction apparatus. By this technique, the time between mixing the reactant solutions to start the renaturation and the commencement of absorbance readings is decreased from 10 seconds for the conventional mixing procedure to several milliseconds.

a. Results

The description and operation of the stopped-flow apparatus are outlined in section VII.6. Since the procedure involved mixing equal volumes of reactant solutions, the ethylene diamine solution and the DNA-Cu^{++} solution were prepared at twice the concentration required in the reaction. The denaturation of DNA in the presence of Cu^{++} was carried out by the normal method, the DNA and Cu^{++} concentrations in the resulting solution being 1.00×10^{-4} M_P and 2.00×10^{-4} M, respectively. The evaporation of EDA was prevented by sealing the reservoir containing the EDA solutions. Once this was done, the system was completely closed and there was no chance of a discrepancy in the EDA concentrations for different reactions. All absorbance measurements were made at 280 nm.

With an EDA/Cu^{++} ratio of 1.5, six renaturation reactions were followed at various time intervals after preparation of the denatured solution. For two of the reactions, a CRO sweep time of

5 seconds was employed which enabled 80% of the reaction to be recorded, while for the other reactions, 100% reaction was observed with a 10 second sweep time. Fig. V-6 shows a CRO trace for a 5 second and a 10 second sweep time. It is noted that the decreasing optical density on renaturation is exhibited as an increasing photomultiplier output on the CRO trace. The line representing the completed reaction was obtained by recording a second sweep after the first was complete. The zero on the CRO trace corresponds to a photomultiplier output of 5.10 volts as indicated by the backing-off voltage which had to be applied to bring the differential amplifier signal to zero.

At EDA/Cu^{++} = 1.25, six renaturation reactions were observed, one using a 10 second sweep time which only recorded 60% of the reaction and the remainder at a 20 second sweep time (85-95% reaction).

Two reactions at EDA/Cu^{++} = 1.0 were performed to enable a comparison to be made between the data obtained by the stopped-flow technique and the conventional mixing technique. However, the maximum sweep time available on the CRO (50 seconds) was only sufficient to follow the first 50% of the reaction and repeated sweeps had to be made to determine the completed reaction position. In so far as a comparison between the two sets of data was possible, the agreement was found to be very satisfactory, as is discussed in detail in section V.5.b.

A preliminary experiment at a much higher EDA concentration

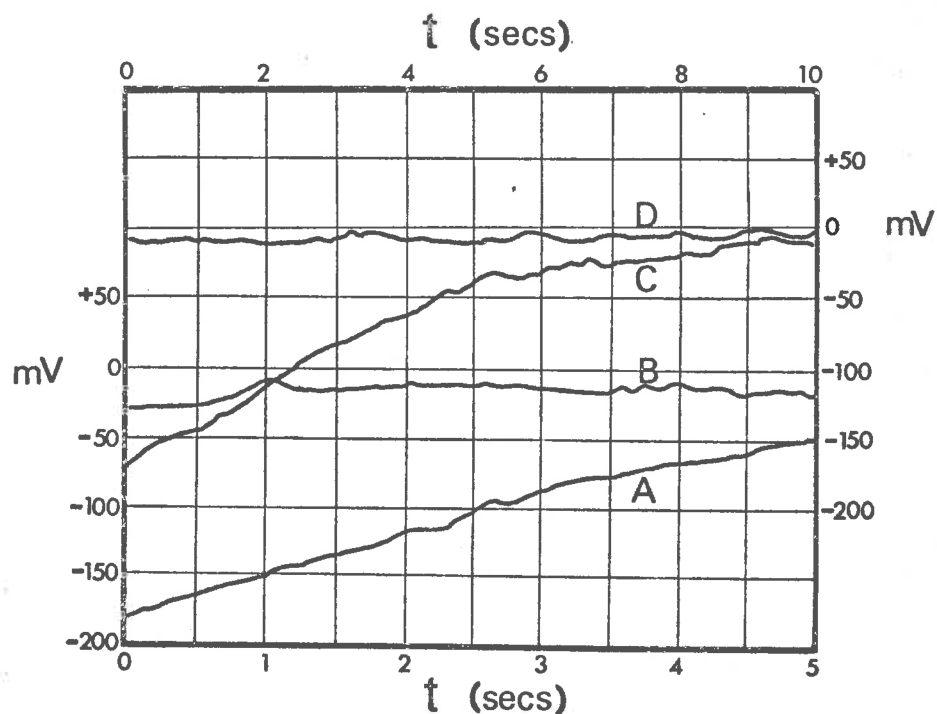

Fig. V-6. Reaction curves observed with stopped-flow rapid reaction apparatus at EDA/Cu^{++} = 1.5

A: CRO trace using a 5 second sweep time. (Left-hand ordinate, and lower time scale)

B: Resweep giving position of completed reaction.

C: CRO trace using a 10 second sweep time. (Right-hand ordinate, and upper time scale)

D: Resweep giving position of completed reaction.

Both reactions at ∿ 210 minutes after denaturation; DNA concentration = 5.00×10^{-5} M_p; Cu^{++} concentration = 1.00×10^{-4} M; EDA concentration = 1.5×10^{-4} M; temperature = 25°C; λ = 280 nm.

($EDA/Cu^{++} \sim 2$) was performed and fig. V-7 shows a renaturation curve thus obtained. It can be seen that the reaction was complete in less than 0.5 seconds. The anomaly in the shape of the curve within the initial 50 msecs is due to an instrumental characteristic; a capacitor in the photomultiplier-CRO circuit required a time interval of 20-30 msecs to be charged to the correct level, resulting in a delay in the CRO signal registering the correct readings.

b. Analysis of the data

Since the potential output of the photomultiplier is proportional to the intensity of the light transmitted through the reaction cell, the relationship between the absorbance at 280 nm of the solution at time t (A_t) and potential at time t (V_t) is given by

$$A_t - A_\infty = \log I_\infty - \log I_t$$
$$= \log V_\infty - \log V_t$$

where I_t is the intensity of transmitted light at time t.

The main source of inaccuracy in the analysis of a set of data was the instability of the photomultiplier output which showed considerable noise and long-term drift. The importance of these factors depended on the time interval over which the data were obtained. Fig. V-7 shows that for a sweep time of 0.5 seconds there was no detectable noise, but in fig. V-6, the trace over 10 seconds shows some random fluctuation. The worst situation was the 50 second trace at $EDA/Cu^{++} = 1.0$, in which continual noise of the

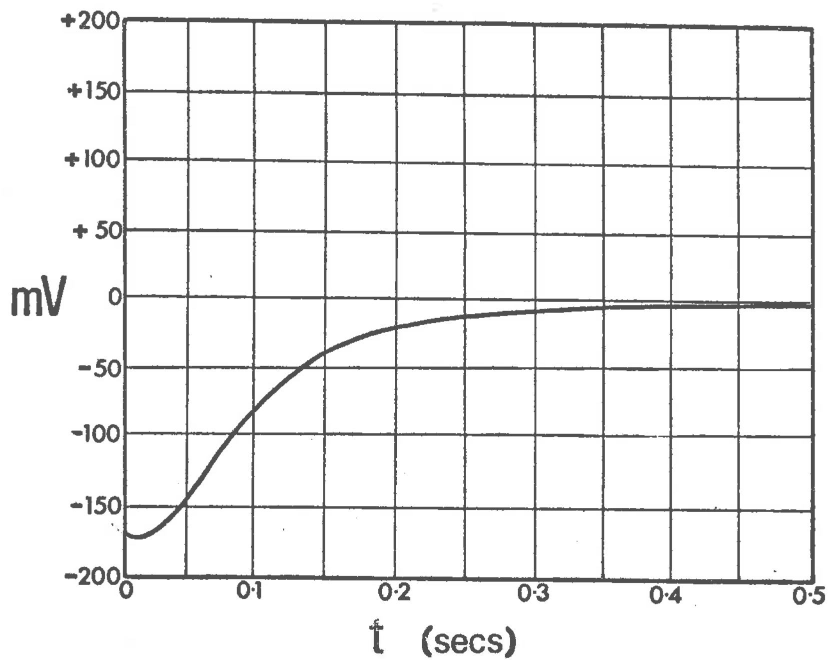

Fig. V-7. Reaction curve observed with stopped-flow rapid reaction apparatus at EDA/Cu^{++} ∿ 2.0

DNA concentration = 5.00×10^{-5} M_P; Cu^{++} concentration = 1.00×10^{-4} M;

EDA concentration ∿ 2.0×10^{-4} M;

time of standing = 60 minutes; temperature = 25°C.

order of 10-15 mV was seen. The error due to these random fluctuations could be minimised by taking the V_t values from a smooth curve drawn through the trace; an uncertainty of $\pm$ 3 mV could then be assigned to each value. If the photomultiplier output when the solution in which the reaction had reached completion was in the light path, was observed over a period of time, superimposed on the random noise was a drift in the average position, on occasions amounting to as much as 50 mV over 2 or 3 minutes. This drift is only significant in the slow reactions where it makes the value of V_∞ uncertain, for the position of V_∞ given by the second sweep relative to the previous V_t values may be in error due to the drift. This error is only small for the reactions at EDA/Cu^{++} = 1.25 but it may be quite large when EDA/Cu^{++} = 1.0, in which case the readings taken after the first 50 second sweep up to t = 3 minutes were considered to be accurate to $\pm$ 20 mV.

For all the reactions observed, the change ($V_0 - V_\infty$) lay in the range 165 $\pm$ 15 mV which corresponds to a total optical density change ($A_0 - A_\infty$) of 0.0146 $\pm$ .0015 O.D. units. There was no correspondence between the total optical density change and the EDA/Cu^{++} ratio, and thus the suggestion from section V.4 that there may be a dependence of the extent of the observed reaction on the EDA/Cu^{++} ratio is not supported by this data over a wider range of EDA concentrations. As the path length of the reaction cell was 2 mm, this value of ($A_0 - A_\infty$) corresponds to a total optical density change in a 1 cm cell of 0.073 $\pm$ .008 O.D. units. This is to be compared

with the expected change at 280 nm accompanying the reaction of 0.095 - 0.100 O.D. units. There are a number of reasons which can be invoked to explain why the observed change is less than the predicted change, such as the path length of the reaction cell, which is only nominally 2 mm, or the accuracy of the monochromator, which may be in error by 5 or 10 nm; other reasons include non-linearity of the photomultiplier or scattered light due to the wide monochromator slit width. Considered in isolation, the low value of $(A_0 - A_\infty)$ could readily be attributed to instrumental error, but it is interesting to note that the logarithm of this value (-1.14 $\pm$ .05) lies within the range of values obtained for the intercepts of the first order plots of the reactions observed by the conventional spectrophotometric technique (-1.30 to -1.05). Since in no case in which the renaturation was brought about by increasing the ionic strength was any discrepancy noticed between the predicted and observed value of $(A_0 - A_\infty)$, it seems that it may be a real property of the renaturation induced by ethylene diamine that the experimentally observed reaction commences at a lower absorbance than predicted. If this is true, then there must be some reaction step which accounts for the decrease in absorbance from 0.095-0.100 to 0.073 $\pm$ .008 and which occurs more rapidly than the reaction observed thus far. This initial step could not be detected by the stopped-flow apparatus, for if a very rapid sweep (5 msec) was made after triggering, the trace obtained was a straight line at a value corresponding to the normal value of V_0. Thus if an initial reaction step is occurring

it must be complete within the 2 or 3 millisecond time interval between mixing the reactant solutions and the triggering of the CRO.

As the values of $(A_t - A_\infty)$ were very small, the data were represented as fractions of the total absorbance change, $\frac{A_t - A_\infty}{A_0 - A_\infty}$, for the kinetic analysis. The second order plots were markedly curved but the first order plots in all cases gave straight lines for some extent of the reaction. Fig. V-8 shows a first order plot for a reaction at EDA/Cu^{++} = 1.5. It can be seen that there is good linear behaviour with the exception of the initial one second of the reaction where the points fall somewhat below the straight line. For all the reactions at EDA/Cu^{++} = 1.5, this behaviour in the initial 1 to 1½ seconds was observed, and at EDA/Cu^{++} = 1.25, there was a similar small deviation from linearity in the first 4-5 seconds. The first order analysis of one of the reactions at EDA/Cu^{++} $\sim$ 2 is shown in fig. V-9. The curved portion over the initial 0.05 seconds is due to the instrumental error discussed above and because of this, it is not possible to detect whether there is an initial non-linearity in the first order plot at this EDA/Cu^{++} ratio also. The observation of the slow reaction at EDA/Cu^{++} = 1.0 using the stopped-flow apparatus was subject to considerable error due to noise and drift in the photomultiplier output, but in spite of this, the data gave satisfactory first order plots, one of which is shown in fig. V-10.

The first order rate constants calculated for all reactions are tabulated in Table V-1. The values in this table illustrate

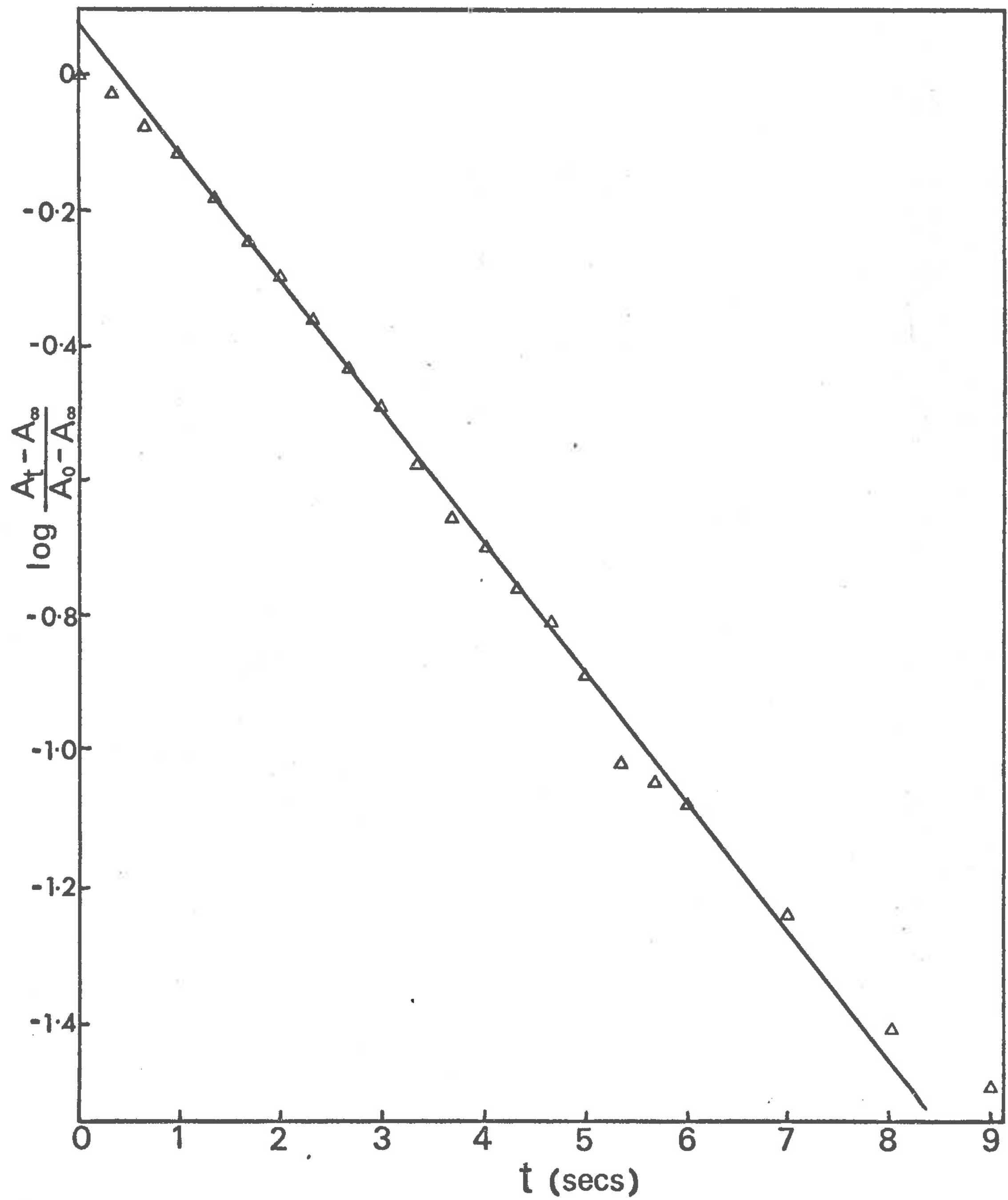

Fig. V-8. First order analysis of data from fig. V-6 (curve C) at EDA/Cu^{++} = 1·5.

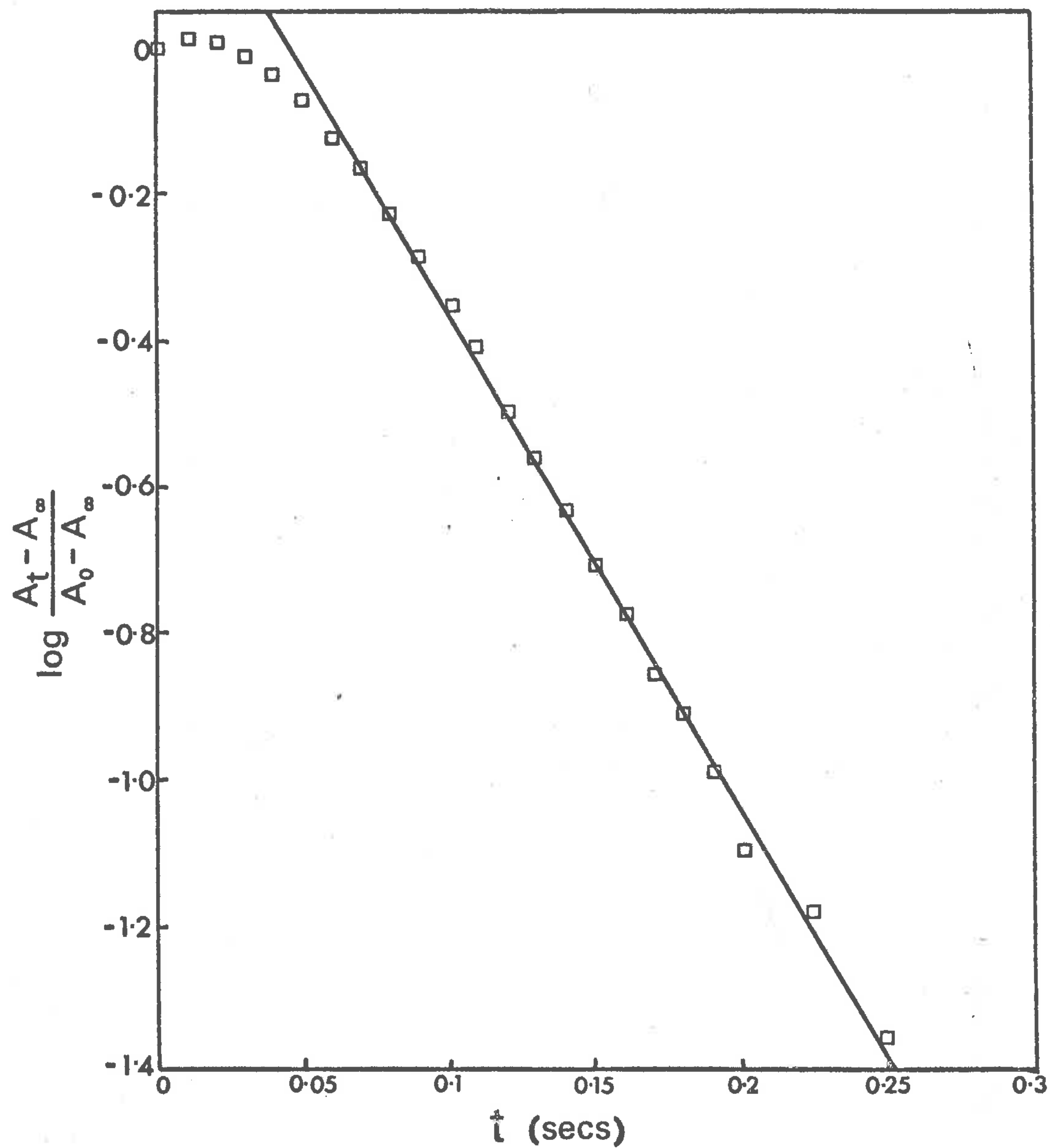

Fig. V-9. First order analysis of data from fig. V-7 at EDA/Cu^{++} ∿ 2.0

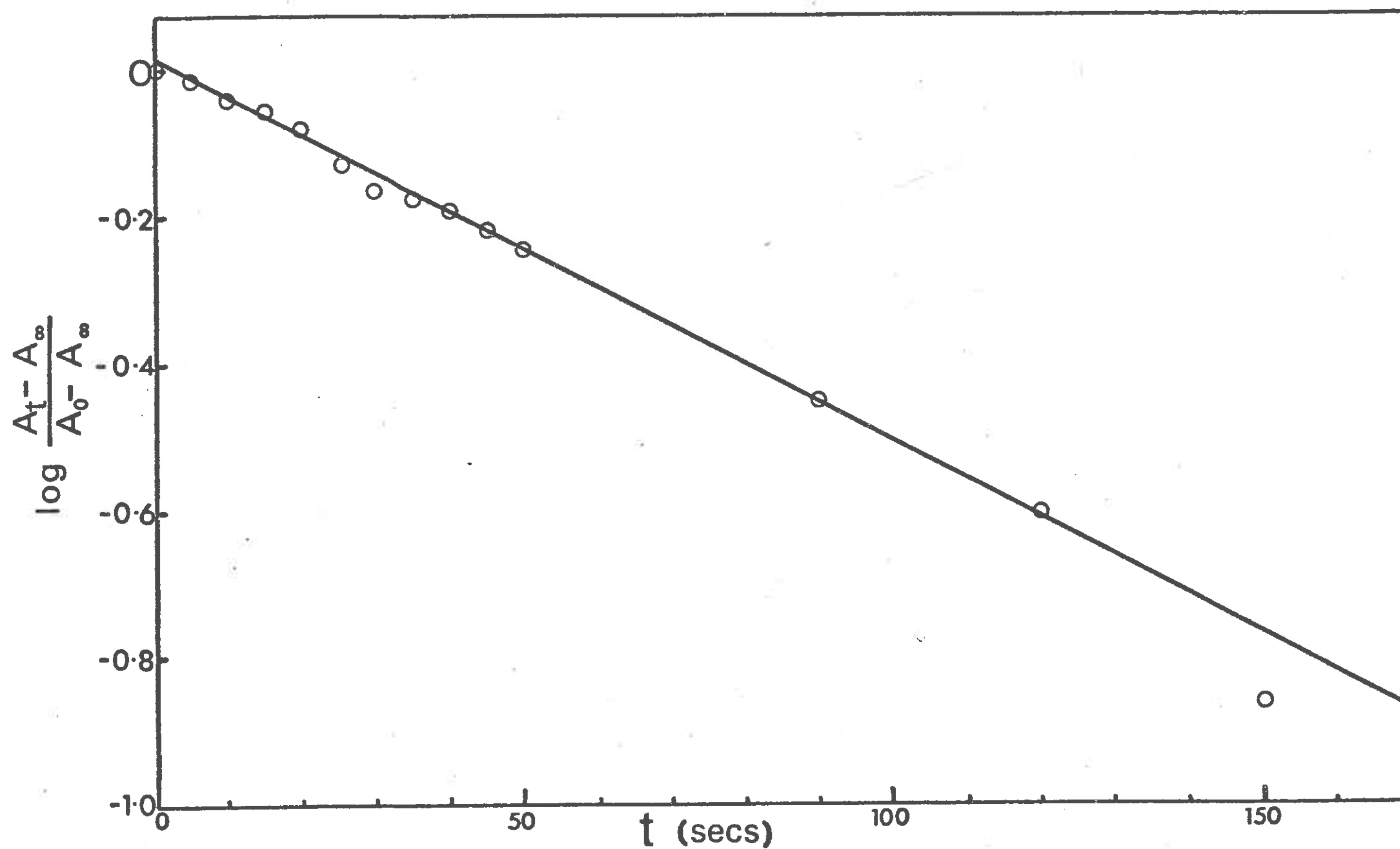

Fig. V-10. First order analysis of a reaction curve at EDA/Cu^{++} = 1.0 as followed by the stopped-flow apparatus.
DNA concentration = 5.00×10^{-5} M_p; Cu^{++} concentration = 1.00×10^{-4} M = EDA concentration; temperature = 25°C.

EDA/Cu^{++} ratio	Time of standing (mins)	First order rate constant, k (sec^{-1})
∿2	60	15.22
	60	16.12
1.5	80	0.689
	80	0.479
	155	0.394
	155	0.444
	210	0.343
	210	0.343
1.25	40	0.190
	40	0.187
	80	0.133
	80	0.089
	95	0.120
	95	0.095
1.0	105	12.1×10^{-3}
	120	15.7×10^{-3}

Table V-1. First order rate constants for reactions at varying EDA/Cu^{++} ratios followed using the stopped-flow rapid reaction apparatus. DNA concentration = 5.00×10^{-5} M_p; Cu/P = 2; temperature = 25°C.

that the renaturation rate is extremely sensitive to the EDA/Cu^{++} ratio, for on increasing the ratio from 1.0 to 2.0, the rate constant is increased by a factor of 10^3. The rate constants at EDA/Cu^{++} = 1.5 show a dependence on the time interval between denaturation and renaturation but in general the time of standing was not varied sufficiently to demonstrate the usual time dependence. When the rate constants at EDA/Cu^{++} = 1.0 are compared with those obtained when the renaturation at the same EDA/Cu^{++} ratio was followed by the conventional spectrophotometric technique (fig. V-4), a close agreement is noted, which indicates that the results obtained by the two different experimental techniques can be compared with confidence.

6. Effect of solution viscosity on the renaturation rate

To study the effect of the bulk viscosity of the solution on the rate of the renaturation brought about by ethylene diamine, the reaction was carried out in a 40% sucrose solution at EDA/Cu^{++} = 1.0 using the conventional slow-mixing spectrophotometric technique. Since for the sake of accuracy it was not desired to prepare either the stock Cu^{++} solution or the stock ethylene diamine solution with 40% sucrose present, the DNA solution was prepared in a 44% sucrose solution so that after a 5% dilution on addition of Cu^{++} and a further 5% dilution on addition of EDA, the final reaction mixture contained 40% sucrose with η_{rel} = 4.06. The reactions observed at various time intervals between denaturation and renaturation followed

first order kinetics for 80-90% reaction, and the rate constants are given in Table V-2. On comparison with fig. V-4, it is clear that the renaturation rate has not been significantly altered by the presence of 40% sucrose. This is in marked contrast to the renaturation brought about by increasing the ionic strength which showed a strong dependence on the solution viscosity.

Reference

1. Hiai, S., J. Mol. Biol., 11, 627 (1965).

Time of standing (mins)	k ($\times 10^{-3}$ sec^{-1})
29	24.95
117	19.34
300	17.62
1270	7.91
1620	9.06

Table V-2. First order rate constants obtained for renaturation in 40% sucrose solution, $EDA/Cu^{++} = 1.0$.
DNA concentration = 5.00×10^{-5} M_P;
Cu/P = 2; temperature = 25°C.

Chapter VI

GENERAL DISCUSSION AND CONCLUSIONS

1. Reaction scheme for the renaturation brought about by increasing the ionic strength

2. Reaction scheme for the renaturation brought about by ethylene diamine

3. The rate-determining step

4. The viscosity dependence

5. The time dependence

6. The renaturation brought about by ethylene diamine

References

Chapter VI

The only report of a kinetic study of the renaturation of the denatured DNA-Cu^{++} complex with which the results of this investigation can be compared is that of Richard and Pacault.[1,2] In agreement with the results presented in Chapter III, these workers found that the renaturation rate depended on the ionic strength, the Cu/P ratio and the time of standing between denaturation and renaturation. However, they did not conclude that the reaction followed first order kinetics even though their first order plot is very similar to those given in Chapter III, and shows good linearity for the initial 80% of the reaction. The aim of Richard and Pacault's study appears to be the establishment of an empirical relationship between the renaturation curves obtained under various conditions and they did not attempt to interpret their results in terms of a reaction mechanism. As mentioned at the beginning of this thesis, the present investigation was aimed at studying the mechanism of the formation of the DNA double helix from the disordered state, and the interpretation of the results from this viewpoint will now be discussed.

1. Reaction scheme for the renaturation brought about by increasing the ionic strength

By application of the known properties of DNA solutions and of the Cu^{++}-DNA system, a reaction scheme can be proposed for the renaturation of the denatured DNA-Cu^{++} complex brought about by increasing the ionic strength; this scheme is summarised in fig. VI-1,

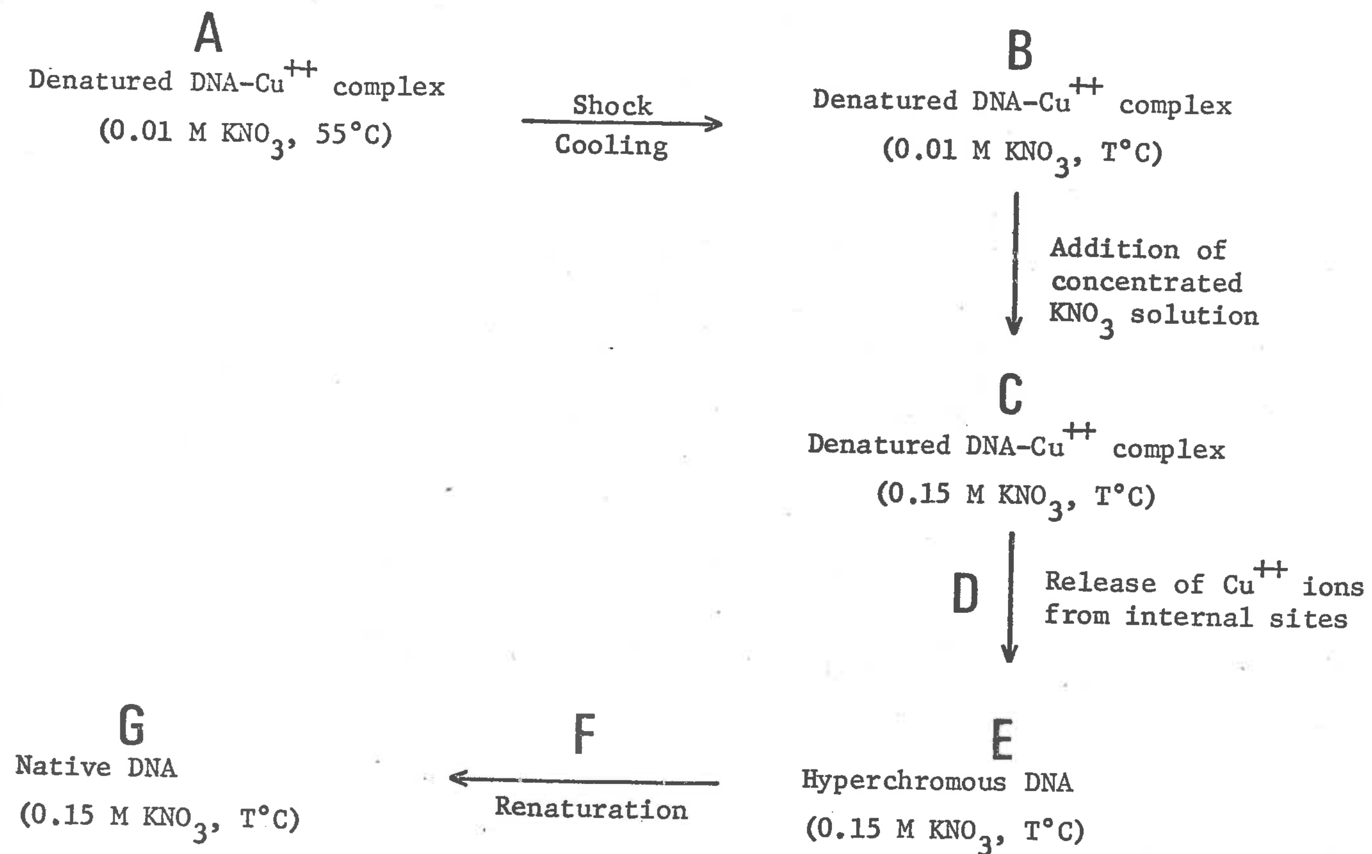

Fig. VI-1. Reaction scheme for renaturation of denatured DNA-Cu^{++} complex at T°C in 0.15 M KNO_3.

and each stage of the reaction as indicated in the diagram is discussed in detail below.

A. The denatured DNA-Cu^{++} complex in 0.01 M KNO_3 at 55°C

The evidence concerning the structure of the complex formed between DNA and Cu^{++} at low ionic strength at a temperature above the melting temperature has been discussed in section II.4. The Cu^{++} ions are bound to internal (base) and external sites on the DNA molecule but the detailed structure of these sites cannot be specified at this stage. In the solution there are equilibria between the free Cu^{++} ions and Cu^{++} ions bound to the internal and external sites. The conformation of the DNA strands in the complex is considered to be similar to that of thermally denatured DNA in so far as the molecule exhibits full hyperchromicity, and is flexible and compact, but the conformational relationship between the complementary strands is not known (section II.4.d).

B. The denatured DNA-Cu^{++} complex in 0.01 M KNO_3 at T°C, the temperature of renaturation

On cooling the solution from 55°C to the renaturation temperature, some change in the conformation of the DNA strands most probably occurs. Assuming that the theory of flexible polyelectrolytes is applicable in this situation, it would be expected that the molecules are less compact at the lower temperature and there may also be some changes in the equilibria between the free and bound Cu^{++} ions on cooling.

C. The denatured DNA-Cu^{++} complex in 0.15 M KNO_3 at T°C

The increase in the ionic strength of the solution has two immediate effects on complex B, immediate in the sense that they occur over a much shorter time scale than the subsequent renaturation reaction. Firstly, the molecule tends to collapse to a more compact configuration because it is known[3] that the extension of the flexible chains of denatured DNA is very sensitive to changes in the ionic strength of the medium. Secondly, the Cu^{++} ions bound to external sites on the DNA molecule are displaced. Since the external sites are defined as the sites at which Cu^{++} binds to native DNA, the evidence for this statement comes from the Cu^{++} ion electrode measurements of Chapter IV and the polarographic measurements of Schreiber and Daune[4] which indicate that a negligibly small amount of Cu^{++} is bound to native DNA at ionic strengths greater than 0.1. Whether the "sandwich" complex (section II.2.c), proposed by Schreiber and Daune to account for their value of 0.055 Cu^{++} ions per nucleotide residue bound to native E. Coli DNA in high ionic strength,[5] is still present when the DNA-Cu^{++} solution is denatured is not known. However, if this complex is present, its effect on the renaturation would most likely be small since only 5% of the DNA molecule is involved and thus, at the present stage of refinement of the renaturation mechanism, the possible presence of this complex will be ignored and it will be assumed that the Cu^{++} ions are completely released from the external sites in 0.15 M KNO_3.

D. The release of Cu^{++} ions from the internal sites

To explain why the renaturation is brought about by increasing

the ionic strength, it is necessary to propose that the equilibrium

$$[Cu^{++} - \text{Internal site}] \rightleftarrows Cu^{++}(\text{free}) + \text{Site}$$

is very dependent on ionic strength. In 0.01 M KNO_3 solution, the equilibrium favours the bound complex (L.H.S.) to an extent such that the complex forms in a sufficiently high concentration for the properties of the DNA to be markedly changed. In ionic strengths $\geqslant 0.1$, the equilibrium is shifted to favour free Cu^{++} (R.H.S.) to such a degree that complete dissociation occurs. There is no species other than DNA present in the solution with any tendency to complex with the Cu^{++} ions and so there can be no driving force for the dissociation other than the shift in the position of the above equilibrium.

E. Hyperchromous DNA in 0.15 M KNO_3 at T°C

Reaction step D by which state C is converted into state E is assumed to involve only a movement of the Cu^{++} ions, and thus the conformation of DNA strands in state E is taken to be identical to their conformation in state C. The name "hyperchromous DNA" is applied to state E rather than the term "denatured DNA" so that no assumption is introduced concerning a relationship between the conformation of the strands in state E and in the thermally denatured state.

F. The renaturation step

Up to this point in the reaction scheme, no step has involved a change in the absorption at 259 nm, with the possible exception of

step D which may be accompanied by a small change due to the slight effect of Cu^{++} on the spectrum of denatured DNA.[6] Thus the spectrophotometric data presented in Chapter III is the experimental observation of the renaturation step F in which the DNA strands are transformed from the hyperchromous conformation (E) to the native conformation (G).

G. Native DNA in 0.15 M KNO_3 at T°C

The evidence that the final state is identical to native DNA was presented in section II.5, and in all the reactions observed throughout this study, the return of the absorbance to the native value leaves no doubt that complete renaturation to the native conformation was occurring.

The ionic strength at which the renaturation is carried out can influence the overall reaction rate in several possible ways. Firstly, it has an effect on reaction step D, for as the final ionic strength is increased, the equilibrium between the free Cu^{++} ions and the Cu^{++} ions bound to the internal sites will more strongly favour the free Cu^{++} ions. The ionic strength also affects the conformation of the complex C which may possibly alter the rate at which the Cu^{++} ions are released from the internal sites, although this cannot be predicted with certainty. However, it is certain that the ionic strength influences the reaction step F. It determines the compactness of the molecular conformation in the initial state E; the concentration of counterions affects the electrostatic potential on the chains and hence the repulsive force between the

chains which has to be overcome on renaturation (cf. ionic strength dependence of annealing renaturation[7-10]); also, the ionic strength has an effect on the repulsion between segments of the same chain and by altering the effective stiffness of the chain may affect the renaturation rate if much intrastrand motion is required. It can thus be predicted that the combination of these effects will result in an increase of the overall renaturation rate at higher ionic strengths, which is experimentally observed.

2. Reaction scheme for the renaturation brought about by ethylene diamine

The reaction mechanism for the renaturation brought about by ethylene diamine is inherently simpler than that induced by increasing the ionic strength. In the former case, the ionic strength remains constant throughout the system and hence the conformational change produced by the ionic strength increase necessary in the latter case is avoided. Thus, the conformation of the strands when the complex is formed is that relevant to the renaturation step.

Fig. VI-2 sets out the reaction scheme for the renaturation by ethylene diamine. The removal of the Cu^{++} ions by EDA from the complex B (step H) can be represented by the reaction

$$[Cu^{++} - \text{Internal Site}] \rightleftarrows Cu^{++}\ (\text{free}) + \text{Site}$$
$$\qquad\qquad\qquad\qquad \downarrow + \text{EDA}$$
$$\qquad\qquad\qquad\qquad [Cu^{++} - \text{EDA}]$$

which shows that as the free Cu^{++} ions are complexed by the ethylene

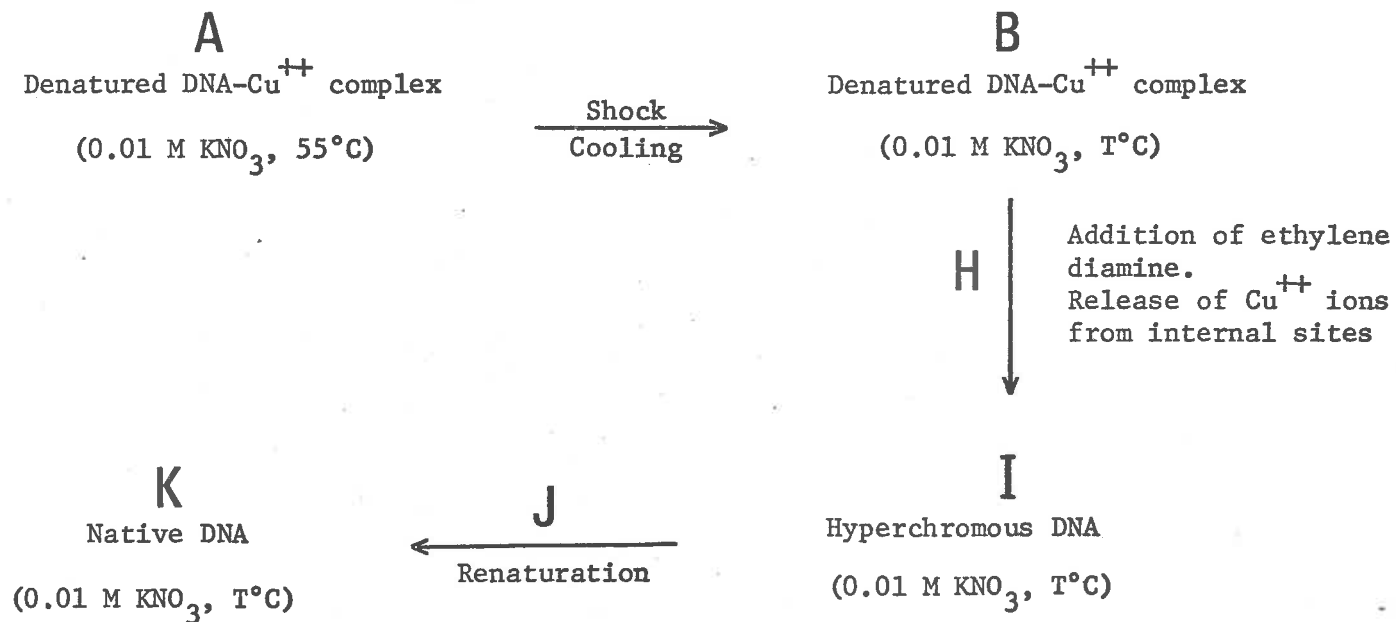

Fig. VI-2. Reaction scheme for renaturation of denatured DNA-Cu^{++} complex at T°C by ethylene diamine.

diamine, the Cu^{++}-DNA complex is made to dissociate. The ethylene diamine ligand may react directly with the Cu^{++} ion while it is still complexed on the internal site. However, since the association constant for the chelation of a second EDA ligand to the Cu^{++}-EDA complex is only a factor of 10 less than the association constant for the first complex,[11] it is likely that direct attack on the bound Cu^{++} ions may not occur until all the free Cu^{++} ions (approximately $0.65-0.75 \times 10^{-4}$ M) are complexed by two EDA molecules. As there may also be a steric barrier to direct attack on the bound Cu^{++}, this mechanism for the Cu^{++} release is considered less probable than the former one, unless high EDA/Cu^{++} ratios are used.

State I differs from the corresponding state E of fig. VI-1 in that, at the lower ionic strength, the conformation of the strands is more extended, the interstrand electrostatic repulsion is stronger and the intrastrand flexibility is less. These differences may well be reflected in differences between the renaturation steps F and J of the two systems.

3. The rate-determining step

When the Cu^{++} ion electrode was used to follow the renaturation brought about by increasing the ionic strength, the observed rate is the rate of reaction step D, viz., the release of Cu^{++} ions from the internal sites. The spectrophotometric method follows step F, the change from hyperchromous to native DNA. Since both methods give rise to the same reaction rate (section IV.4.e) it

follows that the rate of release of Cu^{++} ions from the internal sites of the denatured DNA-Cu^{++} complex determines the rate at which the renaturation can proceed. In the EDA system, the renaturation step, J, would not be expected to be affected in any way by the EDA concentration, and so the marked dependence of the overall renaturation rate on the EDA concentration (section V.4) indicates that step H, the removal of the Cu^{++} ions from the DNA sites, is the rate-determining step. Whereas it was desired to obtain information concerning the conformational rearrangement of the DNA on renaturation, in each system this step is completely masked by the slower preliminary reaction, the release of Cu^{++} ions. Thus the only immediate conclusion which can be made concerning the renaturation step is that it is more rapid than the Cu^{++} ion release under the reaction conditions studied thus far. An indication of the rate of the renaturation step is given by the observation that at EDA/Cu^{++} $\sim$ 2 the total renaturation was complete within 0.3 seconds, and hence the characteristic time for the renaturation step must be at least as short as this. (It is not known at this stage whether at EDA/Cu^{++} $\sim$ 2 the reaction rate is still dependent on EDA concentration or whether the removal of the Cu^{++} ions at the high EDA concentration is sufficiently rapid to allow the renaturation step to be rate-determining. Further experiments should clarify this.)

It is of interest to consider the characteristic times of various other conformational rearrangements of DNA which may bear some relationship to the rearrangement pertinent to this system.

A number of workers, concerned with the relationship between the time required to replicate the DNA content of a cell and the time between cell divisions, have calculated the time required for complete unwinding of the DNA molecule.[12-17] The value calculated depended on the particular model used to describe the unwinding mechanism and on the various parameters inserted such as the viscous resistance of the medium to the unwinding motion. Longuet-Higgins and Zimm[14] for a molecular weight of 10×10^6 suggest the minimum separation time is of the order of 1 second, whereas Freese and Freese[16] for a molecular weight of 35×10^6 calculate 0.07 seconds as the minimum time. Davison[18] has experimentally observed the strand separation on alkaline denaturation of bacteriophage DNAs and at a molecular weight of 25×10^6 the time for complete separation was 1.2-1.4 seconds. Other workers[19-22] have followed the kinetics of denaturation by the accompanying hyperchromic change, a method which does not permit the distinction of the state in which the ordered secondary structure has collapsed from that of complete strand separation. These studies of the acid transition[19] and the alkaline transition[20] by the pH shock method and of the thermal transition by the temperature-jump technique[21,22] gave values for the denaturation half-time ranging from 10^{-2} seconds to 10^3 seconds depending on the molecular weight of the DNA, the ionic strength, the size of the perturbation applied and the relationship between the perturbation and the complete transition, which determines the conformation of the states between which the transition is made.

This last point was most clearly demonstrated by Spatz and Crothers[22] who found that when large temperature perturbations were applied, there was a process occurring in less than 20 millisecs. which resulted in structural disorganisation of the molecule without unwinding, whereas moderate perturbations caused a much slower viscosity-dependent and MW-dependent unwinding process and small perturbations within the transition range gave different kinetic behaviour again.

The above discussion indicates that under certain conditions, major conformational changes have been observed in the millisecond to 100 millisecond range which is of the same order as the time in which the renaturation step of the Cu^{++}-DNA system is believed to occur. However, these conformational changes consist of the formation of a disordered structure from an ordered one, and since a process involving a conformational change in one direction may be subject to different controlling factors from a change in the opposite direction, it is of greater interest to make a comparison with literature reports of the rate of a conformational change accompanying renaturation. The rate of the annealing renaturation[7-10] does not give the required comparison for this is a measure of the rate of diffusion together of separated strands and not of the subsequent conformational change. Elimination of the diffusion step by using cross-linked DNA would be expected to make the conformational change observable but investigations on naturally occurring cross-linked DNA,[23-26] DNA with chemically produced interstrand linkages[27,28] or

DNA cross-linked by a nucleus of base pairs[7] have shown that renaturation is very rapid but no attempt to study the rate has been made. Thus there is no data from any other system concerning the rate of a conformational change forming the double-helical structure which can be compared with the apparent rate for such a process occurring in the Cu^{++}-DNA system and which could therefore assist in understanding the very rapid rearrangement occurring in this system.

The fact that the rearrangement of the disordered strands into the double-helical conformation is limited by the rate of release of the Cu^{++} ions from the complex, points to two possibilities concerning the denatured DNA-Cu^{++} complex. Firstly, the complementary strands are not separated but are in close proximity to each other so that little or no motion towards each other is required for the renaturation step. Secondly, the complementary strands are closely in register, for if they were too far out of register, non-specific base pairs could form on removal of the Cu^{++} ions resulting in a "trial-and-error" matching of the strands which is most probably too slow a mechanism to explain the rapid reaction observed.

The observation that the reaction rate is first order with respect to the DNA concentration is to be expected for a mechanism in which the Cu^{++} release is the rate determining step. The deviation from first order behaviour generally observed in the latter stages of the reaction is not surprising in view of the complexity

of this heterogeneous macromolecular system. The overall rate constant found for the release of the Cu^{++} ions is a combination of rate constants each corresponding to the release of a particular Cu^{++} ion from its site, which may not all be identical due to such factors as the heterogeneity of the sites and the effect of the state of the neighbouring sites on the rate of release from a given site. For instance, it can be postulated that the rate of release of a Cu^{++} ion from a site which is between sites from which the Cu^{++} ions have already been released may be slower than when the adjacent sites are still filled; this would result in a decrease in the overall reaction rate towards the end of the reaction, as is observed. The observation in section III.6 that the extent to which the renaturation in 0.15 M KNO_3 followed first order kinetics decreased from 85% at 5°C to 50% at 35°C suggests another possible explanation for the deviation from first order behaviour; the thermal motion of the molecules at the higher temperatures may cause disruptions from the proper configuration in small regions of the denatured DNA-Cu^{++} complex, perhaps in the form of the strands moving apart or out of register which would result in such regions being difficult to renature.

4. The viscosity dependence

The marked dependence of the rate of the renaturation brought about by increasing the ionic strength on the bulk viscosity of the solution indicates that the rate of release of Cu^{++} ions from the denatured DNA-Cu^{++} complex is a hydrodynamically limited process.

This result is somewhat surprising for it could be expected that the viscosity dependent process is the conformational rearrangement of the DNA strands. However, the Cu^{++} ion electrode measurements (section IV.5) clearly show that the rate of Cu^{++} ion release is decreased with increasing solution viscosity. Thus it appears that reaction step D of fig. VI-1 depends not only on the shift in the equilibrium between the free and bound Cu^{++} ions but also on the rate of diffusion of the dissociated Cu^{++} ions away from the DNA strands.

5. The time dependence

In the discussion thus far no attempt has been made to incorporate the observed time dependence of the renaturation rate into the proposed reaction scheme. There is no indication from the kinetic results of the previous chapters that there is any fundamental change in the reaction mechanism as the time that the denatured solution stands before renaturation is increased; the only property which is observed to change is the rate constant. Thus it appears that at temperatures greater than 5°C, molecular state B of fig. VI-1 and fig. VI-2 must be undergoing some change with time which results in one or more of the subsequent reaction steps proceeding at a slower rate. However, neither spectral nor viscosity measurements, both of which are very sensitive to changes in molecular conformation, were able to detect any change in the denatured DNA-Cu^{++} complex with time (section III.5). A number of

suggestions attempting to explain this phenomenon can be put forward but none of them is satisfactory at this stage.

Since the complex is formed at 55°C and then cooled, it could be thought that the time dependence is due to a slow rearrangement of the strands to the equilibrium conformation at the lower temperature. However Richard and Pacault[1] found that increasing the time at 55°C decreased the subsequent renaturation rate which eliminates a change in equilibrium with a change in temperature as an explanation. The fact that at temperatures less than 5°C the renaturation rate is independent of the time of standing suggests that the process at higher temperatures involves the thermal motion of the DNA strands since the lack of such motion at temperatures near 0°C has been demonstrated.[29] A proposal which incorporates this idea rests on the proposition that in the denatured DNA-Cu^{++} complex the Cu^{++} ions do not bridge between the complementary strands but in some other way prevent the strands from unwinding and separating, perhaps by chelating between sites on the same strand. Such a structure allows relative "slipping" movement of the two strands so that with thermal motion they tend to become more out of register resulting in a slower renaturation. This process would occur at all temperatures greater than 5°C, including the temperature at which the complex was formed, and its effect would increase with increasing temperature, all of which are experimentally observed properties. However this proposal overlooks the vital point that the rate-determining step is not the renaturation step but the

release of the Cu^{++} ions. The process responsible for the time dependence must somehow exert its effect on the Cu^{++} release step. It is unlikely that more Cu^{++} ions become bound during the time of standing because the Cu^{++} ion electrode was unable to detect any change in the free Cu^{++} ion concentration during this period. The Cu^{++} ions may become more firmly bound by small changes in the configuration of the site or by transferring to a neighbouring more favourable site which has become more sterically accessible, but such conjectures have no evidence to support them as yet. Another suggestion is that the diffusion of the Cu^{++} ions away from its internal site is interfered with, as the DNA strands by their thermal motion, in effect succeed in trapping the ions more tightly. The involvement of the thermal motion of the DNA strands in this hypothesis means that it corresponds well with the observed temperature dependence of the process, but it is difficult to see why the random thermal motion can produce such a definite result as an increasing tendency for the Cu^{++} ions to be trapped in the sites.

6. The renaturation brought about by ethylene diamine

While investigating the renaturation brought about by increasing the ionic strength, it was conjectured that the change in the conformation of the DNA strands from that when the complex was formed (state A) to the more compact configuration when the ionic strength was increased (state C) could possibly interfere with the diffusion of the Cu^{++} ions out of the complex and thus explain why this process is the rate-determining step. By carrying

out the renaturation with ethylene diamine, this contraction of the DNA conformation was avoided and at no time during the reaction would the strands be less extended than when the complex was formed. Thus it was thought that under these conditions the rate of Cu^{++} release may no longer be the rate-determining step and the renaturation reaction may be directly observed. However, as discussed in section VI.3, the experiments showed that even in this system the rate of Cu^{++} release was still slower than the conformational rearrangement. In spite of this, the use of ethylene diamine as the renaturing agent offers further possibilities for obtaining information on the Cu^{++}-DNA system and on the renaturation process. The results reported in Chapter V are only preliminary experiments for one of the advantages of the ethylene diamine system is that it should be possible to observe the renaturation under a wide variety of conditions which will assist in elucidating more details of the reaction mechanism and answer some of the questions associated with it. It is not certain whether the small departure from first order kinetics observed in the initial stages of the reaction is significant nor is the explanation for the low values of $(A_0 - A_\infty)$ available at this stage. Another puzzling problem is the lack of dependence of the reaction at $EDA/Cu^{++} = 1$ on the solution viscosity. The diffusion of the Cu^{++} ions away from the internal sites would be expected to be viscosity dependent to the same extent as the similar process in the renaturation brought about by increasing the ionic strength. Even if a direct attack of the EDA on the bound Cu^{++} ions is involved

it would be predicted that the rate would still depend on the diffusion of the Cu^{++}-EDA complex back into the bulk solution.

It may be possible by increasing the EDA/Cu^{++} ratio to reach the situation where the reaction is no longer controlled by the rate of the release of Cu^{++} ions. If this can be done, then the original aim of this project will be achieved; the formation of the double helix from disordered strands may be observed directly and the factors controlling the rate of the process determined.

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Chapter VII

MATERIALS AND METHODS

1. Cleaning of apparatus

2. Preparation of solutions

   a. DNA solutions
   b. $Cu(NO_3)_2$ solutions
   c. KNO_3 solutions
   d. Other solutions

3. Spectrophotometer

   a. Description
   b. Calibration
   c. Use of the instrument for renaturation reactions

4. Procedure for renaturation by increasing the ionic strength

   a. Denaturation
   b. Renaturation
   c. Experiments at 5°C and 15°C

5. The Cu^{++} ion electrode measurements

6. The stopped-flow rapid reaction apparatus

   a. Optical and recording systems
   b. Operating procedure

References

1. Cleaning of apparatus

The general procedure for cleaning all glassware was to stand it for 24 hours in a cleaning mixture consisting of 3% w/v $NaNO_3$ and 3% w/v $NaClO_4$ in concentrated sulphuric acid. This reagent was preferred to the commonly used chromic acid solution because it avoided the possibility of metal ion contamination. The apparatus was then subjected to prolonged rinsing with deionised water, leaching in deionised water, rinsing with distilled water and a final 24 hr leaching in distilled water. With the exception of volumetric glassware, apparatus was dried at 160°C before use.

The silica cuvettes were cleaned by the same procedure and stored in distilled water. When required for use they were rinsed with AR acetone and allowed to dry at room temperature.

Stainless steel apparatus, such as syringe needles and spatulas, were cleaned in chloroform and leached in distilled water.

Polythene vessels used for storage of solutions were washed thoroughly with a hot detergent solution, rinsed and leached in distilled water and steam cleaned for several hours.

The distilled water used for preparation of all solutions and washing of apparatus had a conductivity of 1.5-2.5 × 10^{-6} ohm^{-1} cm^{-1}.

2. Preparation of solutions

a. DNA solutions

The E.Coli DNA used throughout this work was prepared by Mr. I.F. Cullen using the method of Marmur.[1]

A stock solution of DNA containing $4\text{-}6 \times 10^{-4}$ M_p in 10^{-2} M KNO_3 was prepared as follows. Approximately 40 mgm of DNA were placed in a flask with 200 mls of 10^{-3} M KNO_3 and dissolved by gentle shaking at 4°C for at least 48 hours. Sufficient solid KNO_3 to bring the concentration to 10^{-2} M was added. The solution was then centrifuged at 18,000 g for 1 hour and the supernatant solution carefully separated from the small amount of sedimented solid. After addition of approximately 1 ml of AR chloroform, the solution was stored at 4°C. The concentration of the stock solution was determined by carrying out accurately by weight an approximately ten-fold dilution of a sample and measuring the absorbance at 259 nm. The concentration was calculated taking $\varepsilon(P) = 6600$ at this wavelength.

The DNA solutions for use in the reactions were prepared in 100-150 ml quantity by diluting the appropriate weight of stock solution with 10^{-2} M KNO_3 to give the required concentration. These solutions were also stored at 4°C in the presence of chloroform.

b. $Cu(NO_3)_2$ solutions

$Cu(NO_3)_2$ solutions were prepared from AR $Cu(NO_3)_2.3H_2O$ (May and Baker) which had been recrystallised and dried by vacuum desiccation over silica gel. Stock solutions of approximately 2×10^{-3} M in 10^{-2} M KNO_3 were prepared and stored in glass volumetric flasks but the more dilute Cu^{++} solutions which were used for standardisation of the Cu^{++} electrode (section IV.2.b) were stored in steam cleaned polythene vessels to prevent adsorption of Cu^{++}

ions by the vessel walls. The concentration of Cu^{++} in a stock solution was determined by potentiometric titration with a standard EDTA solution (5×10^{-4} M in 10^{-2} M KNO_3) in the presence of sodium acetate buffer (pH 4.8),[2] using the Orion specific cupric ion activity electrode to detect the end point. The accuracy of the standardisation was 0.2%.

c. KNO_3 solutions

All KNO_3 solutions were prepared from AR KNO_3 (British Drug Houses) which had been recrystallised, dried at 160°C and stored in a vacuum desiccator.

d. Other solutions

Sucrose solutions were prepared from analytical grade sucrose (C.S.R.). Standard EDTA solutions were prepared from AR disodium diaminoethane tetraacetate (UniVar). Ethylene diamine solution was prepared from a sample of ethylene diamine (Fluka), purity > 98%, density = 0.90 gm/cc. A solution of approximately 0.1 M in 10^{-2} M KNO_3 was accurately prepared from a weighed sample of liquid, and this solution was carefully diluted to give a solution of approximately 2×10^{-3} M which was stored in a sealed flask.

3. Spectrophotometer

a. Description

The spectrophotometer used throughout was the Gilford Model 2000 automatic multi-sample absorbance recorder in conjunction

with a Beckman model DU monochromator. The photocell output gives a linear response to absorbance from 0 to 3.0 O.D. units which can be read on a digital absorbance indicator or fed into a chart recorder with a continuously variable full scale deflection from 0.1 to 3.0 O.D. units.

The instrument is designed to take four 1 cm cells in a motor driven cell carriage which automatically positions each cell in the light path. The time a cell remains in the measuring position can be controlled manually or can be automatically programmed. Any combination of the four cells can be sequentially measured for a selected time interval up to 45 seconds, and by use of the dwell timing device, any of the cells can be maintained in the light path for periods up to 15 minutes.

The temperature of the cell compartment was maintained at the desired temperature by water from a thermostatted bath circulating through two thermospacer compartments on either side of the cell compartment. The instrument was protected from temperature extremes by a further two thermospacers with circulating water at room temperature positioned to isolate the cell compartment area from the rest of the instrument. This was particularly necessary when working at 5°C to prevent water vapour condensing in any part of the instrument. The temperature of the cell compartment was measured by a thermosensor located in the cell compartment immediately beneath the cell carriage. The output of the thermosensor, which varied linearly with temperature, was calibrated so that the

output displayed on the recorder chart gave the temperature directly.

b. Calibration

The accuracy of the monochromator wavelength selector was checked by Dr. B.S. Chandler prior to use of the instrument. The five lines of a Hg arc lamp in the 250 nm - 450 nm range were found to agree with the literature values to within 0.1 nm.

The slope of the linear absorbance output can be varied, and therefore the instrument must be calibrated by adjusting the absorbance calibration control until the correct reading is obtained for a known standard absorbance. If the response is in fact linear then absorbance measurements throughout the whole operating range should be correct. Such a calibration was performed at the beginning of this study and repeated after installation of a new photomultiplier. The calibration is claimed by the instrument manufacturer to be highly stable, and although this was not tested experimentally with an absorbance standard, the observed stability of the O.D. of solutions measured repeatedly during the course of this work supported this claim.

For the calibration a standard potassium chromate solution containing 0.040 gms of AR potassium dichromate per litre in 0.05 M KOH was employed; the absorbance of such a solution has been established[3] at 25°C in the UV spectral region. The absorbance calibration control was adjusted to bring the measured absorbance of the standard potassium chromate solution at 260 nm into agreement

with the literature value and then the absorbance of the solution was measured every 5 or 10 nm between 240 nm and 400 nm and the deviation from the literature noted. The first time the instrument was calibrated, the deviations lay in the range $\pm$ 0.010, and over the smaller wavelength region 250-285 nm, the readings were correct to $\pm$ 0.005, which was better than 1%. However after installation of a new photomultiplier, the agreement was not as satisfactory. With the reading adjusted to the correct value at 260 nm, deviations over the range 240-400 nm were generally negative, the maximum error being as great as -0.038. Even between 250 nm and 285 nm the deviations ranged from +0.001 to -0.013, maximum error $\sim$ 2%. Hence use of the Gilford Model 2000 for accurate optical density measurements over a large wavelength range does not appear to be justified. However, after calibration of the instrument at 260 nm, absolute absorbance measurements in a restricted wavelength region around this value can be made to an accuracy of 2%.

During this work the spectrophotometer was most frequently used to obtain relative absorbances at a particular wavelength rather than absolute optical densities, and thus the most stringent requirement for the satisfactory performance of this instrument was the linearity of the absorbance output at 259 nm. The accuracy of this property was tested by measuring the absorbances of eight KNO_3 solutions with absorbances spanning the range 0 to 0.3 O.D. units, which were prepared by accurate dilution of a concentrated KNO_3 solution. The O.D. of each solution relative to that of the most

concentrated solution was calculated and compared to the value determined by the spectrophotometer. The agreement was very good, five solutions differing from the calculated absorbance by 0.001 or less and the remaining three by 0.002, 0.003 and 0.004. Hence over this O.D. range (0 - 0.3) the linearity of the instrument is satisfactory although over a more extended range this may no longer be so.

c. Use of the instrument for renaturation reactions

For each renaturation reaction brought about by increasing the ionic strength, four cuvettes were placed in the cell compartment. One contained solvent (1), 10^{-2} M KNO_3. In the second was placed a weighed quantity of 10^{-2} M KNO_3 to which concentrated KNO_3 solution was added by the same technique as that used to start the renaturation (section VII.4.b), to give solvent (2) of the same ionic strength as used in the renaturation, generally 0.15 M KNO_3. The third cell contained a KNO_3 solution whose concentration had been adjusted to give an O.D. at 259 nm relative to solvent (2) of 0.30 - 0.33; this is called the arbitrary reference cell. Prior to each renaturation its O.D. with respect to both solvent (1) and solvent (2) was accurately measured. In the remaining cell, the renaturation was carried out.

Since in all reactions the DNA concentration was 5.00×10^{-5} Mp, the O.D. (259) after 100% reaction was 0.33 and the O.D. change due to the loss of 40% hyperchromicity was 0.13. To observe this change most accurately, the full scale deflection on the absorbance recorder was set to 0.200 O.D. units. With the monochromator slit

width adjusted so that the arbitrary reference cell supplied the zero absorbance base line on the chart, the absorbance of the reaction was traced out relative to the reference cell. Because the absorbance of this cell relative to solvent (2) was known, the chart readings could later be converted to true absorbance readings. Such a procedure was more accurate than using the solvent (2) cell as the chart zero for this would have necessitated a much less sensitive recorder output.

During a reaction an automatic timing cycle was followed in which the temperature was read for 5 seconds, and then the arbitrary reference cell base line for 10 or 15 seconds followed by the absorbance of the reaction cell for a corresponding time. This ensured that no errors were introduced through base line drift. When the slow reactions had to be followed for long periods of time, the dwell timing system was activated after the first 100 minutes of the reaction, and readings of temperature, reference cell and reaction cell were only recorded every 15 minutes.

The time parameter of the reaction was accurately given by the rate of movement of the chart through the recorder. When the reaction was proceeding rapidly, a chart speed of 2 inches/minute was used and this was decreased to ½ inch/minute to follow the slower absorbance changes.

4. Procedure for renaturation by increasing the ionic strength

The general procedure for denaturing DNA in the presence of Cu^{++} and for bringing about the renaturation by increasing the

ionic strength were outlined in section III.2. It is only necessary to give further details on several points in this section.

a. Denaturation

Two practical advantages of the Beckman 250 µl polythene micropipette used for the addition of stock $Cu(NO_3)_2$ solution to the DNA solution were that only one hand was required to fill the pipette and deliver the solution allowing the flask to be agitated with the other hand during the addition, and also successive additions could be made rapidly. Generally, the desired volume of denatured solution was such that 750 µl of Cu^{++} stock solution was required, and the three successive deliveries could be made in 30 seconds. The main disadvantage of the micropipette was the tendency, enhanced by repeated usage, for small droplets of liquid to adhere to the polythene capillary during delivery, causing inaccuracies in the Cu^{++} concentration of the final solution. For the experiments in which the renaturation was brought about by ethylene diamine (Ch. V) a 500 µl microsyringe (Scientific Glass Engineering) was used in the denaturation procedure in place of the Beckman micropipette. Fitted with a repeat adaptor which was calibrated for approximately 370 µl, this microsyringe had good accuracy (·2%). The two successive deliveries required could be performed rapidly but two hands were needed for manipulation of the microsyringe. Since the denaturation was essentially complete in 30-50 seconds after addition of the Cu^{++} at 55°C, it was considered that good mixing of the solution during Cu^{++} addition was desirable, and thus the absence of stirring when

the microsyringe was used was considered a disadvantage.

b. Renaturation

To commence the renaturation, rapid mixing of the concentrated KNO_3 solution with the denatured solution in the cuvette was achieved by forming a layer of the concentrated KNO_3 solution at the bottom of the cuvette using an Agla micrometer syringe (500 µl). The needle of the syringe was bent through an angle of 90° to enable the syringe to be operated horizontally. Since moving the syringe plunger by turning the micrometer caused too much movement of the needle which could disturb the formation of the layer, the syringe was operated by an "end-stop" technique, by slowly moving the plunger with the thumb from its initial position, given by the micrometer reading, until it stopped hard against the syringe barrel. The micrometer reading of the final position had been determined before the syringe was filled. The contents of the cuvette were rapidly stirred for 2-3 seconds with a small teflon disc on a stainless steel rod to commence the renaturation.

c. Experiments at 5°C and 15°C

When the renaturation was carried out at 5°C or 15°C, the spectrophotometer supply bath was required to circulate water at 2-3°C or 14.0-14.5°C around the cell compartment, and this was accomplished by a refrigerator unit circulating cold ethylene glycol through a heat exchanger in the bath. The cell compartment was kept free from condensation during measurements by approximately

20 gms of silica gel in a wire mesh container placed above the cells in an extension of the cell compartment. This was found to keep the cells dry at 5°C provided the silica gel was replaced every few hours.

5. The Cu^{++} ion electrode measurements

For the experiments reported in Ch. IV, the Orion specific cupric ion activity electrode, model 94-29, was used in conjunction with a Radiometer K100 flow-through type saturated calomel reference electrode. The flow of saturated KCl solution from a reservoir through the electrode and down a glass capillary tube was controlled by a fine bore ground glass tap. The liquid junction between the Cu^{++} solution and the KCl solution was formed at the upturned tip of the capillary tube. EMF measurements were made on a Radiometer pH meter 25 with a 10-fold expanded scale. As the Cu^{++} ion electrode was known to be slightly photosensitive, all measurements were made under constant fluorescent illumination with all natural light excluded from the room.

While not in use, the Cu^{++} ion electrode was stored upright with a protective rubber cap over the sensing element, and the calomel electrode was stored with the capillary in distilled water. To prepare for use, the Cu^{++} ion electrode was rinsed in distilled water, wiped dry and a drop of silicone oil on a soft tissue was applied to the sensing element and the lower part of the electrode body which would be immersed in the solution, and the

excess oil was removed. The calomel electrode was rinsed several times in distilled water, dried with a tissue, then several drops of saturated KCl solution were allowed to flow through the electrode and then it was rewashed and dried. A drop of KCl was formed at the tip of the capillary, removed with a tissue, and another drop formed and removed. Then both electrodes were placed in the electrode vessel with the sensing element and the capillary tip just below the surface of the solution, care being taken that no air bubbles adhered to them.

6. The stopped-flow rapid reaction apparatus

a. Optical and recording systems

The main components of the optical system are a 75 watt Xenon arc lamp as the UV light source, a Hilger Watts monochromator, a silica reaction cell of 2 mm path length, and a photomultiplier detector. The photomultiplier output is recorded on a cathode ray oscilliscope (CRO) via a differential amplifier which enables small changes of the order of millivolts in the photomultiplier output to be recorded accurately against the large background photomultiplier output, which is of the order of volts. With the monochromator set at 280 nm and the reaction cell containing completely reacted DNA-Cu^{++}-EDA solution, the slit width of the monochromator was adjusted so that the photomultiplier output was approximately 5.0 volts. A backing-off voltage was then applied to the differential amplifier so that the photomultiplier output was balanced to zero on the CRO

recorder. By this means a scale setting of 50 mV per division on the CRO screen could be used to record the small changes in photomultiplier output during the renaturation reaction.

b. Operating procedure

The ethylene diamine solution and the denatured DNA-Cu^{++} solution are placed in the two reservoirs from which the reactant syringes are filled. By applying a N_2 gas pressure of 40 lbs/sq. inch to a driving piston, equal volumes of the two solutions are forced from the reactant syringes to flow through the reaction cell and into a collection syringe. When the plunger of the collection syringe has moved a certain distance, it hits a stop which halts the flow of the solution through the reaction cell, and at the same time triggers the CRO recorder which then traces the path of the reaction in the cell as detected by the photomultiplier. By knowing the distance between the point of initial mixing of the reactant solutions and the point at which the light beam passes through the cell as well as the velocity of the solution through the cell, the time elapsing between the commencement of the reaction and the first absorbance reading can be calculated to be 2-3 milliseconds. After emptying the collection syringe, fresh samples of the reactant solutions can be forced into the cell and another reaction observed. Generally, traces from two consecutive reactions were stored on the CRO screen and photographed.

The temperature of the solutions was maintained constant by water at 25.0°C circulating through compartments surrounding the

reservoirs, reactant syringes and reaction cell.

References

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2. Orion Cupric Ion Electrode Instruction Manual (1968).
3. Haupt, G.W., J. Opt. Soc. Am., 42, 441 (1952).