DENATURATION OF DEOXYRIBONUCLEIC ACID

by

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CHAPTER I

INTRODUCTION

One of the most striking features of living matter is the ability to reproduce its own kind. This essential property of living material has posed a most challenging question to a large number of the sciences. A cell which is about to divide is capable of supplying the information necessary to direct all the highly specific enzyme reactions that are to occur later in the daughter cells. Similarly, but at a higher evolutionary level, an embryonic cell holds the vast store of information that is needed to produce the array of highly differentiated cells that make up the tissue and gross morphology of a mammal.

Looking at these processes we see that there are two closely related but fundamental problems. Firstly there must be the means of recording this large amount of information. Secondly one must explain how such a store of information can be passed on during the division of a cell or the reproduction of a mammal. This handing on of information is known to proceed for a very large number of generations within any one species without major change.

A similar problem arises if virus multiplication is considered. Here we have a comparatively simple

substance, free from the complex organisation associated with cellular living matter, and yet displaying the same ability of replication. It should be noted, however that in this instance replication can only proceed in the environment of a cell host.

Virus replication therefore, presents a problem similar to that of cellular division. In both cases there must be some mechanism whereby all the necessary information can be recorded and also handed on from one generation to the next. In principle, this problem can be carried down to the molecular level and involves an investigation into the molecular basis of heredity.

It has become clear that the carrier of this genetic information is nucleic acid. This substance, combined with protein as nucleoprotein, is the main constituent of the chromosomes which are intimately linked with cell division. Further, nucleoprotein is in fact the only substance present in many of the simpler forms of virus and bacteriophage. Convincing evidence that it is the nucleic acid rather than the protein that possesses this unique genetic property, comes from the work on the transforming principle of certain bacteria, from the reconstitution experiments on virus and from the mode of bacteriophage replication.

The work of Avery *et al.*[1] conclusively established that the nucleic acid extracted from the

encapsulated strain of pneumococcus would transform an unencapsulated strain into encapsulated types. Further, these transformed bacteria would multiply indefinitely to produce more of the encapsulated strain, proving that the genetic apparatus had been irreversibly changed. Later investigations have revealed many other transformable factors.

Fraenkel-Conrat[2] was able to show that the nucleic acid of tobacco mosaic virus (TMV) also possessed an important genetic function. This virus could be reconstituted from its original nucleic acid and protein. Reconstitution of virus could also be brought about from constituents prepared from two different strains of TMV. The resulting virus, in this case, had biological activity characteristic of the strain supplying the nucleic acid. This type of experimental evidence became all the more convincing when it was shown[3] that the virus nucleic acid itself was infective.

Investigations of the mechanism whereby bacteriophage can infect a bacterial host[4] revealed that the phage nucleic acid, which is contained within a protein shell, is almost the only component that enters the host during infection. This finding again supports the premise that nucleic acid is the genetic determinant.

Two chemically different types of nucleic acid have been recognised, ribonucleic acid (RNA)

and deoxyribonucleic acid (DNA) containing ribose and deoxyribose respectively. The chemical structure of DNA can be represented by a polynucleotide chain with nucleotide attachment through 3',5'-phosphodiester linkages[5] (fig. 1-I). The nucleotides can be made up from any of four bases. These bases are generally adenine, thymine, guanine and cytosine but, 5-methylcytosine or 5-hydroxymethylcytosine sometimes replace cytosine. Chargaff[6,7] has established that in most cases the mole ratios of these bases, in intact unfractionated samples, is such that the adenine/thymine and guanine/cytosine ratios are unity. Recent measurements[8] on fractionated DNA show however, that these ratios can in fact diverge from unity to a small extent.

The X-ray fibre diagrams obtained from DNA by Wilkins *et al.*[9] led Watson and Crick[10] to propose their well known model for DNA. This model essentially consists of a double helix formed from two polynucleotide chains hydrogen bonded together at all the base positions (fig. 2-I). This hydrogen bonded structure becomes possible only when the chains are arranged so that the phosphate groups lie on the outside and the bases on the inside of the double helix. The bases must lie roughly perpendicular to the helical axis. The extensive hydrogen bonding that is postulated in this model will occur only when the pairing of the bases is as shown in fig. 3-I. It necessarily follows that the

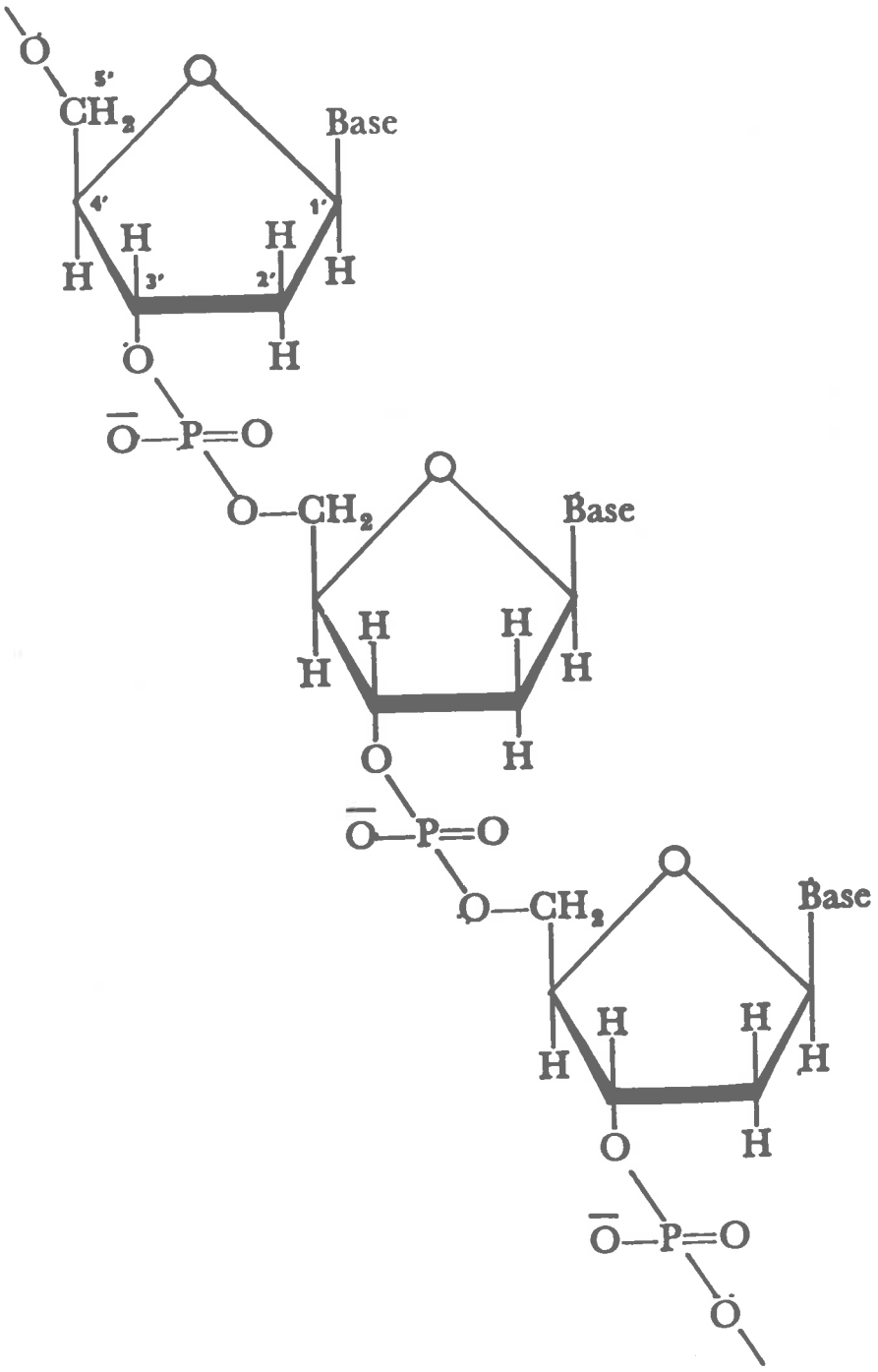

Fig. 1-I. The chemical structure of a polynucleotide chain.

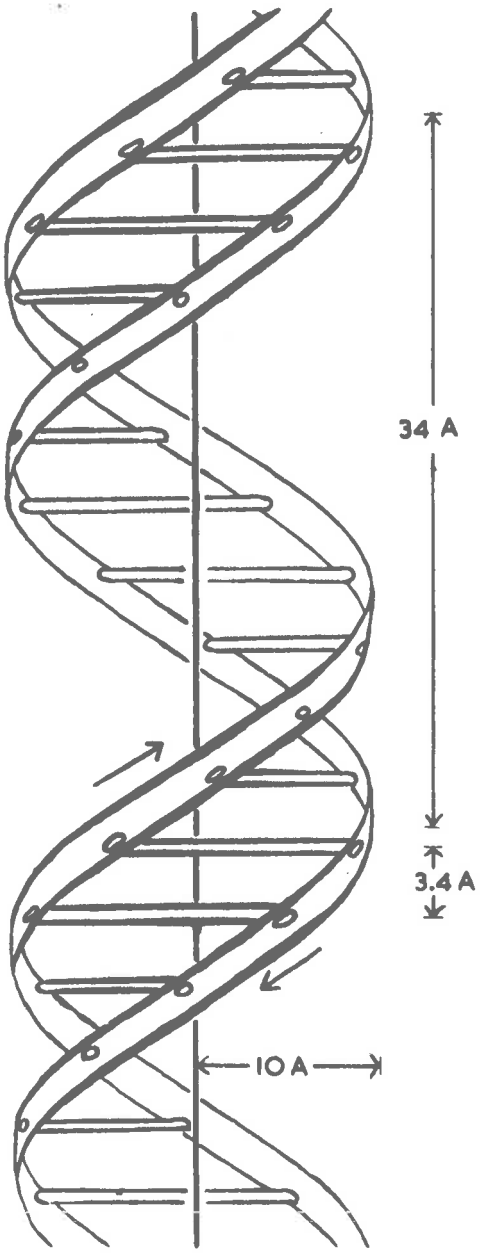

Fig. 2-I. Helical model for DNA proposed by Watson & Crick.

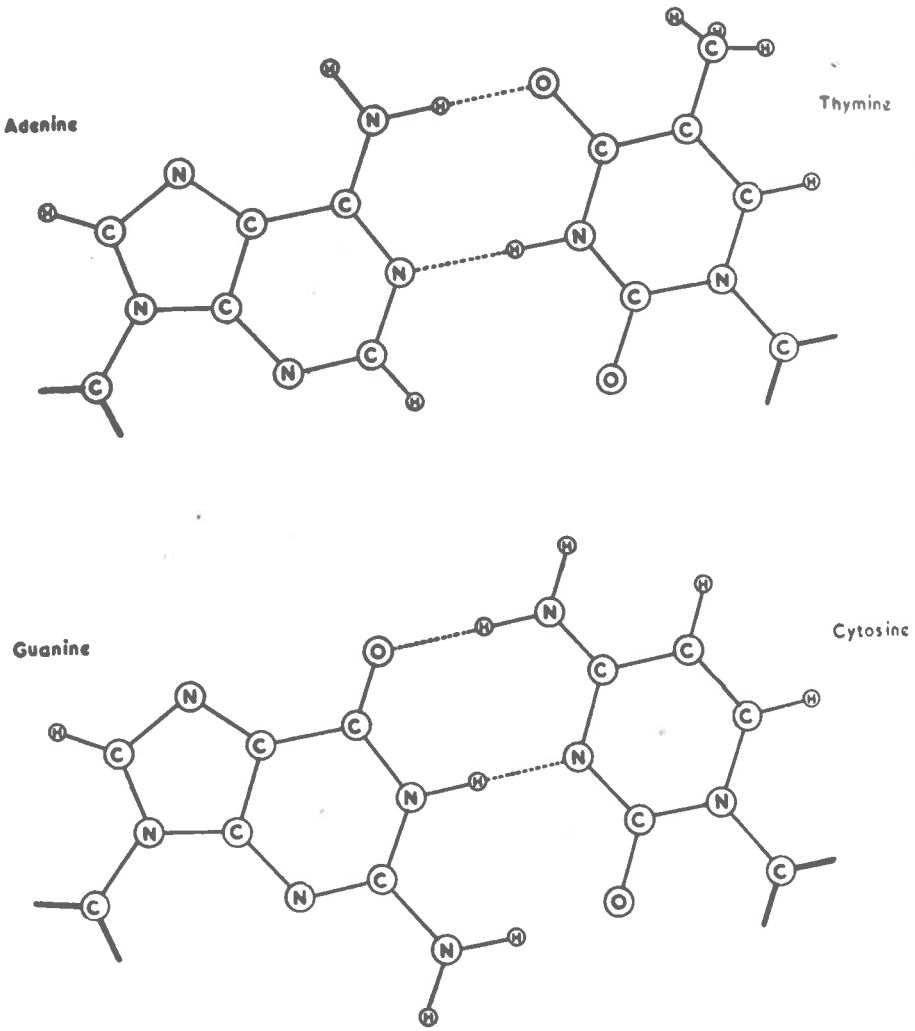

Fig. 3-I. Base pairing postulated by Watson & Crick.

sequence of bases in one chain will determine the sequence in the other polynucleotide strand. This model predicts helical dimensions that agree with those found from the X-ray diagrams and at present, is accepted as depicting the solid state molecular structure of DNA.

A more recent analysis of the available experimental information on the molecular structure of the purine and pyrimidine bases has been made by Pauling and Corey[11]. They postulate that in the case of the guanine-cytosine base pair, three hydrogen bonds are possible without exceeding the accepted bond lengths for N-H...N (fig. 4-I).

Much conjecture has arisen since the Watson-Crick model was proposed concerning its possible biological significance. Once the base sequence in one helical strand is fixed the second helical sequence also becomes defined. If the genetic information was recorded as a base sequence then separation of the DNA double helix with subsequent polymerization to complete the two daughter molecules, would result in two molecules holding the same genetic information. It can be seen that such a process could in principle provide answers to the important questions set out at the beginning of this chapter.

The possibility of the base sequence holding

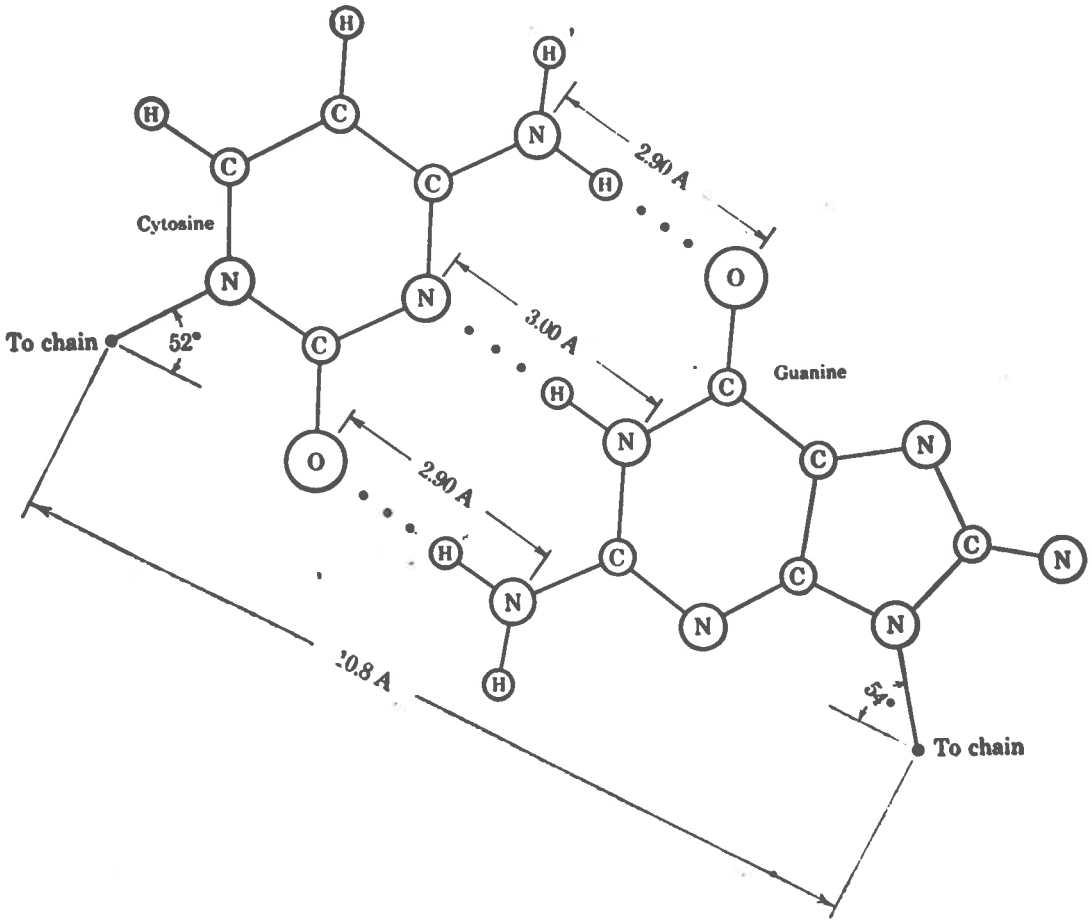

Fig. 4-I. The cytosine-guanine base pair involving three hydrogen bonds, proposed by Pauling & Corey.

genetic information and the duplication of this information by the separation of the DNA double helix has initiated two rapidly developing branches of physicochemical investigation of the nucleic acids.

Although DNA in solution can be classed as a polyanion, the extensive intramolecular hydrogen bonding tends to mask some of the more typical polyelectrolyte properties. Studies of the viscosity[12] and streaming birefringence[13] of DNA as a function of ionic strength have been interpreted in terms of a relatively rigid structure present in solution. Recent work[14] on the variation of viscosity with ionic strength would indicate that the DNA twin helix is slightly flexible in solution. Another important difference from all other polyelectrolytes, except perhaps the synthetic polynucleotides, also arises from the hydrogen bonded base pairs. If by some process these hydrogen bonds were ruptured then the whole structure could collapse, resulting in two randomly coiled but entangled polynucleotide chains. The properties of such a collapsed molecule would presumably be different from the original hydrogen bonded structure and possibly would display the more typical polyelectrolyte behaviour. Much attention has been paid to this facet of the problem and it is clear that the hydrogen bond pairing of the nitrogen ring systems confers on DNA an extra degree of

order not shared by the synthetic polyelectrolytes. In this respect one may speak of a one dimensional crystalline structure for DNA in solution[15] and the breakage of the hydrogen bonds then represents a one dimensional melting point.

The breakage of the hydrogen bonds holding the twin spirals together can be classed as denaturation. Such a process can be brought about by many methods including acid, alkali and thermal treatment. In addition the effect of low ionic strength has been shown to bring about denaturation[14,16,17]. Experiments will be described in the following chapters which are concerned with this denaturation process. It will be shown that denaturation occurs by the simple process of dilution in the absence of salt; moreover, it will be seen that the phenomenon takes place over a relatively small concentration zone.

The importance of studies involving denaturation of DNA are many fold. These studies afford the opportunity to study an extra degree of order not shared by synthetic polyelectrolytes. From a biological point of view denaturation studies are of interest as it is quite likely that denaturation is the first step in a laboratory process to dissociate the twin DNA helix. This would then lead to a better understanding of the

mode of DNA replication in living matter. In a less spectacular way denaturation studies should be of importance in deriving methods for preparing DNA in a form similar to that occurring in nature.

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CHAPTER II

PREPARATION AND CHARACTERIZATION OF CALF THYMUS DNA

The investigations to be described in the following chapters are primarily concerned with a denaturation phenomenon. It is therefore essential to determine whether any denaturation has taken place during the preparation of the DNA samples to be studied. If the samples were denatured during preparation, then the phenomenon to be described later would be diminished in magnitude or perhaps completely masked.

Unfortunatelyit is not possible at present to state whether or not a sample of calf thymus DNA is undenatured, i.e., is in the "native" state. However, we can detect denaturation by comparison with some arbitrarily defined native state. For practical purposes therefore, native calf thymus DNA is defined as that state which is least denatured or which can be purposely denatured to the largest extent.

This empirical state of affairs arises through the lack of a specific biological activity test. It should be mentioned however, that similar methods have been used in the preparation of bacterial DNA without loss of transforming activity[1].

The method used in this investigation for the

detection of any possible denaturation resulting from the preparative proceedure was based on an examination of the hyperchromicity displayed by DNA in the U.V. absorption at 2590 Å.

The calculated extinction coefficient for DNA, obtained by addition of extinction coefficients of the constituent nucleotides, is about 40% higher than that found experimentally for DNA prepared by mild methods. Denaturation of DNA results in an increased extinction coefficient and if denaturation is accompanied by degradation then the calculated extinction coefficient is obtained. The increase in U.V. absorption on denaturation is known as the hyperchromic effect. It follows therefore, that a sample of DNA with the lowest extinction coefficient will be the least denatured.

The reason for the hyperchromic effect is known only in general terms. The absorption at 2590 Å is due to the purine and pyrimidine ring systems[2], and it has been suggested that the spiral stacking of these bases, as proposed in the Watson-Crick structure, results in π-orbital interaction between the rings[3] with a resulting decrease in the U.V. absorption. It has also been suggested[4] that the low absorption results from some type of "shadowing effect", however, this could well originate from the π-orbital interaction already mentioned.

Contrary to these explanations it has been found[5] that stacking of the base pairs is not a necessary requirement for hypochromicity. This conclusion was reached from a study of certain dinucleotide derivatives which show an appreciable hypochromic effect.

Although the origin of the hypochromicity of DNA is not known in detail, it is generally accepted that the extinction coefficient, measured at 2590 Å, is dependent on the degree of denaturation of a DNA solution. The atomic extinction coefficient with respect to phosphorus, $\epsilon(P)$, of DNA prepared by mild methods is in the vicinity of 6600[6]. However, many of the reported values lie in a broad range about this figure. Extinction coefficients as low as 5900[7] and 6000[4] and as high as 7500[8,9] have been reported for DNA. The values obtained by other investigators[3,10-14] fall within the region 6300-6600. In order to determine a more accurate extinction coefficient, nine samples of calf thymus DNA were prepared under slightly varying conditions. The various modifications were designed mainly to test any effect due to the ionic history of a DNA sample. Preparations which were never subjected to low ionic strengths during preparation yielded $\epsilon(P) = 6540$ while samples dissolved in water during preparation gave a value of 6630.

In the investigations to be described in the following chapters, it was desirable to prepare samples

of DNA completely free of sodium chloride; moreover it was essential, in the preparation, to use aqueous solutions of DNA without added salt. To fulfil these conditions it was necessary to carry out the final precipitation with ethanol from a salt free solution rather than from 1M NaCl as commonly employed. The U.V. absorption of various samples subjected to this treatment was compared with measurements made on DNA that had never been dissolved in water during preparation. Table 1-II lists the preparative histories of the nine samples used.

All U.V. measurements were made on solutions of DNA in 0.1 M NaCl in one of two ways, (a) DNA dissolved in water at a concentration of 3×10^{-3} M phosphorus, at 4 C^{o} and later diluted to 5×10^{-5} M with respect to phosphorus and 0.1 M NaCl by the addition of concentrated salt solution before dilution of DNA. In the second method (b) DNA was dissolved directly in 0.1 M NaCl and later diluted with 0.1 M NaCl to 5×10^{-5} M DNA. All $\epsilon(P)$ values refer to the phosphorus concentration, determined on each stock solution used.

Table 1-II

Preparative histories of nine samples of calf thymus DNA.

Preparative history	Dissolutions in water
Preparation 1. Prepared by the detergent method[15] except that final fibres washed in ethanol only. Dried over P_2O_5 in vacuum for 2 days.	2
Preparation 2. As for 1 except that final precipitation was carried out from aqueous solution.	2
Preparation 3. As for 2 except that DNA was redissolved in water and precipitated from saline solution three times then redissolved in water and precipitated in the absence of salt.	4
Preparation 4. Portion of preparation 3 was dissolved and precipitated from aqueous solution.	5
Preparations 5 & 6. As for 1 except that at all stages 0.1 M NaCl was used instead of water. Air dried.	0
Preparation 7. Prepared by the method of Gulland *et al.*[16] and finally dissolved and precipitated from aqueous solution.	2
Preparations 8 & 9. As for 1 except that at all stages 0.0014 M NaCl was used instead of water. Air dried.	0

As no significant difference was noticed between methods (a) and (b) for the samples that had been dissolved in water during preparation, these results were pooled and are given in Table 2-II, about equal numbers of both methods were used. The extinction coefficients of the samples dissolved in water during preparation were found to be 6625 ± 34.

Table 2-II

Atomic extinction coefficients, measured at 2590 Å, of DNA samples dissolved in water during preparation.

Preparation	Number of measurements	$\epsilon(P)$ Pooled results of methods (a) and (b)	Std. error
1	14	6614	26
2	26	6646	23
3	18	6651	20
4	44	6705	12
7	25	6613	22
Average		6625	
		95% confidence limit ± 34	

The results for the DNA samples that had never been dissolved in water during preparation are given in Table 3-II. It is seen that a small but significant difference between methods (a) and (b) was found.

Table 3-II

Atomic extinction coefficients measured at 2590 Å, of DNA samples never dissolved in water during preparation.

Preparation	$\epsilon(P)$	
	(a)	(b)
5	6540	6570
6	6690	6530
8	6670	6570
9	6620	6490
Average	6630	6540

If the sample was dissolved in water as in method (a), before adjusting to 0.1 M NaCl a value of 6630 was obtained in agreement with the results given in Table 2-II. If the sample was initially dissolved in saline solution as in method (b), an average value of 6540 was found. This value is to be regarded as the $\epsilon(P)$ for native DNA, as prepared by the detergent method[15]. It can be concluded that the dissolution of native DNA, $\epsilon(P) = 6540$, in water at a concentration of 3×10^{-3} M results in an irreversible increase in the extinction coefficient of 1.4%, such an increase is compatible with a small amount of denaturation. This small amount of denaturation is unavoidable in any study involving salt free solutions of DNA.

The possible denaturing effect of precipitation with ethanol, which might be in operation in all the results so far given, was checked as follows. The $\epsilon(P)$ was found for a solution of preparation 6 before precipitation with ethanol after the first deproteinisation and corrected for the residual detergent present in the solution. The value found for this sample was between 6580 and 6630 and was not significantly different from the overall mean value.

A source of error in the measurement of the extinction coefficient in the case of DNA is apparent on inspection of the water content of solid samples. For samples initially dried over P_2O_5 the water content displayed a general rise from 5-12% up to 20-30% water. In all the preparations studied, however, there were no predictable changes apparent in the ever changing water content except for the general rise noted above. Air dried DNA samples showed a similar behaviour except for the general rise. Because of this variation in water content any absorptivity measurements which ultimately depend on weighing out DNA will be inviting error. This could occur for instance, if the phosphorus concentration was determined on one solution of DNA and the U.V. measurements made on a further solution prepared at a later date from another weighed out DNA sample.

It is to be concluded that the preparation of DNA by a salt free proceedure is accompanied by a 1.4% increase in the extinction coefficient. As it is imperative to use salt free DNA samples in the work to be described, this small amount of denaturation is unavoidable.

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CHAPTER III

STRUCTURAL CHANGES FOLLOWING DILUTION OF AQUEOUS DNA SOLUTIONS

(1) Flexibility of the nucleate ion.

(2) Conductivity and electrical transport measurements on aqueous DNA solutions.

(3) Possible explanations for the critical concentration phenomenon.

(4) Confirmation of the critical concentration phenomenon by U.V. absorption.

(5) Preliminary conclusions and the design of further investigations

(1) Flexibility of the nucleate ion.

If it is assumed that the Watson-Crick[1] structure for DNA represents the form taken by the nucleate ion in solution, then a rather high degree of stiffness should be exhibited by the polyion kinetic unit. This follows because of the limited configurational positions imposed on the hydrogen bonded base pairs. Large amounts of coiling could destroy the critical distances between these base pairs and therefore the bonds holding the twin spirals together (as proposed by Watson and Crick) would not be present.

The experiments concerned with the flexibility of the nucleate ion have shown that this polyion is not easily deformed and can be best represented, in a qualitative way, by a gently coiled configuration. The following observations support this conclusion. The early measurements of viscosity on DNA solutions[2,3,4] showed that ionic strength had a marked effect on the viscosity, however, it is now known that this sensitivity to salt is due largely to electrostatic interaction effects between the macro-ions rather than to a change in the shape of the nucleate ion. Later work, using very low DNA concentrations, where the interaction effect is reduced, has shown that both the viscosity[5] and streaming birefringence[6] results were consistent with a relatively stiff nucleate ion. Recent viscosity measure-

ments on native DNA[7] show, however, that a small amount of flexibility must be present in the nucleate ion to explain the small but significant variations of viscosity with ionic strength.

The interpretation of light scattering measurements on solutions of native DNA support the above conclusion. The deviations from linear behaviour of the reciprocal scattering envelope at low scattering angles have been explained[8,9] by assuming that the nucleate ions have a highly extended, gently coiled configuration which remains constant over the range of ionic strengths in which the native state of DNA exists.

On the basis of the foregoing results it is now generally concluded that the deoxyribose nucleate ion in solution behaves as a relatively stiff coil and does not undergo the morphological changes with ionisation observed with synthetic polyelectrolytes, however, variations of charge on the DNA ion may produce some changes in flexibility. It is therefore desirable to know the charge on the nucleate ion under various conditions of ionic strength and pH.

The charge on the DNA ion has been previously determined from either membrane potential measurements or from the electrical mobility. Both methods are subject to criticism, the first on the basis of the high

DNA concentrations required to obtain measurable potentials and the lack of reproducibility of the results, the second in that the calculation of charge from electrophoretic mobility measurements requires assumptions concerning the size and shape of the kinetic unit. Values of the charge fractions (net charge per phosphorus atom) obtained from membrane potential measurements[10,11] vary from 0.4 to 0.5 at high ionic strength (0.1 or 0.2) to 0.2 at ionic strengths approaching zero. On the other hand the electrophoretic measurements[12] indicate a value of 0.2 largely independent of ionic strength. These values of the charge indicate that the net negative charge is less than the theoretical value of one electronic charge on every phosphorus atom, a result which has been explained by the association of sodium ions into the DNA kinetic unit. Similar behaviour has been observed with solutions of synthetic polyelectrolytes[13-16], and also for bovine serum albumen[17].

In this investigation the charge on the nucleate ion has been determined from conductivity and electrical transport measurements using the methods described by Huizenga, Grieger and Wall[13] in a study of polyacrylic acid. The electrical properties of DNA solutions were studied as a function of DNA concentration in the absence of added electrolyte and as was expected from the observed denaturation of DNA at low ionic strengths[18,19,7] the results of this investigation are

complicated by the presence of a denaturation phenomenon.

(2) Conductivity and electrical transport measurements on aqueous DNA solutions.

The actual experimental results are shown in fig. 1-III as the specific conductivity and material transport number (the ratio of increase of DNA phosphorus in cathode compartment to the total equivalents of electricity passed). The specific conductivity, as a function of DNA concentration, shows a definite discontinuity over a short range of concentration at 2-3 X 10^{-4} M DNA. The material transport number rises rapidly at concentrations above the discontinuity and finally levels off at a value greater than unity. Material transport numbers greater than one infer that a proportion of the sodium ions are associated with the polyion kinetic unit to such an extent that they are forced to travel with it, resulting in a negative sodium ion material transport number. This result is in agreement with the earlier deductions concerning gegenion association with DNA[10,11,12] and with partially neutralized polyacrylic acid[13]. It can be seen that this gegenion association process only becomes apparent at concentrations above the discontinuity (in future designated as the critical concentration).

These results were combined, using smoothed

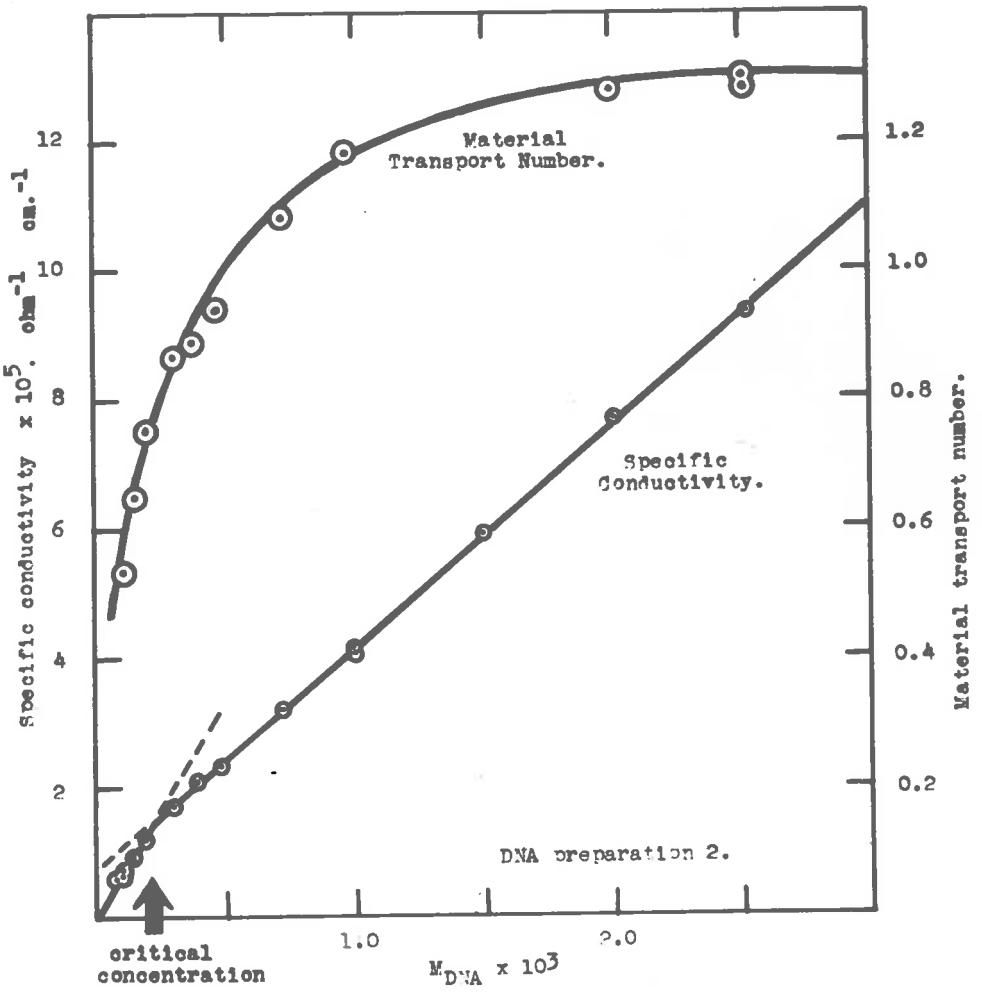

Fig. 1-III. Specific conductivity and material transport numbers of aqueous DNA solutions.

electrical transport values (obtained for preparation 2) and average values of the specific conductivity (obtained for preparations 2,3,4 and 7) to derive the more fundamental electrical properties. Fig. 2-III shows the relationship between total and polyion equivalent conductivity. Although the total equivalent conductivity falls above the critical concentration the contribution from the polyion unit increases. Again this is due to the association of sodium ions which thereby lose their conducting power and results in a net reduction in the total equivalent conductivity. The changes that occur in the charge fraction with DNA concentration are shown in fig. 3-III. A large increase in charge becomes apparent below the critical concentration. The mobility (fig. 3-III), however, decreases in this concentration region. The mobility, in this case, is not dependent on the charge carried by the kinetic unit (mobility $\propto$ polyion equivalent conductivity/charge fraction), but is a true indication of the frictional forces opposing the movement of the polyion unit in solution.

(3) Possible explanations for the critical concentration phenomenon.

Similar changes to those described for DNA solutions have been found for detergent solutions[20-22] which display a critical micelle concentration (CMC). The increased mobility above the CMC has, in this case, been

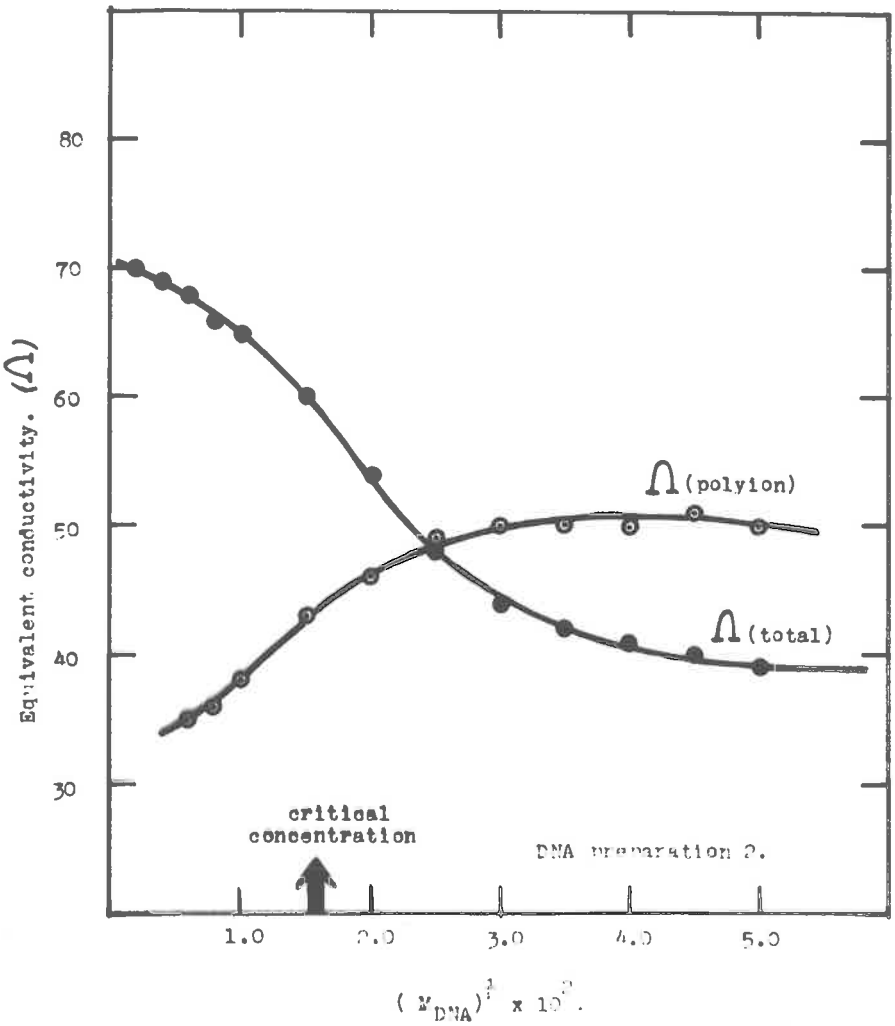

Fig. 2-III. Total equivalent conductivity and nucleate ion equivalent conductivity in aqueous solution.

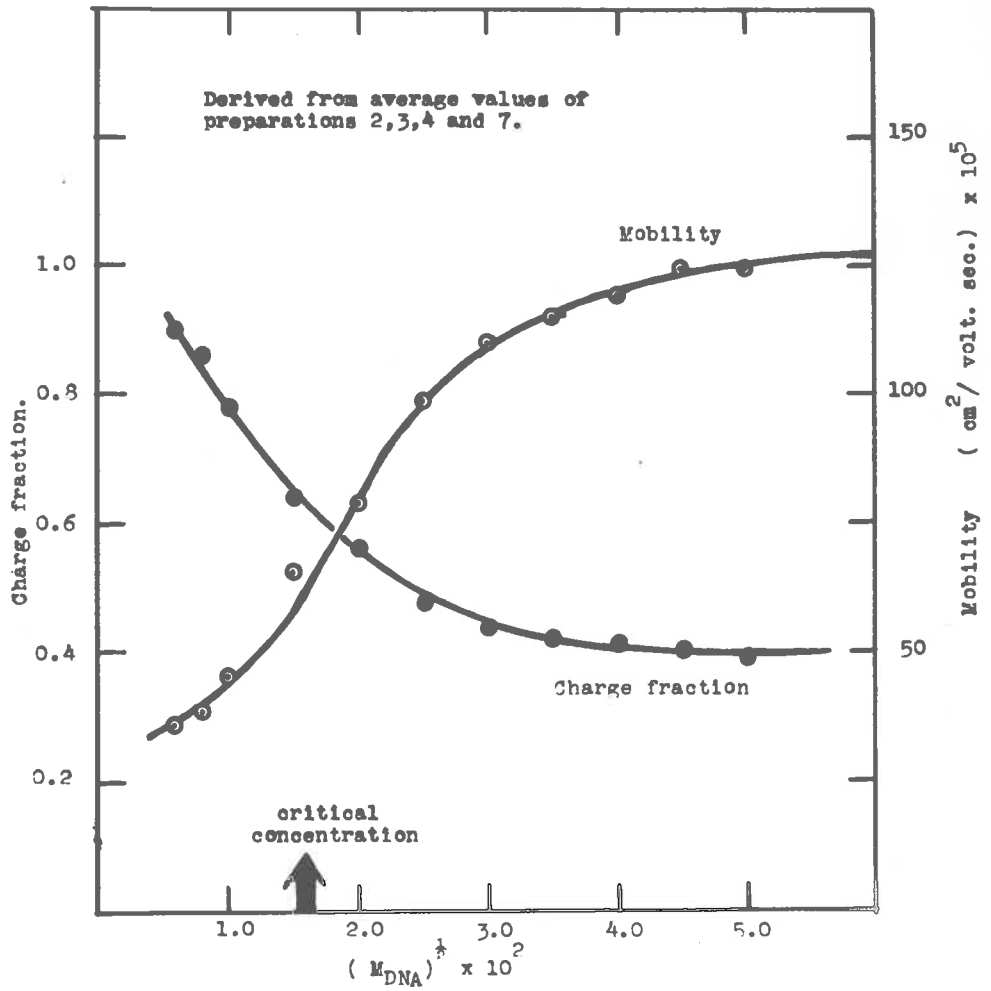

Fig. 3-III. The charge fraction and mobility of the nucleate ion at various concentrations.

explained in terms of aggregation such that the aggregate units (micelles), which form above the CMC, experience less frictional drag than do the same number of molecular components in the unaggregated state.

Apart from the improbable shape changes that native DNA would have to undergo to produce the results given above, it appeared that some type of aggregation phenomenon was also in operation in the case of aqueous DNA solutions. Two alternative explanations appear possible.

(a) The aggregation process may be similar to that found for the detergent ions. Two or more DNA units aggregating in such a way that the resulting kinetic unit offers less frictional resistance than that experienced by the constituent DNA units separately. Such a process should result in a reversible single molecule-aggregate system as there is no reason to suspect that these molecules would be aggregated by a specific type of linkage. This process would also involve a molecular weight change at the critical concentration.

(b) The aggregation phenomenon might be of entirely different origin if the intact DNA unit is classed as the aggregate. Passage through the critical concentration on decreasing the DNA concentration could then represent the disaggregation of the native DNA

double helix by hydrogen bond cleavage between the paired nitrogen bases. This would eliminate the force holding the two spirals in register, and the molecule would then be in what is known as the denatured state. As the hydrogen bonds between the base pairs is thought to be highly specific, such a disaggregation process as described above should be quite distinct from process (a), in being irreversible. Some ambiguity arises when the molecular weight changes are considered. Once the specific hydrogen bonds have been broken there is no direct linkage between the two spirals, but due to the paranemic nature of the interwound strands no decrease in molecular weight can immediately take place. In the case of denaturation by acid[23], alkali[24], thermal[25] and low ionic strength[7] no change in molecular weight has been found, as compared with a native sample. On the other hand once all the hydrogen bonds have been broken unwinding of the two chains appears to be energetically feasible[26]. It is possible that one of the more important factors which determine whether the spirals do unwind is the presence of reformed hydrogen bonds of a nonspecific nature. However, by analogy with acid, alkali, thermal and low ionic strength denaturation it would be expected that process(b) would proceed without molecular weight change.

It can be seen that the two possible explanations given above can be differentiated by the criteria of reversibility and possibly molecular weight change.

(4) Confirmation of the critical concentration phenomenon by U.V. absorption.

Further experimental evidence supports the finding that some structural change occurs in the kinetic unit on diluting below the critical concentration zone. Plots of optical density (measured at 2590 Å) against DNA concentration show a discontinuity[27,28,7] similar to that found for the conductivity of DNA solutions. This discontinuous optical property has been confirmed in the present investigation and the critical concentration agrees with that found from the conductivity measurements. The discontinuity in the U.V. absorption is such that below the critical concentration the extinction coefficient increases.

(5) Preliminary conclusions and the design of further investigations.

In view of the fact that denaturation has already been shown to occur on reducing the ionic strength of DNA solutions[18,19] and by dissolution in water at low DNA concentrations[7] it would appear that explanation (b) involving hydrogen bond cleavage is to be favoured. The increase in extinction coefficient on dilution below the critical concentration would also be compatible with a

denaturation process as outlined in (b), because of the hyperchromic effect described in Chapter II.

If the critical concentration phenomenon does arise from a denaturation process then the electrical properties already described will refer to native DNA at high concentrations only. Such a view results in a charge fraction of 0.40 for the native material in the absence of added electrolyte. This is to be compared with the values already cited, of 0.2 and 0.4 at zero ionic strength. The denatured material (at concentrations below the critical zone) apparently has a much higher charge which appears to approach the theoretical maximum. The mobility of the denatured material has decreased as compared with native DNA. Although the charge increases on passing from the native to the denatured state, the equivalent polyion conductivity decreases. This occurs because of the large mobility decrease accompanying this process.

Although one would expect denaturation to be accompanied by a general molecular collapse, as indeed has been shown for denatured DNA in saline solutions[7], in the present instance (denatured DNA in aqueous solution) this does not appear to be the case. It has been found that denaturation in aqueous solution leads to a highly charged unit and it is thought that, in this

instance, an intra-molecular repulsion effect could counter the collapsing tendency brought about by the rupture of the hydrogen bonds. This could possibly explain the low mobility observed for denatured DNA.

The investigation of the electrical properties associated with the nucleate ion was originally intended as a starting point for a systematic study of the influence of such environmental factors as ionic strength and pH on the charged state of the polyion. However, due to the observations concerning the critical concentration phenomenon, discussed in this chapter, it was imperative to study this apparent structural transition before proceeding with the original investigation. The contents of the following chapters are therefore concerned with investigations designed in an attempt to elucidate the type of structural change responsible for the critical concentration phenomenon.

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CHAPTER IV

ENVIRONMENTAL FACTORS EFFECTING THE CRITICAL CONCENTRATION

(1) Introduction and detailed investigation of the critical concentration by conductivity measurements.

(2) Influence of ionic strength.

(3) Changes effected by variation in gegenion radius.

(4) Influence of dielectric constant.

(5) Effect of added protein and EDTA.

(6) Changes produced by thermal denaturation of DNA.

(7) Sedimentation velocity experiments on aqueous DNA solutions within the critical concentration zone.

(1) Introduction and detailed investigation of the critical concentration by conductivity measurements.

In the previous chapter it was suggested that some type of structural change occurred on diluting an aqueous DNA solution below the critical concentration. The possible mechanisms put forward to explain this phenomenon could, in principle, be resolved by investigations of the reversibility and molecular weight change associated with the process responsible for the critical concentration. Both these methods of approach introduce further problems which complicate what would otherwise be a "yes-no" answer. Because of this difficulty a more accessible approach was initially undertaken which involved a study of the influence of environment on the proposed structural transition. As will be seen this approach, although confirming the initial suggestion that a structural transition was occurring, helped very little in discriminating between the two possible mechanisms. However, the results did supply useful information that was used in the design of later experiments.

Before a study of the effect of environment could be undertaken however, it was necessary to investigate more fully the exact nature of the critical concentration as observed in the conductivity measurements.

The plot of specific conductivity against DNA concentration has been found to exhibit a discontinuity at a critical concentration of 2-3 X 10^{-4} M DNA (fig. 1-III). Fig. 1-IV shows the results of a detailed study of this phenomenon obtained by both increasing and decreasing the polyion concentration. It can be seen that the critical concentration is more accurately represented by a zone of concentration. Both above and below this concentration region a linear relation exists between the conductivity and concentration. The lowest concentrations studied are shown in the inset graph of fig. 1-IV and the highest regions studied were given in fig. 1-III. The reason for the difference in conductivities shown by increasing and decreasing DNA concentrations will be discussed in a later chapter. The average critical concentration (taken as the point at which the two linear portions intersect) for the salt free DNA preparations 2,3,4 and 7 was found to be 21 $\pm$ 3 X 10^{-5} M while the actual zone extended from 4-50 X 10^{-5} M. All these preparations gave values of the equivalent conductivity at infinite dilution of 80 $\pm$ 10. Another preparation (number 1) yielded higher conductivities and a lower critical concentration. The higher conductivities are almost certainly due to the salt impurity in this preparation (see Table 1-II, page 14), the lower critical

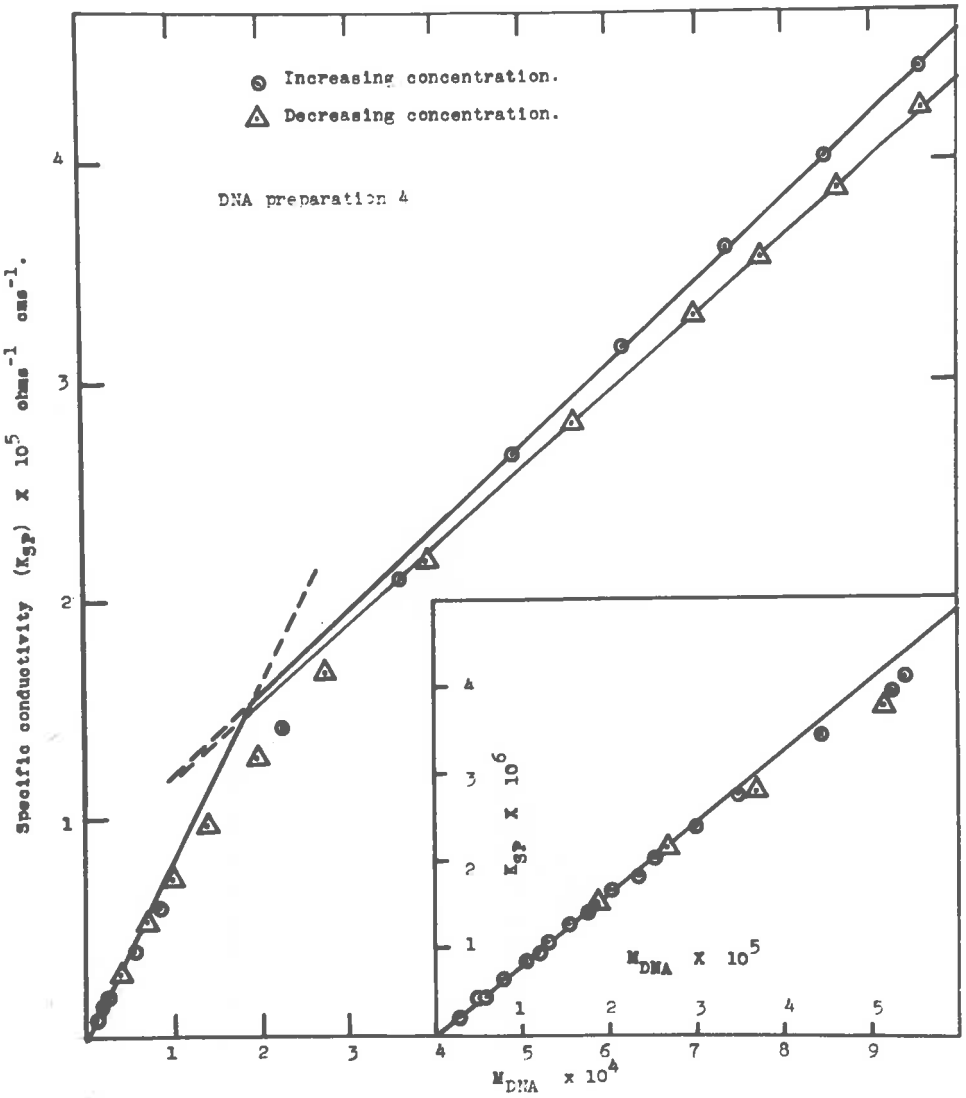

Fig. 1-IV. Specific conductivity of salt free DNA solutions.

concentration is also to be expected in view of the salt impurity (to be discussed later in this chapter).

To show that any possible frequency dependent errors were not responsible for the critical concentration phenomenon, the conductivities measured at 1,2.5, 5,10 and 20 Kc/s. were extrapolated to infinite frequency and compared with the results obtained at 1 Kc./s. No large change in the critical concentration phenomenon was evident from this comparison.

2. Influence of ionic strength.

The relationship between the critical concentration, as determined from conductivity measurements, and the concentration of added sodium chloride is shown in fig. 2-IV. The phenomenon is extremely sensitive to very low salt concentrations, the addition of 2×10^{-5} M NaCl being sufficient to lower the critical concentration by one third. The fact that salt lowers the critical concentration is consistent with both processes that have been proposed to explain the critical concentration phenomenon. If the process arose because of denaturation then the addition of salt should lower the critical concentration. The presence of shielding ions is thought to protect the molecule from possible denaturation[1,2,3] because ion-pair formation should decrease electrostatic

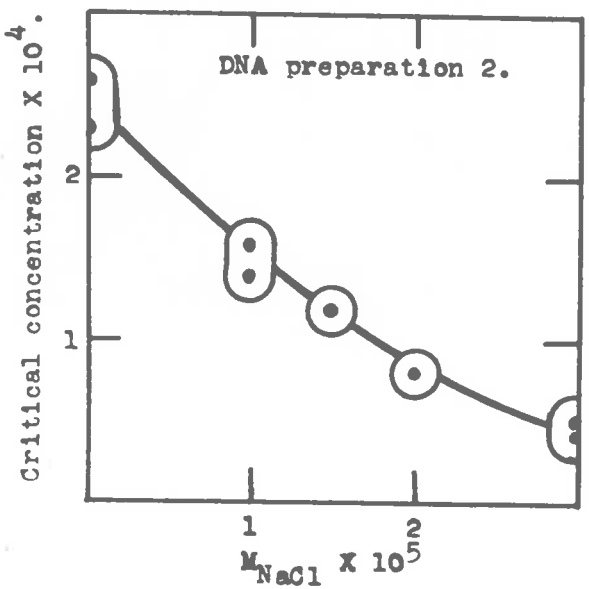

Fig. 2-IV. Dependence of critical concentration on added NaCl.

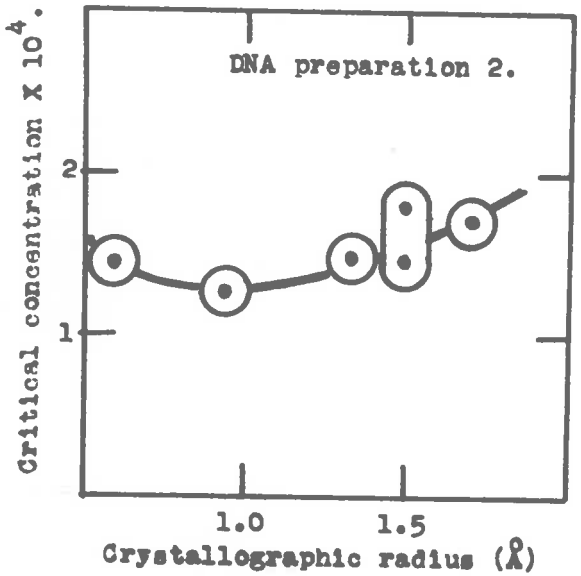

Fig. 3-IV. Dependence of critical concentration on size of added gegenion.

repulsion between the chains and therefore decrease the tendency for hydrogen bond rupture. The alternate, but less likely, explanation of aggregation of two or more DNA molecules at concentrations above the critical zone also receives support from the above finding. A shielding action would tend to lower the repulsive force that must be present between the DNA molecules and therefore increase the possibility of two or more units coming together. The lowering of the critical concentration in the presence of minute amounts of salt excludes any explanations of the phenomenon involving electrostatic interaction between polyions.

As the above experiments show that great changes can be produced by small amounts of added salt it can be seen that measurements on salt free solutions are liable to be irreproducible unless salt impurity is rigorously excluded.

(3) Changes effected by variation in gegenion radius.

Further investigations were made on the effect of changing the cationic radius of added salt, the results can be seen in fig. 3-IV for various monovalent gegenions. Although the differences are not great it appears that sodium ions lower the critical concentration to the greatest extent. If it is assumed that the action of the added salt is that of shielding, then the

minimum in the curve is not wholly unexpected. It is well known[4] that a critical radius exists for ions in aqueous solution, such that ions smaller than a certain size acquire an hydrated sheath which increases the aqueous ionic radius. If lithium ions had such an increased radius then the minimum in fig. 3-IV can be understood because the larger ions will not be as effective in shielding the interchain electrostatic repulsions.

To determine whether the decreased critical concentration following the addition of salt, originated from a shielding action the effect of much larger cations was investigated. The salts used were rosaniline hydrochloride and 5-amino-acridine hydrochloride. Unfortunately the effect of these ions can only be studied over a limited concentration range because of precipitation above certain concentrations. However within this limit the effect of these large ions in lowering the critical concentration was much less than the simple salts at the same concentration. This is to be expected if a shielding action is involved.

(4) Influence of dielectric constant.

In view of the well known relationship between dielectric constant and association in simple electro-

lytes, experiments were made to determine the effect of changes in association between polyion and gegenion on the critical concentration. The changes in dielectric constant were effected by various methanol/water or ethanol/water solvents (fig. 4-IV). It was found that a lowered critical concentration accompanied the lowering of dielectric constant. Again this is consistent with a shielding action for the gegenions. That gegenion/polyion association does increase on lowering the dielectric constant can be inferred from the observed lowering of equivalent conductivity of the DNA in decreasing dielectric constant solvents. In the case of ethanol complete association could be expected in pure ethanol solvent as judged by the ever decreasing equivalent conductivity found with increasing ethanol/water ratios. This was confirmed by direct measurement of DNA in 99% ethanol prepared by dialysis against increasingly stronger alcoholic solutions[5]. The conductivity of DNA in this solution was almost one hundredth that of an aqueous solution. No sudden change was found in the plot of equivalent conductivity versus percentage alcohol solvent as would be expected from the rapid changes found in sedimentation coefficient at 65% ethanol[5].

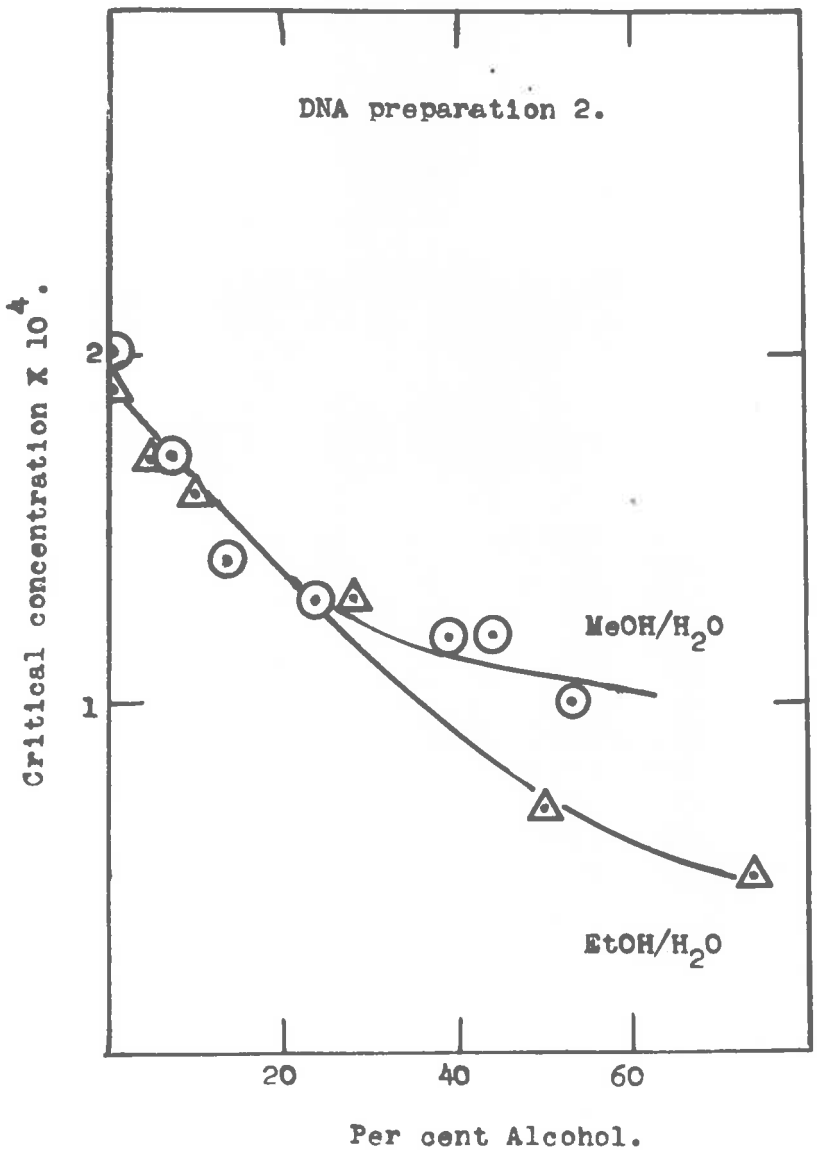

Fig. 4-IV. Dependence of critical concentration on dielectric constant. Dielectric constant varied using EtOH/H_2O & MeOH/H_2O mixtures.

The effect of a small increase in dielectric constant was studied by conductivity measurements on DNA in the presence of 0.5 M glycine. The expected small increase in the critical concentration was found. At concentrations below the critical concentration zone both aqueous DNA and solutions of DNA in 0.5 M glycine appeared to have similar conductivities, however at higher concentrations the specific conductivity of DNA in 0.5 M glycine was found to be much greater than for aqueous DNA (see fig. 5-IV). The reasons for this behaviour will be discussed later in this chapter.

(5) Effect of added protein and EDTA.

A suggestion has been made that protein impurity might be responsible for aggregation of DNA molecules[5]. The residual protein would possibly be present as nucleoprotein. As something of this nature might possibly explain the critical concentration phenomenon, nucleoprotein was deliberately added to DNA solutions and the resulting effect on the phenomenon studied. The effect of added protein was to increase the critical concentration which is the opposite effect to that expected if protein favoured aggregation of DNA. This result does not necessarily eliminate an aggregation mechanism however, because in the present instance the

protein was added as nucleoprotein which might not have the potential aggregation sites shown by a free protein; however it is known that nucleoprotein alone can form gel-like aggregates[7] and therefore it is possible that nucleoprotein may have a residual tendency to form aggregates with DNA. If the critical concentration phenomenon originates from a denaturation process then the presence of protein appears to favour denaturation. Although this is intuitively unexpected from a biological point of view it must be remembered that these arbitrary mixtures (DNP added to DNA) would be most illdefined in structure and would bear little resemblance to the DNA-protein system present in biologically active material.

As aggregation of the nucleate ion could possibly occur through divalent ion bridges, the effect of added ethylene diamine tetra-acetic acid (EDTA) was studied. The presence of EDTA should remove from solution all divalent ions. EDTA at 1×10^{-5} M (disodium salt) had no measurable effect on either the critical concentration or the actual conductivity and therefore divalent ion bridges do not appear responsible for the phenomenon.

(6) Changes produced by thermal denaturation of DNA.

It is now well established that denaturation can result from thermal agitation[8,9]. As the most likely explanation of the critical concentration phenomenon rests on a similar process, it is of interest to determine what effect previous denaturation has on the conductivity. Thermal denaturation was achieved by heating an aqueous DNA solution (3×10^{-3} M) for 30 minutes at 75°C. Previous experiments had shown that this was the minimum heating time required to yield a constant atomic extinction increase (measured in 0.1 M NaCl at room temperature). The change from $\epsilon(P)$ of 6630, measured at 2590 Å, to 7300-7400 was found to be essentially constant for periods of heating up to 200 minutes. No after effects, such as a slow increase in viscosity, were noticed in these thermally denatured salt free solutions. Fig. 5-IV shows a comparison of the conductivity between an initially native sample and the thermally denatured material. It is to be noted that the thermally denatured material has an increased conductivity above the critical concentration only, below the critical region the two solutions have identical values. The overall result is a decrease in the magnitude of the critical concentration if DNA was initially denatured. The fact that the initially

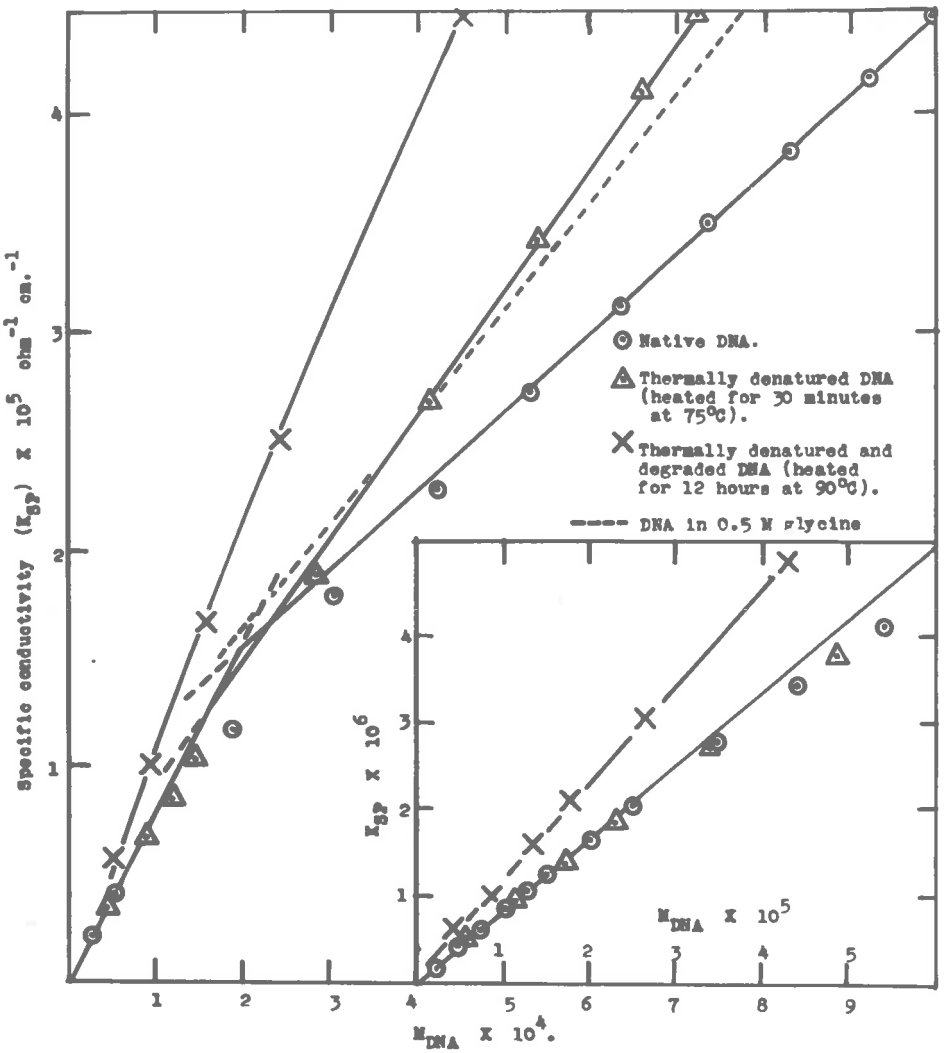

Fig. 5-IV. Conductivity of thermally denatured DNA compared with a native sample.

denatured material has a similar equivalent conductivity to the initially native solution below the critical zone is strong evidence that the critical concentration zone itself originates from a process leading to denatured material below the critical zone. The discontinuity that still exists in the thermally denatured sample may mean that denaturation by heat was not complete, however this appears unlikely from recent studies on thermal denaturation[9]. A more likely explanation appears to be the reformation of hydrogen bonds above the critical concentration. These hydrogen bonds would be of a non-specific type. For comparison another DNA solution was given drastic thermal treatment (90°C for 12 hours), the conductivity of this solution is shown in fig. 5-IV. In this case the discontinuity has been eliminated and the conductivity has increased over the whole concentration range. It is apparent that further changes over and above the normal denaturation process have taken place in this solution. Degradation has certainly accompanied this treatment.

The large increase in conductivity of DNA solutions in 0.5 M glycine (discussed earlier and shown in fig. 5-IV) also occurred above the critical concentration only. In fact the plot of specific conductivity versus DNA concentration resembles very closely the

results given in fig. 5-IV for thermally denatured DNA. It may be inferred that glycine, at this concentration is a denaturing agent for DNA in the absence of added electrolyte.

In Chapter III it has been shown that a discontinuity exists in the plots of optical density versus DNA concentration. As it is probable that this phenomenon arises from the same process that is responsible for the critical concentration in the conductivity measurements, the effect of thermal denaturation on the optical density was also investigated. It was found that the thermal treatment described above completely abolished the discontinuity observed in the U.V. absorption. At all concentrations studied (up to 8×10^{-4} M DNA) the $\epsilon(P)$ in salt free solutions of thermally denatured DNA was constant at 8500 (as measured at room temperature) and was identical with an initially native sample below the critical concentration. Again this would indicate that denaturation by dilution is responsible for the critical concentration. Light scattering investigations have shown[3] that dilution of salt free DNA solutions to low concentrations leads to denaturation with a resultant decrease in root-mean-square end-to-end distance at constant molecular weight.

(7) Sedimentation velocity experiments on aqueous DNA solutions within the critical concentration zone.

As the process responsible for the critical concentration phenomenon appears to originate from denaturation, one would expect to observe two boundaries in the ultracentrifuge for the sedimentation of a salt free solution at concentrations within the critical region. Differences in the sedimentation properties would be expected as the native molecules are thought to be relatively rigid while the denatured or irreversibly changed material, although of the same molecular weight, would not necessarily be restrained to the dimensions imposed on the native structure. The denatured molecule therefore should behave more like a flexible polyelectrolyte.

An investigation of salt free DNA solutions at concentrations within the critical region was hampered by lack of reproducibility. A preliminary study did in fact show the expected double boundaries at concentrations between 2 and 4 X 10^{-4} M DNA. Single boundaries only were observed above and below this concentration zone. Later, when the great sensitivity of the phenomenon to the presence of salt impurity was fully appreciated, the above results could not be re-

produced if salt was rigorously excluded. The leading component if present, could not be resolved although the observed boundary was highly asymmetric on the leading side. These two types of behaviour are illustrated in fig. 6-IV. Fig. 7-IV shows the concentration dependence of the sedimentation coefficient obtained from the later experiments. No rapid change in this property was found at the critical concentration if salt impurity was excluded. The values shown in fig. 7-IV were calculated from the half concentration point of the total asymmetric boundary.

Because of the possible resolution of the two species in the presence of minute amounts of added salt, further experiments were designed and will be given in a later chapter.

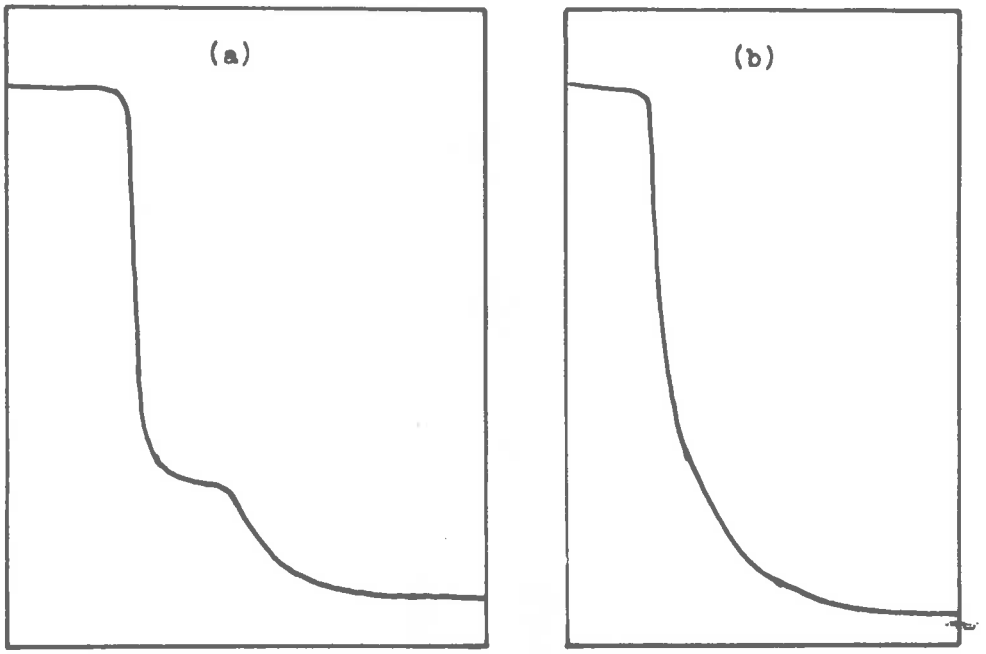

Fig. 6-IV. Sedimentation velocity boundaries of aqueous solutions at concentrations within the critical concentration zone.
(a) The two boundaries observed in preliminary investigations.
(b) Single asymetric boundary obtained in later investigations (salt rigorously excluded).
These tracings are to be considered as inverted plots of DNA concentration versus distance from meniscus (meniscus on left hand side).

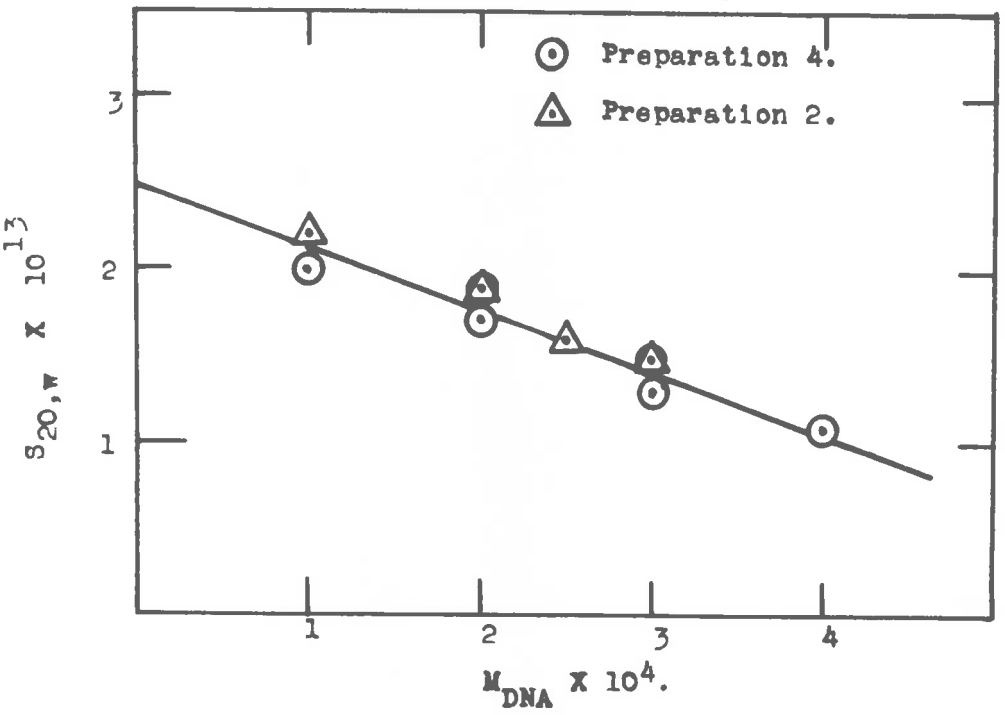

Fig. 7-IV. Sedimentation of DNA in aqueous solution.

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CHAPTER V.

TESTS FOR THE REVERSIBILITY OF THE CRITICAL CONCENTRATION PHENOMENON

(1) Introduction.

(2) Reversible and irreversible components of the structural transition - U.V. absorption.

(3) Preparation of a solid DNA sample previously subjected to dilution below the critical concentration.

(4) Reversible and irreversible components of the structural transition - conductivity measurements.

(5) Tentative explanation of the structural transition in terms of denaturation.

(1) Introduction.

In earlier chapters it has been suggested that the structural transition which gives rise to the critical concentration observed for aqueous DNA solutions could originate from two possible mechanisms - by aggregation of nucleate ions above the critical concentration or by disaggregation of the polyion at concentrations below the critical region. In the former mechanism the native nucleate polyion would be present below the critical concentration while aggregates of native polyions would predominate at high concentrations. The latter explanation would imply that the native material was present at concentrations above the critical region while at lower concentrations denatured DNA would predominate. The two possibilities would be characterised by reversibility and molecular weight change in the first case contrasted with irreversibility and constancy of molecular weight in the second (Chapter III). Three significant observations favour the denaturation mechanism.

(a) Earlier investigations[1,2] have shown that denaturation does occur at low ionic strengths and that aqueous DNA solutions do undergo denaturation[3]. It has also been established that the transition,

like that produced by acid[4], alkali[5] and thermal[6] treatment is not accompanied by significant molecular weight change[3].

(b) The atomic extinction coefficient of aqueous DNA solutions increases at concentrations below the critical zone (which is to be expected if denaturation is involved).

(c) The conductivity behaviour of thermally denatured DNA is very similar to initially native DNA at concentrations below the critical zone but is considerably different from a similar solution above the critical concentration (fig. 5-IV). This would imply that in both cases the kinetic unit is similar below the critical concentration.

Although this evidence favours the process involving denaturation, a final decision cannot be made until either the molecular weight change or the irreversibility associated with the structural transition has been established. An investigation of the possible molecular weight changes accompanying the critical concentration phenomenon would be difficult because such an investigation would involve for instance, a sedimentation study of aqueous DNA solutions where the primary salt effect would be operating. Cavalieri *et al.*[3] have shown that there is no light-scattering molecular weight change when DNA is denatured

at low concentrations in the absence of salt, however these measurements were made after the denatured material had been adjusted to high ionic strength and due to the partial reversibility of the critical concentration phenomenon (to be discussed later) and to the great dependence of the structural transition on salt concentration, this observation cannot be rigorously applied to the polyion present at concentrations below the critical region.

It therefore appeared that experiments concerned with the reversibility or irreversibility of the phenomenon should be the more fruitful. In this chapter investigations will be discussed which employ this method of approach.

(2) Reversible and irreversible components of the structural transition - U.V. absorption.

It is now established that the anomalous low absorptivity (measured at 2590 Å) of native DNA originates from some property of the closely stacked and hydrogen bonded nitrogen bases that occur on the inside of the double spiral structure of DNA (Chapter II). As denaturation involves the destruction of this orderly arrangement of bases it should be accompanied by increased absorptivity. This has been experimentally observed in many investigations of denatured DNA.

However it also appears that this increase in absorptivity can in some respects be a rather insensitive measure of the initial stages of denaturation[3].

An essentially native DNA solution (initially dissolved in water at high concentration) has an atomic extinction coefficient, referred to phosphorus concentration, of 6630 (Chapter II). This value was obtained by dilution of a concentrated aqueous solution to 5×10^{-5} M DNA in the presence of salt to give a final concentration of 0.1 M NaCl. A salt free solution has a similar value[3] at DNA concentrations greater than $9\text{-}10 \times 10^{-4}$ (measured in the absence of added salt). At lower concentrations a discontinuity has been observed (Chapter III) which results in an absorptivity, $\epsilon(P)$, of 8200[3] below the critical concentration zone. Values of 8500 and 8400 - 8000 have been found for similar solutions of preparations 4 and 7 in the present investigation. The nature of the discontinuity is shown in fig. 1-V. This increase in extinction coefficient has been associated with denaturation (Chapter III), however the observed increase is complicated by the fact that addition of salt to such a solution, $\epsilon(P) = 8000 - 8500$, lowers the absorptivity to an intermediate value. Partial reversibility or some other factor arising from the increased ionic strength must therefore be considered in treating the above phenomenon.

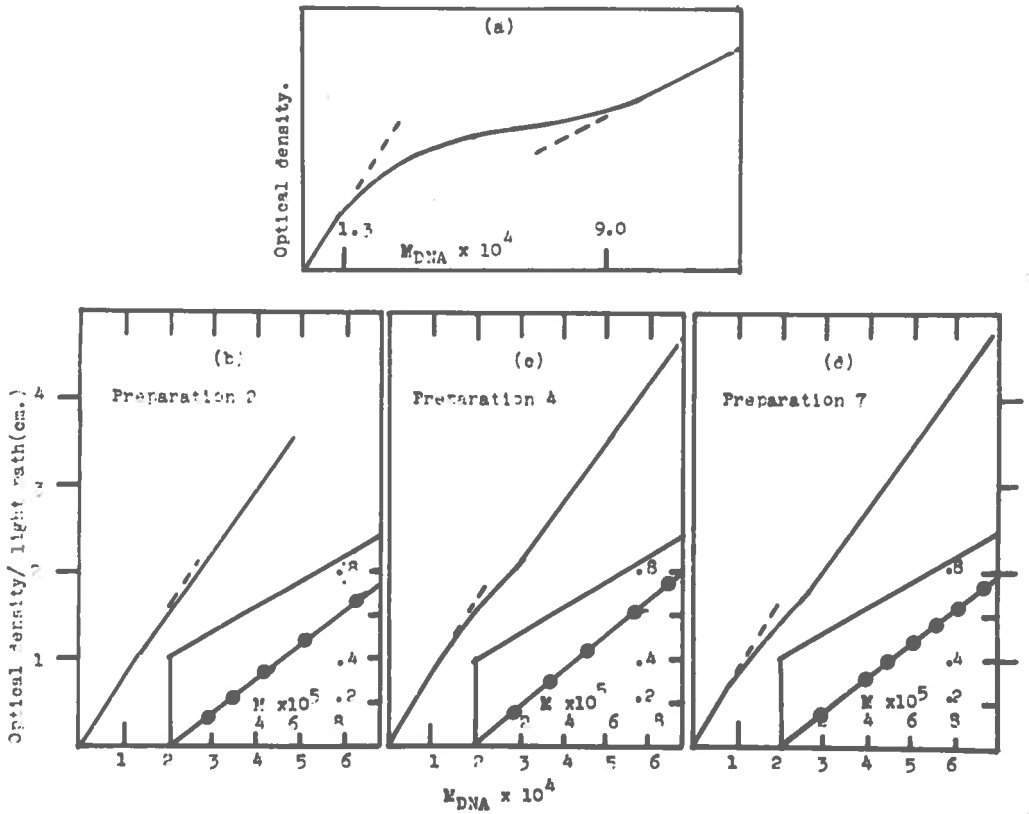

Fig. 1-V. The discontinuity in plots of optical density versus DNA concentration (in the absence of added electrolyte).
(a) Data of Cavalieri et al. (diagram only)
(b,c and d) Optical density of various preparations in water.

As the addition of salt to a solution below the critical concentration results in a partial lowering of the $\epsilon(P)$ it will be assumed that the value so obtained represents the true irreversible increase that has been brought about by dilution below the critical concentration zone.

In previous chapters it was shown that a critical concentration zone exists in plots of specific conductivity versus concentration. It is therefore of interest to compare the concentration zone over which the true irreversible increase in absorptivity occurs, with the zone found from the conductivity measurements. Fig. 2-V shows the true irreversible increase that is observed at low DNA concentrations. In fig. 2-V (a) represents the values obtained at the various DNA concentrations shown, by dilution of stock solution (3×10^{-3} M DNA) in the presence of salt, (b) represents the large partially reversible increase obtained by dilution with water only and (c) was obtained by dilution of the stock solution to the various concentrations shown, followed by the addition of solid salt to 0.1 M NaCl; these solutions were then diluted to 5×10^{-5} M DNA with 0.1 M NaCl and the absorptivity determined. Curve (c) of fig. 2-V represents the true irreversible increase that occurred on dilution of a salt free DNA solution. It can be seen that the irreversible increase begins at

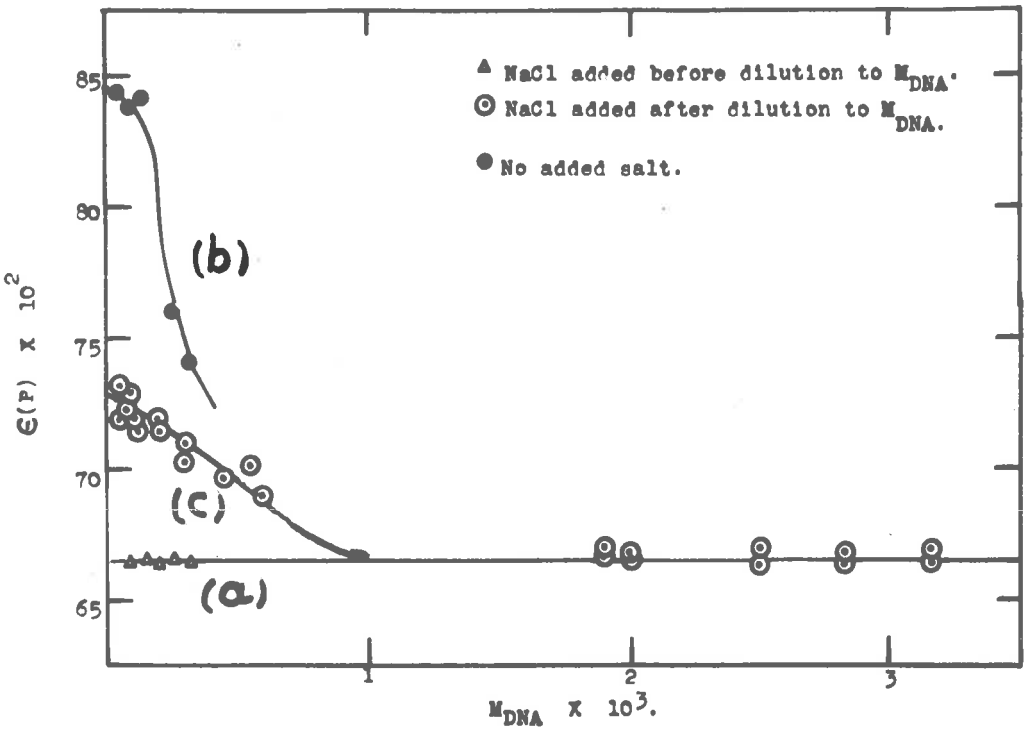

Fig. 2-V. Reversible and irreversible components of the atomic extinction coefficient (measured at 2590 Å).

concentrations lower than 10 X 10^{-4} M DNA. This is in agreement with the partially reversible increase shown in (b). Previous work by Cavalieri et al[3] has shown that this partially reversible increase begins at concentrations below 9-10 X 10^{-4} M DNA. The concentration at which denaturation begins, as judged by the above results, is higher than the upper limit of the critical concentration zone found for the conductivity of salt free DNA solutions (5 X 10^{-4} M).

It is concluded that dissolution of DNA at concentrations below the critical zone and in the absence of salt, results in a 27% increase in absorptivity however, the irreversible increase is only 11% (these figures represent the average of several results). This is to be compared with similar figures of 25% and 11% respectively, reported by Thomas[1] for solutions of DNA at low concentrations. Thermal denaturation results in a slightly higher irreversible increase of 15%[6]. The different irreversible increases reported for denaturation by dilution and by heat, may possibly be due to the temperature dependence of $\epsilon(P)$ for denatured DNA[7]. Similar measurements were made on two further DNA preparations (numbers 1 and 5 as described in Chapter II) which contained salt impurity. The irreversible increases were only 6% and 3% respectively. The smaller increase for the preparations containing salt impurity is

understandable if the great sensitivity of the critical concentration phenomenon to salt is taken into account (Chapter IV).

The above experiments confirm the previous claims that irreversible increases in $\epsilon(P)$ occur in salt free solutions. The change appears to be brought about by a structural transition from essentially native to denatured DNA as the DNA concentration is decreased through the critical concentration zone. It is thought that this transition is similar to that observed on heating saline solutions of DNA, in this case a critical temperature range exists within which denaturation takes place[6,8].

(3) Preparation of a solid DNA sample previously subjected to dilution below the critical concentration.

In order to test for irreversibility of the critical concentration phenomenon observed in the conductivity measurements it is necessary to prepare a solution at high DNA concentration (3×10^{-3} M DNA), which had previously been subjected to dissolution below the critical concentration zone. This was done by freeze drying aqueous solutions (5×10^{-5} M DNA) and redissolving in water to give 3×10^{-3} M DNA. This solution could then be investigated for irreversible changes in conductivity as compared with a solution at a similar

concentration which had not however been first diluted below the critical concentration zone.

Table 1-V

Increased U.V. absorption accompanying denaturation by dilution.

Preparation	$\epsilon(P)$ of DNA solutions (measured in 0.1M NaCl)			
	Initially native solution at 0.003 M DNA (1)[a]		Denatured by one dilution below critical concentration and freeze dried to 0.003 M DNA (4)[a]	
	Solution diluted to 5×10^{-5} M DNA (2)		Solution diluted to 5×10^{-5} M DNA	
	Salt added first	Salt added last	Salt added first	Salt added last
4	6710	7300	7350	7350
7	6610	7350	7400	7380
5[b]	6500	6740	7010	7000

a numbers refer to solutions given in Table 2-V.

b containing salt impurity.

Before any confidence can be placed on such a comparison further factors must be considered. It is necessary to determine just how relevant the observed irreversible increase in $\epsilon(P)$ is to the problem. If for instance, the sample that had been reconcentrated from

below the critical zone is again diluted to 5×10^{-5} M DNA in the absence of salt, would a further irreversible increase in $\epsilon(P)$ occur. The results shown in Table I-V suggest that no further changes accompany a second dilution to below the critical concentration zone. The $\epsilon(P)$ values given in the first two columns in Table 1-V were obtained by the addition of salt before (column 1) and after (column 2) dilution of an aqueous stock solution to 5×10^{-5} M DNA. Column 1 gives the extinction coefficient of the native sample while column 2 shows the irreversible increase that accompanies a single dilution below the critical concentration. The final two columns refer to similar measurements made on a solution denatured by dilution below the critical concentration and followed by freeze drying to give a stock solution of high concentration. Column 3 therefore again represents the irreversible increase due to a single dilution below the critical concentration, (any damaging effect due to the freeze drying process would be expected to show here). Column 4 on the other hand was obtained by dilution to 5×10^{-5} M DNA of the reconcentrated solution before the addition of salt and therefore represents a DNA sample that has been subjected to the influence of the critical concentration phenomenon twice. No further denaturation by dilution was found.

In addition freeze drying had no measurable effect on the extinction coefficient of a concentrated salt free solution of native DNA. Table 2-V shows the various types of solution used in this investigation and the further studies described below.

Table 2-V

Various solutions referred to in the text.

Reference Number	Solution
(1)	Solution of native DNA (dissolved in water at 0.003 M)
(2)	↓ Diluted with water to 5×10^{-5} M DNA
(3)	↓ Freeze dried
(4)	↓ Dissolved in water to 0.003 M DNA
(5)	↓ Adjusted to 1 M NaCl with solid salt
(6)	↓ DNA precipitated with ethanol & dried
(7)	↓ DNA redissolved in water to 0.003 M DNA

From the constancy of the irreversible increase in $\epsilon(P)$ it appears that the denaturation produced by dilution below the critical concentration represents a state that is well defined and not just one of a series of increasing denaturation. The average $\epsilon(P)$ obtained for six different batches of DNA, irreversibly changed by dilution followed by reconcentration, was found to be 7340 ± 60 (measured in 0.1 M NaCl

at room temperature). These figures are shown in Table 3-V.

Table 3-V

Preparation	Number of batches prepared	ϵ(P) of DNA denatured by dilution and reconcentrated by freeze drying (measured in 0.1 M NaCl) at room temperature
2	2	7420
4	1	7450
7	3	7340

A second complication that must be considered in the process involving freeze drying is whether any further irreversible effect is caused by drying, which might not possibly be detected by the absorptivity measurements given in column 3 Table 1-V. Two observations have been made which indicate that something of this nature may occur. Firstly the viscosity of a denatured solution (diluted to 5×10^{-5} M DNA in the absence of salt) is about ten times as great as the viscosity of a similar solution at the same concentration, but after freeze drying. This observation, however, may not be due to the freeze drying process, because the viscosity of an aqueous solution diluted below the critical concentration zone decreases with time[9] as compared with a similar solution reconcentrated by freeze drying followed by dissolution to the same concentration in the absence of salt. Secondly, a sample that had been de-

natured by dilution and reconcentrated was adjusted to 1 M NaCl and precipitated with ethanol. This sample when dissolved in 0.2 M NaCl showed further irreversible changes over and above that shown by a solution denatured by dilution only. The relationship between the various solutions is shown in Table 2-V. The changes in both $\epsilon(P)$ and s_{20} are shown in Table 4-V. Unfortunately it is not possible to precipitate DNA, with ethanol at DNA concentrations of 5×10^{-5} M and therefore one cannot determine if these further irreversible changes, which become apparent on precipitation, arise from the freeze drying or the denaturation processes. It was also observed in the sedimentation velocity experiments on precipitated and redissolved material that non-sedimenting species were present, indicating that precipitation was accompanied by considerable degradation.

(4) Reversible and irreversible components of the structural transition - conductivity measurements.

Before a comparison is made between native DNA and the irreversibly changed sample described above, an earlier observation deserves attention. In a detailed investigation of the critical concentration phenomenon (fig. 1-IV) it was pointed out that a small difference existed between the conductivities obtained by either decreasing or increasing the DNA concentration. As the solution which had been increased in nucleate ion concentration exhibited the higher conductivity, it may

be inferred that at least some irreversibility is to be associated with the critical concentration phenomenon.

Table 4-V

Irreversible changes accompanying precipitation of DNA denatured by dilution followed by freeze drying.

Preparation	Solutions denatured by dilution and then reconcentrated (4)[a]		Solutions denatured by dilution, reconcentrated, precipitated and dissolved in water (7)[a]	
	s_{20}[b]	$\epsilon(P)$[c]	s_{20}[b]	$\epsilon(P)$[c]
2	19	7450	9-10	9900
4	16	7330	11-14	9900
7	17	7340	7-9	8100

a numbers refer to the solutions shown in Table 2-V.

b sedimentation velocity of 2×10^{-4} M DNA in 0.2 M NaCl at 20°C.

c absorptivity measured in 0.1 M NaCl at room temperature.

If the proposed structural transition was completely irreversible no discontinuity would exist, instead the conductivities at high concentrations should be higher and follow the relation between specific conductivity and concentration observed below the critical concentration. However, as the method used to increase the DNA

concentration (fig. 1-IV) is not a true reconcentration of DNA molecules from below the discontinuity (the concentration was increased by addition of 3×10^{-3} M DNA), only a small fraction of the initial molecules present, at concentrations below the critical zone, will contribute to any possible irreversibility observed in fig. 1-IV.

A comparison of the critical concentration phenomenon, as detected by conductivity, between initially native DNA and DNA diluted below the critical concentration followed by freeze drying will now be given. The two solutions are shown as (1) and (4) in Table 2-V. Fig. 3-V gives the conductivity versus concentration plot of these two solutions and for comparison the conductivity of heat denatured DNA, reported earlier, is also shown. At low concentrations, below the discontinuity, the three samples have similar equivalent conductivities which suggest that a similar kinetic unit is involved in each case. This lends strong support to the suggestion that the critical concentration zone arises from denaturation. Below the critical zone we have the three samples in the denatured state - the initially native solution having been denatured by dilution, the sample initially denatured by dilution and freeze dried and finally the heat denatured solution. At DNA concentrations above the

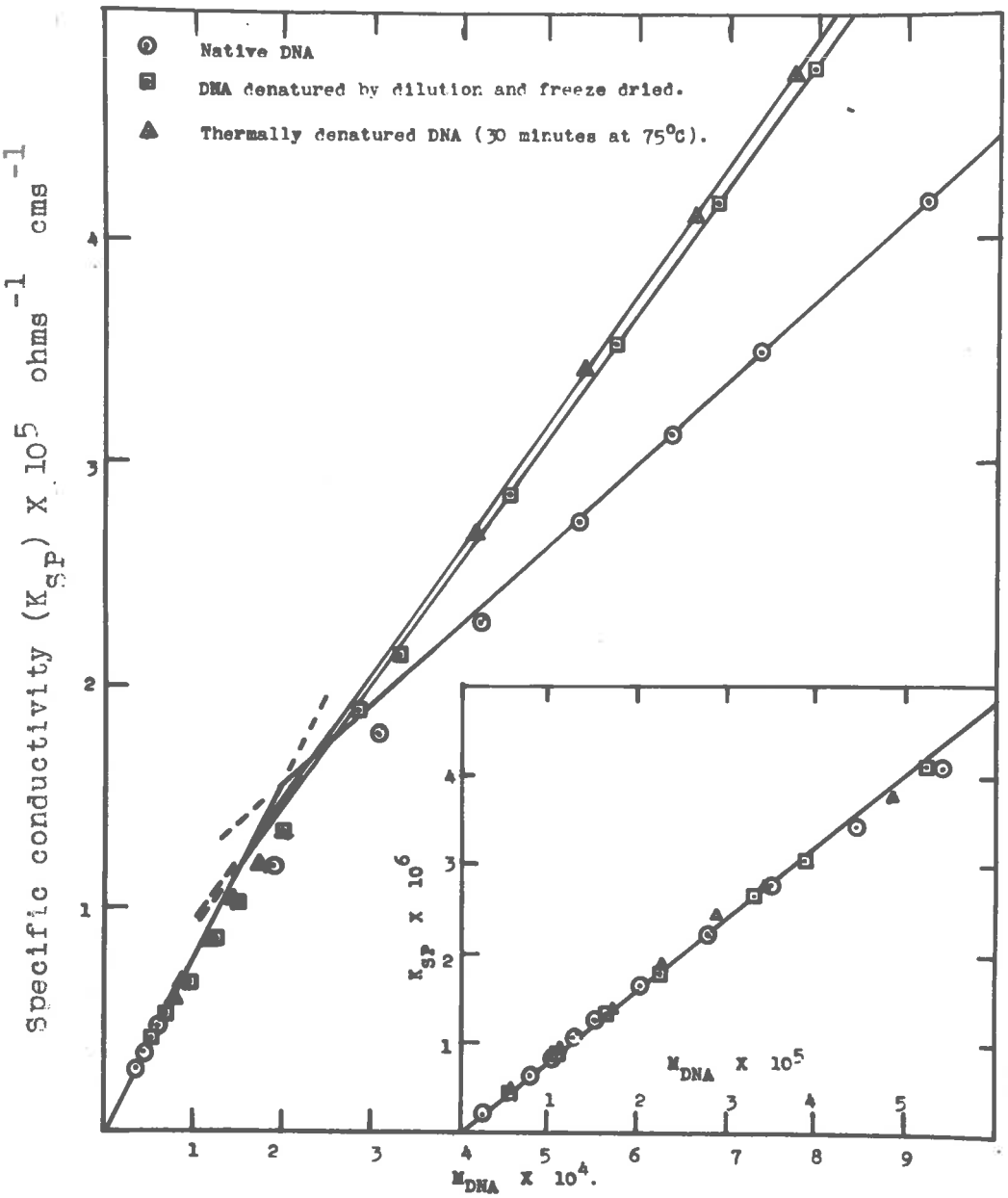

Fig. 3-V. Comparison of the critical concentration phenomena shown by native DNA, thermally denatured DNA and DNA that had previously been dissolved at a concentration below the critical concentration zone.

discontinuity both the thermally denatured sample and the material initially dissolved at low concentration followed by freeze drying, have similar conductivities and conductivity concentration dependence, implying that the kinetic units are similar. This also confirms the suggestion that the critical concentration arises from a denaturation process. In both these cases the conductivity is higher at concentrations above the critical zone, than that shown by native DNA (which consists mainly of DNA never diluted below the critical concentration). If the critical concentration arose entirely from the denaturation of DNA we would expect that the initially denatured solutions would not exhibit a critical concentration at all, however the results in fig. 3-V clearly show that this is not so. Although the magnitude of the phenomenon has been reduced a critical concentration zone still exists. Two explanations appear possible - denaturation may not be complete or denaturation as followed by conductivity is partly reversible. The former explanation does not seem likely. Firstly the absorptivity measurements given in Table 1-V show that a second dilution of a solution initially diluted below the critical concentration and then reconcentrated does not yield any further irreversible increase in $\epsilon(P)$. Secondly the discontinuity

that exists in the plots of optical density (at 2590 Å) versus DNA concentration in the absence of salt, is not present in the denatured samples (fig. 4-V).

The partial reversiblility of this process, as observed in the conductivity measurements, can possibly be explained by the reformation of non-specific hydrogen bonds. These reformed bonds would have to be such that no resemblance to spiral nitrogen base stacking results (to be compatible with the absence of the discontinuity in the optical density measurements). Intermolecular hydrogen bonding may also have to be considered.

(5) Tentative explanation of the structural transition in terms of denaturation.

A tentative explanation of the critical concentration phenomenon can now be put forward.

(a) At high aqueous DNA concentrations (greater than 5×10^{-4} M DNA by conductivity measurements and greater than 10×10^{-4} M by U.V. absorption), an essentially native DNA molecule exists. Hydrogen bond cleavage if it occurs, is reversible. It has been shown (Chapter III) that the kinetic unit possesses a charge fraction (charge/phosphorus atom) of 0.4 under these conditions. A large proportion of the gegenions are therefore held

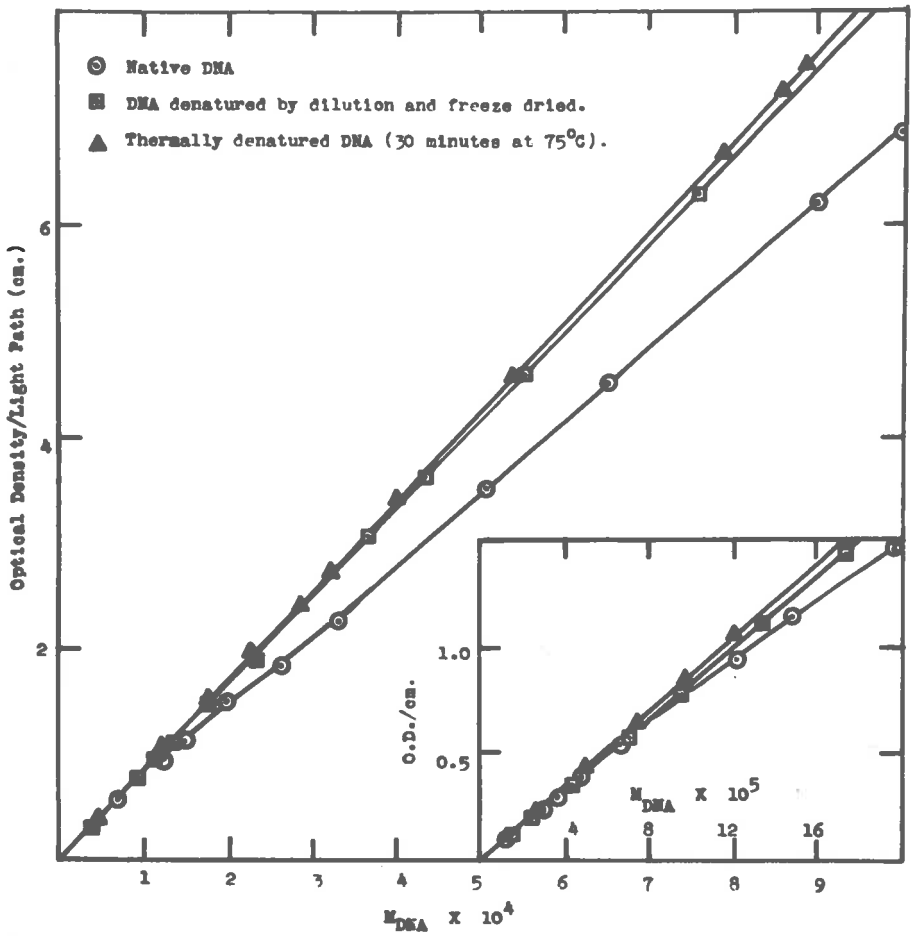

Fig. 4-V. Comparison of the critical concentration phenomena shown by native DNA, thermally denatured DNA and DNA that had previously been dissolved at a concentration below the critical concentration zone.

by the native molecule in aqueous solution. The careful work of Cavalieri et al.[3] has shown however, that the viscosity, as measured in 0.2 M NaCl, of DNA initially dissolved in water at concentrations as high as 20×10^{-4} has already suffered a small but significant decrease as compared with DNA never subjected to low ionic strengths. Similarly it has already been shown (Chapter II) that a 1.5% irreversible increase in $\epsilon(P)$ can be detected when initially native DNA (never dissolved in water during preparation) is dissolved in water at high DNA concentrations. It must therefore be admitted that a small amount of initial denaturation is inevitable when salt free DNA solutions at high concentrations are studied. This will be discussed further in Chapter VII.

(b) Dilution below the critical concentration zone (lower than 4×10^{-5} M DNA in the absence of salt) results in the denaturation of DNA (total hydrogen bond cleavage, or at least a characteristic amount of cleavage). The unit resembles the heat denatured DNA only if the latter is also diluted below the critical concentration in the absence of salt. Solutions of DNA that have been denatured by dilution have a high charge (Chapter III) and it would appear that charge fractions approaching unity are produced by this process. The differencesin other electrical properties

have already been described in Chapter III. It is thought that the high charge on the denatured nucleate ion may possibly counter the collapsing tendency that must result on breakage of the hydrogen bonds.

(c) Solutions reconcentrated from below the critical concentration zone are characterised by partial reversibility of the hydrogen bond cleavage process. The reformed hydrogen bonds would be of a non-specific type bearing no resemblance to "native hydrogen bonds". These non-specific hydrogen bonds would probably be formed at points of closest approach between portions of the polynucleotide chains that were relatively far apart in the native structure. This type of denatured molecule would correspond to the samples thermally denatured in the presence of salt[6].

It should be noted that situation (c) can also be attained by the addition of salt to (b). This follows from the observed dependence of the critical concentration on the presence of small amounts of salt (Chapter IV). Fig. 5-7 gives an illustration representing the proposed structural transition.

If further investigations confirm these findings then in the author's opinion, this situation (b) would be a most fruitful starting point for further processes designed to dissociate the twin polynucleotide strands.

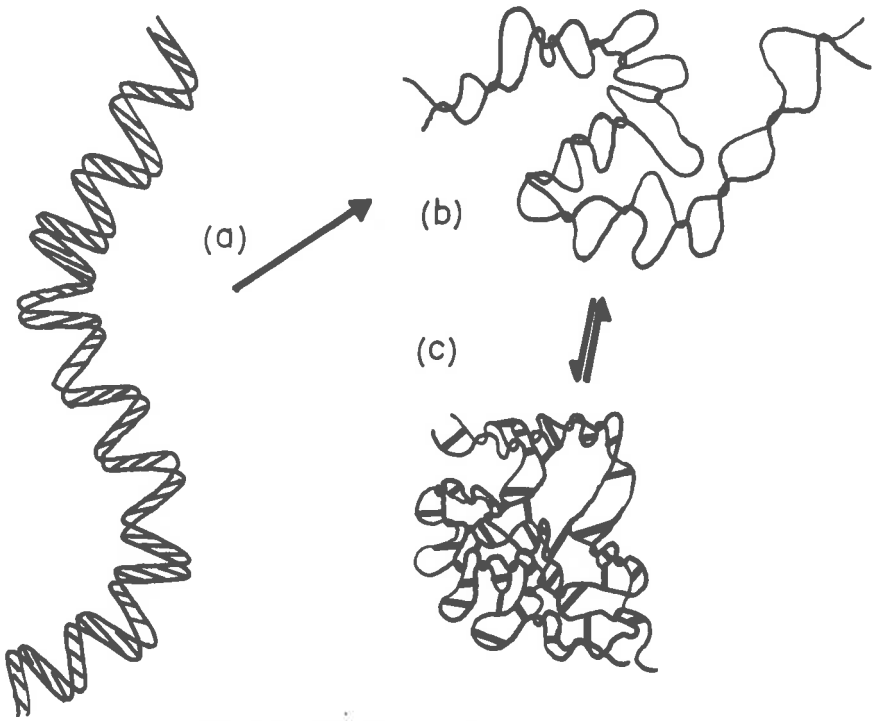

Fig. 5-V. Illustration of the structural transition.

References to Chapter V.

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CHAPTER VI

PROPERTIES OF DNA DENATURED BY DILUTION

(1) Introduction.

(2) Sedimentation properties.

  (a) At high ionic strengths.

  (b) Under the influence of the primary salt effect.

(3) Electron microscopy.

(4) Infra-red spectroscopy.

(1) Introduction.

In the previous chapter it was shown that the critical concentration was partly reversible with respect to conductivity measurements but apparently totally irreversible if followed by U.V. absorption. On the basis of this difference between properties associated with the kinetic unit (conductivity) and those arising from the disposition or interaction of the chromophoric groups (U.V. absorption), a modification of the proposed denaturation process was required. The reversible component of the process was explained by the formation of non-specific hydrogen bonds on the reconcentration of a solution of DNA denatured by dilution below the critical concentration. In the investigations to be discussed in this chapter attempts have been made to compare certain properties of native DNA with DNA denatured by dilution. Unfortunately the concentrations needed for these investigations were above the critical zone, moreover it was necessary to study the two types of DNA in solutions of high ionic strength. Because of the partial reversibility already mentioned and also the great sensitivity of the critical concentration to added salt, the denatured sample studied in these investigations corresponds to denatured DNA which has suffered the partial reversibility which occurs on reconcentrating above the critical zone.

The comparison to be made therefore is between the two structures shown in fig. 5-V, the native sample having the full compliment of specific hydrogen bonds and a rather inflexible configuration and the denatured material containing non-specific hydrogen bonds and having a flexible structure.

(2) Sedimentation properties.

(a) At high ionic strengths.

When a salt free solution of DNA is diluted below the critical concentration zone the following changes are thought to accompany denaturation, - the effective charge increases and the resistance offered to motion of the particle increases. However, these changes were deduced for denatured DNA below the critical concentration and in the absence of salt. Reconcentration of this solution, or the addition of salt, is thought to bring about the formation of non-specific hydrogen bonds and under these conditions the denatured molecule will have different properties. Cavalieri *et al.*[1] have shown that denaturation by dilution results in a decrease in the root-mean-square end-to-end distance at constant molecular weight, as measured in 0.2 M NaCl.

It has already been shown (Chapter IV) that sedimentation experiments carried out on salt free solutions at concentrations within the critical region

resulted in single boundaries only, whereas under these conditions it is believed both the native and denatured molecules coexist. As one would expect from the discussion above, differences in the sedimentation properties between native and denatured DNA further efforts were made to try and resolve the two species by sedimentation velocity.

An objection to the work in salt free solution is that the critical concentration zone may not represent a region of coexistence of both species but rather some change in the structure of either one. To overcome this objection it is necessary to compare the sedimentation properties of separate samples of the two species. This was carried out on native DNA in 0.2 M NaCl and on DNA denatured by dilution followed by freeze drying and adjusting to 0.2 M NaCl. The results of these experiments are shown in fig. 1-VI where the sedimentation coefficient versus DNA concentration is given for both the denatured and the native material. No significant difference in properties was found for the two preparations studied. DNA preparations 7 and 8 were also investigated in this way with the same result (Chapter VII). Similarly mixtures of native and denatured DNA sedimented as one entity. The results shown in fig. 1-VI also indicate that no large difference exists in the sedimentation coefficient concentration dependence. This again is unexpected when ac-

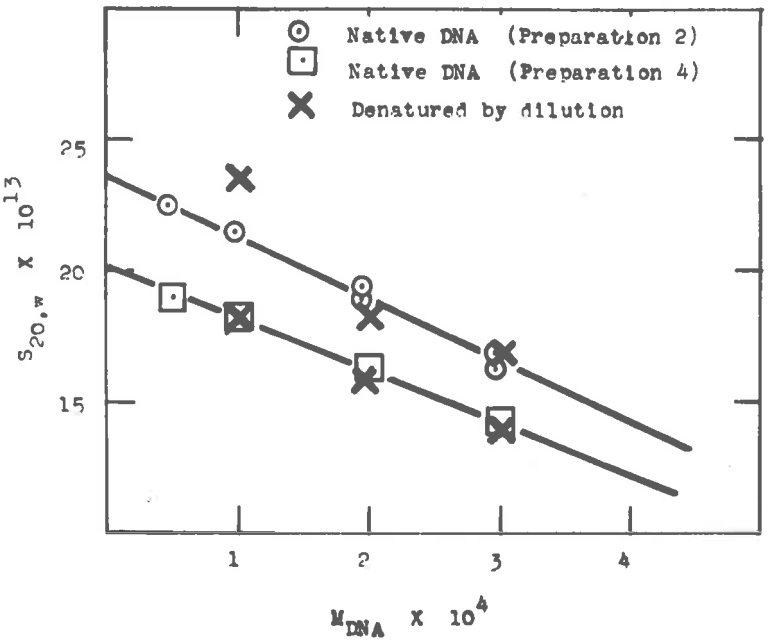

Fig. 1-VI. Sedimentation velocities of native DNA and DNA denatured by dilution. All measurements made in 0.2 M NaCl.

count is taken of the differences in intermolecular interaction that the two species should show. Even at much higher DNA concentrations, where interaction effects are certainly present, a mixture of native and denatured DNA cannot be resolved in the ultracentrifuge. Although no measurable difference in sedimentation velocity could be detected, it does appear that the boundary spreading, exhibited by the denatured material is slightly greater.

No explanation can at present be advanced to explain the similarity in sedimentation properties between native DNA and DNA denatured by dilution. A similar problem has also arisen in the case of denaturation by alkali[2] and thermal treatment[3], where again the native and denatured species appear to have similar sedimentation velocity coefficients. As has already been pointed out[3] it is difficult to put forward a mechanism whereby two species of similar molecular weight but which differ by a factor of three in radius of gyration (a factor of 27 in molecular volume) can sediment at equal rates. In the author's opinion this problem may be resolved by a consideration of possible differences in interaction between solvent and the native and denatured molecules respectively. In a discussion of this problem[3] the above type of interaction

had been assumed of equal magnitude for both native and denatured DNA. A more detailed, but qualitative, discussion of the possible differences in solvent-polyion interaction will be given in Chapter VII.

(b) Under the influence of the primary salt effect.

Preliminary sedimentation experiments on aqueous DNA solutions (Chapter IV) indicated that native and denatured DNA could be differentiated by sedimentation velocity at very low ionic strengths, but not however at zero salt concentration. It has already been shown that no detectable velocity differences are apparent at high salt concentrations (section (a) of this chapter). The following study was designed to explore the above preliminary observations.

An aqueous DNA solution was diluted in the presence of salt to give final concentrations of 2×10^{-4} M DNA and a range of salt concentrations from zero to 0.2 M. The sedimentation velocity boundaries recorded for these solutions are shown in fig. 2-VI. At high ionic strength, 0.2 M NaCl, there is a single symmetrical boundary which will correspond to native DNA. DNA denatured by dilution will not be present in such a solution because the critical concentration zone will be considerably depressed in the presence of 0.2 M NaCl (see fig. 2-IV). As the ionic strength decreases it would be expected that the critical concen-

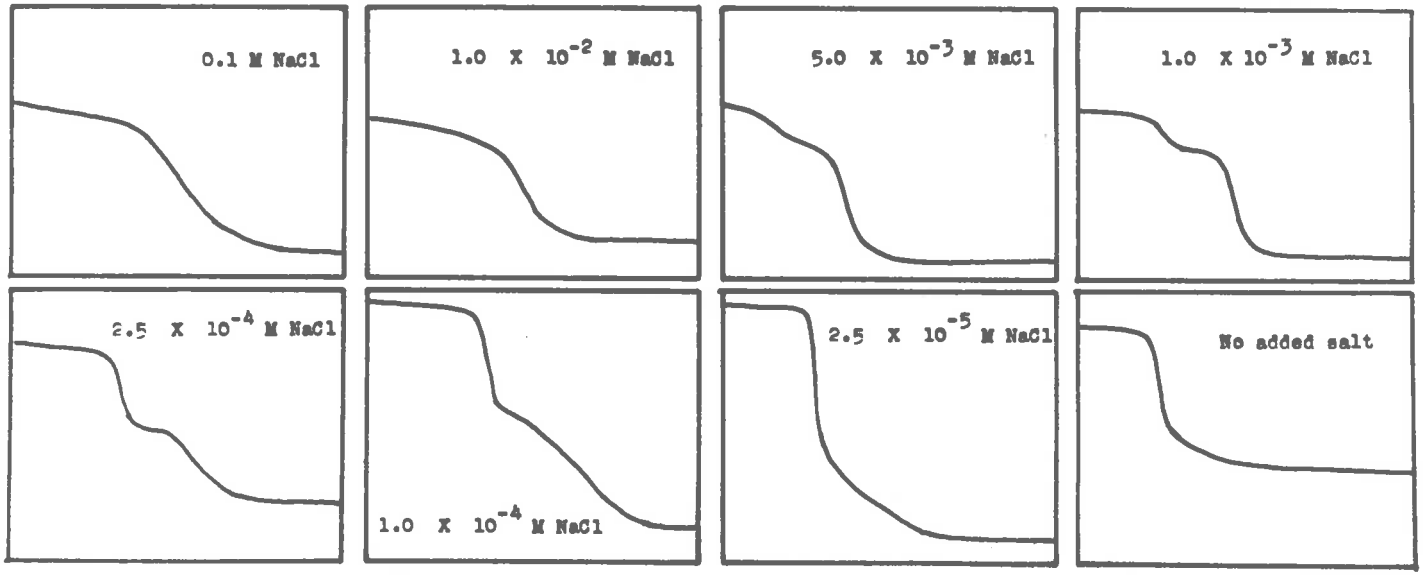

Fig. 2-VI. Sedimentation velocity boundaries obtained at various ionic strengths but at constant DNA concentration (2 X 10^{-4}M). These tracings are to be considered as inverted plots of DNA concentration versus distance from meniscus (meniscus on left hand side).

tration zone should increase (see fig. 2-IV) and eventually reach a concentration of 2×10^{-4} M DNA, which corresponds to the concentrations used in the present experiments. When this occurs the solution will contain a mixture of native and denatured molecules. Fig. 2-VI demonstrates the existence of two sedimentationally distinct species over the range $1\text{-}50 \times 10^{-4}$ M NaCl, at ionic strengths below this one boundary only is observed which however, is asymmetric on the leading edge. From these boundaries (fig. 2-VI) it can also be deduced that with decreasing ionic strength, the slower component increases at the expense of the leading species. At zero ionic strength the leading component is possibly still present and is responsible for the observed asymmetric boundary. Recent investigations by Oth and Desreux[4] have confirmed that low ionic strength allows the separation of the two species in the ultracentrifuge. Double boundaries, at low ionic strengths, have also been reported by Sinsheimer[5] for phage ϕX174 DNA. As this DNA has been found to be single stranded, association of the second boundary with a denatured structure must await a study of the secondary structure exhibited by this interesting type of DNA.

If the two boundaries found in the present investigation are to be associated with native and denatured DNA, then to be compatible with the known dependence of the critical concentration phenomenon on

ionic strength, the leading boundary should correspond to native DNA while denatured DNA should sediment more slowly. This follows from the replacement of the faster by the slower boundary in fig. 2-VI. This appeared to be correct when similar experiments were carried out on solutions of DNA denatured by dilution to 5×10^{-5} M DNA in the absence of salt followed by reconcentration by freeze drying to 2×10^{-4} M. In this case single boundaries only were observed over the entire range of ionic strengths investigated, moreover the sedimentation velocity over the salt concentration range 1-50 $\times 10^{-4}$ M (corresponding to the double boundaries in the previous experiment) was similar to the rate of sedimentation of the trailing boundaries in the first series of experiments. These results are given in fig. 3-VI. Mixtures of native DNA and DNA denatured by dilution, followed by reconcentration also yielded single boundaries at high ionic strengths. Exact agreement between the sedimentation velocities of denatured DNA in the presence of native material and denatured DNA by itself cannot be expected, as the velocity of the denatured species in the presence of native polyion may be changed[6]. Similarly the exact proportions of the two components present in the experiments where two boundaries are recorded, cannot be determined from the present data because of the Johnston-Ogston effect[7].

From these experiments it appears reasonably certain that the native and denatured species can be resolved over a limited ionic strength range and that native DNA sediments with the greater velocity.

From the known dependence of critical concentration on ionic strength (fig. 2-IV) it would not be expected that the critical concentration would be as high as 2×10^{-4} M at the ionic strengths in which the double boundaries are observed. An uncertain extrapolation of fig. 2-IV leads to total disappearance of the critical concentration at a salt concentration of only 5×10^{-5} M, however, as the critical concentration exists as a zone rather than the arbitrary concentrations given in fig. 2-IV, this objection may not be serious. According to the U.V. absorption data for instance, denaturation by dilution (in the absence of salt) begins at concentrations as high as 1×10^{-3} M DNA.

Further experiments were carried out on DNA solutions at various ionic strengths in which the salt concentration was adjusted <u>after</u> dilution in water to 2×10^{-4} M DNA. Single boundaries only were observed during sedimentation and the sedimentation velocity was compatible with denatured DNA (fig. 3-VI). These results again indicate that the critical concentration is higher than that observed by conductivity measurements.

The experiments in which native and denatured

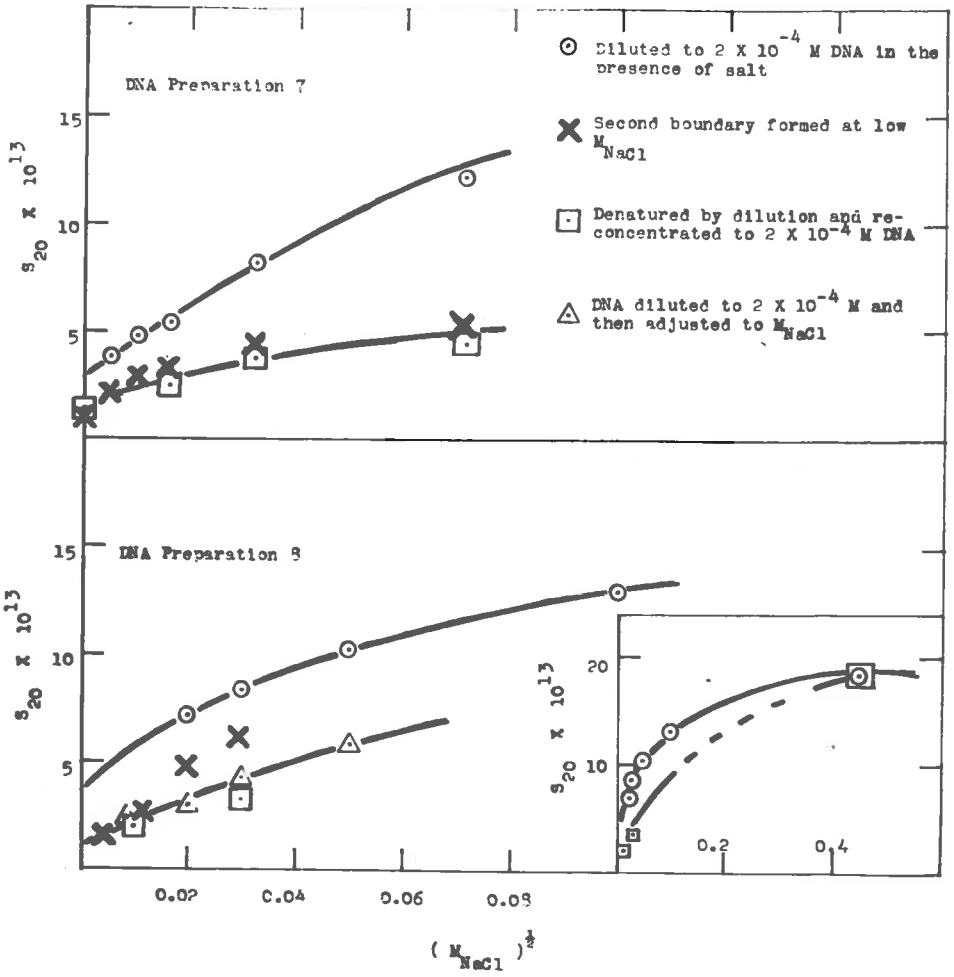

Fig. 3-VI. Sedimentation velocity of DNA at low ionic strengths. Measurements made at 2 X 10^{-4} M DNA.

DNA were resolved over a range of ionic strengths, may possibly be explained in terms of the primary salt effect. This effect decreases the sedimentation velocity of a charged macromolecule because of the potential gradient created by differences in sedimentation velocity between gegenions and polyion[8-11]. The existence of the primary salt effect has been demonstrated in DNA solutions of low ionic strength[12].

It can be seen from fig. 3-VI that the double boundaries do not become apparent until the sedimentation velocities have been greatly reduced. As this marked decrease in velocity is almost certainly due to the primary salt effect it appeared that the differences in velocity, between native and denatured DNA, which became noticeable in this region, also arose from this effect. It has already been concluded that the denatured nucleate ion sediments more slowly than native DNA. It would be expected if this difference arose through the primary salt effect, that denatured DNA would have the higher charge under the condition of very low ionic strength. It has already been shown (Chapter III) that denatured DNA has a much higher charge than the native material (measured at zero ionic strength). The results from Chapter III would indicate that a 2 to 2.5 fold increase in charge follows dilution below the critical concentration (from a charge fraction

of 0.4 to 0.8-1.0). If the primary salt effect was responsible for the observed differences in sedimentation velocity between native and denatured DNA, then a decrease of 2-2.5 fold in sedimentation velocity should be exhibited by the denatured species. Extrapolation of the native and denatured sedimentation velocity curves (fig. 3-VI) to zero ionic strength yields decreases of 1.8 and 3.0 fold for the two respective DNA preparations. This brief calculation ignores any dependence of charge on DNA concentration and relies on two rather inaccurate extrapolations. Although the differences in sedimentation are of the correct sign to be compatible with the influence of the primary salt effect on the two differently charged polyions, a further observation casts considerable doubt on the proposed explanation given above. If the primary salt effect was responsible for the differences in sedimentation velocity then it would be expected that this difference would be at a maximum at zero ionic strength. However, as can be seen from fig. 2-VI the resolution of the two species becomes less well defined below a certain range of ionic strength. This may not be at variance with the above explanation if the critical concentration is higher than 2×10^{-4} M DNA in aqueous solution. Although conductivity measurements indicate a critical concentration of $2\text{-}3 \times 10^{-4}$ M DNA the phenomenon actually occurs over a concentration zone (Chapter IV) and this appears to extend as high as

1×10^{-3} M DNA (Chapter V).

It is to be concluded that sedimentation of DNA under the influence of the primary salt effect, is a very sensitive means of detecting small amounts of denatured DNA in the presence of the native species. This could arise through any of three possibilities. (1) The Johnston-Ogston effect[7] which results in an enhancement of the slower moving species of a mixture[13]. (11) The fact that denatured DNA has a higher atomic extinction coefficient at 2590 Å, than the native material, and that this difference increases with decreasing ionic strength. (111) The equilibrium between native and denatured species which exists at the bulk concentration of these experiments (2×10^{-4} M DNA) will be disturbed during sedimentation due to the range of decreasing concentrations existing at all points in the native boundary[14]. This will certainly lead to the unexpectedly broad boundaries of native DNA. The operation of all or one of these three effects may also explain the absence of a discreet native boundary at zero ionic strength.

(3) Electron microscopy.

Visualisation of single DNA molecules using the electron microscope[15-17] has made possible a direct comparison between native DNA and the material denatured by dilution.

Electron micrographs of the native material

are shown in Plate 1-VI. From a study of many such fields it has become apparent that although the DNA concentration was low enough to allow individual separation of the molecules on drying, a longitudinal aggregating tendency caused many of the fibres to lie together (presumably originating from the drying process). This type of behaviour is shown in Plate 1-VI (fig. 4). In addition a small amount of material appears to have a fibre diameter significantly smaller than that shown by monomolecular strands. The average molecular diameter was estimated (from shadow length and shadow to height ratios) to be 15 ± 8 Å compared with the value of 20 Å reported by Hall[15].

Before comparing the native material with denatured DNA an interesting and perhaps important observation must be discussed. In agreement with Hall[15] it is found that a small amount of the native material terminates in flat patches, Plate 1-VI (figs. 1 and 3). It has been suggested[15] that this is associated with a small amount of denaturation. Further investigations concerned with the terminal patches will be given in Chapter VII.

Plate 2-VI shows the fields obtained for DNA denatured by dilution below the critical concentration. A spectacular change can be seen from the rod-like material to collapsed or melted out DNA. The size of these

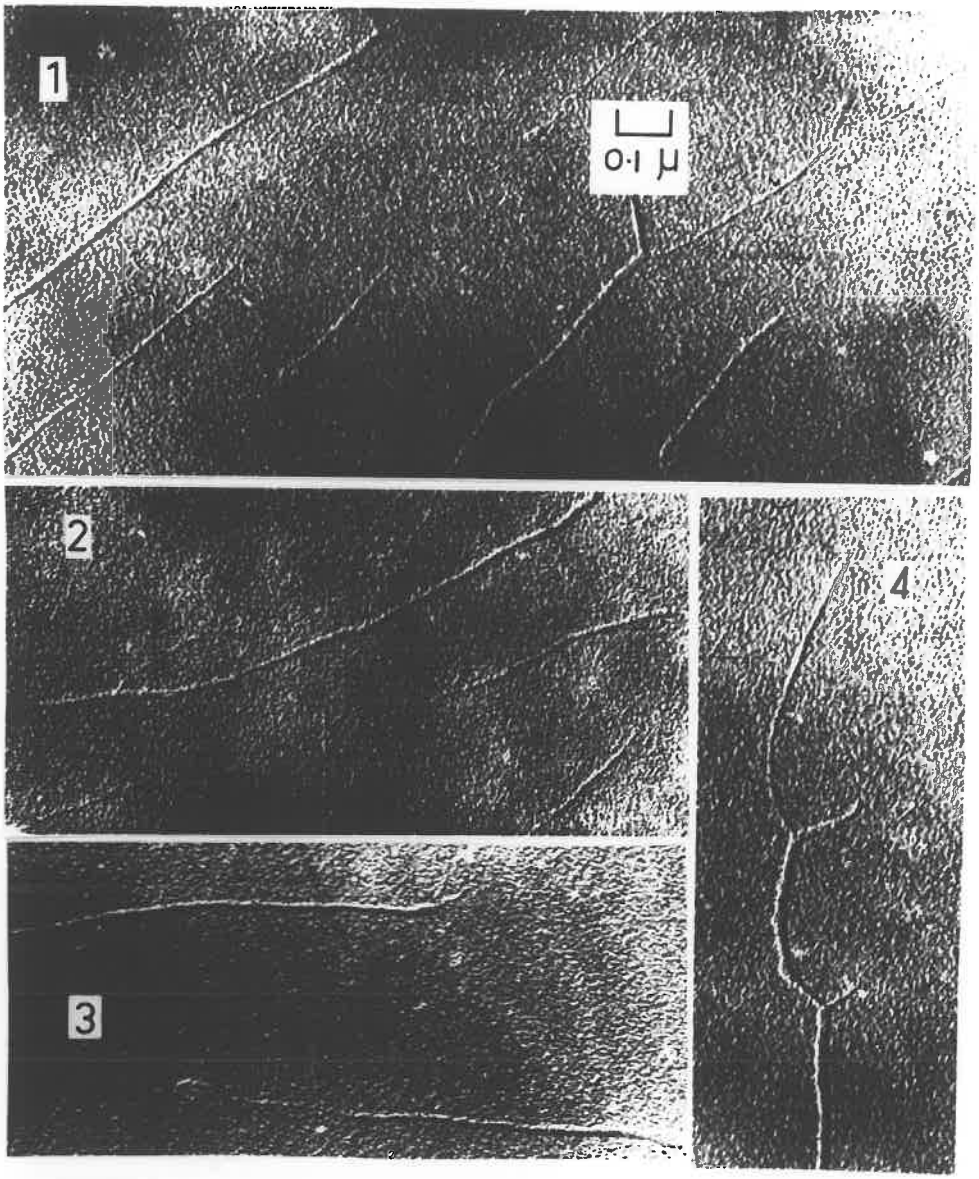

Plate 1-VI. Electron micrograph of native DNA.

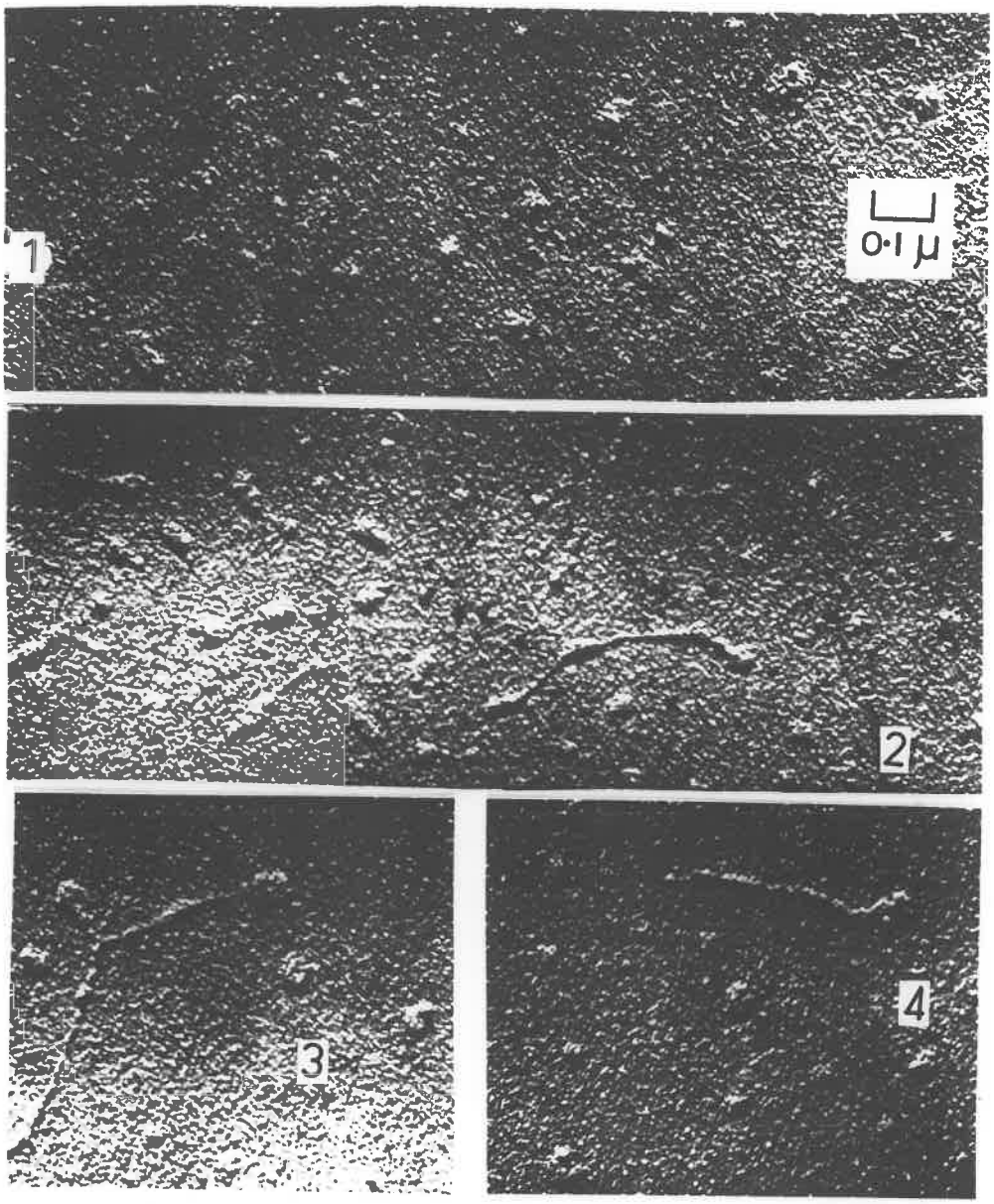

Plate 2-VI. Electron micrograph of DNA denatured by dilution.

collapsed units indicates that coiling of the strands has taken place. Two points of interest should be discussed. Firstly figs. 2,3 and 4 of Plate 2-VI, show remnants of rod-like material which have apparently resisted the denaturation process. It is to be noted that the fields shown in figs. 2-4 are not typical in this respect. A typical field would be such that about one fibre would be found in four fields. Secondly in fig. 1 of Plate 2-VI a completely denatured DNA field is shown where it will be noticed that the collapsed patches are too numerous to correspond to one molecule/patch. This can be seen by comparing the number of collapsed units with the number of unimolecular fibres shown in Plate 1-VI, (both plates obtained under conditions such that the two dimensional concentrations are similar). That these collapsed units can only represent a portion of the original molecules can be seen from figs. 2,3 and 4 of Plate 2-VI. Here presumably unimolecular fibres are melting out into more than one patch. This is at variance with the experimental observation that denaturation is accompanied by no significant molecular weight change. It would therefore appear that the apparent decrease in molecular weight as observed in the electron microscope is an artifact of the method used. Support for this claim has been found in some fields of denatured DNA, here the collapsed units lie in definite lines reminiscent of the original native fibre. The collapsed

units occur at intervals of 1000-2000 Å. These lines of collapsed material are somewhat similar to the patches starting to melt out of the remaining fibres shown in figs. 2 and 3 of Plate 2-VI, except that no interconnecting material remains. The fact that in some instances the denatured patches lie in definite lines although unconnected, indicates that the denatured DNA had a larger molecular weight when in solution than the resulting patches observed in the electron microscope. It is estimated that each patch corresponds to a molecular weight of $2\text{-}4 \times 10^5$ (assuming that a unimolecular fibre melts out into a denatured patch every 1000-2000 Å). As the denatured DNA solution dries on the mica slide each molecule would eventually be surrounded by a small amount of water, and it is quite possible that the observed decrease in molecular weight could occur if some parts of the molecule were held strongly to the mica surface during the final stages of drying and would not allow the necessary movement that should accompany the forces exerted by the receding water phase - fission of the molecule might then occur.

It has already been shown (Chapter V) that a concentrated solution of DNA denatured by dilution (dilution of a native stock solution to 5×10^{-5} M followed by freeze drying to 0.003 M) is further ir-

reversibly changed on precipitation with alcohol and that this process is also accompanied by decreased molecular weight. This is added evidence that denatured material is rather susceptible to degradation on rapid changes from the dissolved to the solid state. Freeze drying does not appear to cause degradation of denatured DNA although decreased viscosity results (Chapter V).

Because of these changes that appear to occur on drying, nothing definite can be concluded concerning the form of the denatured molecule in solution except that it is susceptible to the changes noted above.

(4) Infra-red spectroscopy.

The infra-red spectra of solid samples of native and denatured DNA were studied in an attempt to detect any differences in hydrogen bonding. The two spectra are shown in fig. 4-VI and although differences which could be associated with hydrogen bonding were obtained, the changes were not considered large enough to be of exact interpretation. Only large differences would be expected to yield useful information in view of the sensitivity of this measurement to the moisture content of solid DNA samples[18].

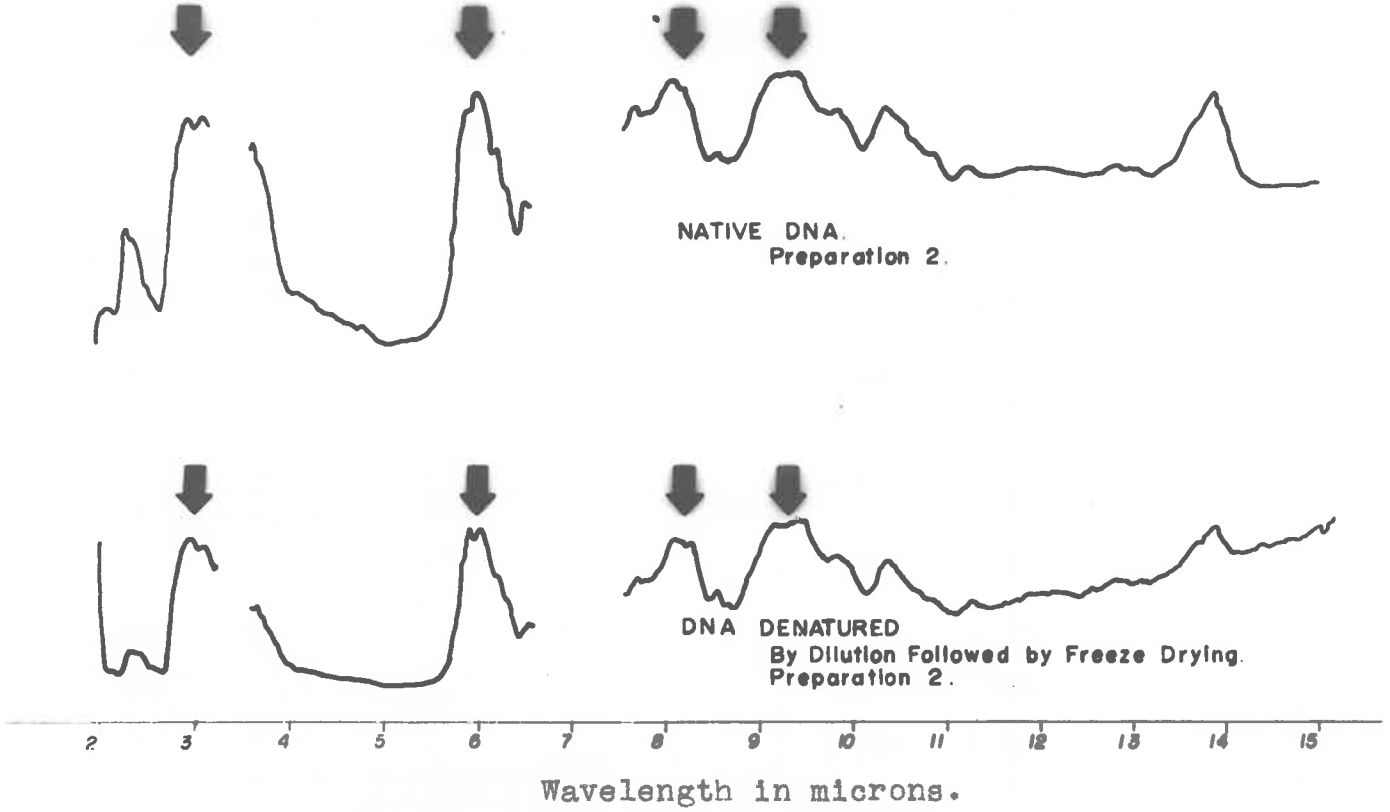

Fig. 4-VI. Infra-red spectra of native DNA and DNA denatured by dilution.

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CHAPTER VII

GENERAL CONCLUSIONS ARISING FROM DENATURATION STUDIES

(1) Evidence for heterogeneity in DNA from denaturation studies.

(2) The small irreversible changes that occur on dissolving DNA in water at high concentrations.

(3) The origin of denaturation by dilution.

(4) Flexibility of denatured DNA - Enzymic synthesis of DNA.

(1) Evidence for heterogeneity in DNA from denaturation studies.

It has been concluded in earlier chapters that the critical concentration phenomenon is a manifestation of a denaturation process which takes place below a certain critical concentration zone. The principal evidence which substantiates this conclusion came from the observed irreversibility of the phenomenon and the finding that thermally denatured DNA was similar to the material formed below the critical concentration zone.

The similarity of this process, which occurs on dilution in a salt free solution, with thermal denaturation of saline DNA solutions is of interest. Heating a saline DNA solution results in a rapid fall of viscosity at temperatures over 80°C[1]. An important paper by Rice and Doty[2] discusses the thermal denaturation process thoroughly. They conclude that denaturation occurs over a critical temperature range, the actual range depending on the initial state of secondary structure order. The more ordered material appears to resist thermal treatment until higher temperatures. In an example of this behaviour they find that denaturation occurs at 93°C and exists over a range of 16°C about this value (fig. 1-VII).

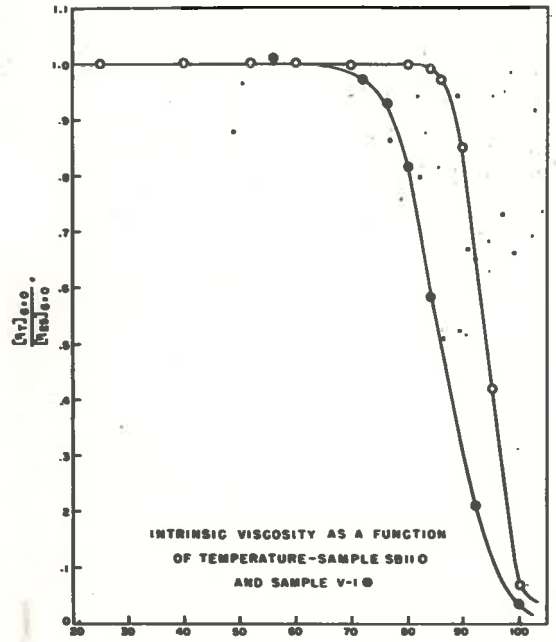

Fig. 1-VII. The rapid decrease in viscosity accompanying thermal denaturation. (Rice & Doty).

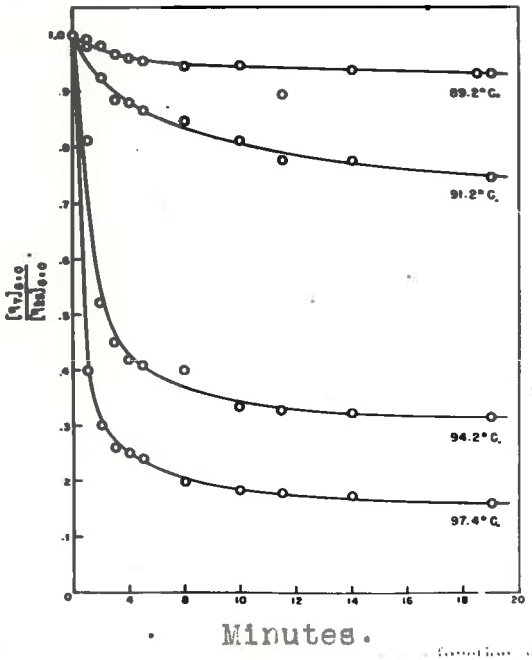

Fig. 2-VII. The fall in viscosity at constant temperature (within the critical temperature zone) as a function of time. (Rice & Doty).

Many of the similarities between the critical concentration and critical temperature phenomena, such as irreversibility, dependence on ionic strength and $\epsilon(P)$ changes, merely serve to show that denaturation is present in each case. However the range of concentrations and temperatures over which denaturation takes place deserves attention. If a saline solution of DNA is heated to 91°C (or at any temperature at which the downward trend in the viscosity is evident) and the amount of denaturation recorded as a function of time, it is found that the process levels off after a number of minutes at a value which depends on the heating temperature and which definitely does not correspond to the final denaturation obtained if the solution is heated to 100°C for a few minutes (fig. 2-VII). An exactly similar process can be deduced for the critical concentration phenomenon; the amount of denaturation depends on the concentration to which the DNA solution had been diluted (fig. 2-V). In both cases the observed irreversibility is dependent on the amount of constraint imposed. Similarly the denaturation that occurs on lowering the ionic strength at low constant DNA concentrations[3-5] occurs over a broad range of sodium chloride concentrations. It should be pointed out however, that the critical ionic strength and DNA concentration zones are directly

related. It has been shown that the critical concentration is dependent on ionic strength (fig. 2-IV). Because of this there will exist, at any constant ionic strength, a critical DNA concentration zone and similarly at any constant DNA concentration a critical ionic strength zone will occur over which denaturation takes place.

As denaturation in the case of DNA denotes the co-operative melting out of all the hydrogen bonds, we would expect the process to occur over a sharp temperature and concentration range, rather than over 16°C and 4-50 X 10^{-5} M for the critical temperature and critical concentration zones respectively. It appears that heterogeneity of hydrogen bond strength can explain this anomaly[2]. If a distribution of base-pair hydrogen bonds existed which varied in bond strength, a series of denaturation temperatures and dilutions would exist, each set of bonds with equal strength melting out co-operatively, but taken as a whole yielding a broad range of critical temperatures and concentrations. The recent studies of Bendich et al.[6] have shown that DNA can be fractionated into samples having small differences in base composition. It therefore appears that heterogeneity of base-pair hydrogen bond strength is a valid explanation of the broad ranges observed in the critical temperature and concen-

tration phenomena. The presence of a similar type of heterogeneity has also been deduced from acid titration studies[7].

Assuming that heterogeneity of hydrogen bond strength does exist, it is pertinent to determine whether this arises from differences between molecules, within any one molecule, or within and between molecules. On the basis of the double boundaries observed at low ionic strengths (Chapter VI) it would appear that two different molecules exist at any one concentration (within the critical concentration region). The simplest conclusion for this observation is as follows. At any one concentration, within the critical region, there exists a mixture of native DNA (having the hydrogen bonds of higher strength) and denatured DNA (which previously contained hydrogen bonds of lower strength). The relative proportions of the mixture would be determined by the magnitude of the hydrogen bond breaking process, which in this case occurs through dilution (or by lowering the ionic strength at constant low DNA concentration). It would thus appear that the heterogeneity arises from differences between molecules, although other possibilities are not excluded.

(2) The small irreversible changes that occur on dissolving DNA in water at high concentrations.

According to the conclusions reached in earlier chapters the process of denaturation by dilution occurs only over a critical concentration zone. The changes associated with the critical concentration phenomenon were not apparent at high concentrations. However certain findings indicate that this is not qualitatively correct.

In Chapter II it was found that the extinction coefficient of DNA in water at an initial concentration of 3×10^{-3} M was 1.4% higher than that determined for samples never dissolved in water of low ionic strength. Cavalieri et al.[4] have likewise found that the viscosity (measured in 0.2 M NaCl) of DNA dissolved in water at 2.4×10^{-3} M DNA is slightly less than similar samples dissolved directly in saline solution.

In a study of any possible relationship between this small irreversible change in structure (resulting from dissolution at high DNA concentrations) and the larger transition that follows dilution through the critical concentration zone, it would be necessary to have on the one hand, DNA which had never been dissolved at low ionic strength and on the other a sample

which was salt free. Clearly this involves experimental difficulties. The preparation of DNA sample 8 (Table 1-II) was designed to approach the above requirements. This sample was never dissolved at low ionic strength and yet would not be expected to contain the usual salt impurity that results from precipitation of DNA from 1 M NaCl solution[8]. As preparation 8 yielded an $\epsilon(P)$ of 6570 it appears that this sample had not suffered the small irreversible increase and yet on dilution below the critical concentration zone denaturation by dilution still took place. In this sample therefore, a comparison can be made between the irreversible changes that accompany solution of DNA at high and low concentrations respectively.

The observation that the sedimentation velocity and sedimentation velocity concentration dependence (measured in 0.2 M NaCl) of essentially native DNA (DNA dissolved in water at high concentration) was similar to DNA denatured by dilution below the critical concentration has already been given (Chapter VI). In view of the small irreversible increase which has already taken place in the native samples used in these experiments, it is of interest to determine whether this small amount of denaturation is responsible

for the apparent similarity in sedimentation properties mentioned above. In fig. 3-VII is shown a comparison of similar sedimentation studies made on preparation 8. The sedimentation velocity and sedimentation velocity concentration dependence are again similar for DNA dissolved in water at high concentration and DNA denatured by dilution. It can also be seen that no difference exists between these two results and those obtained for solutions of preparation 8 which had never been dissolved at low ionic strength. It therefore appears that the original conclusion is valid and that native and denatured DNA sediment at the same rate in 0.2 M NaCl - no explanation for this anomalous behaviour can at present be advanced.

A comparison of the concentration dependence of the samples obtained from preparation 8 (fig. 3-VII) with that observed for other preparations (fig. 1-VI) shows an apparent difference - a definite upward curvature of $s_{20,w}$ with decreasing concentration contrasted with an essentially linear relationship. Although the difference cannot be related to the above problem (two of the samples in fig. 3-VII, having already been dissolved in water) it is of interest to determine why this should be so. It was noticed that preparation 7 also showed a similar but increased curvature of $s_{20,w}$ with concentration (fig. 4-VII)

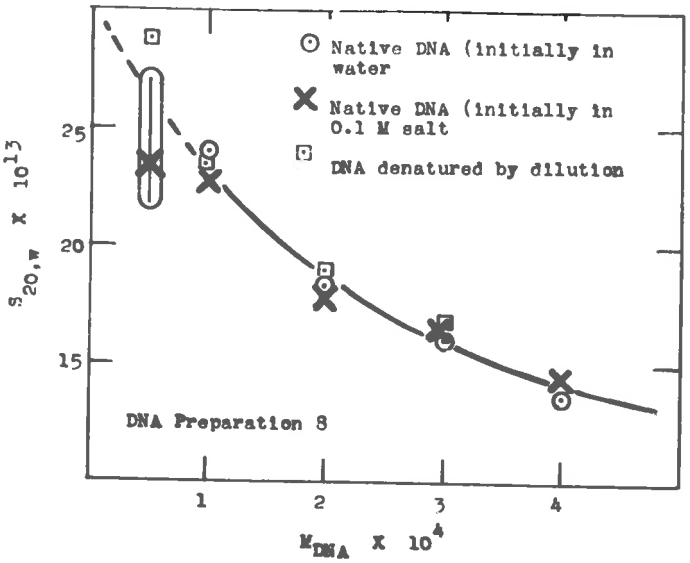

Fig. 3-VII. Sedimentation velocity of native and denatured DNA.
All measurements made in 0.2 M NaCl.

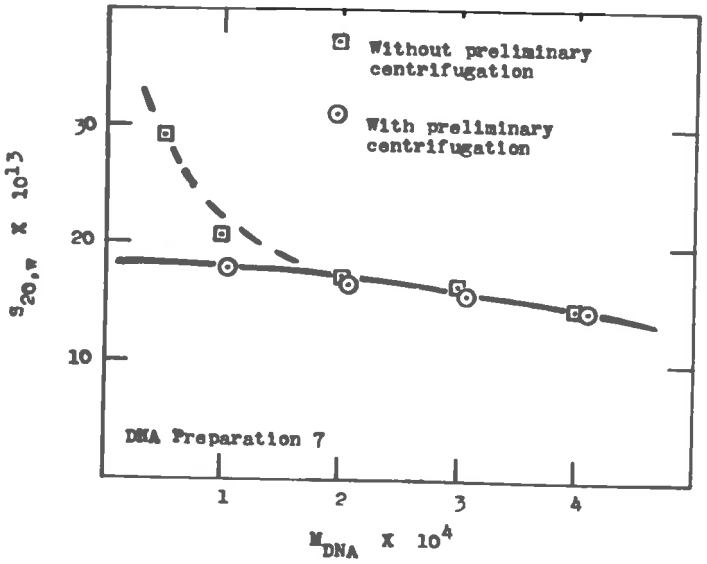

Fig. 4-VII. Sedimentation velocity of native DNA. All measurements made in 0.2 M NaCl.

which could be eliminated by preliminary centrifugation of the stock solution (0.003 M DNA in water). As preliminary centrifugation yielded a gel-like sediment, this would appear to explain the curvature in the $S_{20,w}$ versus concentration results. This was confirmed by visual examination of the sediment obtained in a preliminary centrifugation of a solution of preparation 8 - again a gel-like sediment was observed but in this case in very small amounts. No gel-like material was ever observed on preliminary centrifugation of any of the preparations yielding linear plots of $S_{20,w}$ against DNA concentration.

Heat denatured DNA has been found to give a precipitate with lead ions whereas native DNA remains in solution[9]. In the hope that this test held special virtues with regard to sensitivity in the detection of denatured DNA, an investigation of the interaction of lead ions with DNA was initiated. Although DNA which had been diluted below the critical concentration zone followed by reconcentration did yield a precipitate with lead ions (and therefore confirming the conclusion that the critical concentration was a manifestation of denaturation), no precipitate could be detected on the addition of lead ions to a salt free DNA solution at high DNA concentration.

In the electron microscope studies (Chapter VI)

it was pointed out that native DNA (dissolved in water at high concentrations) had small sections which appeared to result from denaturation and which almost always occurred as a terminal patch (Plate 1-VI). Further investigations have shown that the flat patches are not related to the small irreversible changes that occur when DNA is dissolved in water at high concentration. Plate 1-VII shows electron micrographs of preparation 8, these micrographs were obtained from samples never dissolved in water of low ionic strength - no significant decrease in the numbers or sizes of the terminal patches were observed. Plate 1-VII was constructed mainly to show that the flat patches still exist and bears no resemblance to the relative rarity and average small size of these patches. Some recent electron micrographs of deoxyribonucleoprotein (DNP)[10] show a circular ring, superimposed upon the cylindrical DNP molecular strand. These two observations on DNA and DNP may eventually prove to be of importance in the molecular architecture of the chromosome.

It must be admitted that no definite statement can be made at present on the relationship that possibly exists between the small amount of initial denaturation that occurs on dissolving DNA at high concentrations and that observed on diluting DNA below the critical concentration zone. However on dissolution

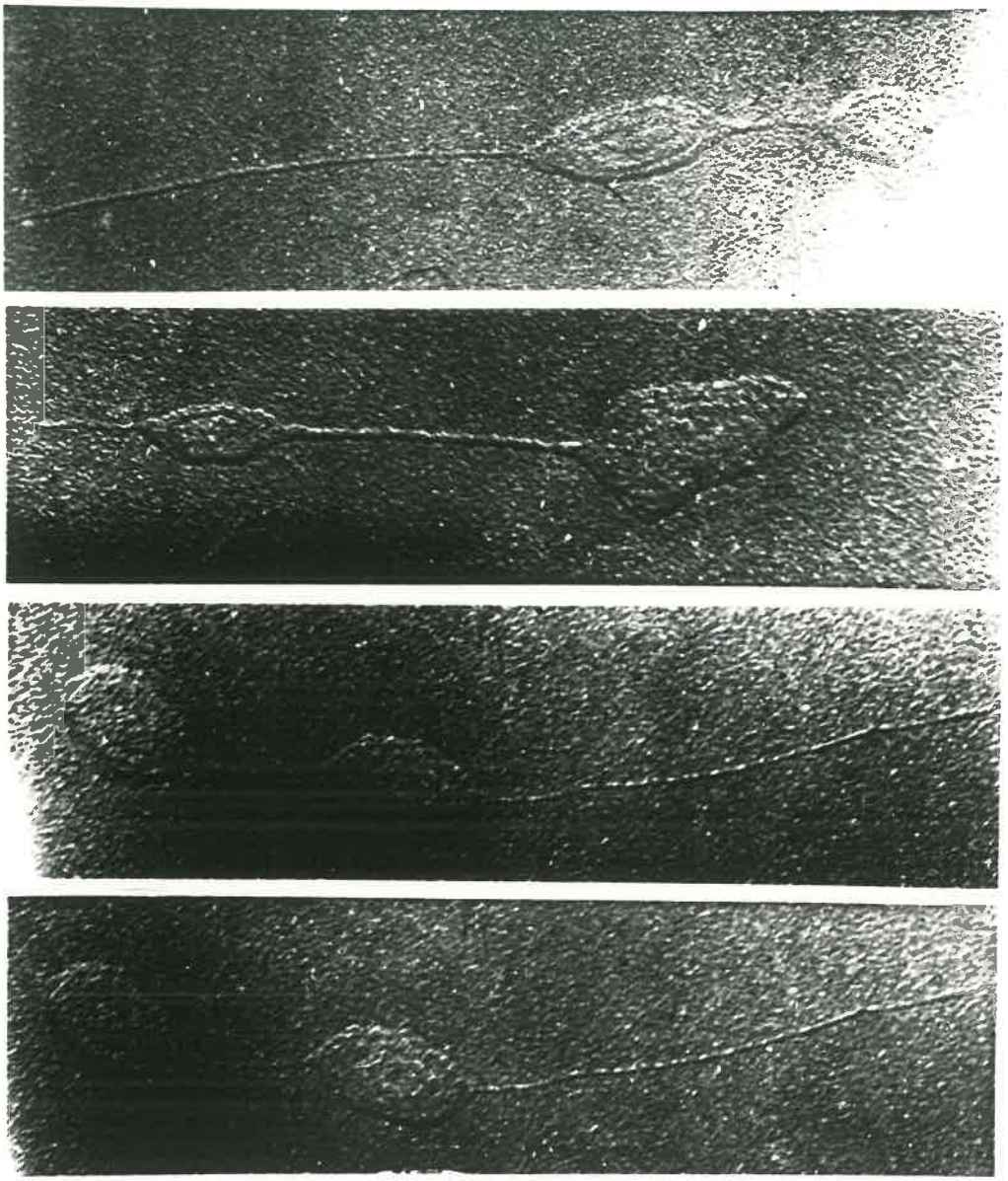

Plate 1-VII. Electron micrograph of native DNA, preparation 8.

of DNA to eventually produce a high DNA concentration, there will necessarily be a stage when a small amount of DNA is in solution at concentrations below the critical zone - these molecules, according to the critical concentration phenomenon, will certainly be denatured.

(3) The origin of denaturation by dilution.

The investigations presented in these chapters have been designed solely to show that the critical concentration phenomenon was one of denaturation. The energetic reason for this process has not as yet been studied, therefore any discussion as to the origin of this phenomenon must of necessity be speculative.

The origin of thermal denaturation can be understood in terms of the increased vibrational and rotational diffusive forces imposed on the base-pair hydrogen bonds by heat. It has been reported[11] that decreased ionic strength would eventually lead to a critical denaturation temperature in the vicinity of room temperature. The origin of denaturation may therefore be similar in the two cases however, the DNA concentration for which the above prediction of denaturation at room temperature was made, is all important in the present instance. Unfortunately Doty et al. make no reference to this concentration, but judging from previous work published by these investigators the

DNA concentration would be very close to the critical concentration zone. If this is so, then their conclusions are compatible with those presented in these chapters. Preliminary investigations have shown that the heating of a salt free DNA solution at high DNA concentration does not result in a drastic decrease in viscosity until quite high temperatures are reached.

In the author's opinion, denaturation by dilution has its origin from the competitive effects of the hydrogen bonding sites between the complimentary bases and between the bases and water. If the tendency for base-base hydrogen bond formation is greater than that for base-water hydrogen bonds, then the native structure will be retained however, if base-water hydrogen bonds become more favourable, denaturation will occur. If such a view is correct then there must be some further process operating which increases the tendency for base-water hydrogen bonding on dilution to the critical concentration zone.

A suggestion has been put forward that denaturation by acid and low ionic strengths originates from a common mechanism involving changes in base-base hydrogen bond strength through pK_a' shifts[4,7]. The proposal that the pK_a' shifts arise from a field effect requires comment. It has been suggested that an ag-

gregation phenomenon[12,13] can give rise to phosphate groups on some molecules which can be considered to reside within a DNA aggregate, because of differences in charge of such internal phosphate groups, as compared with the conventional external phosphate groups, field effects are considered to arise and result in pK_a' shifts. Further confirmation of any possible aggregation process is required before this explanation can be applied to denaturation by dilution.

Cavalieri et al.[4] have made the interesting observation that DNA dissolved in water of pH 7.5 - 8 shows no denaturation at low concentrations (in the absence of salt). This would mean in terms of the critical concentration, that the critical zone had been eliminated or depressed to very low concentrations. If it is assumed that these investigators produced "water" at pH 8 by the addition of NaOH (the pH of a DNA solution is usually 6.5) then it is important to differentiate between protection from denaturation by the pH change and by the effect of the added ions needed to produce the pH increase. Although a pH change from 6.5 to 8 will require very little NaOH, it is also known that the dependence of critical concentration on ionic strength is very great (fig. 2-IV).

Some experiments which are pertinent to this question will now be discussed. Fig. 5-VII shows the

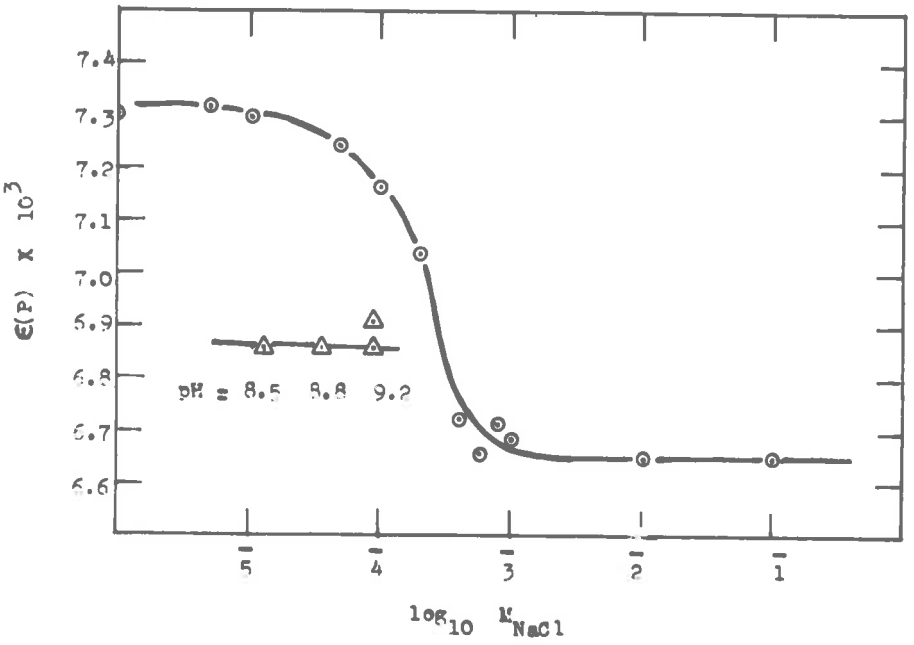

Fig. 5-VII. Irreversible increase in ε(P) associated with low ionic strength and pH. All measurements made at 4 X 10^{-5} M DNA and finally adjusted to 0.1 M NaCl.

ϵ(P) values of various solutions measured at room temperature and in 0.1 M NaCl, which were subjected to the two variables of present interest, pH and ionic strength. It is seen that dilution to 4 X 10^{-5} M DNA in the presence of decreasing amounts of added salt leads to an increase in ϵ(P) from 6650 (at ionic strengths above 1 X 10^{-3} M NaCl) to 7300 (at ionic strengths less than 5 X 10^{-5} M NaCl). If the DNA solution is diluted to 4 X 10^{-5} M DNA at pH 8 the full increase in ϵ(P) to 7300 is not observed, which is in agreement with Cavalieri *et al.*[4] Contrary to these investigators' findings however, an irreversible increase to 6850 is observed. If the ϵ(P) values obtained at pH 8.0 - 9.2 are represented at their approximate ionic strengths (assumed to correspond to M NaCl in fig. 5-VII) then it is seen that the smaller irreversible increase obtained at pH 8.0 - 9.2 cannot be explained solely in terms of ionic strength protection. Although there is a small effect arising from pH this does not appear to be as large as reported by Cavalieri *et al.* The experimental technique used in these investigations concerned with water at pH 8 are of prime importance. In the present investigation all carbon dioxide was rigorously excluded, in addition, pH measurements were never made on the solutions used to measure U.V. ab-

sorption as diffusion of salt from the salt bridge would certainly invalidate any experiments of the above nature.

A mechanism which could possibly explain the competition between base-base and base-water hydrogen bonds is concerned with the "ice-like" structure of water[14-16]. It has been suggested that pure water can be considered as a four co-ordinated lattice with Frenkel defects and lone interstitial water molecules. The presence of a solute, such as DNA, is thought to substantially increase the ordered water[17] component of the liquid surrounding the solute. The water around the DNA molecules would have greatly reduced thermal vibrations and fewer Frenkel defects. It has also been suggested[18] that such an arrangement would result in a water structure (around the DNA molecules) with favourable hydrogen bonding sites. It appears that these proposals can be applied to the mechanism involved in denaturation by dilution. If the lattice ordered water structure increased with dilution of DNA, then eventually the ordered structure would be perfected to such an extent that the strengths of the base-base hydrogen bonds could not compete with the potential base-water sites. The fate of the native DNA configuration, once the base-base bonds were replaced by base-water hydrogen bonds, would depend on

the microscopic picture of the lattice ordered water. According to Pople[19], ordered water is to be considered an average structure and not to be associated with any particular volume of liquid. If this is true then the native configuration would be destroyed by diffusive motion of the polynucleotide strands. If the lattice ordered water is to be considered as a permanent shell, then the native configuration could possibly be preserved (although no base-base hydrogen bonds exist). The latter view would indicate that replacement of the base-water bonds by base-base bonds would lead to retention of the native structure. Although this result does not agree with the irreversibility that has been shown to occur on reconcentrating a solution denatured by dilution, it has been reported[20] that at temperatures approaching $0^{o}C$ the usual acid treatment leading to denaturation, is quite reversible.

Evidence which supports the premise that certain types of macromolecular structures can increase the lattice ordered component of water, comes from proton magnetic resonance (P.M.R.) studies on TMV[21]. In this study it was shown that the line width for water in a TMV solution was broader than that for pure water. The difference in area between the two signals

was interpreted as an increased lattice-order in the water containing the TMV. A P.M.R. investigation of DNA[18] has yielded similar results.

(4) Flexibility of denatured DNA - Enzymic synthesis of DNA.

Denatured DNA in contrast to the native structure, appears to have a high degree of flexibility. At zero ionic strength denatured DNA has a high charge and low mobility (Chapter III), indicating a highly extended molecule. On the other hand at high ionic strengths denatured DNA has greatly reduced dimensions compared with the native structure[4,2,22]. The results of investigations at intermediate ionic strengths are quite compatible with these conclusions. It has been found for instance that alkali denatured DNA exhibits a viscosity increase of about 8 fold on decreasing the ionic strength from 0.165 to 0.0055, and that this is followed by an increase of 2.3 fold in radius of gyration. Ehrlich et al.[22] conclude that at 0.0055 ionic strength the space filling property of denatured DNA is about equal that of the native structure. Viscosity studies of thermally denatured DNA[2] also show the great flexibility of the denatured material.

The recent discovery by Kornberg and co-workers[23-27] of a DNA synthesising enzyme has led to some interesting observations relating to the flexibility

and residual hydrogen bonding in denatured DNA. It has been found that significant synthesis of DNA requires the presence of DNA primer[24]. Further evidence indicates that the synthesised DNA has a base sequence similar to that present in the primer material[28,29]. Recent studies[30] also show that greater synthesis results if denatured rather than native primer is used, moreover phage ØX174 DNA, which has been found to be a single stranded DNA[31], is a better primer than denatured calf thymus DNA[30]. The difference in primer efficiency is evidently due to the energies involved in breaking hydrogen bonds and disentangling the paranemically wound polynucleotide chains.

Apart from the great biochemical importance of the DNA synthesising enzyme, it can be seen that this discovery offers an unprecedented opportunity for the biophysical study of heterogeneity, denaturation and dissociation using various types of synthetic DNA. Use has already been made of a synthetic adenine-cytosine DNA[32] to confirm that heterogeneity of the base-pairs guanine-cytosine and adenine-thymine are responsible for differences in denaturation temperature between DNA samples from various species. A similar investigation has also been reported[33] using the

recently developed technique of density gradient sedimentation[34]. It has been shown that the buoyant density of a DNA solution is determined by the relative proportions of the two base pairs present in the DNA sample.

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CHAPTER VIII

EXPERIMENTAL METHODS

(1) Sedimentation velocity.

(2) Electron microscopy.

- (a) Instrument.
- (b) General considerations.
- (c) Preparation of samples.
- (d) Preparation of solutions.

(3) Electrical conductivity.

- (a) Instrument.
- (b) Conductivity measurements.
- (c) Conductivity water.

(4) Electrical Transport.

(5) Determination of DNA concentration.

(6) U.V. and I.R. absorption.

(1) Sedimentation velocity.

Sedimentation analysis was carried out in a Spinco model E, equipped with an U.V. absorption optical system. All experiments were made at 59,780 r.p.m. The experimental record was converted into plots of photographic blackening versus distance from a reference point using a commercially made automatically scanning photodensitometer (Analytrol Model RB, with a micro-analyzer attachment). The experimental record was made on either Kodak transparency or commercial orthochromatic sheet film. The rotor temperature was recorded with each exposure and all experiments were made at temperatures between 24.6 and 25.2°C. Sedimentation velocity coefficients were computed from the quantity $d(\log x)/dt$, x being the distance of the boundary from the centre of the rotor and dt the time interval between photographs. These results were then corrected to water as solvent at 20°C using the expression $S_{20,w} = \frac{S_t \eta_t}{\eta_{20,w}}$

where S_{20} is the sedimentation coefficient at 20°C

S_t is the sedimentation coefficient at t°C

η_t is the viscosity of the solvent at t°C

$\eta_{20,w}$ is the viscosity of the water at 20°C.

(2) Electron Microscopy.

(a) Instrument.

The electron microscope used in these investigations was the Metalix 1190, fitted with the improved objective lens supplied by the manufacturer. Some of the work was carried out after a modified electron gun had been installed. The converted microscope was equivalent to the Philips EM/100 instrument. A resolution of 20-25 Å could be demonstrated for the samples used in this investigation. Instrument magnification was obtained by calibration with the well known and tested Dowex spheres of 2570 Å diameter.

(b) General considerations.

The techniques recently described by Hall[1,2,3] have been used throughout this investigation. As the method has not been fully described by the above investigator, a detailed account of the method used in this instance will be outlined below.

For ideal microscopy near the resolution limit of the electron microscope, certain criteria must be fulfilled. Firstly there must be sufficient electron absorbing contrast between the macromolecule and the support. Secondly, changes in contrast in the support with distance must be of a much smaller magnitude than those arising from the macromolecule. Finally for interpretation of the resulting micrographs, the macro-

molecules must be well separated from one another on the support. Sufficient contrast is obtained by shadowing the sample with platinum, however the supporting film is not enhanced in contrast because it is not present during this operation. Separation of the molecules has previously been achieved using very dilute solutions, this however leads to a troublesome impurity/sample ratio. In the present method a relatively concentrated DNA solution is sprayed onto the hydrophilic surface of freshly cleaved mica. The hydrophilic surface causes spreading of the droplet before evaporation can take place and a low two dimensional concentration results.

(c) Preparation of samples.

Reference spheres[4] are sprayed onto a selected area of a freshly cleaved mica slide[5], using a commercial throat nebulizer backed with a low pressure air pump. The DNA solution is then sprayed onto the same area in such an amount that approximately 50% of the surface is covered with evaporated droplets. The mica slide is placed at such a distance from the sprayer that a steady state is set up between droplets hitting and droplets drying on the slide. If the population of undried droplets becomes too large the droplets will merge.

The slides are then shadowed with platinum (4 cms. of 0.1 mm. wire at a distance of 7-10 cms. and at a shadow to height ratio of 5:1 to 10:1). The shadowing apparatus should be pumped down for at least three hours for best results. The thickness of the platinum film was found to be most critical, a film which was just visible to the naked eye is of optimum thickness. Thicker films do not allow sufficient resolution of the macromolecular shape while thinner films do not offer sufficient electron contrast to enable accurate focusing of the image to be made. A collodion film is spread over the shadowed area (3-5 drops of 1% collodion in amyl acetate). The dried film plus shadowing is then floated off the mica at an air/water interface. The DNA itself is lost from the specimen at this stage as it dissolves in the water. The film (containing a replica of the DNA and the shadowing) is lowered onto a set of electron microscope grids by lowering the air/water interface.

(d) Preparation of solutions.

It is essential in high resolution electron microscopy to eliminate all sources of impurity. Because the examination of DNA in the native state necessitates an environment of high ionic strength, a volatile salt must be used. A solution of A.R. 0.1 M ammonium acetate/0.05 M ammonium carbonate has been

suggested[1], and found in this investigation, to be free from non-volatile impurities.

(3) Electrical conductivity.

(a) Instrument.

The bridge[6] was a simple Jones type instrument without the Wagner earthing circuit. The oscillator which supplied set voltages from 0.1-10 volts at 1.0-20 K.c.p.s., was isolated from the bridge by an astatically wound, shielded transformer and since the supply was not resistively connected to ground, one side of both the cell and the decades could be earthed. Amplification of the signal was effected by a linear-logarithmic amplifier which became linear near the null point. The signal was then fed to a C.R.O. for visual determination of the null point.

(b) Conductivity measurements.

All conductivity measurements were made, unless otherwise stated, at 1.0 volt and 1.0 Kc.p.s. The conductivity measurements described in the earlier chapters were made primarily to investigate a discontinuity in the dependence of the specific conductivity on the DNA concentration. As this discontinuity occurs at quite low concentrations it is necessary to measure very small conductivities. The lowest possible concentration that can be studied is dependent on how accurately the water correction is known. Accordingly the following

method was adopted for the measurement of specific conductivities at low DNA concentrations.

A conductivity cell was constructed (cell constant 0.02) with a volume of 10-12 mls. The cell was provided with an inlet tube situated at the bottom through which carbon dioxide free nitrogen could be passed. A known volume of conductivity water was weighed into the cell and the conductivity measured (always less than 1 X 10^{-6} ohm^{-1} $cm.^{-1}$). Nitrogen which had been saturated with water at 25°C was bubbled through the solution to displace the carbon dioxide dissolved in the water. This resulted in a conductivity of 0.4-0.2 X 10^{-6} $ohms^{-1}$ $cm.^{-1}$. The conductivity of the water was then determined precisely. A known volume of water was withdrawn followed by the addition of an equal volume of concentrated DNA solution, (0.003 M). This was mixed with the water in the cell with a stream of nitrogen bubbles. The conductivity was then determined. Further increases in DNA concentration were made by successive withdrawal and addition of DNA solution and concentrated stock solution respectively.

As the measurements at low DNA concentrations were made under conditions such that most of the water was that initially present in the cell, the large water corrections necessary in this work, were known more precisely.

The calculation of DNA concentration using the above method, assumed that no volume change occurred on mixing a concentrated DNA solution with a more dilute solution or with water and further, any volumetric errors will be additive. To show that large errors did not arise in this method the true concentration was determined after twenty successive increases in concentration. It was found that calculated concentration was in the two cases tested, 2.5% and 1.0% higher than the true concentration as found by phosphorus analysis. Such an error would not alter any of the conclusions reached in earlier chapters. Considerable errors arose however from a tendency in the more concentrated solutions, to hold small bubbles of nitrogen. This became noticeable at concentrations above 0.001 M. Accordingly measurements above this concentration were made on separate degassed solutions.

In all the measurements on DNA solutions there were small increases in conductivity with time. The increases were similar to that observed for conductivity water itself and was presumably due to ions being leached from the glass vessel. Tests showed that the critical concentration was quite independent of any time effect.

(c) Conductivity water.

The water was first demineralised by the exchange process followed by distillation in a large pyrex still. The water at this stage had a conductivity

of 1 X 10^{-6} ohm^{-1} $cm.^{-1}$. Finally the water was distilled in a small pyrex vessel at a slow rate and collected in a separating funnel. The water was taken from this reservoir when needed through an ungreased glass tap. The final conductivity was only slightly less than that observed after the first distillation, however, when freed from carbon dioxide was always between 0.2 and 0.4 $ohms^{-1}$ $cm.^{-1}$. This low conductivity could only be obtained if rain water was initially used. Tap water was found to be unsuitable unless an initial distillation from alkaline permanganate was carried out. It is thought that the source of the trouble with tap water was due to River Murray water which makes up, in part, the tap water of Adelaide.

(4) Electrical Transport.

The transference cell was of the two compartment type[7], the compartments being separated by a coarse glass frit. The current passed during transport was measured as the potential drop across a known resistance. The quantity of electricity used in the transport experiments never exceeded that necessary for the transport of 1/10 of the polyion. Unlike the transport experiments described for polyacrylic acid[7] the passage of DNA through the glass frit was accompanied by electro-osmosis, which was opposed by an equal and

opposite gas pressure. The level of the liquid in both compartments was kept constant throughout the experiment by reference to a graduation mark.

At the conclusion of an experiment the DNA solution in each compartment was washed out with 1/10 N NaOH followed by water then 1/10 N HCl and finally more water. The concentrations of the initial solution and the final solutions were determined by phosphorus analysis. The treatment of the results followed that suggested by Huizenga *et al.*[7] except that the change in polyion concentration was known directly by analysis rather than from a consideration of differences in total weight and changes in gegenion concentration.

(5) Determination of DNA concentration.

All DNA concentrations were determined by phosphorus analysis using a modification of the methods of Jones *et al.*[8] and Griswold *et al.*[9]. The concentration of DNA may also be measured from the more easily determined extinction coefficient at 2590 Å, however, such a proceedure involves assumptions concerning the extent of denaturation. It was found that accurate U.V. measurements necessitated the averaging of many readings on a single stock solution.

Although accurate determinations could be made by phosphorus analysis (0.5%), certain disadvantages of this method should be pointed out. It was found that

the extinction coefficient of the coloured complex was variable from day to day. Because of this, at least three standards had to be included in each determination. The accuracy of the results was also very sensitive to the volumes of added reagent. It is of interest to note that the standard flasks used in the above method suffered very little change in volume after having been boiled some 80 times. The change in volume before and after three years of use was 0.04 mls. in 24.97 mls. (average of six flasks).

(6) U.V. and I.R. absorption.

The U.V. measurements were made with the "Unicam SP500". This instrument was tested with respect to wavelength calibration and photo-electric cell response using a solution of potassium chromate[10]. At a wavelength of 2750 Å the absorbance was found to be 0.776 as compared with 0.780 and 0.793 found for "Carey"[11] and "Beckman"[10] instruments respectively.

The I.R. absorption spectra were obtained on mulled samples of DNA in Nujol. The instrument used was the "Grubb Parsons S-4", fitted with a sodium chloride prism.

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Summary of a Thesis entitled "Denaturation of Deoxyribonucleic Acid" presented by Ross B. Inman, for the Degree of Ph.D. in the University of Adelaide. October, 1959.

Rapid changes of specific conductivity, electrical transport and atomic extinction coefficient have been found on dilution of aqueous salt free DNA solutions through a critical concentration zone. This phenomenon was essentially irreversible and was explained in terms of a structural transition from native DNA at high concentrations to denatured material below the critical zone. Within the critical concentration zone both species appear to coexist and were resolvable by ultracentrifugation at low ionic strengths. Coexistence of both species was taken to mean that hydrogen bond strengths were heterogeneous.

Further evidence that the critical concentration phenomenon originated from a denaturation process came from electron microscopy studies, where it was shown that a structural transition did in fact accompany dilution below the critical concentration. A comparison between thermally denatured material and DNA diluted below the critical zone using electrical conductivity and absorptivity measurements further confirmed the proposed explanation. Both the charge and mobility (derived from conductivity and electrical

transport) were determined for native and denatured DNA. Denatured DNA in the absence of salt, appeared to be highly charged and extended.

The proposed explanation for the critical concentration phenomenon involved the irreversible rupture of specific hydrogen bonds existing in the native structure. However, on reconcentrating such a solution (or by the addition of salt) a further structural transition was observed, which was reversible, and was thought to be brought about by the formation of non-specific hydrogen bonds. Native hydrogen bond rupture possibly occurs through the competitive effect of water-base hydrogen bonding which may arise because of an increase in the semi-ordered water structure around the polyions.

Although essentially native DNA existed above the critical concentration zone, it was evident that a small amount of denaturation had taken place on dissolution of DNA in water at concentrations as high as 0.003 M.

To the author's knowledge this thesis does not contain material previously submitted for a degree in any University excepting however, when due reference is made in the text.

Ross B. Inman.