DEOXYPENTOSE NUCLEIC ACIDS

XIII. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: A TEST FOR THE IRREVERSIBILITY OF THE CRITICAL CONCENTRATION PHENOMENON

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SUMMARY

Evidence has been reported which indicates that dilution of a salt free DNA solution below a critical concentration zone ($4\text{-}50 \cdot 10^{-5}$ *M*) results in denaturation. This process is irreversible with respect to the specific hydrogen bonds present in the native structure, but is thought to be partially reversible with respect to non-specific hydrogen bonds. The non-specific hydrogen bonds would form above the critical concentration zone between polynucleotide strands at points of close approach. This latter state can be reached by reconcentration to above the critical concentration zone and also by addition of salt to solutions below the critical zone.

The structural change resulting from dilution below the critical concentration zone appears similar to that observed for saline DNA solutions which undergo denaturation when heated through a critical temperature range. In both instances the broad ranges of denaturation constraint can be interpreted in terms of hydrogen bond strength heterogeneity.

INTRODUCTION

Previous measurements of the electrical transport number and conductivity of aqueous solutions of DNA[1,2] have been interpreted in terms of a denaturation process. The critical concentration zone that is observed in the variation of the specific conductivity with DNA concentration is now considered to arise from denaturation by dilution. Above this zone (greater than $50 \cdot 10^{-5}$ *M* DNA) essentially native DNA exists, while at concentrations lower than $4 \cdot 10^{-5}$ *M* the denatured structure predominates. An alternative, less likely, explanation of the critical concentration phenomenon involves aggregation of DNA molecules above the critical zone[1]. It is improbable that specific binding would be involved in such an aggregating system and therefore it would be expected that such a system would be completely reversible on dilution and reconcentration through the critical concentration. The more likely

Abbreviation: DNA, deoxyribonucleic acid.

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process of denaturation would be irreversible in view of the specific nature of the hydrogen bonds, which break during denaturation and are not reformed on restoring the original conditions.

Although the explanation involving denaturation must be favoured in view of the tendency for denaturation at low ionic strength[3-5], the ultimate decision on this question rests on testing the reversibility or irreversibility of the critical concentration phenomenon. An endeavour has been made in the present investigation to apply such a test. Although complicating factors are present, it appears that the process is essentially irreversible and can therefore be satisfactorily explained by a denaturation process.

EXPERIMENTAL

A description of the preparative histories of the calf thymus DNA samples used in this investigation has already been given[6]. The conductivity bridge was identical to that already described[1] and the technique used to determine the critical concentration has also been reported[2]. All DNA concentrations were determined by phosphorus analysis.

RESULTS AND DISCUSSION

It is now well 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 DNA double helix. As denaturation involves the destruction of this orderly arrangement of bases, it should be accompanied by increased u.v. absorption. This has been experimentally observed in many investigations on the denaturation of DNA. However, it also appears that the increase in absorptivity can in some respects be a rather insensitive measure of the initial stages of denaturation[5].

An essentially native DNA solution (initially dissolved in water at high DNA concentration) has an atomic extinction coefficient referred to the phosphorus concentration, $\varepsilon(P)$, of 6660 (see ref. 6). This value was obtained by dilution of a concentrated aqueous solution to $5 \cdot 10^{-5}$ *M* DNA in the presence of salt to give a final sodium chloride concentration of 0.1 *M*. A salt free solution has a similar value at high concentrations[5] (greater than $9\text{–}10 \cdot 10^{-4}$ *M* DNA). At lower DNA concentrations a discontinuity has been observed[1,5,7,8] in the variation of $\varepsilon(P)$ with concentration, which results in an extinction coefficient of 8200 (see ref. 5) 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. This increase in extinction coefficient has been associated with denaturation[1,2,5]; however, the observed increase is complicated by the fact that the addition of salt to such a solution lowers the absorptivity to an intermediate value. It would therefore appear that both irreversible and reversible changes comprise the structural transition responsible for the critical concentration. As the addition of salt to a solution below the critical concentration zone results in a partial lowering of the extinction coefficient, it will be assumed that the value so obtained represents the true irreversible increase brought about by dilution below the critical zone.

Previous studies[1,2] have shown that a critical concentration zone also exists in

the variation of the specific conductivity with DNA concentration. It is of interest to compare the critical zone over which the irreversible increase in u.v. absorption occurs with that found from the conductivity measurements. Fig. 1 shows the irreversible increase in extinction coefficient that is observed at low DNA concentrations. Curve a (Fig. 1) represents the values obtained at the various DNA concentrations by dilution of stock solution ($3 \cdot 10^{-3} M$ DNA) in the presence of salt; curve b represents the large, partially reversible increase, that accompanies dilution in the absence of salt; and curve c was obtained by dilution of a stock solution with water only, followed by the addition of solid salt to yield 0.1 M NaCl, the solution being then diluted to $5 \cdot 10^{-5} M$ DNA with 0.1 M NaCl and the absorption determined. Curve c thus represents the irreversible increase that accompanies dilution of a salt free solution below the critical concentration zone. It can be seen that the irreversible increase begins at concentrations lower than $10^{-3} M$ DNA. This is in agreement with the partially reversible increase shown in curve b. Previous work by CAVALIERI, ROSOFF AND ROSENBERG[5] has shown that this partially reversible increase begins at concentrations below $9\text{–}10 \cdot 10^{-4} M$ DNA. As the critical concentration phenomenon is at least in part irreversible it appears that the process originates from denaturation.

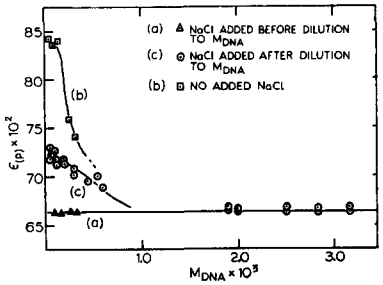

Fig. 1. Increase in atomic extinction coefficient accompanying dilution of DNA.

The concentration at which denaturation begins, as judged by the above results, is higher than that found from the conductivity measurements. It is concluded that dissolution of aqueous DNA solutions at concentrations below the critical concentration in the absence of salt results in a 27 % increase in extinction coefficient. The irreversible component, however, is only 11 %. These figures represent the average of several results. These values are to be compared with those of 25 % and 11 % respectively reported by THOMAS[3] for solutions at low DNA concentration and ionic strength. Thermal denaturation results in an irreversible increase of 15 % (see ref. 9). Differences in extinction coefficient of denatured DNA (usually measured at room temperature) could possibly arise because of the temperature dependence of the u.v. absorption of the denatured species[10]. Similar measurements were made on two further DNA preparations (preparations 1 and 5) which contained salt impurity; the irreversible increases were only 6 % and 3 % respectively. The smaller increases are understandable when the great sensitivity of the critical concentration to salt is considered[2].

The above experiments confirm the previous conclusions that irreversible increases in extinction coefficient occur in salt free solutions at low DNA concentrations. The change appears to originate from a structural transition from essentially native to denatured DNA as the polyion 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[9].

In order to test the irreversibility of the critical concentration phenomenon observed in the conductivity measurements, it is necessary to prepare a solution at high DNA concentration ($3 \cdot 10^{-3}$ *M*), which had previously been subjected to dissolution below the critical zone. This was done by freeze drying aqueous solutions ($5 \cdot 10^{-5}$ *M* DNA) and redissolving in water to give $3 \cdot 10^{-3}$ *M* DNA. This solution could then be studied for irreversible changes by conductivity measurements as compared with solutions at a similar concentration not first diluted below the critical zone.

Before any confidence can be placed in such a comparison, further factors must be considered. It is necessary to determine just how relevant the observed irreversible increase in extinction coefficient is to the problem. If, for instance, the sample that had been diluted and reconcentrated from below the critical concentration is again diluted to $5 \cdot 10^{-5}$ *M* DNA in the absence of salt, would a further irreversible increase in extinction coefficient occur? The results shown in Table I suggest that no further changes accompany a second dilution below the critical concentration zone. The atomic extinction coefficients given in Table I were obtained in the following way:

TABLE I

IRREVERSIBLE INCREASE IN ATOMIC EXTINCTION COEFFICIENT ACCOMPANYING DILUTION BELOW THE CRITICAL CONCENTRATION

Preparation	*ε(P) of DNA solutions (measured in 0.1 M NaCl)*			
	Initially native solution at $3 \cdot 10^{-3}$ M DNA (1) Solution diluted to $5 \cdot 10^{-5}$ M DNA (2)*		*Denatured by one dilution below critical concentration and freeze dried to $3 \cdot 10^{-3}$ M DNA (4**) Solution diluted to $5 \cdot 10^{-5}$ 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**	6500	6740	7010	7000

* Numbers refer to solutions shown in Table II.
** Preparation 5 contained salt impurity.

The ε(P) values given in the first two columns were obtained by the addition of salt *before* (column 1) and *after* (column 2) dilution of an aqueous stock solution to $5 \cdot 10^{-5}$ *M* DNA. Column 1 gives the ε(P) of the native sample, while the second column 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 zone followed by freeze drying to give the stock solution at $3 \cdot 10^{-3}$ *M* DNA. Column 3 therefore again represents the irreversible increase associated with a single dilution below the critical concentration (any damaging effect due to the freeze drying procedure would show up here). Column 4, on the other hand, was obtained by dilution of the reconcentrated solution to $5 \cdot 10^{-5}$ *M* DNA *before* the addition of salt and therefore represents a DNA sample subjected twice to the influence of the process operating at the critical concentration. No further denaturation by dilution was found. In addition freeze drying had no measurable

effect on the $\varepsilon(P)$ of a concentrated aqueous solution of native DNA. Table II shows the various solutions used in this investigation. From the constancy of the irreversible increase in $\varepsilon(P)$ it appears that the denaturation produced by dilution below the critical concentration represents a well defined state and not just one of a series of denatured states. The average $\varepsilon(P)$ obtained for six different batches of DNA, irreversibly changed by dilution followed by freeze drying, was found to be 7340 $\pm$ 60 (measured in 0.1 M NaCl at room temperature). These figures are shown in Table III.

TABLE II

DNA SOLUTIONS REFERRED TO IN THE TEXT

Reference number	*Solution*
(1)	Solution of native DNA dissolved in water at $3 \cdot 10^{-3}$ M DNA
(2)	Solution (1) diluted with water to $5 \cdot 10^{-5}$ M DNA
(3)	Solution (2) freeze dried
(4)	DNA from (3) dissolved in water to give $3 \cdot 10^{-3}$ M DNA
(5)	Solution (4) adjusted to 1 M NaCl with solid salt
(6)	DNA precipitated from (5) with ethanol and dried
(7)	DNA from (6) redissolved in water to give $3 \cdot 10^{-3}$ M DNA

TABLE III

ATOMIC EXTINCTION COEFFICIENT OF DNA DENATURATED BY DILUTION

Preparation	*Number of batches prepared*	*$\varepsilon(P)$ of DNA denatured by dilution followed by freeze drying (measured at room temperature in 0.1 M NaCl)*
2	2	7420
4	1	7450
7	3	7340

A second complication that must be considered is whether any further irreversible effect is caused by freeze drying, which might not be detected by the absorption measurements given in Table I. Two observations have been made which indicate that something of this nature possibly occurs. Firstly, the viscosity of a denatured solution (diluted to $5 \cdot 10^{-5}$ M DNA in the absence of salt) is about 10 times as great as that of a similar solution obtained by freeze drying and dilution to $5 \cdot 10^{-5}$ M. However, this anomaly may not be due to the freeze drying process, because the viscosity of an aqueous solution diluted below the critical concentration appears to decrease with time[11], as compared with a similar solution reconcentrated and rediluted. Secondly, a sample that had been denatured by dilution and reconcentrated was adjusted to 1 M NaCl and precipitated with ethanol. This sample when dissolved in 0.2 M NaCl gave further irreversible changes over and above that shown by a solution denatured by dilution only. The relationship between these various solutions is shown in Table II. The changes in both $\varepsilon(P)$ and S_{20} are shown in Table IV. Unfortunately it is not possible to precipitate DNA with ethanol at DNA concentrations of $5 \cdot 10^{-5}$ M and therefore it is not possible to determine whether 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 study just mentioned that u.v. absorbing, non-sedimenting species were present in the precipitated and

redissolved material, thus indicating that precipitation of denatured and freeze dried DNA is accompanied by degradation.

TABLE IV

IRREVERSIBLE CHANGES FOLLOWING PRECIPITATION OF DNA PREVIOUSLY DENATURATED BY DILUTION

Preparation	*Solutions denatured by dilution and then reconcentrated (4)**		*Solutions denatured by dilution, reconcentrated, precipitated and redissolved (7)**	
	S_{20}**	$\varepsilon(P)$ ***	S_{20}**	$\varepsilon(P)$ ***
2	19	7450	9–10	9900
4	16	7330	11–14	9900
7	17	7340	7–9	8100

* Numbers refer to solutions shown in Table II.
** Sedimentation of $2 \cdot 10^{-4}$ *M* DNA in 0.2 *M* NaCl.
*** Absorption measured in 0.1 *M* NaCl at room temperature and 2590 Å.

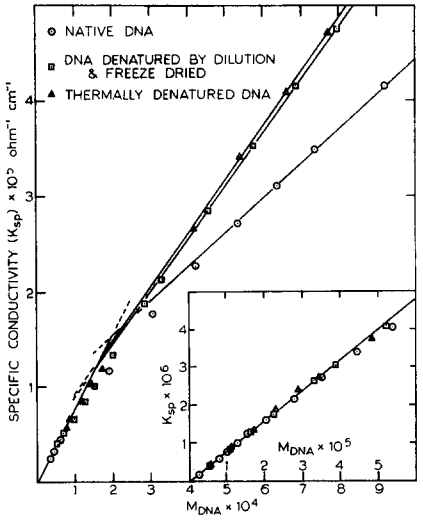

Fig. 2. Comparison of the critical concentration phenomena displayed by native DNA, DNA denatured by dilution and thermally denatured DNA solutions. Inset plot shows the conductivity at the lower concentrations.

A comparison of the critical concentration phenomenon, as judged by conductivity measurements, between initially native DNA and DNA diluted below the critical concentration followed by freeze drying, will now be given. A description of the two solutions used in this comparison (1 and 4) is given in Table II. Fig. 2 shows the variation of conductivity with concentration of the two solutions in addition to that of heat denatured DNA reported earlier[2]. At low concentrations, below the critical concentration zone, the three samples have similar equivalent conductivities, which suggests that a similar kinetic unit is involved in each case. This lends strong support to the suggestion that the critical concentration originates from a structural transition from native to denatured DNA. Below the critical zone the three samples are all in the denatured state, viz., the initially native sample which has been denatured by dilution, the sample initially denatured by dilution and freeze dried, and the thermally denatured sample. At DNA concentrations above the critical zone both the

thermally denatured solution and the material initially dissolved below the critical zone followed by freeze drying, have similar conductivities and conductivity concentration dependence, implying again that the kinetic unit of the freeze dried solution is in the denatured state. In both cases the conductivity is higher, at concentrations above the critical zone, than that shown by native DNA solutions.

If the critical concentration phenomenon arose entirely from the denaturation of DNA it would be expected that initially denatured solutions would not display any discontinuity at all in the conductivity measurements; however, as can be seen from Fig. 2, this is not so. Although the magnitude of the effect is reduced, the discontinuity is still apparent. Two explanations appear possible, denaturation may not be complete; or denaturation as followed by conductivity measurements is partly reversible. The former explanation does not seem likely, because the absorptivity measurements given in Table I show that a further dilution below the critical concentration does not yield further irreversible increases in $\varepsilon(P)$. Also, the discontinuity that exists in plots of O.D. (measured at 2590 Å) versus DNA concentration in the absence of added salt is not present in the denatured samples (Fig. 3).

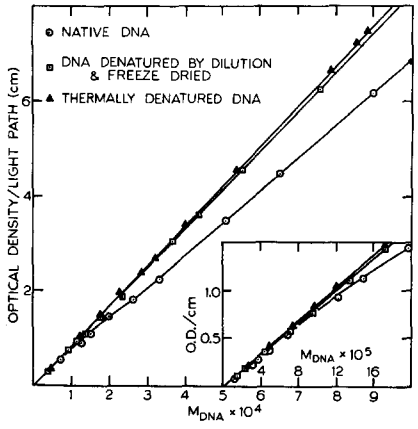

Fig. 3. U.V. absorption of native DNA, DNA denatured by dilution and thermally denatured DNA. Inset plot shows the O.D. at the lower concentrations.

The partial reversibility 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 resulted (to be compatible with the absence of the discontinuity in the u.v. measurements). Intermolecular hydrogen bonding may also have to be considered.

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

1. At high aqueous DNA concentrations (greater than $50 \cdot 10^{-5} M$ by conductivity measurements and greater than $100 \cdot 10^{-5} M$ by absorptivity measurements), an essentially native DNA molecule exists. Hydrogen bond cleavage, if it occurs, is reversible. It has been shown that under these conditions the kinetic unit possesses a charge fraction (charge per phosphorus atom) of 0.40. A large proportion of the gegenions are therefore held by the nucleate ion in aqueous solution. The careful work of CAVALIERI, ROSOFF AND ROSENBERG[5] has shown, however, that the viscosity,

as measured in 0.2 M NaCl, of DNA, initially dissolved in water at concentrations as high as $20 \cdot 10^{-4}$ M DNA, has already suffered a small but significant decrease as compared with a similar sample never dissolved in solvents of low ionic strength. Similarly it has been shown[6] that a 1.5 % increase in ε(P)s accompanies dissolution of native DNA in water at concentrations as high as $3 \cdot 10^{-3}$ M. It must therefore be concluded that a small amount of denaturation is inevitable when salt free DNA solutions at high concentration are studied.

2. Dilution below the critical concentration zone (lower than $4 \cdot 10^{-5}$ M DNA) in the absence of salt results in the denaturation of DNA (total hydrogen bond cleavage, or rupture of more than a critical number of bonds). The kinetic unit resembles the heat denatured material only if the latter is also diluted below the critical concentration zone in the absence of salt. Solutions of DNA that have been denatured by dilution have a high charge[1] and it would appear that charge fractions approaching unity are produced by this process. The differences in other electrical properties have already been described[1]. It is thought that the high charge on the denatured species may possibly counter the collapsing tendency that must accompany denaturation and allow the denatured molecule when dissolved in the absence of salt to take up an extended configuration, not unlike the native species.

3. Solutions reconcentrated from below the critical concentration zone are characterized 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". The bonds could be formed at points of closest approach between portions of the polynucleotide chain 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[9].

It should be noted that situation described in 3 can also be attained by the addition of salt to the species discussed in 2, without the need for reconcentrating the DNA solutions. This follows from the known dependence of the critical concentration on ionic strength[2]. Fig. 4 gives an illustration of the proposed structural transition occurring at the critical concentration zone. If further investigations confirm the above findings, then in the authors' opinion situation (2) should be a most fruitful starting point for a further process designed to dissociate the twin polynucleotide strands.

An examination of Fig. 2 shows that denaturation by dilution occurs over a

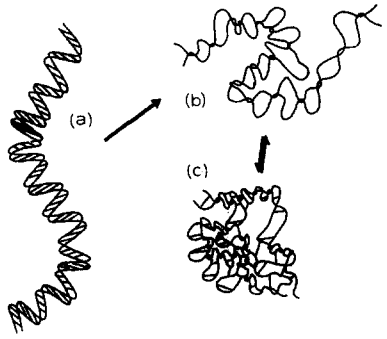

Fig. 4. Illustration of the proposed structural transition accompanying dilution below the critical concentration zone (a) ⟶ (b), and reconcentration to higher concentrations (b) ⟶ (c).

broad concentration range ($4\text{-}50 \cdot 10^{-5}$ *M*), which would not be entirely compatible with the cooperative melting out of all the native hydrogen bonds. Moreover, it can be seen from Fig. 1 that the amount of denaturation (measured as an increase in ε(P)) is dependent on the concentration. Dilution to concentrations within the critical concentration zone produces various amounts of denaturation, which are always less than that produced by dilution to below $4\text{-}5 \cdot 10^{-5}$ *M* DNA. This is taken to mean that the hydrogen bond strengths of the native structure are heterogeneous, the weaker bonds permitting some denaturation at the higher concentrations within the critical zone, while the stronger bonds do not rupture until lower DNA concentrations. This state of affairs is quite analogous to the critical temperature zone within which native saline DNA solutions undergo denaturation[9], the existence of a broad temperature zone having been, in this instance, interpreted in terms of hydrogen bond heterogeneity. As with denaturation by dilution, the amount of thermal denaturation depends on the applied constraint, the weaker hydrogen bonds melting out at the lower temperatures within the critical temperature zone[9]. These observations, coupled with the demonstrated double boundaries in sedimentation experiments on DNA solutions within the critical concentration zone at low ionic strength[12], would indicate that the hydrogen bond strength heterogeneity is on an intermolecular scale.

No explanation can yet be given for the energetic reason for denaturation by the apparently simple process of dilution in the absence of salt. One line of approach will be to explore the possibility that the process involving hydrogen bond cleavage (and also non-specific hydrogen bond formation) is influenced by the condition of the "ice-like" structure of the water surrounding the DNA molecules. It can be imagined, for instance, that denaturation by dilution could result from the competitive effect of water–base hydrogen bonding on the native base–base hydrogen bonds. Native DNA is thought to have an ordering effect on the surrounding water[13,14] and, if this effect was large enough under the condition of high dilution of DNA, then the above possibility would be feasible.

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