DEOXYPENTOSE NUCLEIC ACIDS

XII. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: CHANGES PRODUCED BY ENVIRONMENT

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(Received January 11th, 1960)

SUMMARY

An investigation of salt free DNA solutions has yielded further information on the critical concentration phenomenon reported earlier. This phenomenon appears to arise from denaturation by dilution below a certain critical concentration. The critical concentration can be arbitrarily set at $21 \pm 2 \cdot 10^{-5}$ M DNA for several salt free DNA preparations. Conductivity measurements show, however, that the structural transition actually occurs over a concentration zone extending from 50 to $4 \cdot 10^{-5}$ M DNA. The process is at least in part irreversible.

The effects, on the phenomenon, of ionic strength, gegenion radius, dielectric constant, EDTA and thermal denaturation were investigated. The results reported on the effect of thermal denaturation in particular support the premise that the critical concentration originates from a denaturation process. These findings are in agreement with the changes observed by CAVALIERI *et al.*[4], who find a large decrease in mean end-to-end distance on dilution of salt free DNA solutions to low concentrations. This change was not accompanied by a light scattering molecular weight change.

INTRODUCTION

In a previous paper changes of the charge, mobility and transport number of the DNA kinetic unit were reported as a function of the polyion concentration in salt free solution[1]. The rapid changes of these properties over a small concentration zone could be explained by two alternative mechanisms; one involving aggregation of two or more DNA polyions and the other involving the dissociation of the DNA double helix yielding, most probably, two entangled polynucleotide chains. The latter process is usually referred to as denaturation. Denaturation must be the more likely explanation in view of the reported denaturation at low ionic strength[2,3] and in dilute aqueous solution[4]. Experiments are described in this investigation which were designed to test the effect of various environmental factors on the critical concentration phenomenon.

Abbreviations used: DNA, deoxyribonucleic acid; EDTA, ethylene diamine tetra-acetic acid.

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EXPERIMENTAL

The samples of calf thymus DNA used in this investigation have already been described[5]. All DNA concentrations were determined by phosphorus analysis on stock solutions ($3 \cdot 10^{-3}$ *M* DNA in water). The stock solutions were first centrifuged to remove dust particles.

Conductivity measurements were made with the bridge already described[1]. In the study of the conductivity at various DNA concentrations, increases in concentration were effected in the conductivity cell in the following way. Successive additions of a concentrated stock solution ($3 \cdot 10^{-3}$ *M* DNA) were made to a known volume of water or DNA solution by first withdrawing an equal volume of liquid from the cell. The contents were then mixed by a stream of purified nitrogen (free from carbon dioxide). Dilution of a concentrated solution was made in a similar way, by removal of solution and the addition of an equal volume of water. The former method of preparing solutions (*i.e.*, by increasing the concentration) was in general preferred, since this procedure enabled the large water corrections, which were necessarily incurred in the investigation, to be known more precisely at the lower concentrations. This is so because such solutions will still contain a large proportion of the water of known conductivity which was present in the cell at the beginning of the experiment.

The method used to calculate the resulting DNA concentration after withdrawal and addition of known volumes of DNA solution assumed that no volume change occurred on mixing. No large errors came about through this assumption. In the two experiments where a check was made on the final concentration after, in one case 18 and in the other 20, successive increases in concentration, the final calculated concentrations were in error by 1.5 % and 2.0 % respectively as, judged by phosphorus analysis on the final solutions. An error of this magnitude does not alter the conclusions reached in this investigation.

The determination of the critical concentration was found by measurements of the specific conductivity at various increasing DNA concentrations, 15 to 20 successive increases in concentration were needed to define the critical concentration for the comparisons made in this and later investigations.

All the conductivities reported for DNA were obtained by subtraction of the conductivity contributions of the water and of any additive. The conductivity of the water was always less than $0.5 \cdot 10^{-6}$ ohms^{-1} cm^{-1} and had an average value of $0.35 \cdot 10^{-6}$ ohms^{-1} cm^{-1}.

Sedimentation experiments were made on a Spinco Model E Ultracentrifuge equipped with an u.v. absorption optical system. The photographic records were converted into plots of photographic density *versus* distance from the centre of the rotor using an Analytrol photodensitometer.

RESULTS AND DISCUSSION

Detailed investigation of the conductivity at various DNA concentrations

The variation of specific conductivity with DNA concentration has been found to exhibit a discontinuity at a critical concentration of $2\text{–}3 \cdot 10^{-4}$ *M* DNA[1]. Fig. 1 shows the results of a detailed study of this phenomenon. The difference between the conductivities obtained by increasing and decreasing the DNA concentration will

be discussed later. The inset plot of Fig. 1 represents the lower concentration range. It can be seen that the critical concentration is more accurately represented by a zone of concentration; above and below this zone a linear relation appears to exist. According to an earlier investigation[1] the linear relation above the critical zone is

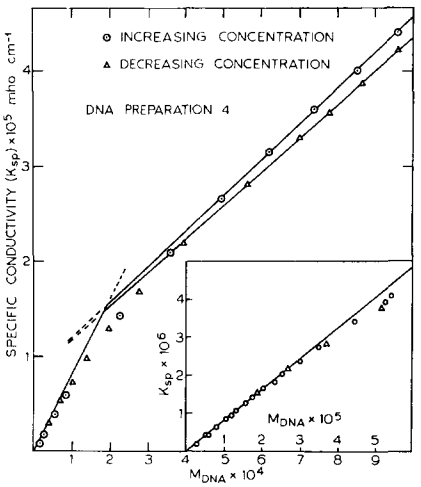

Fig. 1. Specific conductivity of salt free DNA solutions at various concentrations. DNA preparation 4. (a) Obtained by progressive increase in concentration (addition of concentrated stock solution). (b) Obtained by progressive dilution of DNA solution with water. Insert graph shows the linear relation that exists below the critical concentration zone.

preserved up to at least $2.5 \cdot 10^{-3}$ M DNA. The average critical concentration (the point at which the two linear portions intersect) for the salt free preparations 2, 3, 4 and 7 (see ref. 5) was found to be $21 \pm 2 \cdot 10^{-5}$ M while the actual zone extended from $4\text{–}50 \cdot 10^{-5}$ M. All these preparations gave values of 80 ± 10 for the equivalent conductivity at zero concentration. Another preparation, number 1, gave higher conductivities and a lower critical concentration. The higher conductivity is almost certainly due to the salt impurity in this preparation[5] and the lower critical concentration is also to be expected in view of the dependence of the critical zone on ionic strength, to be discussed later.

To show that any possible frequency dependent errors were not responsible for the critical concentration phenomenon, conductivities were measured at 1, 2.5, 10, and 20 kc/sec and the values obtained extrapolated to infinite frequency and compared with the results obtained at 1 kc/sec. From this comparison it was evident that change in frequency had little effect on the critical concentration phenomenon.

A test for the reversibility of the phenomenon was carried out by making conductivity measurements on DNA solutions prepared by both increasing and decreasing the DNA concentration (Fig. 1). The small but significant increased conductivity of the plot obtained when increasing the DNA concentration (curve (a), Fig. 1) over that obtained on decreasing the concentration (curve (b), Fig. 1) is consistent with irreversibility. A small amount of reversibility cannot, however, be excluded, because the method used to increase the DNA concentration (addition of concentrated DNA solution) tends to mask any irreversible changes that occur at the critical concentration. Calculations show that increasing the concentration from below the

critical concentration zone to $1 \cdot 10^{-3}$ *M* (by addition of concentrated DNA solution) results in a solution containing only 3.8 % of the original DNA present at concentrations below the critical zone. Using an equivalent conductivity of 80 for the DNA present that had been below the critical concentration and 43.5 for the remainder (obtained from the conductivity of DNA never diluted below the critical zone, Fig. 1, curve (b)), results in a specific conductivity for the mixture (Fig. 1, curve (a)) at $1 \cdot 10^{-3}$ *M* of $0.45 \cdot 10^{-5}$ ohm^{-1} cm^{-1} as compared with the experimental value of $0.46 \cdot 10^{-5}$ ohm^{-1} cm^{-1}. Due to the small magnitude of the observed irreversibility and due to the uncertainty in DNA concentration after many concentration changes it may only be concluded that the process must be, at least in part, irreversible, but not necessarily totally irreversible. The question of total irreversibility must await a more sensitive test.

Because the critical concentration does show some irreversibility, it would appear that the phenomenon is, at least in part, a manifestation of denaturation which occurs on dilution through a critical zone of concentration.

Influence of ionic strength

The relationship between the critical concentration, as determined by conductivity measurements, and the concentration of added sodium chloride is shown in Fig. 2. The phenomenon is extremely sensitive to low salt concentrations, the addition of $2 \cdot 10^{-5}$ *M* NaCl being sufficient to lower the critical concentration by one-third. The fact that salt lowers the critical concentration is compatible with both processes that have been proposed to explain the phenomenon. If the critical zone resulted from denaturation, then the addition of salt should lower the critical zone, because the presence of shielding ions is known to protect the polyion in this respect[2-4], by

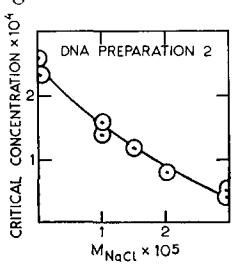

Fig. 2.

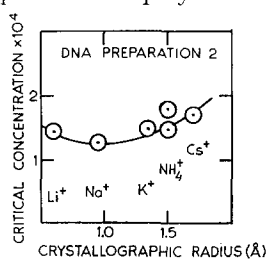

Fig. 3.

Fig. 2. Dependence of critical concentration on added sodium chloride. DNA preparation 2.
Fig. 3. Dependence of critical concentration on crystallographic radii of added monovalent gegenions. DNA preparation 2.

decreasing the electrostatic repulsion between the charged sites on the molecule. The alternative, less likely, explanation of the phenomenon involving aggregation of two or more DNA molecules at concentrations higher than the critical zone also receives support from the findings concerning the effect of salt. A shielding action would tend to lower the repulsive forces that must be present between the charged polyions and would therefore increase the possibility of aggregation. The results given in Fig. 2 indicate that marked changes in this critical concentration can be produced by the addition of small amounts of salt; it is therefore evident that investigations of aqueous DNA solutions are liable to be irreproducible unless salt impurity is rigorously excluded.

Further investigations were made on the effect of variations in size of the cation and the results are given in Fig. 3 for monovalent ions. Although the effect of cation radius is not large, it appears that the sodium ion lowers the critical concentration to the greatest extent. If it is assumed that the action of the added salt is one of shielding, then the minimum in the curve shown in Fig. 3 is not unexpected. It is known that a critical radius exists for ions in solution such that ions smaller than a certain size acquire an hydrated sheath which increases the aqueous radius of the ion[6]. If the lithium ion has such an increased radius, then the minimum in the curve can be understood.

To determine whether the decreased critical concentration, which follows the addition of salt, originated from a shielding action, the effects of much larger ions were studied. The salts used were rosaniline hydrochloride and 5-aminoacridine hydrochloride. Unfortunately the effect of these ions can only be studied over a limited concentration range due to precipitation of a DNA complex at relatively low ionic strengths. However, within this limit the effect of the large cations, in lowering the critical concentration, was much less than that shown by the small gegenions. Such a result is to be expected if a shielding action is involved.

Influence of dielectric constant

In view of the well known relationship between dielectric constant and association in simple electrolytes, experiments were made to determine the effect of changes in association between gegenion and polyion on the critical concentration phenomenon. The changes in dielectric constant were effected by various methanol–water and ethanol–water solvents. It was found that the critical concentration was lowered on decreasing the dielectric constant. Again this result is consistent with a gegenion shielding action. That association of the gegenions by the polyion increases on lowering the dielectric constant was inferred from the observed lowering of the equivalent conductivity of DNA with decreasing dielectric constant. Complete association could

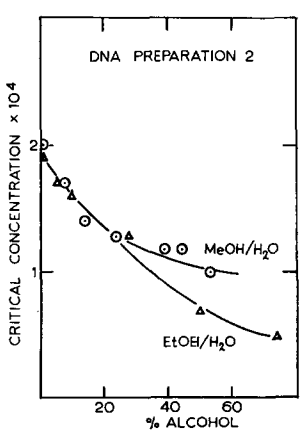

Fig. 4. Dependence of critical concentration on dielectric constant. Dielectric constant varied by the use of MeOH–H_2O and EtOH–H_2O mixtures. DNA preparation 2.

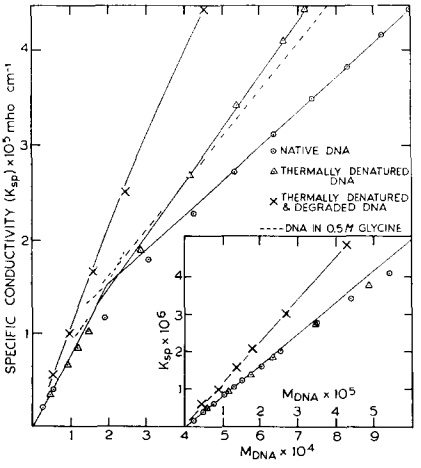

Fig. 5. Comparison of the critical concentration phenomena displayed by an initially native sample, thermally denatured DNA and DNA in 0.5 *M* glycine. Insert graph shows the lower concentration region. DNA preparation 2.

be expected in pure ethanol solvent as judged by the decreasing equivalent conductivity of DNA in increasing ethanol–water solvent ratios. This was confirmed by direct measurement of the conductivity of DNA in 98 % ethanol prepared by dialysis of an aqueous solution against increasingly stronger alcohol solutions[7]. The conductivity of DNA in such a solution was about 0.01 times that of an aqueous DNA solution. No sudden change was found in the variation of the conductivity with alcohol concentration as would be expected from the observed rapid change in sedimentation constant at 65 % ethanol[7].

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

The effect of added EDTA

As aggregation of the nucleate ion could possibly occur through divalent ion bridges, the effect of the addition of EDTA was studied. The presence of EDTA should remove from solution all divalent ions which might be present as trace impurities. EDTA at $1 \cdot 10^{-5}$ *M* (disodium salt) had no effect on either the critical concentration or the specific conductivity of DNA. Divalent ion bridges therefore would not appear responsible for the critical concentration phenomenon.

The effect of thermal denaturation

It is now well established that denaturation of DNA 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 critical concentration zone. Thermal denaturation was achieved by heating an aqueous DNA solution ($3 \cdot 10^{-3}$ *M* DNA) for 30 min at 75°. Previous experiments had shown that the above conditions were just maximal in yielding a constant increase of the atomic extinction coefficient (as measured in 0.1 *M* NaCl at room temperature). The increase in extinction coefficient from 6660 (measured at 2590 Å) to 7300–7400 was found to be essentially constant for periods of heating up to 200 min. No after effects, such as a slow increase in viscosity, were ever noticed in the thermally denatured salt free DNA solutions.

Fig. 5 shows the comparison between the critical concentration phenomena shown by initially native and thermally denatured DNA solutions. It is to be noted that, above the critical concentration zone, the thermally denatured material has increased conductivity compared to the initially native material; below this zone, however, the two solutions have similar conductivities. The overall result is a decrease in the magnitude of the critical concentration phenomenon for the thermally denatured solution. The observation that the two solutions have similar conductivities below the critical zone only is strong evidence that this zone itself also originates from a denaturation process. The small discontinuity that still exists in the variation of conductivity with concentration for the thermally denatured material (Fig. 5), may mean that thermal denaturation, under the conditions described earlier, was not

complete and that further denaturation takes place on dilution below the critical zone. However, this appears to be an unlikely explanation in view of the constancy of the extinction coefficient and also from the similarity of this property with that obtained from thermal denaturation in salt solutions. A more likely explanation for this remaining discontinuity may arise from the possible reformation of non-specific hydrogen bonds above the critical concentration. In Fig. 5 there is also shown, for comparison, the results of measurements made on a DNA solution subjected to drastic thermal treatment (90°, for 12 h). The conductivity of this solution does not show a discontinuity but rather, the conductivity has increased over the entire concentration range. It is evident that some degradation in addition to denaturation has taken place in this solution.

The large increase in conductivity of DNA in 0.5 *M* glycine (discussed earlier and shown in Fig. 5) also occurred only above the critical concentration. Furthermore, the variation of conductivity with concentration for the thermally denatured material and for DNA in 0.5 *M* glycine are similar. It may thus be inferred that denaturation has taken place in glycine solutions at concentrations above the critical zone, and 0.5 *M* glycine is to be considered a denaturing agent for DNA at zero ionic strength.

It has been reported that a discontinuity exists in the variation of O.D. (at 2590 Å) with concentration of DNA in the absence of added electrolyte[1,4,10,11]. As it is probable that this phenomenon arises from the same process that is responsible for the discontinuity in the conductivity measurements, the effect of thermal denaturation of the O.D. of DNA was also investigated. It was found that the thermal treatment described above completely removed the discontinuity observed in the dependence of the u.v. absorption on concentration. At all concentrations up to $8 \cdot 10^{-4}$ *M* DNA the extinction coefficient of the thermally denatured solutions was constant at 8500 (measured in the absence of salt at room temperature) and identical with that of an initially native solution below the critical concentration zone. Again this would indicate that denaturation by dilution is responsible for the critical concentration phenomenon.

Light scattering investigations[4] have shown that aqueous DNA solutions at low concentration are denatured and display a decreased root-mean-square end-to-end distance at constant molecular weight. In view of this finding, and that presented in the present investigation, it appears that denaturation by dilution can explain satisfactorily the critical concentration phenomenon.

Sedimentation studies

Since it would appear that the critical concentration is produced by denaturation of the DNA, it would be expected that two boundaries would be observed in sedimentation velocity experiments at DNA concentrations within the critical zone where both native and denatured DNA coexist. The presence of both native and denatured DNA together would be expected from the known heterogeneity of calf thymus DNA. Differences in sedimentation velocity would be expected, as the native species is relatively rigid, while denatured DNA, although it has the same molecular weight, would not necessarily be restrained to the dimensions imposed on the native structure.

An investigation of the sedimentation of DNA solutions at concentrations within the critical concentration zone has shown that the formation of two boundaries is very dependent upon the salt concentration of the solution being studied[12]. With so-

lutions from which salt had been rigorously excluded and which have a DNA concentration of $2 \cdot 10^{-4}$ M, sedimentation analysis shows (Fig. 6a) that, whilst no resolution into two distinct boundaries occurs, a diffuse leading edge is obtained in addition to a hypersharp trailing edge. This observation suggests the presence of more than one component. In 0.2 M sodium chloride, with the same DNA concentration, no further resolution was obtained, although the denatured material would still be present. At a sodium chloride concentration of $2.5 \cdot 10^{-4}$ M, however, resolution into two boundaries was observed[12] (Fig. 6b). Although the reason why this resolution only occurs

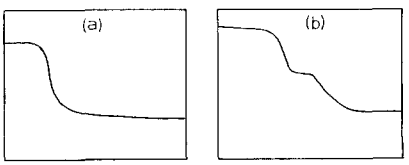

Fig. 6. Tracings of sedimentation velocity boundaries of DNA solutions. Tracings to be considered as inverted plots of DNA concentration.

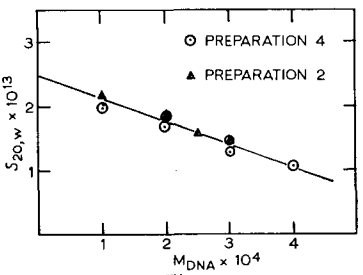

Fig. 7.

over a limited salt concentration is, at present, not known, the observation nevertheless confirms the theoretical prediction of more than one boundary. A study of the sedimentation velocity of fully denatured DNA suggests that the slower moving component corresponds to denatured DNA.

In the absence of salt, it was observed that no rapid change of the sedimentation coefficient occurred on dilution through the critical concentration zone. The values of $s_{20,w}$ (Fig. 7) show a linear relationship with concentration with no change in slope at the critical concentration. The values given in Fig. 7 were estimated from the half concentration point of the total asymmetric boundary.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the financial assistance given to one of us (R.B.I.) by the Anti-Cancer Committee of South Australia, the Commonwealth Scientific and Industrial Research Organisation and the Imperial Chemical Industries of Australia and New Zealand Ltd.

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