# DEOXYPENTOSE NUCLEIC ACIDS

## XIV. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: PROPERTIES OF DNA DENATURED BY DILUTION

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SUMMARY

Sedimentation studies indicate that native DNA and DNA denatured by dilution with water below the critical concentration zone have similar sedimentation coefficients and sedimentation coefficient concentration dependence. This is similar to the findings for alkali and thermally denatured DNA.

Electron microscope investigations show that denaturation by dilution is attended by large morphological changes; however, it is thought that the observed molecular weight decrease is an artifact of the method used in the preparation of DNA samples for electron microscopy.

### INTRODUCTION

It is generally accepted that the term denaturation[1-3] as applied to DNA,* should refer to the process involving the rupture of the specific hydrogen bonds that exist between the nitrogen bases in the native twin helix. A current problem of biological interest concerns the possibility of dissociating the constituent polynucleotide strands of the intact molecule. The first step of such a process would presumably involve the breakage of the hydrogen bonds holding the two strands in register. Studies involving the denaturation of DNA should therefore be pertinent to the above problem. Once denaturation has occurred the two polynucleotide strands are no longer bonded together. Because, however, of the plectonemic nature of the twin helix, the two strands cannot immediately dissociate. In the complete absence of any hydrogen bonding between the two strands, disentanglement by diffuse motion must take place before true dissociation can be said to have occurred. Calculations show that this dissociation is energetically feasible[4].

Experimental evidence reported for alkali[5], thermal[6] and low ionic strength denaturation[7] showed that denaturation is not accompanied by significant molecular weight change. It is therefore of interest to determine what factor is responsible for

Abbreviation: DNA, deoxyribonucleic acid.

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the stability of the undissociated denatured state. If enough hydrogen bonds were present, in the denatured unit, then it could be imagined that diffusive motion would not lead to dissociation. Studies on denatured DNA[5,6] have led to the suggestion that under certain circumstances residual hydrogen bonds may exist. Two types of such residual hydrogen bonding may have to be considered. Some native hydrogen bonds having high bond strength may resist the denaturation process (evidence has already been reported[6,8] showing that hydrogen bond strength is heterogeneous in the native structure). Furthermore, it is possible that non-specific hydrogen bonds reform under some conditions of denaturation[5].

Previous communications from this laboratory have dealt with investigations of salt free DNA solutions and it was concluded that an essentially native structure existed at high DNA concentrations only. Dilution through a critical concentration zone was accompanied by denaturation. The critical denaturation zone was found to be $4\text{–}50 \cdot 10^{-5}$ *M* DNA for salt free solutions. Below this concentration zone the molecule was thought to be devoid of native hydrogen bonds. However, reconcentration of such a solution or the addition of salt was believed to result in the formation of a number of non-specific hydrogen bonds. In the present investigation some properties of DNA denatured by dilution to the critical concentration zone followed by reconcentration, will be compared with native calf thymus DNA.

## EXPERIMENTAL

The preparations of calf thymus DNA used in this investigation have already been described[9]. Samples of DNA denatured by dilution were prepared in the following manner. A native stock solution of DNA ($3 \cdot 10^{-3}$ *M* in water) was diluted with water to $5 \cdot 10^{-5}$ *M*, taking care to avoid ionic impurities. This solution was then freeze dried and redissolved to give $3 \cdot 10^{-3}$ *M* DNA in water. The process of denaturation in water, with or without subsequent freeze drying, results in an atomic extinction coefficient with respect to phosphorus concentration (measured in 0.1 *M* NaCl at room temperature) of $7340 \pm 60$, compared with the average value of 6660 for essentially native samples. All DNA concentrations were determined by phosphorus analysis on each stock solution. Sedimentation velocity experiments were made with a Spinco model E ultracentrifuge equipped with an u.v. absorption optical system. The experimental records were converted into plots of photographic density *versus* distance with an Analytrol photodensitometer. All the sedimentation experiments were made in 0.2 *M* NaCl at temperatures close to 25°, the sedimentation velocities were then corrected to water as solvent at 20°.

The electron microscope used was a Metalix 1190 fitted with the latest type of objective lens supplied by the manufacturer. Some of the later work was carried out with an improved electron gun. The converted instrument was equivalent in performance to the Philips EM-100 electron microscope. The technique described by HALL[10–12] was followed in the preparation of the samples for microscopy. Samples of native DNA were prepared by dilution of an aqueous stock solution to $2\text{-}5 \cdot 10^{-5}$ *M* DNA with a solution containing 0.1 *M* ammonium acetate and 0.05 *M* ammonium carbonate. The denatured material was prepared by dilution of a salt free stock solution to $2\text{-}5 \cdot 10^{-5}$ *M* DNA with water. The sprayed specimens were shadowed with platinum at shadow to height ratios between 5 : 1 and 10 : 1.

## RESULTS AND DISCUSSION

### *Sedimentation*

When a salt free solution of DNA is diluted below the critical concentration zone, denaturation[13] is accompanied by an increase in the effective charge and an increase in the resistance offered to motion of the kinetic unit. However, these changes were deduced for denatured DNA below the critical concentration and in the absence of salt. Reconcentration of such a solution, or the addition of salt, is thought to bring about the formation of non-specific hydrogen bonds and under these conditions the denatured kinetic unit would have quite different properties. For example, CAVALIERI, ROSOFF AND ROSENBERG[7] have shown that denatured DNA at high ionic strength has a decreased root-mean-square end-to-end distance (at constant molecular weight) as compared with a native sample.

Earlier investigations[14] showed that sedimentation velocity experiments on salt free DNA solutions yielded only a single boundary at DNA concentrations within the critical zone, although at these concentrations it is believed that native and denatured species coexist. It would be expected, from the discussion above, that the two species should exhibit different sedimentation properties, therefore further efforts have been made to resolve the two species by sedimentation.

An objection to the sedimentation experiments in salt free solution is that the critical concentration zone may not represent a region of coexistence of two species, but rather a change in property of either one. To overcome this objection it was necessary to compare the sedimentation properties of separate samples of both native and denatured DNA. This was carried out by a comparison of essentially native DNA (prepared by dilution of a salt free stock solution, at $3 \cdot 10^{-3}$ *M*, to various DNA concentrations in the presence of salt to give 0.2 *M* NaCl) with DNA denatured by dilution, which was prepared by dilution of a similar stock solution to $5 \cdot 10^{-5}$ *M* DNA in the absence of salt followed by freeze drying and dissolution to $3 \cdot 10^{-3}$ *M* DNA. The various dilutions of the denatured material were then made by diluting in the presence of salt to give 0.2 *M* NaCl. The results of these experiments are shown in Fig. 1, where the variation of the sedimentation coefficient with concentration is given for both native DNA and DNA denatured by dilution. No significant difference in the sedimentation behaviour for the native and denatured material was found in these two preparations. A similar result (shown in Fig. 2) was found for a further DNA

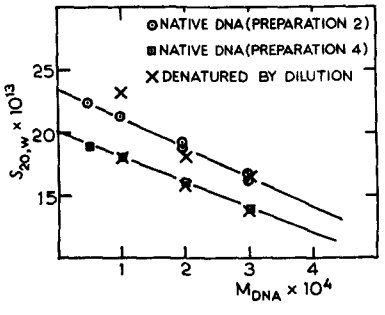


Fig. 1. Sedimentation coefficient of essentially native DNA and DNA denatured by dilution. All measurements made in 0.2 *M* NaCl at 25.0°.

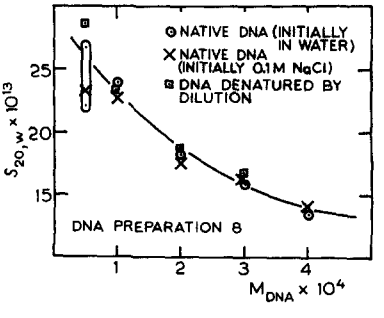


Fig. 2. Sedimentation coefficient of native DNA, essentially native DNA and DNA denatured by dilution.

preparation (see below). Mixtures of native and denatured DNA could not be resolved in further sedimentation experiments in 0.2 *M* NaCl; even at high DNA concentrations where differences in interaction would be expected, the two species sedimented together. The results shown in Figs. 1 and 2 also show that no significant difference in the sedimentation velocity concentration dependence exists between the native and denatured species of any one preparation. Although no differences in velocity were apparent in these experiments, the denatured material exhibited a more rapidly spreading boundary than native DNA.

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 investigations concerned with alkali[5] and thermal[6] denaturation, where the native and denatured species also cannot be resolved by sedimentation. As has already been pointed out[6], 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 authors' opinion this problem may ultimately 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[6] the above type of interaction was assumed to be of equal magnitude for both DNA species.

Two further factors related to the above problem deserve attention. In contrast to native DNA the denatured species appears to have a high degree of flexibility. At zero ionic strength denatured DNA has a high charge and low mobility[13], indicating a highly extended molecule. On the other hand, at high ionic strengths denatured DNA has greatly reduced dimensions compared with the native unit[5-7]. The results at intermediate ionic strengths are quite compatible with these findings. It has been found, for example, that alkali denatured DNA exhibits a viscosity increase of about 8-fold on decreasing the ionic strength from 0.165 to 0.0055. (see ref. 5) and that this is followed by an increase of 2.3-fold in radius of gyration. EHRLICH AND DOTY[5] conclude that, at an ionic strength of $5.5 \cdot 10^{-3}$, the space filling properties of denatured DNA are about equal to that of the native structure. Viscosity studies on thermally denatured material also confirm that denatured DNA has a high degree of flexibility[6]. In view of the flexibility and relative stiffness of the denatured and native units respectively, it would be expected, accepting the observation that the two units have similar sedimentation velocities at high ionic strengths, that differences in this property would arise at lower salt concentrations. Preliminary investigations[15] concerned with sedimentation at low ionic strengths (but not at zero salt concentration) have already shown that resolution of the two species is possible; however, it is thought that this arises directly from the differences in charge rather than from the related shape change that occurs in the denatured unit.

Although the process of denaturation by dilution has been shown to occur over a critical concentration zone ($4\text{-}50 \cdot 10^{-5}$ *M* DNA), there is evidence which indicates that a small amount of denaturation accompanies dissolution of DNA in water at very much higher concentrations. An irreversible increase in atomic extinction coefficient (measured at 2590 Å in 0.1 *M* NaCl) of 1.4% has been reported[9] on dissolving DNA in water to give a final concentration of $3 \cdot 10^{-3}$ *M*. Similarly, solutions of DNA in water at concentrations as high as $2\text{-}3 \cdot 10^{-3}$ *M* DNA suffer a small but significant irreversible decrease in viscosity (measured in 0.2 *M* NaCl), as compared

with a sample of DNA never dissolved at low ionic strength[7]. In view of these findings it is pertinent to determine whether the small amount of denaturation inherent in the essentially native salt free samples used in this and earlier investigations (stock solutions of $3 \cdot 10^{-3}$ *M* DNA in water) is responsible for the similarity of sedimentation velocity between essentially native DNA and DNA denatured by dilution. An investigation of preparation 8 yielded information on this problem. This preparation had never been dissolved at low ionic strengths during preparation[9] and had therefore not suffered the small amount of denaturation discussed above. However, the precipitation of this preparation with ethanol was carried out at relatively low ionic strength (0.0014 *M* NaCl) and therefore the occluded salt impurity should be low. This preparation exhibited the typical critical concentration phenomenon[13,14] shown by the earlier salt free DNA preparations (precipitated at zero ionic strength). Preparation 8 can therefore be used to determine whether or not the small amount of denaturation which accompanies dissolution in water at high DNA concentrations has any bearing on the apparent similarity of sedimentation velocity of native DNA and DNA denatured by dilution to very much lower concentrations (below the critical zone). Fig. 2 gives a comparison of the sedimentation velocities shown by three solutions relevant to the present problem: (a) DNA initially dissolved in 0.2 *M* NaCl (having not suffered the small irreversible increase in $\varepsilon(P)$), (b) DNA initially dissolved in water at $3 \cdot 10^{-3}$ *M* DNA (having suffered the small irreversible change) and then diluted in the presence of salt to give 0.2 *M* NaCl, and (c) a solution of DNA diluted in water below the critical concentration, followed by freeze drying and redissolution in 0.2 *M* NaCl. It can be seen that no large difference in sedimentation velocity or sedimentation velocity concentration dependence is apparent in these three solutions. Therefore the small amount of denaturation that follows dissolution in water at high DNA concentrations cannot be held responsible for the similarity in sedimentation velocity between native DNA and DNA denatured by dilution in water below the critical concentration zone.

An interesting difference is to be noted between the sedimentation velocity concentration dependence shown in Figs. 1 and 2. Preparation 8 yields an increasing concentration dependence with decreasing concentration compared with the linear dependence exhibited by preparations 2 and 4. Although this cannot be related to the problem just discussed (two of the solutions shown in Fig. 2 having already been dissolved in water), a similar phenomenon was also found for preparation 7 (see ref. 9) which, however, was of even greater magnitude. Preparations 7 and 8 yielded a gel-like sediment on preliminary centrifugation (at 13,000 rev./min) of an aqueous stock solution ($3 \cdot 10^{-3}$ *M* DNA). This preliminary treatment of preparation 7 reduced substantially the curvature in the concentration dependence of the sedimentation velocity. No gel-like sediment was observed after preliminary centrifugation of stock solutions of preparations 2 and 4 (shown in Fig. 1). The results given in Figs. 1 and 2 were obtained after preliminary centrifugation of the stock solutions.

*Electron microscopy*

Visualization of single DNA molecules using the electron microscope[10–12] has made possible a direct comparison between native DNA and DNA denatured by dilution. The results of an investigation of native DNA confirm the previous findings of HALL[10–12]; however, the average molecular diameter was found to be $15 \pm 8$ Å,

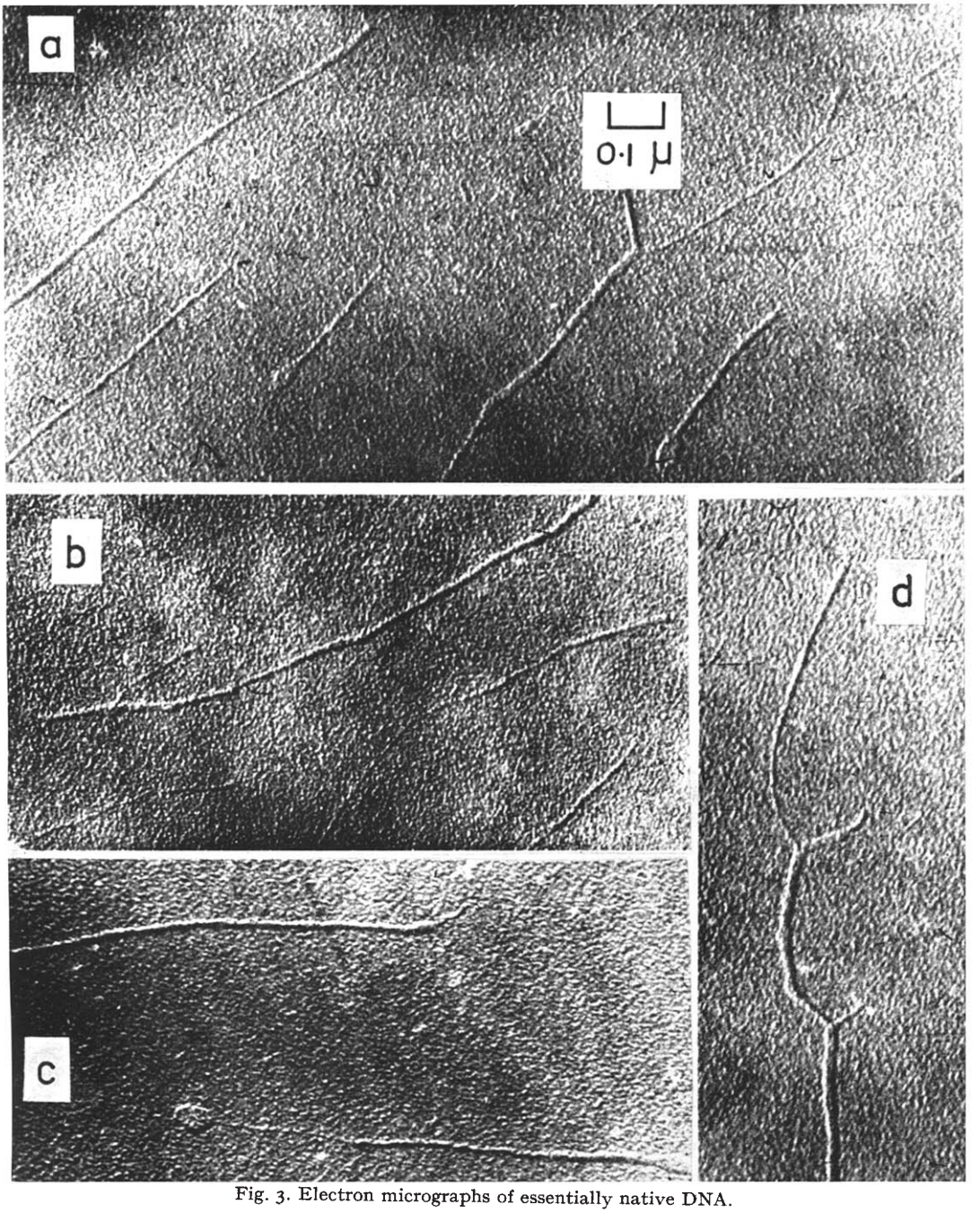


Fig. 3. Electron micrographs of essentially native DNA.

compared with the value of 20 Å given by HALL. Electron micrographs of the native material are shown in Fig. 3. From a study of many such fields it has become apparent that, although the initial DNA concentration was low, on drying, a longitudinal

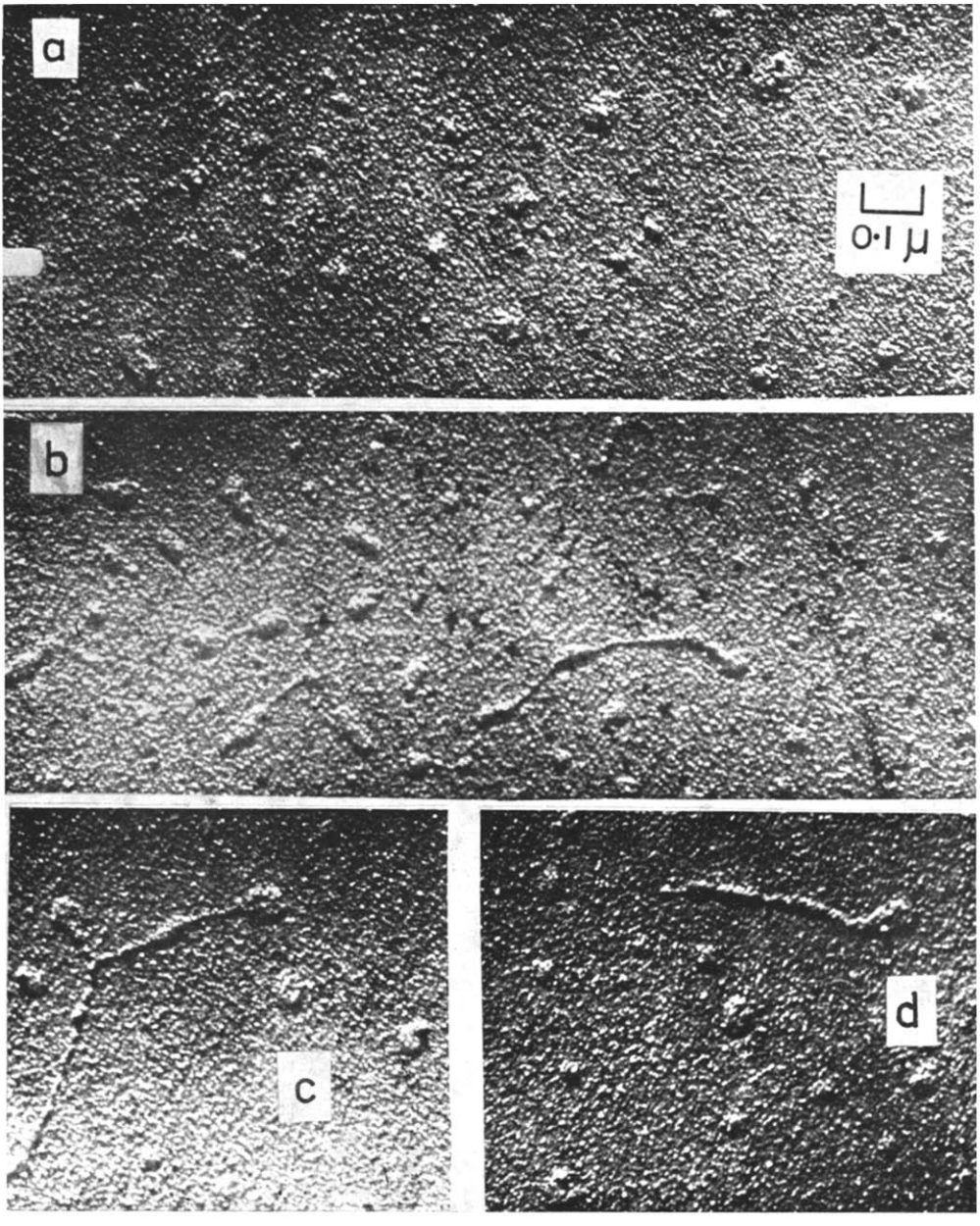


Fig. 4. Electron micrographs of DNA denatured by dilution.

aggregating tendency caused many of the molecules to lie together. This type of behaviour is shown in Fig. 3d. In addition a small amount of the native material would appear to have a molecular diameter smaller than that consistent with the diameter of the DNA double helix.

Before comparing the native material with denatured DNA, an interesting and

perhaps important observation must be discussed. In agreement with HALL it is found that a small amount of the native DNA terminates in what appears to be flat patches (Fig. 3, a and c). It has been suggested that this phenomenon is to be associated with a small amount of denaturation[12]. However, electron microscope studies on DNA preparation 8 (a preparation never dissolved at low ionic strength) again yielded the characteristic flattened terminal patches and occasional patches along the native strand. Although these flat patches could be due to a small amount of denaturation inherent in calf thymus samples prepared by the detergent method[16], it is possible, in view of a somewhat similar observation made on calf thymus nucleoprotein[17], that this phenomenon may be a true feature of the molecular architecture of the chromosome.

Fig. 4 shows the fields obtained for DNA denatured by dilution below the critical concentration. A spectacular change from the rod-like material to collapsed or "melted out" DNA can be seen. The size of the patches indicates that coiling of the denatured strands has taken place. Two points of interest should be discussed. Firstly, Fig. 4 b, c, d, show remnants of rod-like material which have apparently resisted the denaturation process. It is to be noted that Fig. 4 is not typical in this respect; an average field would yield about one undenatured portion to every four fields examined. Secondly, in Fig. 4a, a completely denatured DNA field is shown and in this it will be noticed that the collapsed patches are too numerous to correspond to one patch for every native DNA molecule. This can be seen by comparing the number of collapsed units with the number of unimolecular strands shown in Fig. 3 (both figures represent samples with roughly equal two dimensional concentrations). That each of these collapsed units can only represent a portion of the original native molecules can also be seen from Fig. 4b,c,d, here presumably unimolecular strands are melting out into more than one collapsed unit. This observation is at variance with the constancy of molecular weight attending denaturation. It would therefore appear that the observed molecular weight decrease (found in the electron microscope studies) is an artifact of the method used to prepare the samples for microscopy. Support for this claim was found in further fields of denatured DNA, in which it was noticed that some of the collapsed units lay in lines reminiscent of the original native molecule. The collapsed units occurred at intervals of 1000–2000 Å, and were similar to those shown in Fig. 4 b and c, except that no interconnecting material was observable. The fact that in some instances the denatured patches lie in definite lines although unconnected, indicates that the denatured material has a larger molecular weight when in solution than the resulting specimens observed by electron microscopy. It is estimated that each patch corresponds to a molecular weight of $2\text{-}4 \cdot 10^5$ (assuming that a native molecule melts out into a denatured unit every 1000–2000 Å). As the denatured material dries on the mica slide each molecule will eventually be surrounded by a small amount of water and it is possible that the observed decrease in molecular weight could come about if some parts of each molecule were held more strongly to the mica surface during the final stages of drying. If this were so, then the forces accompanying the receding water phase could possibly bring about rupture of covalent bonds and hence a decrease in molecular weight. It has already been reported that, when a DNA solution, denatured by dilution followed by freeze drying and dissolution to $3 \cdot 10^{-3}$ $M$, is precipitated from 1 $M$ NaCl with ethanol, a further irreversible change occurs and that this is accompanied by decreased molecular weight[18]. This is added evidence that

the denatured material is rather susceptible to degradation on rapid changes from the dissolved to the solid state.

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# DEOXYPENTOSE NUCLEIC ACIDS

## XV. THE SEDIMENTATION OF CALF THYMUS DEOXYRIBONUCLEIC ACID IN 95% ETHANOL

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SUMMARY

The sedimentation properties of calf thymus DNA dissolved in ethanol have been studied and sedimentation coefficient distributions have been obtained. Evidence is presented for the collapsed state of the DNA molecule in ethanolic solution compared with aqueous solutions.

Abbreviation: DNA, deoxyribonucleic acid.