that the greatest amount of type 2 was isolated from low temp. preparations, where the least decomposition would be expected to take place. Possibly, the low temp. preparations contain the highest concn. of the decomposing agent. The decomposition of type 1 to type 2, which must occur in crude preparations or on the column, is different from the inactivation directly observed for the isolated LDH of type 1. This inactivation was associated with the appearance of fluorescence and no spectral changes. From the resulting products the mechanism of the decomposition of type 1 to type 2 can be reconstructed. Initially, half of the flavin is dissociated and the binding of the heme is altered so that half or more of the activity is lost and the spectral properties are changed. Further destruction can take place without dissociation of the remaining flavin (or increase in fluorescence).

Alteration of the binding of the heme does not necessarily cause inactivation. This is evident from the facts that type 2 had been isolated with a high TN (6000/flavin) and that the heme was observed to become irreducible by lactate without decrease in activity.

ACKNOWLEDGEMENT

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DENATURATION OR DISSOCIATION OF DEOXYRIBONUCLEIC ACID IN DILUTE SOLUTION

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SUMMARY

Ultracentrifugal analysis of dilute solutions of deoxyribonucleic acid at low ionic strength shows that the sedimentation boundary is composed of two molecular species at pH values lower than 5. The proportion of each component varies from 100 to 0 % in the pH range from 5 to 4.

Combination of the sedimentation and viscometric data at pH 7 and 4 indicates that one component is constituted of native molecules and the other one of denatured material.

The sedimentation pattern proves also that the denaturation process is "all-or-none" in these conditions.

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INTRODUCTION

Since the publication by WATSON AND CRICK[1] of their twin helix model for deoxyribonucleic acid (DNA) and their hypothesis on the mechanism of DNA duplication in living cells by a simple dissociation of the double stranded model, many investigations have been published in connection with this important problem.

In principle, the two parts of the DNA molecule being held together presumably by hydrogen bonds only, introduction of hydrogen bond-rupturing agents ought to be sufficient to split the molecule into two halves.

STACEY AND ALEXANDER[2], using urea to rupture the hydrogen bonds, have shown that the light-scattering molecular weight drops to about one half after this treatment DOTY AND RICE[3,4,5], on the other hand, did not find, in the presence of 8 *M* urea, a significant decrease in molecular weight. DOTY and his collaborators have interpreted many of their experimental results on the basis of a "denaturation" of the DNA molecule (change of shape at constant molecular weight) and have drawn attention to the importance of the ionic strength of the medium. THOMAS[6] and several other authors have shown also that an important increase in the u.v. adsorption at 260 mμ is observed when the molarity of NaCl in the solution is decreased. This effect has been related to hydrogen bond ruptures in the twin helix model. The same effect is observed when the pH is lowered; it occurs at higher pH for lower salt concentrations.

In this paper, we report results obtained on DNA solutions at low ionic strength by ultracentrifugal analysis and viscometry with variable gradient. Our aim was to see whether in these special conditions the DNA molecules are dissociated or only denatured.

EXPERIMENTAL

DNA samples from calf thymus and liver were prepared by the dodecylsulphate method of KAY, SIMMONS AND DOUNCE[7]. We have found that this procedure yields high molecular weight material with a protein content of less than 1 %, provided that the treatment with the detergent is repeated 3 times.

The DNA samples were always dissolved in redistilled water (0.15 g/100 ml) and dialysed in the cold against redistilled water for 48 h. Solutions were made up by mixing the required amount of stock solution with acetate buffer at the desired pH.

Sedimentation analyses at 0.01–0.1 % DNA were performed in the large rotor, equipped with a 30-mm cell, of the Spinco model L Ultracentrifuge.

Viscosity measurements were made in function of shear rate in a capillary viscometer with variable pressure described elsewhere[8]. Concentrations of DNA were measured by optical density at 260 mμ in a Beckmann model DU spectrophotometer, the extinction of a 1 % solution in 0.2 *M* NaCl at pH 7 being taken as equal to 213.

RESULTS

(1) *Ultracentrifugal analysis*

For DNA concentrations of 0.04 %, in acetate buffer 0.002 *M* and in the pH range 4–5, we found for all the samples studied that the sedimentation picture resolved into two well-separated components of variable proportions. This is shown in Fig. 1.

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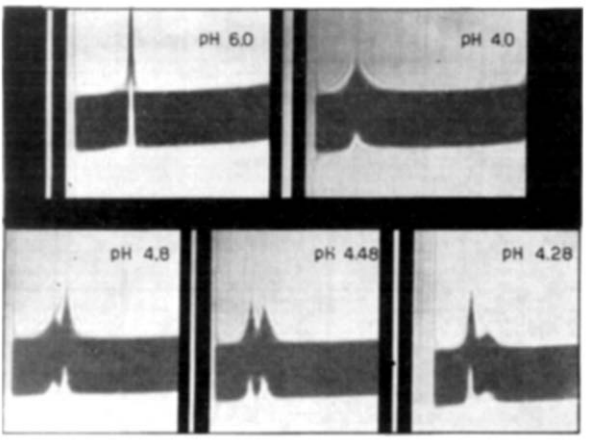

Fig. 1. Sedimentation boundary at various pH values for T-DNA-43. Sedimentation proceeds from left to right.

Table I gives the results for several samples.

The variations of S_1 and S_2 as a function of pH for the 3 samples and for the ionic strength 0.0025 are given in Fig. 2. The same behaviour is observed in the 3 cases; there is only a small variation of S_1 from pH 7 down to about pH 4.6, but when the pH is decreased further both S_1 and S_2 increase rapidly. This is to be expected since it is below pH 5 that the ionisation of cytosine and adenine begins;

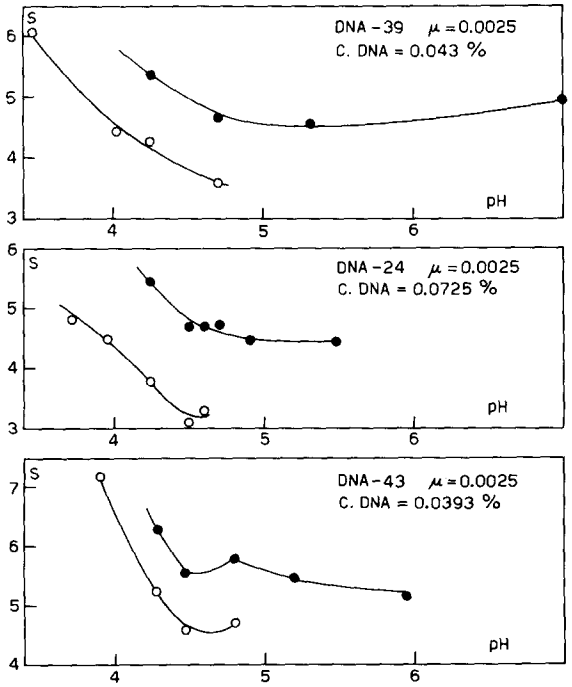

Fig. 2. Sedimentation constants in function of pH.

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TABLE I

The number following DNA refers to its intrinsic viscosity (100 ml/g) at zero shear rate in 0.2 *M* NaCl; L = liver and T = thymus. The proportion of each component has been estimated from the corresponding surfaces on the sedimentation picture enlarged 10 times; the accuracy is ± 5%.

Sample	*DNA concn.*	*Ionic strength* μ	*pH*	*Fast component*		*Slow component*	
				%	S_1	%	S_2
T-DNA-39	0.043	0.0025	7.0	100	4.95	0	—
T-DNA-39	0.043	0.0025	5.31	100	4.55	0	—
T-DNA-39	0.043	0.0025	4.70	57	4.64	43	3.60
T-DNA-39	0.043	0.0025	4.25	20	5.35	80	4.26
T-DNA-39	0.043	0.0025	4.02	0	—	100	4.41
T-DNA-39	0.043	0.0025	3.47	0	—	100	6.07
T-DNA-39	0.043	0.004	4.46	57	5.27	43	4.42
T-DNA-39	0.043	0.0005	5.00	53	3.83	47	2.84
T-DNA-39	0.043	0.0005	4.75	35	4.15	65	2.91
T-DNA-39	0.043	0.0005	4.32	10	—	100	3.68
T-DNA-39	0.0258	0.0025	7.0	100	5.90	0	—
T-DNA-39	0.0129	0.0025	7.0	100	7.87	0	—
T-DNA-39	0.0065	0.0025	7.0	100	10.3	0	—
T-DNA-39	0.043	0.0025	4.0	0	—	100	4.41
T-DNA-39	0.0322	0.0025	4.0	0	—	100	7.80
T-DNA-39	0.0215	0.0025	4.0	0	—	100	11.8
T-DNA-39	0.0107	0.0025	4.0	0	—	100	14.3
L-DNA-24	0.0725	0.0025	5.48	100	4.43	0	—
L-DNA-24	0.0725	0.0025	4.90	100	4.48	0	—
L-DNA-24	0.0725	0.0025	4.70	100	4.75	0	—
L-DNA-24	0.0725	0.0025	4.60	50	4.73	50	3.30
L-DNA-24	0.0725	0.0025	4.50	40	4.68	60	3.07
L-DNA-24	0.0725	0.0025	4.25	20	5.45	80	3.77
L-DNA-24	0.0725	0.0025	3.95	0	—	100	4.49
L-DNA-24	0.0725	0.0025	3.72	0	—	100	4.80
T-DNA-43	0.0393	0.0025	5.95	100	5.16	0	—
T-DNA-43	0.0393	0.0025	5.20	100	5.49	0	—
T-DNA-43	0.0393	0.0025	4.80	80	5.80	20	4.71
T-DNA-43	0.0393	0.0025	4.47	55	5.54	45	4.57
T-DNA-43	0.0393	0.0025	4.28	35	6.31	65	5.26
T-DNA-43	0.059	0.0025	4.28	80	5.44	20	4.96
T-DNA-43	0.0196	0.0025	4.28	0	—	100	10.0
T-DNA-43	0.0393	0.0025	3.96	0	—	100	7.21
T-DNA-55	0.061	75% H_2O 25% Diox	—	70	1.80	30	1.01
T-DNA-55	0.061	62% H_2O 38% Diox	—	50	1.88	50	1.23
T-DNA-55	0.061	50% H_2O 50% Diox	—	10	2.68	90	2.13

consequently the net charge on the molecule decreases and the sedimentation coefficient increases because the charge effect remains important at low ionic strength.

The proportion of the slow sedimenting component as a function of pH is plotted in Fig. 3 for the 3 samples. The results for T-DNA-39 and T-DNA-43 are very similar, complete separation into two components occurring between pH 5 and 4. For the L-DNA-24 sample the range of pH appears to be substantially reduced. The difference could be due to the smaller average molecular weight of the liver sample, which is about half that of the other two.

For the T-DNA-39 sample, several results as a function of the ionic strength are plotted on Fig. 4 (see also Fig. 3): an increase in ionic strength shifts the separation into two components towards lower pH. This is similar to the displacement of the

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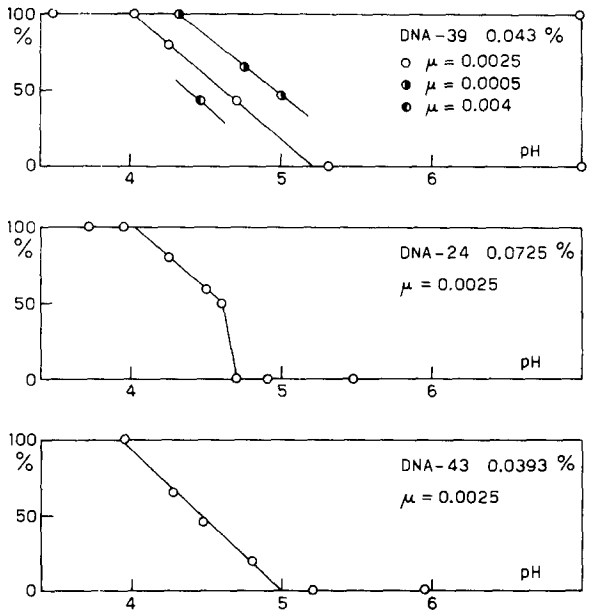

Fig. 3. Percentage of the low sedimenting component in function of pH.

titration curve as a function of salt concentration shown by COX AND PEACOCKE[9]. The effect of DNA concentration at constant pH and constant ionic strength on the proportion of the two components is shown in Fig. 5 for the sample T-DNA-43: when the DNA concentration is decreased by a factor of 3 a 100% change is observed. With a DNA concentration of more than 0.07 % there is 100 % of the fast sedimenting species and with a DNA concentration of less than 0.02 % there is 100 % of the slow one.

The relation between the sedimentation constant and the DNA concentration for the two distinct components is evident only at pH 7 and 4 where one single component is observed. In Fig. 6, we have plotted $1/S$ as a function of the total DNA concentration of T-DNA-39 for the two pH values mentioned. The curious feature of these results is that the values of $1/S$ yield almost the same extrapolation

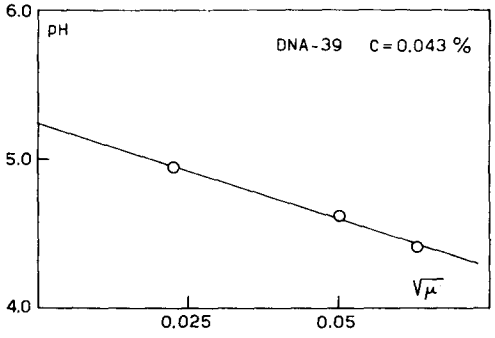

Fig. 4. Influence of ionic strength on the pH corresponding to 50 % of each component.

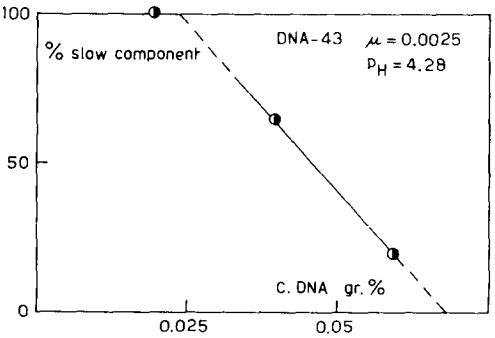

Fig. 5. Influence of the total DNA concentration on the composition of the mixture.

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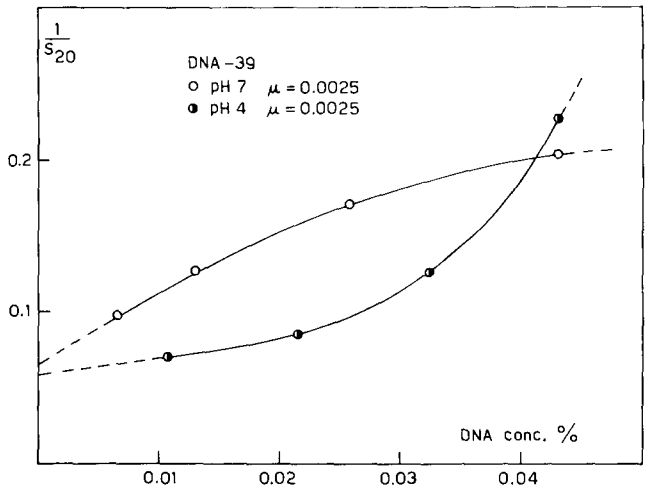

Fig. 6. Dependence of the sedimentation constants on DNA concentration at pH 7 and 4.

for the two pH, the curvature of the $1/S$ plot *versus* concentration being concave at pH 4 and convex at pH 7.

Three results obtained with the T-DNA-55 sample and listed in Table I show that the same kind of effect is observed when the dielectric constant of the solution is reduced by addition of an increasing amount of dioxane.

(2) *Viscometry*

For some of the samples, we measured the viscosities as a function of the shear stress at various pH. The extrapolations at zero shear and zero concentration give the intrinsic viscosities $[\eta]$ listed in Table II.

TABLE II

T-DNA-39				*T-DNA-43*	
Ionic strength 0.0025		*Ionic strength 0.2*		*Ionic strength 0.0025*	
pH	$[\eta]$	*pH*	$[\eta]$	*pH*	$[\eta]$
6.0	77	6.2	39	6.5	73
5.0	75	5.6	40	5.1	60
4.95	69	4.5	38	4.8	52
4.7	58	4.05	36	4.58	44
4.45	42	3.75	33	4.3	36
4.18	25	3.25	25	4.0	20
4.0	21	—	—	3.18	8
3.7	19	—	—	—	—

Fig. 7 shows the shear dependence of the viscosity of T-DNA-43 for a concentration of 0.00472 at various pH. We use here the plot $1/\eta_{rel.}$ *versus* pressure, which has been previously found[8] to be linear in the range of velocity gradient used (from 10 to 150 sec^{-1}). One sees that the influence of the pressure becomes negligible at pH 4.3. The intrinsic viscosities as a function of pH are plotted in Fig. 8 for two

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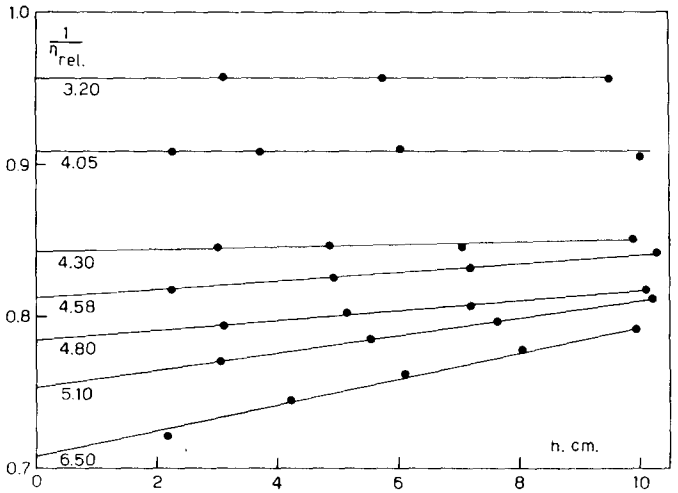

Fig. 7. Shear stress dependence of the relative viscosities at various pH.

samples. The effect of the ionic concentration is clearly shown in this figure; the decrease of $[\eta]$ at 0.2 M occurs one pH unit lower than at 0.0025 M. We observe a quantitative difference between the two samples at 0.0025 ionic strength, the change of $[\eta]$ with pH being sharper for the T-DNA-39 sample. This could perhaps be explained by a difference in molecular weight distribution of the two samples.

The experimental facts may be summarized as followed:

(1) For DNA concentrations of 0.05 % and ionic concentrations of 0.0025, the ultracentrifugal analysis shows the gradual appearance of a second slower component when the pH drops to below 5. This second component increases at the expense of the original one as the pH is lowered to 4, and at this pH and for values still lower only this component remains; its sedimentation picture reveals a much broader distribution than that of the original one at pH 7.

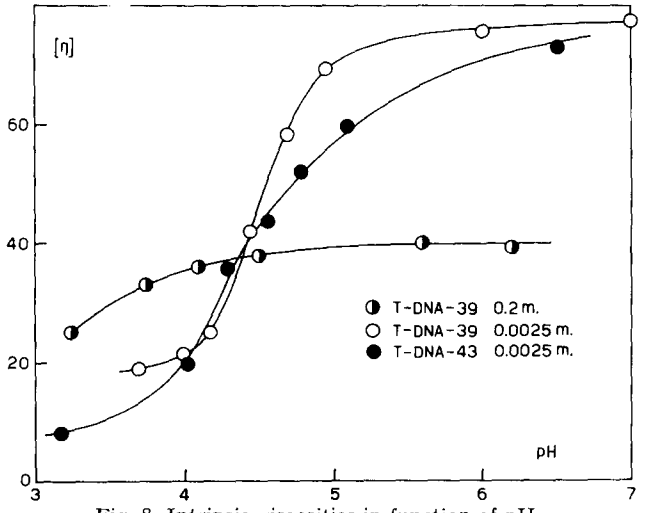

Fig. 8. Intrinsic viscosities in function of pH.

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(2) At any given pH between 5 and 4, the amount of the slow component increases when (a) the ionic strength decreases, (b) the total DNA concentration decreases, (c) the dielectric constant decreases.

(3) At pH 4 and 7, the sedimentation constants extrapolated at zero DNA concentration are almost identical.

(4) The shear rate dependence of the viscosity is pronounced at pH 7 and becomes almost non-existent at pH 4.

(5) The intrinsic viscosities at zero shear rate decrease sharply in the pH range 5–4.

DISCUSSION

To interpret the results that have been presented here several hypotheses may be considered:

(1) There is a progressive hydrolysis of the phosphoester bonds under these conditions of pH and ionic strength, and consequently a decrease in molecular weight. This hypothesis can be ruled out because it is very difficult to imagine any degradation process leading to two discrete distributions of molecular weights. Moreover, there is no change of composition of the mixture during the sedimentation run and solutions centrifuged immediately after preparation or 24 h after yield the same result.

(2) There is a dissociation of the twin helix molecule with decrease of the molecular weight to one half.

(3) There is denaturation of the DNA involving change of the shape of the molecule (coiling) without change in molecular weight; one component would correspond to the native state (hydrogen-bonded, double helix model) and the other one to the denatured state (almost not hydrogen-bonded, but still double-stranded molecule, more flexible and with a more compact configuration). This hypothesis has been proposed by DOTY AND RICE[3,4,5] on the basis of results obtained by light scattering. However, these authors have pointed out that throughout their experiments the DNA has never been subjected to low salt concentration. We, however, have decreased the salt concentration in order to keep the interactions between molecules and inside one molecule as large as possible and to allow the two polynucleotidic chains to repel each other when the total DNA concentration is low enough.

If we attempt to find out whether, the experimental results presented in this paper are compatible with a denaturation or with a dissociation process, we are facing a difficult problem. It could be solved by measurements of the molecular weights at pH 7 and 4 in the same conditions of ionic strength (0.0025). Unfortunately, we have not been able to use the light-scattering technique in this case because the level of the scattering is much too low at this ionic strength. The only alternative, therefore, is to combine the data obtained from viscometry and sedimentation analysis.

To calculate the molecular weight M from $[\eta]$ and S, we have the following expressions:

for free-draining random coil[10]:

$$S = \frac{R^2 \, (1 - \bar{V}\varrho)}{3{,}600 \, \eta_0 \, [\eta]} \tag{1}$$

for matted coil[11]:

$$S = \frac{M^{2/3} \, (1 - \bar{V}\varrho)}{[\eta]^{1/3} \, \eta_0 \, N_a} \, 2.5 \cdot 10^6 \tag{2}$$

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for elongated rod-like particle[12]:

$$S = \frac{M^{2/3}(1 - \bar{V}\varrho)}{[\eta]^{1/3}\eta_0 N_a}(\ln 2p)^{2/3}\ 1.05\cdot 10^6 \tag{3}$$

with

$$100[\eta]\varrho^* = Ap = \frac{p^2}{3.26 \ln 2p} \quad \text{for } p \gtrsim 50 \tag{3'}$$

In all these relations; S is the sedimentation; M, molecular weight; $[\eta]$, intrinsic viscosity; V, partial specific volume of the solute = 0.55; ϱ, density of the solvent; ϱ^*, hydrodynamic density of the particle; η_0, viscosity of the solvent; N_a, Avogadro's number; p, axial ratio of the molecule = length L/diameter $2r$; R, root mean square end-to-end distance of the random coil model.

Equation (1) is independent of M and is useless for our purpose. Moreover, this relation has never been quantitatively checked by the experimental data. For instance, in the case of the sample T-DNA-39, the values of M and R measured by light scattering at pH 7 in 0.2 M NaCl are respectively $4\cdot 10^6$ and $5\cdot 10^{-5}$ cm, and S and $[\eta]$, in the same conditions, are respectively $21\cdot 10^{-13}$ sec and 39 in 100 ml/g; if we calculate S from eqn. (1), we obtain $S = 9\cdot 10^{-13}$ which is far from the experimental value of $21\cdot 10^{-13}$.

Table III lists the calculated values of M by means of relations 2 and 3.

TABLE III

pH	*Ionic strength*	$[\eta]$	p	$S\ 10^{13}$	$M\ 10^{-6}$ *rel. 2*	$M\ 10^{-6}$ *rel. 3*	$M\ 10^{-6}$ *light scattering*
7	0.2	39	350	21	6.3	3.6	4.0
7	0.0025	77	500	17	6.5	3.5	—
4	0.2	36	340	22	6.5	3.5	—
4	0.0025	21	250	19	4.0	2.4	—

From the table we see that it is only in the case of pH 4 at low ionic strength that the molecular weight, calculated with a given equation, decreases. But if we consider the value measured by light scattering, we see that eqn. (3) gives the best fit for the three first cases and eqn. (2) for the last one (pH 4, 0.0025 M). Moreover, if we take into account the shear stress dependence of the viscosities (important shear effect for the three first cases and no effect for the last one), we must conclude that the choice of relation 3 for the calculation of M in the three first cases and of relation 2 for the last one is justified.

Consequently, the molecular weight probably remains constant and independent of pH and ionic strength; we are thus dealing with a denaturation process without evidence of dissociation of the double helix molecule. This confirms the conclusion of DOTY AND RICE[3,4,5] obtained from light-scattering data at higher ionic strength. The fact that we are able to observe two separated components at low ionic strength proves that the denaturation is an "all-or-none" process; in other words, when a few hydrogen bonds are broken in a given molecule, the remaining ones are easily and rapidly split. The new molecules coexist with the native ones if the rate of reformation of the hydrogen bonds is much slower than the rate of denaturation. When

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the reversibility of the process is studied, one finds that by bringing the solution back to pH 7, the sedimentation constant is higher than the original one and that the distribution of the sedimentation constants is apparently somewhat broader. The viscosity of a solution exposed to pH 4 and brought back to pH 7 rises immediately to about half its original value and continues to rise slowly during several days, reaching about 75 % of its original value after three weeks. Hydrogen bonds probably reform at random and the molecules reorganize themselves extremely slowly. Some experiments indicate that the rise of η_{sp}/C towards the original value is more rapid at lower DNA concentration.

Finally, a last question arises: why is it not possible to observe the two components at higher ionic strength in the pH range from 4 to 3 for instance? The reason is probably the following: a high salt concentration reduces the differences between the sedimentation constants of the two kinds of molecules in the range of DNA concentration accessible by the ultracentrifugal analysis and one observes only a broadening of the sedimentation boundary.

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