Structure and Properties of Rat Thymus Deoxyribonucleoprotein

SOLUBILITY PROPERTIES AND EFFECTS OF PRECIPITATION

By RUTH F. ITZHAKI*

Department of Radiotherapeutics, University of Cambridge

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1. Deoxyribonucleoprotein was prepared from rat thymus and was studied chiefly by the method of electric birefringence. The birefringence depends on the electrical and optical properties of the molecules and on their volume; the decay time of birefringence (after the removal of the electric field) depends on molecular length. 2. A comparison was made of the properties of deoxyribonucleoprotein redissolved after precipitation in 0.15M-sodium chloride with those of the original material, the main object being to find if structural changes have occurred in the former. As a preliminary, the dependence of the solubility of the deoxyribonucleoprotein on the concentration of added salt was investigated, and the birefringence properties were also studied after the addition of sodium chloride at low concentrations, after the alteration of pH and after dialysis. 3. It was found that precipitation of deoxyribonucleoprotein occurs in two fractions, beginning at about 0.4mM-sodium chloride. The solubility is minimal at about 0.15M-sodium chloride. 4. The electric birefringence and decay time of the deoxyribonucleoprotein decrease with increasing concentration of added sodium chloride, and the birefringence also decreases after dialysis. Prolonged dialysis leads to precipitation. 5. The properties of deoxyribonucleoprotein redissolved after precipitation with 0.15M-sodium chloride differ considerably from those of the original deoxyribonucleoprotein. This is attributed to some type of disorganization process occurring during precipitation. It is concluded that the organization of the original structure is very specific.

Studies have been made in this Laboratory on dNP† gels and their dilutions, chiefly by the method of electric birefringence (Itzhaki, 1962, 1966*a,b,c*). This method gives information about the optical and electrical properties of the molecules and their length. Centrifugation of the diluted gels was found to produce a gel phase and a non-gelatinous solution phase, showing that the dNP was incompletely dispersed. However, the birefringence measurements indicated that the units oriented in the electric field were of the same range of lengths at all dNP concentrations and in both gel and solution phase, i.e. they were independent of the state of dispersal of the dNP. It was therefore suggested that, on dilution, the dNP gels broke into clusters of variable size containing associated particles, the extent of association probably depending on concentration. In the electric field the clusters were more or less broken so that the individual particles were oriented. The structure of the individual particle was

tentatively suggested to be a lateral aggregate of single dNP molecules, dissociating to a limited extent on dilution. The satisfactory reproducibility of the results and the stability of the material have been described (Itzhaki, 1966*a*).

dNP is known to be insoluble in 0.15M-sodium chloride. It was thought that precipitation of dilutions of dNP gels by added salt solution would therefore be a way of purifying and of storing the material, since storage by freezing and freeze-drying has proved unsatisfactory (Itzhaki, 1966*a*). Purification was of interest because the preparation procedure, though carried out under strictly controlled conditions, was intentionally very simple and mild, the original aim being to prepare material retaining a 'native' structure. Indeed, it was found later that the properties of the dNP depended markedly on conditions of preparation (Itzhaki, 1966*c*).

The present paper describes the properties of reprecipitated dNP. As a preliminary, it was necessary to study the variation of the solubility of the dNP with concentration of added salt, and

* Present address: Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, 20.

† Abbreviation: dNP, deoxyribonucleoprotein.

to find the effects on the birefringence properties of varying the salt concentration and pH and of dialysis.

Solutions of reprecipitated dNP were, in fact, less stable than the original material, and the structure was apparently irreversibly altered. Some but not all of the properties resembled those of dNP after the addition of enzymes, and it is suggested that a gross disorganization process, possibly involving lateral disaggregation into intact sub-units, occurs when the dNP is precipitated with 0.15M-sodium chloride.

MATERIALS AND METHODS

dNP preparation. dNP was prepared from rat thymus as described previously (Itzhaki, 1966a,c). In brief, the tissue was homogenized in 0.15M-NaCl in 5mM-phosphate buffer, pH 7.4 (solution A), and the mixture was centrifuged. The resulting precipitate was rehomogenized in solution A, the mixture centrifuged and the precipitate homogenized in double-distilled water. The mixture was then filtered through gauze and dialysed overnight against double-distilled water. The final product, which was an elastic opaque gel of concentration usually within the range 0.2–0.3% DNA, was diluted usually 10–50-fold in solutions of 10 μ M-Na₂HPO₄–40 μ M-NaOH, pH 6.8 (solution B). The dilutions were gelatinous at concentrations greater than about 0.01% DNA but are referred to as solutions. (For convenience, all dNP concentrations are given in terms of DNA.)

DNA, RNA and protein analyses. DNA concentrations were estimated either from E_{260} values, $E_{1\text{cm}}^{1\%}$ at 260m μ being taken as 210, or by the diphenylamine method (Dische, 1955). RNA was estimated by the Schmidt & Thannhauser (1945) method. Protein was estimated by a micro-biuret method (Itzhaki & Gill, 1964).

Acid-soluble fractions. Measurements of the acid-solubility of the dNP were made as follows. 2N-HClO₄ was added to dNP solution to give a final concentration of 0.67N, and the mixture was kept at 0° for 15 min. It was then centrifuged at 3000g for 15 min. The DNA concentration of the supernatant was calculated from E_{260} , $E_{1\text{cm}}^{1\%}$ at 260m μ being taken as 270; the protein was measured by the micro-biuret method, correction being made for the very small HClO₄ blank.

Metal-ion analyses. Ca²⁺ and Na⁺ concentrations were measured by flame photometry (with a Unicam SP.900 photometer). Analysis of Ca²⁺ was made on material extracted by 0.05N-HClO₄ at 70° overnight (R. F. Itzhaki, unpublished work). Analysis of Na⁺ was made directly on dNP solutions.

Dialysis procedure. Visking dialysis tubing was boiled for about 10 min. in distilled water and was then rinsed several times in distilled water. During dialysis, the liquid outside the bag was stirred by a magnetic stirrer but the bag was secured to reduce agitation, since vigorous shaking had been found to affect dNP solutions (Itzhaki, 1966a).

Electric birefringence. In this method, birefringence is induced in a solution through orientation of solute molecules in an electric field. Details of the method and apparatus have been given elsewhere (Itzhaki, 1966a,c).

In brief, the birefringence (Δn) at a given field strength (E) depends in a complex way (Tinoco, 1955) on the volume-fraction of the molecules, on their dipole moment and on their optical and electrical polarizability. Thus a decrease in birefringence of a given system reflects a decrease in molecular size or charge.

On removal of the electric field, the molecules disorient through Brownian motion and the birefringence decays exponentially. For ellipsoidal molecules, the relaxation time, T , is related to the molecular length, $2a$, thus:

$$1/6T = 3kT'(-0.5 + \ln 2a/b)/8\pi\eta a^3$$

where $2b$ is the length of the equatorial axis, η the solvent viscosity, T' the absolute temperature and k the Boltzmann constant. Thus T depends strongly on molecular length but only slightly on axial ratio.

Graphs of $\log \Delta n$ against $\log E$ were found to be approximately linear, with gradient m , over the voltage range 200–600 v (field strength 370–1100 v/cm.), values of Δn and of m increasing with increasing dNP concentration. For convenience, the birefringence is given here as $\Delta n/\lambda$, where λ is the mean wavelength of the incident light, and quoted values of Δn are for a voltage of 250 v. (Clearly, for two samples with similar values of m , comparison of Δn anywhere in the linear range would give similar results.) The accuracy of measurement of Δn was about $\pm 2\%$.

The birefringence decay curves were fitted approximately by the sum of two exponentially decaying terms of decay times T_1 and T_2 (where $T_2 \geq 20T_1$ usually). These times were measured by a simple analogue method, the accuracy being within about $\pm 17\%$ for T_2 and $\pm 30\%$ for T_1 (Itzhaki, 1966a).

RESULTS

Except for dialysed reprecipitated samples, the nucleoprotein preparations usually contained 36–39% nucleic acids. The Na⁺ concentration was 0.1–0.18M (after correction for the Na⁺ in solution B) in dNP solutions of 0.01% DNA, and the Ca²⁺ concentration was 13–25 μ M.

Precipitation by sodium chloride, calcium chloride and magnesium chloride. Solutions of sodium chloride were added to dNP solutions to give final concentrations of 0.1M–1.0M. The mixtures were kept at 0° and, after centrifugation at 3000g for 15 min., the E_{260} values of the supernatants were measured.

On addition of sodium chloride to give final concentrations between about 0.5M and 3M, there was no visible precipitation, but gelatinous sediments were obtained on centrifuging; between 3M and 30M, gelatinous suspensions formed slowly. To find if there was a time effect, the E_{260} values of supernatants obtained from samples kept for 4 hr. and 21 hr. in 1M- and in 10M-sodium chloride before centrifugation were compared; at each concentration, the values for the two times were almost identical, showing that the action was complete within 4 hr. At final sodium chloride concentrations above about 30M, fibrous precipitates formed, the action being complete within 30 min.

Fig. 1 shows a composite graph of the percentage of the total DNA in the soluble fraction against sodium chloride concentration for different precipitation times (30 min.–21 hr.) and different dNP concentrations (0.007–0.015% DNA). The onset of insolubility is at about 0.4–0.5 mM-sodium chloride.

The results of Fig. 1 agree with those of Oth & Desreux (1957), who studied the solubility of calf thymus dNP in sodium chloride solution. They obtained a two-component curve, precipitation beginning at 0.1 mM- and reaching a maximum at about 0.14 M-sodium chloride. However, only about 40% of the dNP was precipitated in the first stage (below about 10 mM-sodium chloride), whereas in the present work the amount was 60–70% of the total.

Addition of calcium chloride and magnesium chloride to final concentrations of 0.11 mM caused immediate formation of gelatinous suspensions in dNP solutions of 0.02% DNA.

Dependence of birefringence properties on salt concentration. Addition of solutions of sodium chloride to dNP solutions in the ratio 1 : 100, to give

Fig. 1. Variation of solubility of dNP with concentration of NaCl solution.

final concentrations of 0.24–0.8 mM-sodium chloride, caused a progressive decrease in birefringence and, at the higher salt concentrations, a decrease also in decay time and in gelatinousness. Table 1 shows the changes in birefringence properties. The changes were not due to a pH effect, the dNP–sodium chloride mixtures being at pH 6.6–6.8 and the original dNP solutions at pH 6.7–6.9.

O’Konski (1960) suggested that DNA is oriented in an electric field through polarization of its ion atmosphere, and has shown that addition of salt to such a system would decrease the birefringence. If dNP is oriented, at least in part, through a similar mechanism, this would explain the above changes in Δn . The decrease in T_2 reflects, presumably, a contraction or a decrease in hydration of the dNP particle, due to the salt.

Measurements were made also at lower ionic strength by diluting dNP gels with distilled water. Values of birefringence were usually 90–110% of values for dilutions in solution B (Table 2). The variability may have been due to differences in extent of shaking on diluting the gels (this can affect Δn by 10–15%), but was not due to a pH difference, pH values being the same for dilutions in both solvents.

Dependence of birefringence properties on pH. The pH of the dNP solutions was adjusted by adding an appropriate volume of 10 MN-hydrochloric acid or 10 MN-sodium hydroxide slowly with shaking. To avoid appreciable increases in ionic strength, only a relatively small range of pH values was used. Also, addition of hydrochloric acid to final concentrations above 0.1 MN led to precipitation of the dNP. Table 3 shows that lowering the pH caused a decrease in Δn and in T_2 and that an increase in pH, at least to pH 7.5, caused a small increase in Δn and no change in T_2 .

A limited comparison can be made with the results of Houssier & Fredericq (1966), who studied

Table 1. Changes in the electric birefringence of dNP solutions after addition of sodium chloride

Expt. no.	Sample	Concn. of dNP (%)	Concn. of Na ⁺ (mM)			Birefringence 10 ³ (Δ <i>n</i> /λ) (cm. ⁻¹)	Birefringence (% of control)	<i>T</i> ₂ (msec.)	<i>T</i> ₁ (msec.)	<i>m</i>
			Initial	Added	Total					
1	dNP control	0.0103	0.14	0	0.14	6.22		100	2	0.52
	dNP+NaCl	0.0103	0.14	0.24	0.38	5.35	86	100	2	0.54
2	dNP control	0.0091	*	0		6.48		100	1	0.50
	dNP+NaCl	0.0091	*	0.30		5.21	81	83	1	0.46
3	dNP control	0.0098	0.13	0	0.13	5.11		126	2	0.54
	dNP+NaCl	0.0098	0.13	0.39	0.52	3.31	65	100	2	0.49
4	dNP control	0.0104	0.17	0	0.17	6.54		90	2	0.44
	dNP+NaCl	0.0104	0.17	0.45	0.62	4.22	64	72	2	0.43
5	dNP control	0.0095	0.17	0	0.17	6.50		86	2	0.47
	dNP+NaCl	0.0095	0.17	0.80	0.97	3.09	48	63	2	0.65

* Concn. not measured.

Table 2. *Comparison of the electric birefringence properties of dNP diluted in water and in 10 μ M-disodium hydrogen phosphate-40 μ N-sodium hydroxide (solution B)*

Expt. no	Diluent	Concn. of dNP (% DNA)	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	$\left(\frac{\Delta n/\lambda \text{ in water}}{\Delta n/\lambda \text{ in soln. B}}\right) \times 100$	T_2 (msec.)	T_1 (msec.)	m
1	Solution B	0.0151	10.6		100	2	0.55
	Distilled water	0.0156	9.85	93	100	2	0.50
2	Solution B	0.0086	5.85		100	1	0.52
	Double-distilled water	0.0087	5.85	100	100	2	0.52
3	Solution B	0.0162	11.0		100	2	0.60
	Double-distilled water	0.0164	11.4	104	100	2	0.42

Table 3. *Electric birefringence properties of dNP at different pH values*

Expt. no.	Sample	Concn. of dNP (% DNA)	pH	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	Birefringence (% of control)	T_2 (msec.)	T_1 (msec.)
1	dNP control	0.0115	6.63	8.29		100	2
	dNP in 0.075 mN-HCl	0.0115	6.20	5.90	72	72	2
	dNP in 0.1 mN-HCl	0.0115	5.92	5.23	63	72	2
2	dNP control	0.0120	6.80	7.08		100	2
	dNP in 0.1 mN-NaOH	0.0120	7.52	7.70	109	100	2

changes in the electric birefringence properties of calf thymus nucleohistone on changing the pH and ionic strength. Their material was prepared by a different method, and the 'gel-fraction' used was obtained by centrifugation of the original nucleohistone in 1 mM-sodium chloride, the suspension being dispersed finally in water; also, much higher electric field strengths were used. However, qualitatively their results are similar, Δn and T decreasing with increasing ionic strength. The authors relate these changes to the decrease in rigidity. In the present work, however, the particle length is independent of the state of dispersal (i.e. whether in gel or solution phase) and independent of concentration, and does not change on very vigorous shaking of the solution, although this decreases the gelatinousness (Itzhaki, 1966a). Thus the same units are oriented whatever their degree of interaction, though clearly the latter property determines the solution rigidity.

Houssier & Fredericq (1966) found also a decrease in Δn when the pH was adjusted both below and above pH 7, values at pH 6.0 and pH 7.5 being similar to those found in the present work. However, they obtained a decrease in T at the higher as well as at the lower pH value.

Dialysis of dNP solutions. Prolonged dialysis of dNP solutions against distilled or double-distilled water led to precipitation. After limited dialysis, the solutions were found to be less gelatinous and more turbid; on their being kept there was often a further change in these properties, and in some cases

precipitation occurred. These effects were more marked at lower dNP concentrations.

Changes were less on dialysis of dNP solutions against 10 μ M-EDTA, and even less against solution B, than on dialysis against water. To check that this was not due to a pH difference (solution B being at pH 6.8, the EDTA solution at pH 5.9 and distilled water at about pH 5.3), dialysis against solution B was compared with that against a solution of the same ionic strength but at lower pH (10 μ M-disodium hydrogen phosphate-40 μ N-sodium chloride, pH 5.5); the changes in the two dialysis residues were found to be similar.

There was no significant loss of DNA nor of protein after dialysis against distilled water. The Na⁺ content of the dNP solutions decreased after dialysis against distilled water to 30-40% of the original value, but the Ca²⁺ content increased to 170-190%. The birefringence of the dNP solutions decreased after dialysis, values being about 55-80% of the original value. There were no changes in T .

The above effects are probably due merely to loss of Na⁺ ions (plus anions) rather than to loss of a particular multivalent cation, as otherwise changes would be greatest on dialysis against EDTA. (Presumably solution B acts by removing part of the excess of residual salt in the dNP.) However, the birefringence changes are unexpected, since, judging by the effects of added salt, removal of salt should increase Δn and T . Possibly the salt loss, though rather small, causes some sort of denaturation.

Table 4. *Changes in the properties of dNP after precipitation in 0.15M-sodium chloride*

In this experiment, two samples of dNP solution were precipitated separately in 0.15M-NaCl; one of these was to be used for analyses and so was redissolved to give a solution of relatively high dNP concentration. Sample P_1 was prepared by mixing 22.5 ml. of dNP solution with 2.5 ml. of 1.5M-NaCl, and sample P_2 by mixing 9 ml. of dNP solution with 1 ml. of 1.5M-NaCl. Both mixtures were kept at 4° for 5 hr. and were then centrifuged at about 1000g for 20 min. The supernatants, which contained less than 4% of the 260m μ -absorbing material, were removed and the precipitates were drained well. The precipitate of sample P_1 was dissolved in 22 ml. of double-distilled water. The precipitate of sample P_2 was squeezed lightly on filter paper and was then dissolved in 30 ml. of double-distilled water. Control samples of the original unpurified dNP are referred to as C_1 and C_2 .

(a) Birefringence properties

Sample	Conc. of dNP (% DNA)	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	Birefringence (% of original value)	T_2 (msec.)	m
C_2	0.0061	4.05		120	0.52
P_2	0.0059	2.27	56	30	0.43

(b) Analyses

Sample	Concn. of DNA	Conc. of protein	Recovery of DNA (% of original content)	Recovery of protein (% of original content)	Solubility in NaCl (% of total DNA)	Acid-soluble DNA (% of total DNA)	Acid-soluble protein (% of total protein)	Concn. of Ca ²⁺ (μ M)	Concn. of Na ⁺ (mm)
C_1	0.0255	0.0436			< 4	~ 1	16	44	
P_1	0.0254	0.0399	98	90	< 2	~ 1	14		
C_2	0.0061				< 4			~ 13	~ 0.12*
P_2	0.0059		78†		< 2			~ 6	~ 0.35

* Including the Na⁺ content of solution B (0.06 mm).

† Presumably material was lost when the original precipitate was drained on filter paper.

Alternatively, the changes may be related to the increase in Ca²⁺ concentration. The dNP must have a great ability to bind this ion, which must have come from the external liquid or from the dialysis tubing.

Reprecipitation of dNP

General. Solutions of 1.5M-sodium chloride were added slowly, with vigorous stirring, to dNP solutions in the ratio 1 : 10. The mixtures were kept at 4° for known periods and were then centrifuged at about 1000g for 20 min. The precipitates obtained were dissolved in distilled or double-distilled water, giving slightly cloudy non-viscous solutions; these are referred to below as reprecipitated dNP. In initial experiments the latter were dialysed, to remove excess of salt, against water for several hours at 4° and finally against solution B. In some cases precipitation occurred during dialysis.

Birefringence changes. Birefringence properties were measured in samples that had not precipitated and values of Δn and of T were found to be considerably lower than those of control dNP. However, it was found later that dialysis of control dNP caused similar changes (see under 'Dialysis of dNP solutions'), although the relative decrease in bi-

refringence properties was smaller than that of the reprecipitated dNP and formation of suspensions was less rapid. Subsequent studies were therefore made on undialysed material.

Birefringence measurements showed that values of Δn and of T were lower in the reprecipitated undialysed dNP than in the control dNP at the same concentration. The changes varied greatly in different experiments, Δn decreasing to between 25% and 90% of the original value and T_2 to between 10% and 65%. Table 4 gives an example. The higher values of Δn (80–90%) were usually associated with low values of T_2 (20–30%). Attempts were made to find the cause of this variability by altering the following conditions during reprecipitation, but in no case was a correlation found: (1) length of time the dNP was kept in 0.15M-sodium chloride (30 min.–22 hr.); (2) initial concentration of dNP solution (0.008–0.032% DNA); (3) final concentration of reprecipitated dNP solution; (4) initial salt concentration (in several experiments the original dNP was precipitated by mixing with an equal volume of 0.30M-sodium chloride); (5) extent of stirring on mixing the salt and dNP solutions; (6) use of distilled or double-distilled water to dissolve the purified dNP; (7) rate of addition of sodium chloride.

During the application of the voltage pulses to reprecipitated dNP solutions, a suspension formed between the electrodes and the light transmitted decreased to 50–90% of the initial value. Occasionally therefore values of Δn could be calculated only for the initial signals, before precipitation became appreciable. With control dNP, including dialysed samples, the final light transmission was usually 96–100% of the initial value, and 90–100% with dNP plus added sodium chloride.

Recovery of DNA and protein. The recovery of DNA in the reprecipitated undialysed dNP was usually 96–99%, and the recovery of protein usually 80–90% (DNA/dNP ratio being 0.39–0.42, and for the original material 0.37–0.38). When the redissolved material was dialysed, the recovery of protein was only 73–75%, although the DNA recovery was 90–100% (DNA/dNP ratio being 0.44–0.49), i.e. there was a greater loss of protein than of DNA during precipitation and dialysis.

Solubility. In studies on the effects of added enzymes on dNP (Itzhaki, 1966b), the changes in solubility in 0.67N-perchloric acid and in 0.15M-sodium chloride were followed as a simple and rapid assay of enzyme action. Since some of the changes in reprecipitated dNP resembled those due to added enzymes, the solubility properties of the reprecipitated dNP were measured for further comparison. However, it was found that the values were similar to those for control dNP (see Table 4), although the latter formed fibrous suspensions in 0.15M-sodium chloride and in 0.67N-perchloric acid, whereas suspensions formed by reprecipitated dNP were thin and non-fibrous.

Also, in samples prepared for metal analyses by heating in perchloric acid, those with reprecipitated dNP were almost clear, whereas with control dNP thin suspensions were present.

Salt content and pH. The Ca^{2+} content of the reprecipitated dNP was 40–80% of that of the original control dNP; the Na^{+} content was about two- to five-fold higher than that of the original control dNP. Addition of sodium chloride to reprecipitated dNP (the salt concentration being increased from 0.38mM to 0.46mM) caused no change in Δn , but decreased T_2 further, from 38% to 20% of the control (a much greater change than would be expected with control dNP). Also, after addition of the salt the reprecipitated dNP was even more unstable in the electric field. Reprecipitated-dNP solutions were found to be at pH 6.3–6.5.

To find the effect of simultaneously decreasing the pH and increasing the salt content, the birefringence properties of samples of control dNP were measured after addition of hydrochloric acid and sodium chloride, in one experiment to concentrations of 75 μN and 0.8mM respectively, pH 5.7, and in the

other to 10 μN and 0.8mM respectively, pH 6.38. The first mixture resembled reprecipitated dNP in its appearance, and also was so unstable in the electric field that Δn could not be measured. T_2 , measured from an initial pulse, was found to be 63% of the control. In the second sample, the instability was much less marked; values of T_2 and Δn were 72% and 50% of control values respectively.

Precipitation in 0.15M-sodium chloride at pH 7.4. dNP was precipitated also by addition of 0.1 vol. of 1.5M-sodium chloride–50mM-phosphate buffer, pH 7.4 (thus giving a suspension of dNP in solution A). The products were clearer and more gelatinous than those obtained by precipitation in unbuffered sodium chloride and changes in birefringence properties were usually less, though still variable, Δn decreasing to 68–83% of the control value usually, and T to 38–72%. Also, there was less precipitation on application of the electric field. Again protein was lost, the ratio DNA/dNP being 0.40–0.43, and for controls 0.38.

dNP gels. dNP was reprecipitated from the gel state, an equal volume of 0.3% sodium chloride being added to ensure adequate mixing. The products resembled control dNP in their appearance and in their stability in the electric field, but values of birefringence and decay time were lower, the former decreasing to 60–90% and the latter to 50–80%. However, these changes, and also the loss of protein, were less than those in dNP reprecipitated from solution.

DISCUSSION

The general decrease in birefringence and decay time of dNP after reprecipitation suggests that a decrease in particle size has occurred (although, as discussed above, this and the observed decrease in gelatinousness are not necessarily related). There are four possible causes: enzymic action, increased salt content, decreased pH or a disaggregation process.

Comparison of reprecipitated dNP with enzyme-treated dNP. Addition of deoxyribonuclease and proteolytic enzymes to dNP causes degradation of their respective substrates, and the dNP–enzyme mixtures resemble reprecipitated dNP in their appearance and in the type of precipitate formed on addition of perchloric acid or of 0.15M-sodium chloride (Itzhaki, 1966b).

Degradation could occur during reprecipitation if enzymes were present as impurities from the preparation and if their action were more rapid on suspended dNP than on dNP in solution. dNP does show an aging effect in solution that may be due to enzyme impurities (Itzhaki, 1966a,b), and a more rapid action on suspended dNP could occur if, as suggested by Shooter, Davison & Butler (1954), the

enzymes are occluded on the fibrous debris present, as this would presumably be precipitated with the dNP.

Trypsin was found to act more rapidly on dNP suspended in 0.15M-sodium chloride than on dNP in solution, as measured by the rate of solubilization of the DNA component in 0.15M-sodium chloride [free DNA (and probably highly degraded dNP) is soluble in 0.15M-sodium chloride solution (Itzhaki, 1966b)]. However, after extensive trypsin action on dNP in solution, Δn increased and T decreased slightly. Limited trypsin action, on the other hand, decreased Δn but did not affect T , and again, in contrast with the precipitation process, made the dNP appreciably soluble in 0.15M-sodium chloride. [For example, an amount of trypsin causing a decrease in Δn to about 65% solubilizes 44% of the DNA after 5 min. suspension in added 0.15M-sodium chloride (total digestion time 40 min. at 0°).]

The rates of action of deoxyribonuclease on dNP in solution and in suspension have not been compared. However, on addition of deoxyribonuclease to dNP in solution both Δn and T decrease (Itzhaki, 1966b), but, for changes in Δn and T comparable with those in reprecipitated dNP, the fraction of the DNA that becomes acid-soluble is much greater.

Thus, if dNP is degraded on reprecipitation, there are no small fragments such as those produced by enzymes; also, there is no extensive separation of the DNA and protein components.

Comparison of reprecipitated dNP with dNP containing added salt. Table 1 shows that, with control dNP even at the highest added salt concentration (almost a sixfold increase compared with a maximal fivefold increase on precipitation), T_2 decreased only to about 73%. Thus the higher salt content alone cannot account for the frequently greater decrease in T and the variable changes in Δn on reprecipitation.

Comparison of reprecipitated dNP and dNP at lower pH. Table 3 shows that even at pH 6.0 T_2 for control dNP decreased only to 72%, so the lower pH of reprecipitated dNP solutions cannot be the sole cause of their change in properties. Also, dNP precipitated in solution A was obtained at pH 6.7–6.8, yet it differed considerably from controls. However, the effects of simultaneously decreasing the pH and increasing the salt content of control dNP show that these factors when combined may account for the instability, though not for the greater decrease in T , on reprecipitation of dNP.

Disaggregation of dNP on reprecipitation. Longitudinal disaggregation of dNP in 0.15M-sodium chloride is unlikely; covalent links would not be broken and, anyway, the dNP is not linked longitudinally (Itzhaki, 1966b) by say protein or metal ions (except possibly at loop-points, so that the

overall particle length is unaffected). Also, the great variation in values of T (expressed as a percentage of the original) for reprecipitated dNP in different experiments suggests that any longitudinal links dissociable in 0.15M-sodium chloride must be both numerous and broken at random, which seems improbable. Conversely, the constancy of values for T for control dNP (Itzhaki, 1966a) indicates that the unit oriented in the electric field is not a random longitudinal aggregate linked by secondary bonds.

If, however, the dNP has the lateral aggregate structure previously described (Itzhaki, 1966a), a decrease in Δn , without accompanying changes in solubility properties, could result from extensive lateral disaggregation into intact sub-units, i.e. single dNP molecules. Lateral disaggregation would decrease T also to some extent. For example, a change in axial ratio from 10:1 to 100:1 at constant molecular length would decrease T to about 50%. Possibly the cementing material between sub-units is protein; some protein is in fact solubilized in 0.15M-sodium chloride, though the loss is rather small. Loss of Ca^{2+} is unlikely to be the cause, as studies on control dNP with added EDTA suggest that metal ions are not important structurally within the dNP particles (Itzhaki, 1966b).

A further decrease in T could be due to contraction of the dNP sub-unit because of the relatively high salt content, the action of added sodium chloride indicating a greater sensitivity than that of the original aggregate to this factor.

The decay time depends only on molecular size, but the birefringence depends on size and charge. Thus the occasional high value of Δn combined with a low value of T suggests that the particle size may have decreased and the charge simultaneously increased during purification. If part of the DNA were revealed by displacement or removal of a small amount of protein, the particle charge and thence Δn would increase. The variability of the values of Δn and T would then be due to a variable extent of removal of protein (and therefore of disaggregation and of exposure of DNA) and to a variable salt content and pH.

A similar process of lateral disaggregation has been suggested to occur when dNP ages, and also when proteolytic enzymes are added to dNP; in the latter case this would be before damage to the protein of each sub-unit and subsequent release of DNA (Itzhaki, 1966b). Disaggregation in 0.15M-sodium chloride would then explain the more rapid action of added trypsin on dNP in suspension in 0.15M-sodium chloride than in solution (Itzhaki, 1966b). Also, displacement (as opposed to removal) of some protein in 0.15M-sodium chloride might account for subsequent loss of protein on dialysis of purified dNP.

Bernstein & Mazia (1953) found that a progressive decrease in stability accompanied repeated precipitation of sea-urchin sperm dNP by 0.15M-sodium chloride. However, they lost about 50% of the DNA and of the histone and 80% of the non-histone protein in the first precipitation.

When histones are fractionated with increasing concentrations of sodium chloride, the 'lysine-rich' fraction (Ia or F₁), comprising 20% of the total histones, is removed first (Phillips, 1962). Thus part of this fraction may have been removed or displaced during precipitation in 0.15M-sodium chloride in the present work. It is noteworthy that Littau, Burdick, Allfrey & Mirsky (1965) found that removal of 'lysine-rich' histones from thymus nuclei loosened the structure of the dense chromatin, giving a diffuse fibril network; they suggested that this fraction cross-links DNA molecules, but that 'arginine-rich' histone lies along the length of the DNA molecule.

Since the properties of the dNP are irreversibly changed merely by the mild treatment of precipitation in salt solution, the original organization of the dNP particle must be fairly specific, i.e. not easily regained once disrupted, and unlikely to have been formed by chance during preparation. Also, the fact that some of the properties of dNP after aging and after limited enzyme action resemble those of reprecipitated dNP suggests that in all three cases the dNP may be in a state of disorganization or denaturation, but that in the reprecipitated dNP this disorganization may be at a much more advanced stage.

In the preparation of dNP, the tissue is homogenized in solution A and finally dialysed against water. Since precipitation of dNP from solution by 0.15M-sodium chloride and solution A and also dialysis lead to irreversible changes, this subsequent lability must be attributed to the difference in concentration between the dNP in solution and

that in the tissue homogenate (10–50-fold lower concentration in solution). This is supported by the finding that much smaller changes occur on precipitation of dNP from the gel state (i.e. at high concentration) than from solution. The fact that there are changes in the former case may be due to the previous removal, during the preparation, of other cellular components that may protect the dNP. That the concentration of dNP is the main factor in producing and maintaining stable and 'native' material is supported by studies on preparations made at various dNP concentrations (Itzhaki, 1966c) and on the aging of dNP in the gel and solution state (Itzhaki, 1966a).

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