Structure and Properties of Rat-Thymus Deoxyribonucleoprotein

A COMPARISON OF THE ELECTRIC BIREFRINGENCE AND OTHER PROPERTIES OF DEOXYRIBONUCLEOPROTEIN PREPARED BY VARIOUS METHODS

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1. A comparison has been made of the properties of dNP (deoxyribonucleoprotein) prepared in various ways from rat thymus. The variables studied were: (a) extent of homogenization; (b) concentration of various metal ions; (c) concentration of EDTA; (d) extent of dialysis; (e) concentration of dNP. The chief method used for studying the dNP was that of electric birefringence; this gives information about the optical and electrical properties of molecules oriented in an electric field and about their length. 2. The extent of homogenization during the preparation affects the birefringence properties only slightly and the solubility properties of the constituent DNA not at all. However, the protein content of the dNP decreases after repeated homogenization. 3. In the presence of added Ca^{2+} , Mg^{2+} or Na^+ an insoluble product is obtained. 4. The presence of added EDTA affects the birefringence properties only slightly. 5. In the final stage of preparation, both very limited dialysis and extensive dialysis (in terms of volume ratio) cause partial insolubility of the final product. 6. dNP prepared at low concentrations resembles in some respects dNP prepared at higher concentrations and then subjected to limited enzyme action but it is not degraded (as by extensive enzyme action); ultracentrifuge studies of the constituent DNA confirm that no degradation of the DNA has occurred. 7. It is concluded that dNP prepared at low concentrations or by extensive dialysis is denatured and that preparation of 'native' material is favoured by maintenance of a high dNP concentration and by fairly limited dialysis.

Although dNP* and DNA have been studied for over 90 years, relatively little is known about the dNP complex. There is still controversy about the best method of preparing dNP (see review by Davison, Conway & Butler, 1954) and about whether or not material obtained as a gel is in a 'native' state (see review by Peacocke, 1960). Various difficulties have been associated with studies on dNP gels: first, relatively few physico-chemical methods could be used owing to incomplete dispersal of the gel on dilution; secondly, the dNP tended to be very unstable and such measurements as could be made were not very reproducible.

A series of studies has been made in this Laboratory on dNP prepared from rat thymus (Itzhaki, 1962, 1966a,b). Preparations were made under strictly controlled conditions and the procedure was very mild as it was hoped that this might prevent damage to 'native' structures. The final product was, in fact, a gel and centrifugation studies on dilutions of the gel showed the presence of a substantial gel phase, i.e. the dNP was not

completely dispersed. Despite this it was decided to attempt an investigation by the method of electric birefringence. It was found that use of this method obviated difficulties associated with dispersal of the dNP on dilution as, at all concentrations and in both gel and solution phase, units of a similar range of lengths were oriented in the electric field. Also, the results were reproducible from one preparation to another and the material was reasonably stable.

On the basis of these results it was suggested that dNP gels, consisting of a network of particles, dispersed progressively on dilution to form reversibly associated gel clusters. In the electric field, all clusters were more or less broken so that single particles were oriented. The particle structure was tentatively suggested to be a lateral aggregate of single dNP molecules, dissociating to a limited extent on dilution.

Since it was thought that differences in reproducibility and stability of dNP prepared by previous workers might be related to differences in preparative procedures, a study was made of the

* Abbreviation: dNP, deoxyribonucleoprotein.

changes in the dNP properties caused by altering certain conditions during preparation. The basic procedure used here and by most other workers for preparing dNP consists in homogenizing the tissue in 0.15M-sodium chloride and centrifuging; in general (though not here) the homogenization-centrifugation process is repeated a further four or five times before the final homogenization of the precipitate in water. Variants include the addition of arsenate, citrate or EDTA as enzyme inhibitors. Further modifications were introduced by Zubay & Doty (1959). They described a method for preparing single dNP molecules and considered that dNP gels were preparation artifacts whose formation was favoured by maintenance of a high dNP concentration during preparation. They suggested that other contributing factors were: delay before the final homogenization in water of the dNP precipitate, and use of too low a speed for the final homogenization.

This paper compares the properties of dNP prepared at various concentrations and by different total periods of homogenization, and prepared in the presence of added metal ions and of added EDTA. It was of interest also to follow the method of Zubay & Doty (1959), especially as some of their conditions had already been studied as separate factors.

The properties of the dNP depended greatly on dNP concentration during preparation and on salt concentration during dialysis at the final stage of preparation: at low values of the former and at relatively extreme values of the latter, the products were unstable. Comparison of the birefringence properties of these products with those of the standard dNP preparation described here suggests that the former had undergone some type of denaturation process.

MATERIAL AND METHODS

Buffer and solution. Solution A: 0.15M-NaCl in 5mm-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$), pH 7.4. Solution B: 10 μM - Na_2HPO_4 in 40 μN -NaOH, pH 6.8.

Dialysis. Visking dialysis tubing (24/32 in.) was cleaned by soaking in three changes of distilled water for about 20 min. No ultraviolet-absorbing material was released from the washed tubing when used for a mock dialysis of double-distilled water against ten times the volume of the same liquid. As a further check that the cleaning was adequate, three preparations (of types I and V dNP; see below) were carried out with dialysis tubing which had previously been boiled for about 10 min. in distilled water and then rinsed thoroughly (and in one case subsequently soaked for 2 hr.); it was found that the products did not differ from those prepared with unboiled tubing.

Unless otherwise stated, the dialysis procedure in all preparations was as follows: the dialysis bag was placed in a stoppered flask containing double-distilled water and the flask was shaken very gently on a mechanical agitator for

30 min. The double-distilled water was then changed and the dialysis system was kept stationary overnight.

Preparation of dNP. The standard preparation (type I) has been described in detail by Itzhaki (1966a). In brief, frozen rat thymus glands were minced with scissors and then homogenized in solution A (1.3:10, w/v) with the masticator of an MSE emulsifier operated at about 6500 rev./min. The mixture was centrifuged at 2000g for 20 min. and the precipitate thus obtained was homogenized in solution A and centrifuged as before. The precipitate was then homogenized in double-distilled water (with the same volume as that of solution A). After filtration through Terylene gauze, the mixture was dialysed overnight against double-distilled water (1:10, v/v, changed once).

The basic procedure of homogenizing the tissue twice in salt solution and once in water, followed by filtration, was the same in all types of preparation except type II. Differences in the preparations lay either in the composition of the salt solution or in the treatment of the filtrate of the third homogenate in water; this filtrate is referred to in all types of preparation as homogenate 3 whether or not the solution used for the two previous homogenizations differs from solution A. Table 1 summarizes the various types of preparation, all of which were carried out at 4°. For comparison, type I dNP was usually prepared simultaneously from the same batch of tissue by treating homogenate 3 in the prescribed way.

All the final products other than the more dilute ones of type V were diluted for birefringence measurements in solution B to concentrations of 0.005–0.02% DNA. Although in general dilutions were gelatinous, they will be referred to here as solutions.

Analyses. DNA was estimated either from the ultraviolet absorption at 260 m μ , $E_{260}^{1\%}$ being taken as 210, or by the diphenylamine method (Dische, 1955). Protein was estimated by a micro-biuret method (Itzhaki & Gill, 1964).

Measurements of the solubility of the DNA component of the dNP in 0.15M-NaCl solution were made as follows: 1.5M-NaCl was added to dNP solution to give a final concentration of 0.15M 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 the 260 m μ absorption.

The acid-solubility of the DNA component of the dNP was measured by adding 2N-perchloric acid to dNP solution to give a final concentration of 0.67N. The mixture was kept at 0° for 15 min. and centrifuged as above. The DNA concentration of the supernatant was calculated from $E_{260\text{ m}\mu}^{1\%}$, $E_{260}^{1\%}$ being taken as 270.

Calcium and sodium analyses were carried out by flame photometry, the former being estimated in material extracted by 0.05N-perchloric acid at 70° overnight (R. F. Itzhaki, unpublished work). Sodium estimations were made directly on the dNP solutions.

Electric birefringence

Theory. The basis of the method is the induction of birefringence in a solution through orientation of solute molecules in an electric field. The difference in refractive index (Δn) in directions parallel and perpendicular to the electric field (E) is related to E thus: $\Delta n = B\lambda E^2 = K\lambda n^2 E^2$ (Kerr law), where B and K are constants depending on the system, λ is the wavelength of the incident light and n is

Table 1. *Summary of different procedures used for preparing dNP from rat thymus*

Standard procedure: Frozen rat thymus glands were minced and then homogenized for 2 min. in solution A (0.15 M-NaCl in 5 mM-phosphate buffer, pH 7.4, 1:3:10, w/v). The mixture was then centrifuged at about 2000g for 20 min. and the precipitate thus obtained was homogenized in solution A as before. The mixture was then centrifuged. The resulting precipitate was homogenized for 2 min. in double-distilled water (of volume equal to that of solution A) and the mixture was filtered through gauze and then dialysed overnight against double-distilled water (1:10, v/v, once changed). Zubay & Doty (1959) procedure: 1g. of frozen rat thymus glands was minced and then homogenized for 2 min. in 10 ml. of 'standard solvent' (75 mM-NaCl in 24 mM-EDTA, pH 8), in which one drop of octan-2-ol had previously been homogenized. The mixture was filtered through gauze and then centrifuged at about 400g for 10 min. The precipitate thus obtained was homogenized for 1 min. in the 'standard solvent' containing octan-2-ol, and was then centrifuged. The cycle of homogenizing the precipitate and centrifuging was repeated a further six times (homogenizing for 1 min. at the third cycle and 30 sec. subsequently). The final precipitate was homogenized in 15 ml. of double-distilled water; the mixture was diluted immediately to 50 ml. with double-distilled water and was stirred for 1 hr. It was then dialysed overnight against 0.7 M-potassium phosphate buffer, pH 6.8 (approx. 200 ml. changed three times).

Alteration in procedure from that of type I

Type of dNP	In homogenization	In salt solution	In treatment of homogenate 3
I		Standard procedure	
II	As in type I then homogenized four more times	—	—
III	—	Sometimes 0.15 M-NaCl + CaCl ₂ or MgCl ₂ , otherwise solution B	Dialysed against CaCl ₂ , MgCl ₂ or NaCl
IV	—	Sometimes 0.15 M-NaCl + EDTA, otherwise solution B	Dialysed against EDTA
V	—	—	Diluted with water
VI	—	—	Dialysed extensively
VII	Reagents and procedure according to Zubay & Doty (1959)		

the refractive index in the absence of the field. Tinoco (1955) has derived the following expression for the birefringence produced by orientation of ellipsoidal molecules:

$$\Delta n(t \rightarrow \infty) = (2\pi/15)(g_1 - g_2)VE^2(p_1 - p_2 + q)$$

t is the time after the start of application of the field; g_1 and g_2 are the optical polarizabilities per unit volume along the symmetry and transverse axes of the ellipsoid: these depend on refractive index and on a form factor (L) which depends only on the axial ratio of the ellipsoid; V is the volume fraction of ellipsoids in solution; p_1 and p_2 are functions of L and of the permanent dipole moments and the permittivities along the symmetry and transverse axes; q is the induced dipole term and depends on the electrical polarizabilities per unit volume and on L . Thus Δn is related to molecular size, shape and charge.

On removal of the electric field, the molecules disorient through Brownian motion, the time taken depending on their length. The relaxation time (T) of decay of birefringence is related to the molecular length $2a$ thus: $T = 1/6D$ (where D is the rotational diffusion constant) and for ellipsoidal molecules, $D = 3kT'(\ln 2a/b - 0.5)/8\pi\eta a^3$ (where $2b$ is the equatorial axis, η is the viscosity of the solvent, T' is the absolute temperature and k is the Boltzmann constant). Thus T depends strongly on molecular length but only slightly on width.

Kerr cell. Three Kerr cells were used. These consisted of rectangular vessels of strain-free glass containing pairs of vertical rectangular electrodes of length 1.75, 3.95 and 7.95 cm. respectively. The electrodes were gold-plated and

their supports were fixed to the insulating (Tufnol) lids of the cells. The electrode separation was adjusted by an external fine screw with graduated scale and was checked by a travelling microscope. The usual separation was 0.54 cm. The cells were precooled and the liquid under investigation was poured in to a height such that on insertion of the lid assembly the electrodes were covered. The unit was then placed in a holder containing ice and water on the optical bench. The cells were cleaned by thorough rinsing and the electrodes by rubbing with wet filter paper, followed by rinsing.

Optical and electrical system. The light-source was a 12 v, 100 w quartz-iodine bulb. A lens system produced a gently divergent beam incident on to a polaroid set at 45° to the direction of the electric field. The polarized beam passed through a slit and then between the electrodes in the Kerr cell. After transmission through a second slit and a second polaroid crossed with respect to the first, the beam was focused by a lens on to the photomultiplier (E.M.I. 6097B) which was coupled to the oscilloscope (Telequipment, type D31). The latter displayed the electrical and optical signals simultaneously and these were photographed on Ilford HPS 35 mm. film.

To reduce heating and electrophoretic effects, the electric field was applied as a pulse; the latter was generated by a multivibrator (triggered by the oscilloscope camera switch), amplified and led to a cathode follower. The final pulse was rectangular, of height up to 600 v and length 22 msec.

Measurement of Δn and T . The light-intensity (I)

transmitted through the second polaroid on application of the electric field is proportional to $\sin^2$ (const. Δn). I was measured by the method of Benoit (1951) in which the oscilloscope trace deflexion (proportional to I) is compared with that produced by rotation of the second polaroid through known angles (ϕ) from extinction (I then being proportional to $\sin^2\phi$). This calibration was carried out before and after application of the voltage pulses.

It was found that the Kerr law was not obeyed, Δn increasing less rapidly than with the square of the field. This indicated that conditions were approaching saturation. Graphs of $\log \Delta n$ were plotted against $\log E$ and were found to be approximately linear, with gradient m , over the voltage range 200–600 v, values of Δn and m increasing with increasing dNP concentration. Quoted values of Δn (for convenience, of $\Delta n/\lambda$) are for 250 v. (For two samples with similar values of m , clearly comparison of Δn anywhere in the linear range gives similar results.) The accuracy of measurement of Δn was $\pm 2\%$.

Initially, semi-logarithmic plots were made of the birefringence decay curves and were found to be 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). Later, these times were measured by a simple and rapid analogue method in which the intensity decay curve was compared with standard voltage decay curves produced by two condenser-resistor circuits of known parameter (Itzhaki, 1966a). The accuracy was within about $\pm 17\%$ for T_2 and $\pm 30\%$ for T_1 .

RESULTS AND DISCUSSION

Standard preparation (type I dNP). Type I dNP was obtained as a rigid, opalescent gel of concentration usually between 0.2 and 0.3% DNA. (For convenience all concentrations of dNP gels or solutions are given in terms of DNA.) The yield was about 0.02g. of DNA/g. wet tissue. The ratio

DNA/dNP was usually about 0.36–0.39. The sodium concentration (after correction for the sodium in solution B) was within the range 0.1–0.12M and the calcium content 13–25 μ M in dNP solutions at 0.01% DNA. Type I gels and solutions were stable at 4° for at least 5 days, the only change being a slight decrease in birefringence (Itzhaki, 1962, 1966a). The reproducibility was satisfactory, values of birefringence at a given concentration differing by less than $\pm 15\%$ in different preparations.

Preparations made by repeated homogenization (type II dNP). Two preparations were made in which the tissue was minced, homogenized in solution A and centrifuged a total of six times. The sixth precipitate was then homogenized in water, filtered and dialysed as in type I preparations. The final products were gels, similar in appearance to those of type I; however, they dispersed less readily on dilution.

Table 2(a) shows that the birefringence of type II dNP is slightly higher than that of type I (values of m being the same). However, the increase may be attributable merely to the more vigorous shaking required to dilute the type II gels; this could increase Δn by up to 10% (Itzhaki, 1966a). The difference in values of T_2 is within the error of measurement.

The absence of any decrease in Δn and T for type II dNP indicates that no decrease has occurred in particle size, i.e. the longer total period of homogenization and of preparation has not caused degradation of the dNP. As a further check, the solubility of the DNA component of type II dNP in 0.67N-perchloric acid and in 0.15M-sodium

Table 2. Comparison of properties of type I dNP and of dNP prepared by repeated homogenization of tissue in 0.15M-sodium chloride (type II dNP)

(a) Birefringence properties							
Expt. no.	Type of dNP	Concn. of dNP during measurement (% DNA)	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	$\frac{\Delta n/\lambda \text{ of type II dNP}}{\Delta n/\lambda \text{ of type I dNP}} \times 100$	T_2 (msec.)	T_1 (msec.)	m
1	I	0.0130	7.70		100	2	0.48
	II	0.0130	8.80	114	112	2	0.48
2	II	0.0180	14.2*		100	2	

* For type I dNP, the range of values of $\Delta n/\lambda$ at this concentration is ~ 12 –15.

(b) Composition and solubility properties of type II dNP					
Expt. no.	Type of DNA	$\frac{\text{DNA}}{\text{dNP}} \times 100$	Fraction of DNA soluble in 0.15M-NaCl (% of total DNA)	Fraction of DNA soluble in 0.67N-HClO ₄ (% of total DNA)	Fraction of protein soluble in 0.2N-HCl (% of total protein)
1	I	37	< 3	~ 1	81
	II	42	< 2		94
2	II	43	< 2	4	

chloride solutions was measured, solubility measurements having previously been used as a rapid and simple assay of the degradative action of added enzymes (Itzhaki, 1966b). Table 2(b) shows that the solubility was in fact as low as that of type I dNP.

The protein content of type II dNP relative to its DNA was about 20% lower than that of type I dNP (see higher ratio, DNA/dNP, in Table 2b). To find whether histone or non-histone protein was lost, the solubility of the protein in 0.2N-hydrochloric acid was measured (by extracting twice at 0°): the high value found for type II dNP (Table 2b) indicates that the protein lost was mostly non-histone.

Preparations made in the presence of added calcium, magnesium and sodium (type III dNP). When preparations were made in the presence of 1mM-calcium chloride or 1mM-magnesium chloride (in all three homogenates and during dialysis), the final product was a white precipitate. This could not be dissolved even after dialysis against double-distilled water for 24hr. With preparations made in the presence of 0.1mM-calcium chloride or by dialysis against 2mM-sodium chloride, the final product was inhomogeneous, consisting of regions of gel surrounded by liquid. On dilution, the material dispersed immediately, giving a very cloudy, non-viscous liquid. Values of birefringence were about 16% and 25% respectively of that of type I dNP. Optical signals were too low to permit measurement of T . Expt. 2 of Table 3 gives another example of material prepared by dialysis against sodium chloride. The change in birefringence was smaller, probably because sodium chloride was not added to homogenate 3 and also dialysis was less extensive.

dNP is precipitated from solution (about 0.01% DNA) by sodium chloride at or above 0.4mM; at lower salt concentration, Δn and T decrease (R. F. Itzhaki, unpublished work). The concentration required to produce precipitation of dNP in the gel state is unknown. The initial sodium concentra-

tion of type I homogenate 3 is about 20mM (see the section on type VI dNP). When the homogenate 3 is dialysed against sodium chloride during preparation, the loss of sodium is small, presumably, and the residual sodium chloride is sufficiently high to cause partial insolubility of the dNP and to decrease the birefringence of the dNP dilutions. It is surprising, though, that despite the large decrease in Δn in the type III dNP of Table 3 there is no decrease in T .

Precipitation of dNP from solution occurred at lower concentrations (expressed as moles/l.) of calcium chloride and magnesium chloride than of sodium chloride (R. F. Itzhaki, unpublished work). Also, on dialysis of dNP solutions against double-distilled water, the dNP did not lose calcium but actually increased its calcium content by a factor of 1.7–1.9 (in four experiments), presumably by binding it from the external liquid or from the dialysis bag. This may happen also during the preparation, on dialysis of dNP against calcium chloride or against water. Since the final calcium concentration in a type I gel is about 0.5mM, the fact that partial precipitation of the dNP occurs after dialysis against 0.1mM-calcium chloride, during a type III preparation, supports this idea. The irreversible insolubility after dialysis against 1mM-calcium chloride shows that the dNP binds calcium very strongly: possibly the calcium forms intermolecular cross-links.

Preparations made in the presence of EDTA (type IV dNP). Preparations made by dialysing homogenate 3 against low concentrations of EDTA (0.1mM adjusted to pH 5.9 or 0.01mM at pH 5.3) yielded gels of normal appearance. At higher EDTA concentrations (1mM or 2mM adjusted to pH 7.1 or 6.2) the products tended to be less rigid gels than usual (as judged by eye) but they were not inhomogeneous. In one case a type III sample was prepared at the same time by dialysing a portion of homogenate 3 against 4mM-sodium chloride (to compare the effect of the sodium in EDTA with that in a normal salt). This yielded a

Table 3. Comparison of electric birefringence properties of type I dNP and of dNP prepared in the presence of EDTA (type IV dNP)

Expt. no.	Type of dNP	Concn. during birefringence measurements (% DNA)	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	$\Delta n/\lambda$ of sample $\Delta n/\lambda$ of type I dNP $\times 100$	T_2 (msec.)	T_1 (msec.)	m
1	I	0.0166	8.30		113	2	0.69
	IV*	0.0170	6.64	80	100	2	0.62
2	I	0.0165	8.62		72	2	0.69
	IV*	0.0156	7.60	88	72	2	0.74
	III†	0.0159	2.99	35	72	2	0.59

* Dialysed against 1mM-tetrasodium EDTA in 1mM-HCl, pH 7.

† Dialysed against 4mM-NaCl.

very weak, inhomogeneous gel. Values of birefringence were slightly lower for the type IV dNP, the decrease depending on EDTA concentration, and considerably lower for the type III dNP than for type I dNP (Table 3). (Values of m were similar.) Changes in T were within the error of measurement. These results suggest that the EDTA acts partially as a salt. However, the lower rigidity of the type IV products probably cannot be attributed to this, as, judging by the results of this and of the previous section, an amount of salt sufficient to produce material of as low a rigidity would cause also appreciable inhomogeneity and turbidity and would decrease Δn considerably. The low rigidity may, however, be related to the metal-chelating properties of the EDTA and, if so, suggests that metal links may be involved in forming the normal gel network of type I dNP.

The effect of repeated application of the voltage pulse to solutions of type I dNP had previously been studied (Itzhaki, 1966a) to find if any permanent changes were caused by the electric field. It was found that at higher concentrations (above about 0.01% DNA) the birefringence increased, after application of about 10 pulses at 200v, by about 10 or 15% of the original value. The increase was attributed to breakage of residual inter-particle links which enabled individual particles to orient themselves more completely. With type IV dNP the increase was 70%. This suggests that a more complete breakage occurs here and is explicable if inter-particle links involve metals and if, because of the EDTA, these are more labile than in type I dNP.

As to the action of EDTA in inhibiting deoxyribonuclease I (the pH being sufficiently high, probably, at the higher EDTA concentrations to inhibit deoxyribonuclease II also), one can predict the changes which should, accordingly, be found in type IV dNP. Addition of deoxyribonuclease I to type I dNP causes a decrease in Δn , and at lower dNP concentrations (about 0.005% DNA), a concurrent decrease in T ; at higher concentrations (about 0.02% DNA), T decreases only at a later stage (Itzhaki, 1966b). Thus, if deoxyribonuclease were present as an impurity during preparation, one would expect type IV dNP to have higher values of Δn , and possibly of T , than type I dNP. Since this is not the case, it seems very unlikely that appreciable deoxyribonuclease action occurs normally in type I preparations. As further evidence, less than 1% of the 260m μ -absorbing material in type I dNP is soluble in 0.67N-perchloric acid, yet after even limited action due to added deoxyribonuclease the percentage rises appreciably. To check that no loss of potentially acid-soluble degradation products had occurred during dialysis in the type I preparations, the

ultraviolet absorption of the external dialysis liquids was measured; this showed that no significant amount of DNA was present (detection limit equivalent to about 0.5% of the DNA inside the dialysis bag).

Preparations of dNP at low concentrations (type V dNP) and preparations with extensive dialysis (type VI dNP). Preparations were made in which samples of type I homogenate 3 were diluted 5–20 times with double-distilled water and were then dialysed against double-distilled water. The more concentrated samples of this type were obtained as weak gels. Those at lower concentrations were only slightly (or not at all) gelatinous and were always less so than type I dilutions at the same dNP concentration. Also, type V dNP was far more turbid and, when diluted in distilled water for concentration measurements, tended to be precipitated.

To find if the lower rigidity of type V dNP was associated with any decrease in gel content, samples of undiluted types V and I dNP were centrifuged at or above 70000g for about an hour. It had previously been found that centrifugation of type I gels caused a separation into a gel sediment and a clear non-viscous supernatant of very small volume, the latter containing less than 5% of the total DNA (Itzhaki, 1966a). Similar results were obtained with type V dNP (Table 4) (but as might be expected the supernatant occupied a relatively larger volume). The centrifugation behaviour of an undiluted type IV gel and a type IV–V product (Table 4) resembled those of types I and V respectively. Thus, despite the lower rigidity of types V and IV–V dNP, which suggests a decrease in number or strength of inter-particle links, the proportion of the material sedimenting as a gel is unaffected.

The birefringence of type V dNP was much lower than that of type I (Table 5). (In Expt. 4, owing to the higher values of m for type V dNP, the change in Δn is less at high fields than the value given.) However, values of Δn for type V dNP at a given concentration were not reproducible; their magnitude depended somewhat on concentration of dNP during the preparation (Table 5), but even with products prepared at similar concentrations values varied by up to $\pm 50\%$. The decay patterns of type V dNP resembled those of type I.

It was found that the protein content of type V dNP, relative to its DNA, was usually slightly lower than that of type I dNP, the ratio DNA/dNP being 0.39–0.40. The calcium content of type V was about 2.3–2.4 times (in two experiments) that of the corresponding type I dNP.

Type V dNP resembles type I dNP which has been subjected to limited action of deoxyribonuclease or trypsin, the solutions in both cases

Table 4. *Centrifugation of undiluted type I and type V dNP*

Expt. no.	Type of dNP	Concn. (% dNP)	Centrifugal force (g)	Centrifugation time (min.)	Concn. in supernatant (% of DNA)
1	I	0.272	70000	30	5
	V	0.026	70000	30	4
2	I	0.280	105000	45	2
	V	0.0317	105000	45	6
3	I	0.211	105000	90	3
	V	0.0400	105000	90	1
4 ^a	IV	0.226	105000	90	2
b	IV-V	0.0165	105000	45	4

* This preparation was made in the presence of 2mM-EDTA in 0.15M-NaCl, pH 7.0, and was finally dialysed against 10 μ M-tetrasodium EDTA, pH 5.3. Sample (a) was obtained from an undiluted portion of homogenate 3 and sample (b) from a portion diluted 1:10.

Table 5. *Electric birefringence properties of dNP prepared at high and at low concentrations (types I and V dNP)*

Expt. no.	Type of dNP	Concn. during dialysis in the preparation (% DNA)	Concn. during birefringence measurements (% DNA)	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	$\frac{\Delta n/\lambda \text{ of type V dNP}}{\Delta n/\lambda \text{ of type I dNP}} \times 100$	T_2 (msec.)	T_1 (msec.)	m
1	I	0.330	0.0223	11.5				
	V	0.0185	0.0185	< 1	< 9*			
2	I	0.308	0.0149	9.8		100	2	
	V	~0.020	~0.015	< 2	< 20			
3	I	0.272	0.0086	5.85		100	2	0.50
	V	0.026	0.0078	2.14	37*	100	2	0.53
4	I	0.233	0.0163	11.1		100	2	0.60
	V	0.054	0.0162	4.88	44	100	2	0.79

* Not corrected for concentration difference.

being less gelatinous, more turbid and having lower values of Δn but not of T than untreated type I dNP (Itzhaki, 1966b). For further comparison the solubility properties of type V dNP were examined. It was noted that the precipitates formed in 0.15M-sodium chloride and in 0.67N-perchloric acid were thinner and less fibrous (as with enzyme-treated type I dNP) than with control type I dNP; however, values of soluble fraction of the constituent DNA of type V were as low as those of control type I dNP (less than 4%), whereas after even limited deoxyribonuclease or trypsin action the solubility of the DNA of type I in the perchloric acid or the sodium chloride respectively increased appreciably. Measurement of the ultraviolet absorption of the external dialysis liquids used in the preparation showed that no significant loss of DNA had occurred (detection limit equivalent to about 4% of the total DNA), i.e. there was apparently no degradation at this earlier stage. Thus the changes in type V dNP are probably not caused by enzymic impurities (see also the Appendix).

It was thought that the difference between type I and type V dNP might be due merely to a difference in ratio of salt concentration to that of dNP; this might occur if the percentage loss of salt differed during dialysis in the two preparations. To test this, first the salt content was maintained at a higher value than in previous type V preparations, by limiting the extent of dialysis or by not dialysing the diluted homogenate 3. However, the final products were cloudy and inhomogeneous and values of Δn for their dilutions were less than about 20% of that of type I solutions. In other experiments the salt content was reduced to a lower value than before by carrying out extensive dialysis. The products were white suspensions. Thus the difference between types I and V dNP is not due solely to a difference in loss of salt.

Since, however, the extent of dialysis during preparation appeared to be an important factor, the effect of more exhaustive dialysis on an undiluted (type I) homogenate 3 was studied: the volume ratio was about eight times that normally

used and the external dialysis liquid was stirred by magnetic stirrer (the bag being suspended by threads to reduce agitation). The final products (type VI) were weak, rather inhomogeneous gels containing much suspended white matter. On dilution they gave turbid, non-viscous liquids from which, in some cases, precipitation occurred after standing for a day. Values of Δn were about 25% of those of type I solutions.

In a further experiment, an attempt was made to bring type V and type IV dNP to the same final salt concentration during preparation. The obvious concentration to choose was that of solution B, since all products were diluted with this solution. An undiluted portion of homogenate 3 and another portion diluted 1:10 with solution B were both dialysed against relatively large volumes of solution B. The undiluted sample (type VI) yielded an apparently normal gel. The product from the diluted portion (type V) was rather cloudy, though less so than most type V products. Values of Δn for the types VI and V solution were 56% and 29% respectively of that of type I dNP. Values of T were approximately the same for all three samples.

From these results one can calculate the limits of salt content within which a soluble, homogeneous dNP product can be obtained. The sodium chloride content of a type I dNP gel is about 2mm; therefore in a type VI preparation the maximum decrease in salt content during dialysis would be to about one-eighth of this value, i.e. to about 0.25mm. Type I homogenate 3 forms a precipitate if it is not dialysed, its salt content being about 20mm (calculated from the sodium concentration of solution A and the dilution factor of the second tissue precipitate on homogenizing in water). Thus the limits are between about 20mm- and 0.25mm-sodium chloride.

The limits are uncertain for type V preparations, but clearly the changes in the products are related to the lower concentration of dNP, not merely to an incorrect salt content. A similar dependence on dNP concentration and similar changes in birefringence properties are found during the aging of type I dNP, and also when the latter is dialysed (Itzhaki, 1966a, and unpublished work). In both cases, Δn decreases but T does not change; aging is more rapid in dNP solutions than in dNP gels and is more rapid also at higher temperatures. Prolonged dialysis leads to precipitation of the dNP and this occurs also on keeping the solutions after they have been subjected to limited dialysis, the changes again being greater at lower dNP concentrations. It was previously suggested that, in both cases, some type of disorganization or denaturation (but not longitudinal breakage) of the dNP particle had occurred and it seems likely

that type V dNP has undergone a similar process. The higher calcium content of type V dNP may be related to a disorganization process but whether the increase is a result of the latter (or even a cause) is uncertain.

The general resemblance of types V and VI dNP suggests that the latter, also, is denatured during preparation, presumably because of the extensive dialysis.

Zubay-Doty type preparation (type VII dNP). A preparation was made by the method of Zubay & Doty (1959), but rat thymus was used instead of calf thymus and the MSE emulsifier (operated at 6500 rev./min.) was used instead of a Waring Blendor because the amount of material was too small for the latter. However, the relative volumes of tissue to suspending liquid were kept the same as in the Zubay-Doty method.

The final product was a gelatinous, cloudy liquid. A sample was centrifuged at 70000g for 30min., as in the procedure of Zubay & Doty (1959), and this separated into a gel sediment occupying about 0.4 of the volume and a clear, non-viscous supernatant which contained only about 12% of the total DNA. In contrast, Zubay & Doty obtained not less than 85% of their material in the supernatant. The explanation for this discrepancy is unknown. The only possible difference in procedure was in the blending speed: that used by Zubay & Doty (1959) is not given but in Zubay & Doty (1956) the Blendor is said to have been operated at or below half-speed and the latter is rated at 6000 rev./min. Thus the speed used here (6500 rev./min.) is slightly higher, which should not, on their reasoning, favour gel formation. Alternatively the difference may be due to the use of rat thymus here.

Birefringence measurements were made on both the supernatant fraction and on a dilution of the unfractionated type VII dNP. Table 6 gives results for these samples and for types I and V dNP made on the same day (the type V being prepared at a concentration similar to that of the type VII dNP).

The birefringence properties and the appearance of the unfractionated type VII dNP resemble those of the type V dNP. Since the only common factor in the two preparative methods is the low value of dNP concentration, the material is not affected greatly by the special conditions of the type VII method (the presence of EDTA, the maintenance of a relatively high pH, the prolonged stirring of the final homogenate and the dialysis against phosphate buffer). Also, the high proportion of material in the gel phase here suggests that, as in type V dNP, the main changes are within the particles and that the interaction properties are affected to a lesser extent.

Table 6. *Comparison of electric birefringence properties of type I dNP and of dNP prepared by the method of Zubay & Doty (type VII dNP)*

Type of dNP	Concn. during dialysis in the preparation (% DNA)	Concn. during dialysis in the preparation (% DNA)	DNA dNP $\times 100$	Birefringence $10^3(\Delta n/\lambda)$ (cm. ⁻¹)	$\frac{\Delta n/\lambda \text{ of sample}}{\Delta n/\lambda \text{ of type I dNP}} \times 100$	T_2 (msec.)	T_1 (msec.)	m
I	0.233	0.0163	35	11.1		100	2	0.60
VII	0.046	0.0162	39	5.2	47	100	2	0.77
(unfractionated) VII*		0.0049		< 0.8	< 7 (< 13)†			
(supernatant) V	0.054	0.0162	39	4.88	44	100	2	0.79

* Obtained by centrifuging undiluted material at 70000g for 30 min.

† Corrected, approximately, for concentration difference.

The birefringence of the supernatant obtained by high-speed centrifugation of the type VII dNP is very low. The same is found with supernatants obtained on centrifuging undiluted type I gels (Itzhaki, 1966a) and indicates that, in both cases, the molecules or particles in the solution phase are much smaller than those forming the gel phase (the latter apparently being oriented individually and not within gel clusters).

Comparison of types I, V and VII dNP has been made also by electron microscopy and supports the ideas discussed. Micrographs of type I dNP show particles consisting of bundles or networks of molecules with a certain degree of internal organization and alignment, whereas those of type V dNP and of the supernatant of the type VII dNP show smaller particles somewhat disorganized internally, resembling trypsin-treated type I dNP (R. F. Itzhaki & A. J. Rowe, unpublished work).

The Zubay & Doty (1956, 1959) preparative method has been studied also by Dounce & O'Connell (1958), who maintain that gel formation (which they consider to be associated with 'native' material) is prevented in this method by excessive shear or by the action of proteases. The present work indicates that shear rates comparable with those used by Zubay & Doty (1956, 1959) are not in fact harmful and that the apparent denaturation of the type VII dNP is probably not due to enzyme action.

Dounce & Hilgartner (1964) have suggested that the dNP gels which they prepare from calf thymus and rat liver are formed by covalent linkage of the DNA molecules at their ends to residual protein, the latter molecules being linked to each other by disulphide bonds. Comparison of type I dNP with their material is difficult because the composition, i.e. ratio DNA/dNP and ratio

residual protein/total protein, of their dNP is not given (although they refer mainly to 'histone- and globulin-extracted' dNP). Clearly, differences in composition may affect greatly the properties of the dNP. Indeed, as might be expected, the gelation properties they describe resemble those observed in the present study in material containing dNP which is probably at least partly dissociated. Dounce & Hilgartner (1964) consider that the presence of elasticity, and the formation of long 'threads' on withdrawing glass rods inserted in the material, are criteria of gel formation, although the latter phenomenon is certainly not shown by most gels and is in fact shown by several non-gel materials. Both characteristics are shown here by thymus homogenates in 0.15M-sodium chloride after addition of detergent, i.e. in the first stage of preparation of DNA, and by type I dNP in the presence of 5M-urea. However, the property of elasticity but not of 'thread' formation is shown by type I dNP gels.

Studies with added enzymes (Itzhaki, 1966b) do not support the structure proposed for dNP by Dounce & Hilgartner (1964): extensive action of Pronase, subtilisin and trypsin on type I dNP does not cause a decrease in particle length (as measured by relaxation time of birefringence), indicating that protein molecules do not link the constituent DNA molecules end to end unless they do so at or near loop-points. Also, the effects of heating dNP gels and shaking dNP solutions show that the main gel inter-links are weak secondary bonds, not covalent bonds (Itzhaki, 1966a). Further, the results with type II dNP show that loss of what is presumably residual protein does not affect the dNP properties (or certainly not the gelation or birefringence properties) to any extent.

On the other hand, type II dNP does not

resemble in its properties the dNP studied by Doty & Zubay (1959), the protein of which they believe to be almost wholly histone. Clearly, as discussed above, the critical factor producing differences between type I or type II dNP and the dNP studied by Zubay & Doty (1959) is the dNP concentration during the preparation. This may account also, in part, for the difference between the Zubay & Doty (1959) and the Dounce & Hilgartner (1964) types of dNP, although the latter authors give no details of concentration. Presumably, the other factor in all three types of dNP is that of composition. As to the role of end-links, further work is needed to show whether or not in dNP DNA molecules are inter-linked by protein at or near loop-points.

In conclusion, although there is no criterion of 'nativeness' of dNP, comparisons of stability, reproducibility and birefringence properties of the products show that the type I method for preparing dNP is the best of the preparative methods tried here.

The satisfactory stability and reproducibility of type I dNP has been described by Itzhaki (1966a). In contrast, types V and VI dNP are unstable, especially when diluted, and their properties are non-reproducible. Birefringence measurements as such are difficult to interpret unambiguously; however, comparison of changes in these properties caused by the different preparative methods, with those produced by enzymes added to type I dNP, indicates that types V, VI and VII dNP are denatured. Therefore denaturation occurs if, during preparation, the dNP concentration is low and if dialysis is extensive. A high residual salt concentration is deleterious also, as shown by production of wholly or partially insoluble material after very limited dialysis against water and after dialysis against sodium chloride, calcium chloride or magnesium chloride solution (as in type III preparations).

Absence of visible gel formation or a very low rigidity may be related to denaturation but, on the other hand, the gel content of types V and VII dNP, as obtained by centrifugation, is almost as high as that of type I. Also, type IV gels tend to be weak yet the properties of their dilutions differ only slightly from those of type I.

The similarity of types I and II dNP shows that the total time of preparation is not an important factor (at least when the dNP concentration is high), indicating either that degradative enzymes are not present or that they are not acting; the results with type IV dNP confirm the absence of enzyme action and show therefore that addition

of EDTA is unnecessary. The properties of type II dNP indicate also that the extent of homogenization is unimportant, i.e. that homogenization does not cause degradation and, conversely, that the simplicity and rapidity of the type I method are further advantages (although excess of protein is not removed).

A loss of protein is associated with changes in other properties only at low dNP concentrations, as in type V dNP, and as on purification of type I dNP solutions: it had previously been found that purification by precipitation in 0.15M-sodium chloride caused irreversible changes in the dNP and there was an associated loss of protein (R. F. Itzhaki, unpublished work). Since the original material is prepared by homogenization of tissue in 0.15M-sodium chloride, the purification changes must be related to the low dNP concentration in solution.

Thus both the present results with types V and VII dNP and the previous studies on purification of dNP support the idea that, at lower concentrations, dNP is less stable and less resistant to damage (whether or not the loss of protein is relevant). It is well known that DNA and proteins are more resistant at high concentration to denaturation. Also, since dNP is present at a high concentration in chromosomes, one might expect maintenance of a high concentration during preparation, as in type I preparations, to favour production of 'native' material.

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REFERENCES

- Benoit, H. (1951). *Ann. Phys.* **6**, 651.
- Davison, P. F., Conway, B. E. & Butler, J. A. V. (1954). *Progr. Biophys.* **4**, 148.
- Dische, Z. (1955). In *The Nucleic Acids*, vol. 1, p. 287. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Dounce, A. L. & Hilgartner, C. A. (1964). *Exp. Cell Res.* **36**, 228.
- Dounce, A. L. & O'Connell, M. (1958). *J. Amer. chem. Soc.* **80**, 2013.
- Itzhaki, R. F. (1962). *Nature, Lond.*, **194**, 1241.
- Itzhaki, R. F. (1966a). *Proc. Roy. Soc. B*, **164**, 75.
- Itzhaki, R. F. (1966b). *Proc. Roy. Soc. B*, **164**, 411.
- Itzhaki, R. F. & Gill, D. M. (1964). *Analyt. Biochem.* **9**, 401.
- Peacocke, A. R. (1960). *Progr. Biophys. biophys. Chem.* **10**, 56.
- Tinoco, I. (1955). *J. Amer. chem. Soc.* **77**, 4486.
- Zubay, G. & Doty, P. (1956). *J. Amer. chem. Soc.* **78**, 6207.
- Zubay, G. & Doty, P. (1959). *J. molec. Biol.* **1**, 1.