Some Physical and Chemical Properties of Trypsin-Digested Nucleoprotein

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A DNA-peptide complex that is soluble in 0.2 M-sodium chloride can be prepared by trypsin digestion of calf thymus nucleoprotein. The trypsin-digested nucleoprotein molecule contains about 70% of DNA and 30% of peptides by weight, and consists of one DNA molecule associated with arginine-rich peptides. A series of trypsin-digested nucleoprotein preparations differing only in molecular weight were prepared by blending. The intrinsic viscosity and average sedimentation coefficient were determined for each of these preparations. Then the DNA was isolated from each preparation and the hydrodynamic measurements were repeated on the DNA. From a comparison of these results it was concluded that the presence of the complex-forming peptides causes a large decrease in intrinsic viscosity of the DNA and an increase in sedimentation coefficient. In addition, the hydrodynamic data indicate that the DNA-peptide complex behaves like a coil in solution but is more compact than the same length of DNA. The 'melting' profiles, streptomycin precipitation curves and maximum viscosities obtained with ethidium bromide binding for the trypsin-digested nucleoprotein are similar to those of purified DNA, and markedly different from those of undigested nucleoprotein. These findings suggest that the peptides are not strongly associated with the DNA, and that secondary valency forces are involved in the binding.

Electrostatic and secondary valency bonds probably both contribute to the binding of protein to DNA in specifically joined nucleohistone complexes (Chargaff, 1963; Busch, 1965; Akinrimisi, Bonner & Ts'o, 1965). To remove these proteins from the DNA, high salt concentrations are frequently employed to weaken electrostatic interactions, and the protein is removed by shaking with an organic solvent (Sevag, Lackman & Smolens, 1938) or detergent is added to bind and solubilize the protein during subsequent ethanol precipitation of the DNA (Kay, Simmons & Dounce, 1952; Simmons, Chavos & Orbach, 1952). A combination of these methods may be used (Marmur, 1961). Lipophilic reagents with chelating properties have been successfully employed (Kirby, 1964), and phenol is much used for the isolation of viral DNA (Mandell & Hershey, 1960). These techniques never seem to remove all of the protein, however, though in the best preparations only traces (Kirby, 1957) or from 0.1 to 0.6% (Salser & Balis, 1967) remain.

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Traces of protein could be of importance in linking DNA molecules together (Taylor, 1963).

Another approach to the problem of protein removal is to digest it away with proteolytic enzymes. A major feature of the Schwander & Signer (1950) procedure for the isolation of DNA is a 10-day period of stirring at 0°, during which time it is likely that endogenous proteolytic enzymes contribute to the digestion of the concentrated nucleoprotein gel. Butler, Phillips & Shooter (1957) have employed chymotrypsin to remove traces of protein from DNA, and Champagne, Mazen & Pouyet (1964) have shown that only about one amino acid residue per 1000 nucleotides remains after extensive deproteinization employing both chymotrypsin and organic solvents. Massie & Zimm (1965) and Davern (1966) have used Pronase for the isolation of high-molecular-weight bacterial DNA.

To remove protein from chromosomal material of very high molecular weight, proteolytic enzyme digestion would appear to be particularly suitable, since degradation by shear (Levinthal & Davidson, 1961) may be avoided. Since most nucleohistone fractions contain a high proportion of basic amino acids (Murray, 1965), it is logical to suppose

that trypsin might be particularly efficacious in the digestion of nucleoprotein, since it catalyses the hydrolysis of peptide bonds for which the carbonyl carbon is contributed by arginine or lysine (Bergmann & Fruton, 1941).

We decided to extend the studies of the removal of protein with proteolytic enzymes, and this paper describes the action of trypsin on calf thymus nucleohistone. The general approach that we have taken to the problem of what trypsin does to the nucleoprotein has been to prepare first a suspension of nucleoprotein in dilute salt by high-speed blending. On treatment of this with trypsin, we find many properties of the nucleoprotein are changed. From the trypsin-treated nucleoprotein the DNA may then be isolated by a detergent method. Therefore three types of preparations are available for our study: the original nucleoprotein (TNH-O*), the trypsin-treated nucleoprotein (TNH-T) and the DNA derived from the latter.

We have examined the amino acid composition, the solubility properties and the hydrodynamic properties of both types of nucleoprotein preparations and the DNA isolated from them. An attempt was made to assess the strength of interaction between the DNA and the peptides that remain associated after trypsin treatment by studying the streptomycin precipitation characteristics, ethidium bromide binding properties and thermal melting curves for the nucleoprotein preparations and the DNA.

The properties of trypsin-treated nucleoprotein have been investigated previously by Cohen (1945). The original formulation of this problem was suggested to us by him, and he has contributed many ideas along the way.

MATERIALS AND METHODS

Preparation of nuclei. The method of Allfrey, Mirsky & Osawa (1957) was used for the isolation of calf thymus nuclei. The nuclei were either used immediately or frozen and then thawed when needed for preparation of the nucleoprotein.

Determination of nucleic acids and proteins. The diphenylamine test of Dische (1955) was used for DNA determinations. Calf thymus DNA was employed as a standard. The weight of the DNA was based on its phosphorus content (King, 1932) with the assumption that pure DNA contains 10% of phosphorus. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Calf thymus histone purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) was used as the standard.

Amino acid analyses. Duplicate amino acid analyses were performed on a Beckman 120C amino acid analyser (Spinco Division, Beckman Instruments, Palo Alto, Calif., U.S.A.). The nucleoprotein samples were hydrolysed for 24 hr. at 110° in 6N-HCl in sealed but not evacuated tubes. A 2 ml. sample of a digest containing between 0.03 and 0.3 ml. of

each amino acid was applied to each column. The buffers used for the long and short columns are described in the manufacturers' instruction manual entitled *Preparation of Reagents* (March, 1966) A-TB-020C, p. 3.

Lanthanum precipitation. One vol. of a 2% solution of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ in 0.1M-sodium acetate buffer, pH 4.0, was added to 10 vol. of the nucleoprotein solution, containing approx. 200 μg . of DNA/ml. in 0.2M-NaCl-10mM-sodium phosphate buffer, pH 7.0. The mixture was placed in an ice bath for at least 10 min. Then it was centrifuged at 600g for 10 min. The supernatant solution was removed with a Pasteur pipette, and Lowry and diphenylamine determinations were made on it.

La^{3+} forms a white precipitate with the reagent for the Lowry determinations. This precipitate was removed from the solution by centrifugation. Then the extinction of the supernatant solution was measured. Such a procedure had no effect on the amount of colour produced by calf thymus histone.

Hydrodynamic studies. All viscosity measurements were made at approx. 27° and corrected to 27.00°.

(a) The rotating-cylinder viscometer described by Schumaker & Bennett (1962) was used to obtain the results in Fig. 1.

(b) The viscosity changes during digestion of the nucleoprotein with trypsin (Fig. 2) were measured in Ostwald viscometers mounted side by side in a 27° water bath.

(c) The intrinsic viscosities of the nucleoprotein and DNA preparations were calculated from measurements made in a three-bulb capillary viscometer with average velocity gradients of 53, 113 and 174 sec^{-1} . For the ethidium bromide-nucleoprotein studies a similar viscometer having average velocity gradients of 30, 60 and 90 sec^{-1} was employed. The procedure of Schumaker & Marano (1960) was used for the extrapolations.

The procedure of Rosenbloom & Schumaker (1963) was used for sedimentation measurements. These measurements were made in a 4–6% (w/v) sucrose gradient at 35 600 rev./min. in a Beckman-Spinco model E analytical ultracentrifuge equipped with ultraviolet optics. A 30 mm. cell allowed the use of DNA concentrations of approx. 10 μg /ml. The measurements were made at room temperature and the sedimentation coefficients corrected to $S_{20,w}$.

Streptomycin precipitation. Cohen (1946) has shown that streptomycin combines with nucleic acids to produce polymeric compounds whose size depends on the ratios of the bivalent base to the multivalent nucleate. Above a limiting ratio, precipitate forms. To 2 ml. of a nucleoprotein (or DNA) solution in 2mM-NaCl, containing about 35 μg . of DNA/ml., streptomycin (Pfizer Laboratories, Los Angeles, Calif., U.S.A.) was added to give the desired S/P ratio (the ratio, in moles, of streptomycin to DNA phosphate). Water was then added to give a total volume of 3 ml. This mixture was stirred and placed in ice for 10 min. and then centrifuged for 10 min. at 600g. The extinction at 260 μ of the supernatant solution was measured and compared with the extinction of the nucleoprotein (or DNA) solution to which only water had been added (no streptomycin). This extinction ratio was plotted against the S/P ratio. The above procedure was repeated over a range of S/P values from zero to the point at which no further precipitation was observed. [All extinction readings were corrected for turbidity according to the method of Englander & Epstein (1957).]

* Abbreviations: TNH-O, intact thymus nucleoprotein; TNH-T, trypsin-digested thymus nucleoprotein.

RESULTS

Disruption of nuclei and solubilization by blending. The procedure was carried out at 4°. Nuclei from the calf thymus gland were filtered through cheese-cloth to remove any large aggregates. The DNA concentration was determined by dissolving a measured volume of the suspension of nuclei in 0.1N-sodium hydroxide and measuring the extinction at 260m μ , by using a specific extinction coefficient of 20ml./mg.cm. (Rubenstein, Thomas & Hershey, 1961).

The suspension was adjusted to a concentration of approx. 200 μ g. of DNA/ml. The nuclei were then disrupted by the addition of 1ml. of 0.2M-sodium citrate buffer (pH 8.0)/100ml. of the suspension of nuclei; 15min. was allowed for complete disruption to occur.

The disrupted nuclei were treated in a Waring Blendor connected to a Powerstat. The speed at which the blender is operating and the length of time of blending both affect the viscosity of the disrupted nuclei (Fig. 1).

Preparation of nucleoprotein. The disrupted nuclei used for preparation TNH-O(A) were blended at 8000rev./min. for 5min., which corresponded to a rheostat setting of 60v. The disrupted nuclei used for preparation TNH-O(35) were blended at 5500rev./min. for 2min. For each of the preparations, 17.6ml. of 1M-sodium chloride was added/100ml. of blended nuclei, which brought the concentration to 0.15M-sodium chloride. The precipitated material was allowed to stand for 30min., then centrifuged at 1000g for 15min. The precipitate was redissolved in 2mM-sodium citrate buffer, pH 8.0, by stirring magnetically for 15min.

Any undissolved gel was broken up by being run through a 10ml. blow-out pipette. The precipitation and centrifugation were then repeated except that the final precipitate was redissolved in 10mM-sodium EDTA, pH 7.0. The final solution was then dialysed exhaustively against water. This procedure is a modification of that developed by Chargaff (1955).

Protein content, amino acid composition and viscosity of the intact nucleoprotein. The protein (Lowry)/DNA (diphenylamine) ratio in seven different preparations of the nucleoprotein TNH-O ranged from 1.44 to 1.71, with an average value of 1.58.

The amino acid composition for TNH-O is listed in Table 1, where it is compared with the data of Crampton, Moore & Stein (1955) for calf thymus histone. (We have adjusted their values to the same scale, and rounded-off to integral numbers. We do not list glycine in Table 1 because it is produced in large quantity as a breakdown product during digestion of the DNA.)

The intrinsic viscosity of the nucleoprotein in 0.7mM-sodium phosphate buffer, pH 7, was 24dl./g. of DNA. When the phosphate concentration was increased to 1.0mM, the intrinsic viscosity was 22.5dl./g. of DNA. In 1.5mM-phosphate the intrinsic viscosity decreased to 18dl./g. of DNA, and in 10mM-phosphate the value had decreased to 5dl./g. of DNA. The same kinds of results have been obtained by Zubay & Doty (1959), which they suggest reflect aggregation of the nucleoprotein with increasing salt concentration. In 2M-sodium chloride, a concentration at which dissociation occurs, the intrinsic viscosity was 29dl./g. of DNA.

Fig. 1. Effect of blender treatment on the reduced viscosity of thymus nucleoprotein in 2mM-sodium citrate buffer, pH 8.0: (a) effect of blender speed (plotted as rheostat setting, in volts); (b) effect of the length of time of blending at a rheostat setting of 60v, which corresponds to a speed of 8000rev./min. The viscosity measurements were made with a rotating-cylinder viscometer at a shear gradient of 20sec.⁻¹.

Table 1. *Amino acid analyses*

Values are rounded to integers and adjusted to appropriate totals to facilitate comparisons. The results for Crampton *et al.* (1955) calf thymus histone are taken from a review article by Tristram & Smith (1963). Of the materials studied in the present work, TNH-O is the original nucleoprotein, TNH-T is the nucleoprotein treated for 24 hr. with trypsin and then exhaustively dialysed against water, supernatant is the supernatant solution after centrifugation for 5 hr. at 114000g, and pellet is the pellet formed on centrifugation of nucleoprotein. The results taken from Phillips & Simson (1962) refer to the precipitate from trypsin digestion of arginine-rich histone and the arginine-rich histone starting material.

Amino acid	Amino acid composition						
	Crampton <i>et al.</i>	Present work				Phillips & Simson (1962)	
	(1955) Histone	TNH-O	TNH-T	Supernatant	Pellet	Precipitate	Starting material
Lys	60	63	29	22	7	5	8
His	8	8	4	4	2	1	2
Arg	33	39	22	12	12	6	13
Asp	19	20	24	14	8	6	4
Thr	23	18	18	9	4	4	6
Ser	23	16	14	9	4	3	4
Glu	33	39	34	25	9	13	11
Pro	20	25	16	14	3	3	4
Ala	55	44	34	25	8	15	13
Cys	0	0	0	0	0	0	0
Val	25	26	22	14	8	8	5
Met	3	3	4	0	0	0	0
Ile	18	18	15	10	6	15	12
Leu	31	36	27	19	10		
Tyr	10	8	6	1	2	3	1
Phe	8	8	9	7	3	3	2
Total	369	371	278	185	86	85	85

Digestion of the nucleoprotein with trypsin. Nucleoprotein was dissolved in 10mM-sodium borate-2mM-sodium citrate buffer, pH 7.0, so that the final DNA concentration was about 200 $\mu\text{g./ml.}$ Then water was added to a portion of the nucleoprotein solution (TNH-O) and the same volume of a trypsin solution was added to the second portion (TNH-T) so that the final ratio of enzyme to DNA would be 1:20 by weight. Trypsin (twice-crystallized, salt-free; purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was used in these experiments. The two portions of the nucleoprotein solution were placed in a 27° water bath for approx. 8 hr. For preparation TNH-T(A), viscosity measurements were made during the digestion and the amount of protein removed from the DNA was determined by lanthanum precipitation (Fig. 2). After an 8 hr. digestion the nucleoprotein solutions were cooled to 4° and dialysed against 10mM-sodium chloride.

Fig. 2 shows that on trypsin treatment there occurs an immediate doubling of the viscosity, which then continues to rise for the next 12-24 hr. Peptide release is rapid also, and it continues to rise during this time-interval.

Solubility of the trypsin-treated nucleoprotein. In marked contrast with the original nucleoprotein,

Fig. 2. Effect of trypsin digestion on the viscosity and protein solubilization. For viscosity measurements, the digestion proceeded in Ostwald viscometers in a 27.0° water bath. ●, Reduced viscosity of nucleoprotein (172 $\mu\text{g.}$ of DNA/ml.) dissolved in 10mM-sodium borate-2mM-sodium citrate buffer, pH 7.0; ○, reduced viscosity of the same solution containing 1 $\mu\text{g.}$ of trypsin/20 $\mu\text{g.}$ of DNA. In a separate experiment were measured the changes in the amount of protein in the La^{3+} supernatant solution during the digestion of nucleoprotein with trypsin. Δ, Protein in solution containing nucleoprotein (172 $\mu\text{g.}$ of DNA/ml.) and trypsin (1 $\mu\text{g.}/20 \mu\text{g.}$ of DNA); ▲, protein in solution containing only nucleoprotein (172 $\mu\text{g.}$ of DNA/ml.). Each of the solutions was in 10mM-sodium borate-2mM-sodium citrate buffer, pH 7.0; the temperature was 27.0°.

the trypsin-treated material is soluble at all concentrations of sodium chloride. Thus hydrodynamic studies could be performed at sufficiently high salt concentrations, e.g. 0.2M-sodium chloride, to minimize electrostatic interactions, and permitted less ambiguous interpretation of the results in terms of size, shape and molecular weight of the trypsin-digested nucleoprotein.

Protein and amino acid composition of the trypsin-digested nucleoprotein. The DNA in the nucleoprotein solutions (TNH-O and TNH-T) is entirely precipitated by lanthanum; 94% of the protein in the solution of the undigested nucleoprotein (TNH-O) is also precipitated by this procedure, but only approx. 33% of the protein in the solution of 24hr.-trypsin-digested nucleoprotein (TNH-T) is precipitated.

To test by an independent method that a portion of the trypsin-digested protein was still in the form of a complex with the DNA, we used a centrifugal isolation procedure. A 6ml. sample of TNH-T dissolved in 0.2M-sodium chloride-10mM-sodium phosphate buffer, pH 7.0, was placed in each of two centrifuge tubes. These tubes were then placed in the 40.3 rotor of the Spinco model L preparative ultracentrifuge and centrifuged for 5 hr. at 40000 rev./min. (114000g). Samples of the supernatant solutions were used for Lowry and diphenylamine determinations.

All but 2% of the DNA in the nucleoprotein solutions (TNH-O and TNH-T) sediments under these conditions. In the undigested nucleoprotein solutions (TNH-O) 92% of the protein sediments with the DNA. In the digest nucleoprotein solutions (TNH-T) only 31% of the protein sediments.

For preparation TNH-T(A) the amount of digested protein in the form of a complex with the DNA was determined by means of lanthanum precipitation and also by ultracentrifugation. The results of the two procedures were nearly identical, and the average value was 0.44 μ g. of protein/ μ g. of DNA. For the other preparations only the lanthanum precipitation procedure was used to determine the amount of protein attached to the DNA. These preparations were found to have an average of 0.41 μ g. of protein/ μ g. of DNA.

The amino acid compositions of similarly prepared TNH-T, the dialysed supernatant and the centrifugally separated trypsin-digested nucleoprotein are given in Table 1.

Preparation of a molecular-weight series of trypsin-digested nucleoproteins and DNA. The trypsin-digested nucleoprotein TNH-T(35) was blended to obtain the nucleoprotein preparations other than TNH-T(A). TNH-T(41) was obtained by blending at 6500 rev./min. for 5 min., TNH-T(70) at 11500 rev./min. for 5 min. and TNH-T(90) at 14800 rev./min. for 10 min.

The hyperchromicity produced by the addition of alkali is the same for all the samples measured, so the sedimentation and viscosity results probably do not reflect a denaturation due to the Waring Blender treatment.

DNA was isolated from a portion of the nucleoprotein preparations by a modification of the procedure of Kay *et al.* (1952) as developed by Borenfreund, Fitt & Bendich (1961). However, we substituted our trypsin digestion procedure for theirs. The DNA preparations isolated from TNH-T(35), TNH-T(41), TNH-T(70), TNH-T(90) and TNH-T(A) were designated DNA(35), DNA(41), DNA(70), DNA(90), and DNA(A) respectively.

Effects of unattached peptides on hydrodynamic studies of DNA. The solutions of trypsin-digested nucleoprotein contained unattached peptides as well as a DNA-protein complex. We attempted to separate these unattached peptides from the solutions containing the complex. Ultracentrifugation and lanthanum precipitation effected such a separation; however, in both cases the complex had become insoluble. Therefore we had to use the solutions containing both the unattached protein and the DNA-protein complex for our hydrodynamic measurements. Thus it became important to determine whether or not the unattached peptides influenced the hydrodynamic behaviour of our preparations. We used the ultracentrifugation separation method to prepare a solution containing only the unattached peptides. This solution was

Fig. 3. Variation of the reduced viscosity of trypsin-digested nucleoprotein with shear gradient. ●, TNH-T(35); △, TNH-T(A); ○, TNH-T(41); ×, TNH-T(70); □, TNH-T(90). Straight lines were used to extrapolate to zero shear gradient. The $\eta_{sp.}/c$ value at zero shear gradient was considered to be the intrinsic viscosity in terms of dl./g. of DNA. If this intrinsic viscosity value is divided by 1.41, or by 1.44 for TNH-T(A), the intrinsic viscosity is then expressed in units of dl./g. of DNA plus protein.

Table 2. *Intrinsic viscosity, sedimentation and molecular-weight values for DNA and trypsin-digested nucleohistone*

$[\eta]$ for DNA is expressed as dl./g. of DNA, and $[\eta]$ for TNH-T as dl./g. of DNA plus protein. Molecular weights of DNA were calculated by the method of Schumaker (1959) with β values from Eigner & Doty (1965), and molecular weights for TNH-T by the method of Schumaker (1959) with β values from the preceding column. In the last column each molecular weight for TNH-T was calculated from the molecular weight of the DNA isolated from it plus the weight of the attached protein.

Sample	DNA			TNH-T				
	$[\eta]$	$S_{50\%}$	$10^{-6}M$ from S , $[\eta]$ and β	$[\eta]$	$S_{50\%}$	$10^{-6}\beta$	$10^{-6}M$ from S , $[\eta]$ and β	$10^{-6}M$ from protein content and M of DNA
(35)	40.0	16.4	5.48	17.4	24.2	2.12 2.50 2.80 3.10	9.80 7.58 6.43 5.50	7.74
(A)	35.0	13.2	3.68	11.8	21.8	2.12 2.50 2.80 3.10	6.80 5.33 4.52 3.85	5.30
(41)	23.0	12.9	2.52	10.0	16.6	2.12 2.50 2.80 3.10	4.37 3.44 2.90 2.49	3.55
(70)	23.7	12.7	2.48	7.8	15.6	2.12 2.50 2.80 3.10	3.52 2.74 2.32 1.98	3.50
(90)	15.0	10.3	1.60	5.7	12.3	2.12 2.50 2.80 3.10	2.17 1.66 1.44 1.22	2.25

added to a solution of free DNA and the viscosity was determined as 41 dl./g., at zero shear gradient and a DNA concentration of 20 μ g./ml. The viscosity of a solution containing only DNA (20 μ g./ml.) was 40 dl./g. at zero shear gradient. (The solvent for the viscosity measurements was 0.2M-sodium chloride-10mM-sodium phosphate buffer, pH 7.0.) So the intrinsic viscosity of DNA, which was one of the most sensitive properties measured in these studies, was not significantly influenced by the presence of these unattached peptides.

Comparison of the molecular weights of the nucleoprotein and the DNA isolated from it. For each of the nucleoprotein and DNA preparations, viscosity (Fig. 3) and sedimentation measurements were made, and the molecular weights of the preparations were calculated from these results (Table 2). Weight-average molecular weights were computed from the intrinsic viscosities and the entire sedimentation coefficient distribution of each sample, by the method of Schumaker (1959). A digital computer was used for the computations. If the values of $S_{50\%}$ were used, molecular weights calculated by the method of Mandelkern & Flory (1952) and Scheraga & Mandelkern (1953) were

close to those obtained by using the entire distribution.

Inspection of the data in Table 2 shows that, if a value of about 2.40×10^6 is chosen for the Scheraga & Mandelkern (1953) β function for the TNH-T preparations, then the molecular weights so calculated agree with those calculated from the molecular weights of a single piece of DNA and its associated protein.

Concentration- and shear-dependence of the viscosity of nucleoprotein solutions. Viscosity measurements for TNH-T(A) and the DNA isolated from it were made in 0.2M-sodium chloride, and the intrinsic viscosities were determined from double extrapolation plots (Fig. 4) to be 17 ± 1 and 38 ± 1 dl./g. respectively. The concentration- and shear-dependence for TNH-T are much less than for DNA.

The aggregated intact nucleoprotein TNH-O(A) is precipitated in 0.2M-sodium chloride. However, in 10mM-sodium chloride-10mM-sodium phosphate its intrinsic viscosity is 3 dl./g. In this salt solution the DNA isolated from the nucleoprotein has an intrinsic viscosity of 45 dl./g.

The densitometer tracings from which the sedimentation coefficients for the TNH-T preparations were calculated all have a very flat plateau

Fig. 4. Reduced viscosity of solutions of TNH-T and DNA isolated from the TNH-T as a function of the shear gradient and the DNA concentration. The solvent was 0.2M-NaCl-10mM-sodium phosphate buffer, pH 7.0. The DNA concentrations were: ●, 76.7 $\mu\text{g./ml.}$; □, 42.8 $\mu\text{g./ml.}$; ■, 21.7 $\mu\text{g./ml.}$. The nucleohistone concentrations were: ▽, 74.4 $\mu\text{g. of DNA/ml.}$; ○, 37.2 $\mu\text{g. of DNA/ml.}$; ▼, 18.6 $\mu\text{g. of DNA/ml.}$. The continuous lines that rise from left to right represent contours of constant shear gradient. The continuous lines that descend from left to right represent contours of constant DNA concentration. From these lines curves were determined for the variation of $\eta_{sp.}/c$ with DNA concentration at zero shear gradient (×) and for the variation of $\eta_{sp.}/c$ with shear gradient at zero DNA concentration (Δ). The point at which these curves meet on the $\eta_{sp.}/c$ axis is the intrinsic viscosity in units of dl./g. of DNA.

region with no material accumulated at the bottom of the cell. These results indicate that there are no significant amounts of large aggregates present.

'Melting' curves and curves of precipitation by streptomycin. The intact nucleoprotein undergoes its thermal transition at a higher temperature than DNA (Fig. 5). The 'melting' curve for a 1:1 mixture (by extinction) of DNA and intact nucleoprotein is intermediate between the separate curves for DNA and intact nucleoprotein. Bonner & Huang (1963) have obtained a similar curve for a material they believe to be a mixture of nucleoprotein and DNA. The 'melting' curve for TNH-T is similar to that for DNA. TNH-T does not behave like the intact nucleoprotein or the mixture of DNA and intact nucleoprotein.

The intact nucleoprotein is precipitated with streptomycin much more readily than DNA (Fig. 6). A 1:1 mixture (by extinction) of DNA and intact nucleoprotein gives a precipitation curve that is intermediate between the separate curves for DNA and intact nucleoprotein. The precipitation curve for TNH-T, however, can be superimposed on that

Fig. 5. Variation in the relative extinction as a function of the temperature of solutions containing DNA or intact nucleoprotein. ○, TNH-T; ●, DNA; ×, TNH-O; △, mixture of DNA and TNH-O. The middle curve is calculated for a mixture of DNA and TNH-O from the averaged normalized values obtained from the other two curves. For the mixture, 50% of the extinction was caused by DNA and 50% by TNH-O. The solvent was 1mM-NaCl-10mM-sodium phosphate buffer, pH 7.0.

Fig. 6. Streptomycin precipitation curves for DNA, intact nucleoprotein, trypsin-digested nucleoprotein and mixtures of DNA and intact nucleoprotein. ●, DNA; ○, TNH-T; △, 1:1 mixture of DNA and TNH-O; ----, curve for mixture of DNA and TNH-O calculated from the averaged normalized values obtained from the DNA (●) and TNH-O (×) curves. The S/P ratio is the ratio of moles of streptomycin to DNA phosphate.

Fig. 7. Effect of ethidium bromide on the viscosity of TNH-T. The solvent employed was 0.2M-NaCl. The concentrations of TNH-T were 29.6, 54.9 and 75.4 $\mu\text{g./ml.}$ Curve B is before the addition of ethidium bromide, and curve A is after the addition of 100 $\mu\text{g.}$ of ethidium bromide/ml. Lines and extrapolation procedure are as in Fig. 4.

for DNA. Thus both the 'melting' profiles and the streptomycin precipitation curves indicate that TNH-T is a molecule much like DNA and is not a mixture of DNA and intact nucleoprotein.

Effect of ethidium bromide on the viscosities of TNH-O, TNH-T and DNA. Ethidium bromide intercalates with DNA, causing a stiffening of the helix and an increase in the viscosity (Le Pecq, 1965; Le Pecq & Paoletti, 1967). A large excess of ethidium bromide causes the viscosity of our DNA to double and that of TNH-T to increase threefold (Fig. 7). The same concentration of dye causes precipitation of TNH-O. As the concentration of dye is raised from zero, the viscosity of TNH-O increases about 50%, and then levels off before precipitation begins.

DISCUSSION

Chemical composition. The nucleoprotein that we employed as our starting material is different in its physical and chemical properties from the well-characterized nucleoprotein described by Zubay & Doty (1959). Their material has approximately equal weights of protein and nucleic acid, whereas ours contains about 1.6 parts of protein to 1 part of nucleic acid. Zubay & Doty (1959) repeatedly

homogenized the calf thymus tissue and centrifuged from a solution of 75mM-sodium chloride-24mM-sodium EDTA, pH 8. This cycle was repeated six times. Our material was a homogenate of nuclei in 2mM-sodium citrate, subsequently precipitated in 0.15M-sodium chloride. This cycle was repeated only once, which may account for the greater abundance of protein in our sample.

The amino acid composition of the TNH-O is similar to that of the nucleoprotein analysed by Crampton *et al.* (1955).

After treatment with trypsin, about a quarter of the protein was lost during dialysis, including at least half of the arginine and lysine. About half of the original protein is present as soluble non-diffusible peptides, and the remaining quarter appears to be bound to the DNA. These free and bound fractions are different in their amino acid compositions; the bound peptides have a lysine/arginine ratio about 0.6, whereas for the free peptides this ratio is 1.8. This clearly indicates preferential binding to the DNA of a fraction of the peptides.

Approx. 0.9 amino acid residue/nucleotide is bound to the DNA. Out of every ten residues bound, about two are arginine or lysine, two are glutamic acid and aspartic acid (or their respective amides) and there is one each of alanine, leucine, isoleucine and valine. The bound peptides appear to be more like histone B than histone A (Crampton *et al.* 1955) in overall composition.

Minimum degree of polymerization. If trypsin cleaves protein at bonds adjacent to every lysine and arginine residue, then the minimum number-average degree of polymerization of the bound peptide would be about 4.7. However, some of the potentially susceptible sites might not be available for tryptic hydrolysis if portions of the protein were firmly attached to the DNA, or if there were sequence peculiarities. It is noteworthy that Phillips & Simson (1961) have found that 25% of the arginine-rich (lysine/arginine ratio 0.6-0.8) histone is precipitated when digested with trypsin. The amino acid composition of their precipitate is compared with our bound histone (the pellet) in Table 1: it contains considerably less arginine and lysine, and more alanine and glutamic acid or glutamine. It is plausible to suppose that much of the bound histone in our experiments is a fraction similar to their precipitate, except that our material has been partially protected during the trypsin digestion by being bound to the DNA and hence contains more arginine and lysine. Indeed, if the starting material used by Phillips & Simson (1962), an arginine-rich histone isolated by an acid-ethanol extraction procedure, is compared in amino acid composition with our pellet, the agreement is considerably greater (Table 1). They estimate that

the average molecular weight of the precipitated peptide is about 5000.

The quantities of bound lysine and arginine in the pellet are sufficient to neutralize less than 25% of the negative charge on the 'backbone' phosphate groups. As much as 25% more could be neutralized by potential α -amino groups, depending on the extent of tryptic digestion.

Solubility characteristics. The solubility properties of TNH-O and the Zubay & Doty (1959) nucleoproteins are similar; both are soluble in 0.7 mM-phosphate, and begin to aggregate above 1 mM. During trypsin treatment, however, the nucleoprotein rapidly becomes soluble at all concentrations of sodium chloride. This behaviour also contrasts with the solubility properties of mixtures of DNA and synthetic basic polypeptides such as polylysine and polyarginine. In 0.1 M-sodium chloride, polylysine of degree of polymerization 7 at a polylysine/DNA (amino acid residue/phosphate) ratio 0.45, will cause precipitation of over 20% of the DNA (Fig. 1a of Lang & Felsenfeld, 1966).

This contrast in solubility behaviour suggests that the synthetic peptides and intact histones neutralize extended regions of contiguous negative charge on the 'backbone' phosphate groups of DNA, whereas the TNH-T peptides only partially and intermittently neutralize the negative charge in any local regions.

Precipitation with streptomycin. The basic amine, streptomycin, causes precipitation of TNH-O at S/P ratios below 1, whereas DNA is not comparably precipitated until the S/P ratio reaches 3. Probably this difference in behaviour reflects the firm binding of polypeptides to the negative charges on the 'backbone' phosphate groups of the intact nucleoprotein. Thus a lower streptomycin concentration is needed to neutralize any remaining negative charges in TNH-O and, perhaps, to cross-link molecules.

The precipitation characteristics of TNH-T are identical with those of DNA rather than intermediate between DNA and TNH-O. Evidently the positively charged arginine and lysine residues remaining on TNH-T are not successfully competing with streptomycin for negative charges on the 'backbone' phosphate groups.

Number of pieces of DNA in TNH-T. The unusual solubility properties of TNH-T permit physicochemical studies at salt concentrations sufficient to suppress most of the intermolecular electrostatic interactions found at low salt concentrations. Therefore reliable sedimentation and viscosity results can be obtained for the series of TNH-T preparations blended for increasing times. To convert these into molecular weights, values for the β -function of Scheraga & Mandelkern (1953) are required for TNH-T. We would expect β

values close to 2.5×10^6 for coiled molecules (Flory, 1953; Eizner & Ptitsyn, 1962; Eigner & Doty, 1965). A maximum molecular weight, however, may be computed by assuming the minimum permissible value for β of 2.12×10^6 (Scheraga & Mandelkern, 1953). A minimum molecular weight for the TNH-T may be also computed from the known molecular weight of one piece of DNA and the ratio of protein to nucleic acid. These values are presented in the last two columns of Table 2. These minimum and maximum values are not far apart, indicating that TNH-T contains only one DNA molecule.

In 0.7 mM-phosphate, the Zubay & Doty (1959) nucleoprotein also is composed of one piece of DNA in the form of a complex with protein. As the concentration of phosphate is increased above 1 mM, however, their material begins to aggregate as determined by light-scattering (Zubay & Doty, 1959). Our trypsin-treated nucleoprotein, on the other hand, appears to remain as a single piece of DNA with associated peptides at a salt concentration of 0.2 M.

Molecular shape of TNH-T. For a homologous series, the molecular-weight exponents in the equations relating the sedimentation coefficient, S , and the intrinsic viscosity, $[\eta]$, to molecular weight, M :

$$S = K_S M^{a_S} \quad (1)$$

$$[\eta] = K_\eta M^{a_\eta} \quad (2)$$

(where K_S , a_S , K_η and a_η are constants) can be theoretically related to the configuration of the molecule. Log-log plots of $[\eta]$ or $S_{50\%}$ versus M were made for TNH-T, by using for M the values given in column 8 of Table 2 and at a β value of 2.50×10^6 . The slope of the plot of $\log S$ versus $\log M$ was found to be 0.45, whereas that of the plot of $\log [\eta]$ versus $\log M$ was found to be 0.66 (Fig. 8). For coils these slopes should lie in the range 0.40–0.50 for the $\log S$ relation and in the range 0.50–0.80 for the $\log [\eta]$ relation (Flory, 1953). Rod-like molecules, on the other hand, would have a slope of the plot of $\log [\eta]$ versus $\log M$ of 1.8 and a slope of the plot of $\log S$ versus $\log M$ of 0.2 (Nishihara & Doty, 1958). Thus the $[\eta]$ - S - M relationships indicate that TNH-T behaves like a coil in solution, provided that we can assume that the blended samples form a homologous series.

Molecular size of TNH-T. The molecular dimensions of the coiled TNH-T molecules are smaller than those of the DNA they contain. A size may be computed from hydrodynamic considerations. The function $[\eta]S/(1 - \bar{v}\rho)$ can be shown to be proportional to the square of the radius of the equivalent hydrodynamic sphere (Flory, 1953). Thus a simple proportion will yield the percentage change in radius. For TNH-T(A), the decrease in radius is found to be 20%.

Fig. 8. Relationship between the intrinsic viscosity, sedimentation coefficient and the molecular weight for trypsin-digested nucleoprotein. $\times$, $[\eta]$ versus M_w ; $\bullet$, $S_{50\%}$ versus M_w . The molecular weights were calculated by the method of Schumaker by using a β value of 2.40×10^6 . The data are also presented in Table 1. The intrinsic viscosity is presented as dl./g. of DNA and protein.

From hydrodynamic studies (Giannoni & Peacocke, 1963) and light-scattering measurements (Zubay & Doty, 1959), the thymus nucleoprotein isolated by the method of Zubay & Doty (1959) also appears to be a more compact coil than the DNA that may be isolated from it. On the other hand, the binding of polylysine to DNA causes a small increase in the intrinsic viscosity of the complex (Scruggs & Ross, 1964). Though this may, at least in part, be caused by aggregation rather than by expansion of the molecules, nevertheless it indicates a difference in mode of interaction of the synthetic polylysine with DNA from that observed with intact nucleoprotein and TNH-T.

Ethidium bromide binding studies. Like DNA, the trypsin-treated nucleoprotein stiffens when the dye, ethidium bromide, intercalates between the bases. The TNH-O molecule shows a much smaller total viscosity increase, and is saturated at considerably lower ethidium bromide concentrations, which implies that much of the TNH-O molecule is protected from dye insertion by associated protein. Evidently those proteins that protect the TNH-O molecule from dye insertion have not been left intact during trypsin treatment.

Thermal stability. Intact nucleoprotein (Lee, Walker & Peacocke, 1963), and DNA plus poly-arginine or polylysine (Olins, Olins & Hippel, 1967) show enhanced thermal stability when compared with DNA. TNH-T does not, and its 'melting' properties are very similar to or identical with those

of DNA. If increased thermal stabilization were simply a question of 'damping' of net negative charges, an intermediate curve might be expected. Evidently the TNH-T peptides are associated with the DNA in such a manner that neither the stacking forces between the bases are increased nor are the electrostatic repulsions on the 'backbone' phosphate groups effectively neutralized.

Model for TNH-T. We now have enough information to suggest a model for TNH-T. The streptomycin precipitation data, ethidium binding data and lack of enhanced thermal stability suggest that the peptides are only weakly bound to the DNA in TNH-T. This is not inconsistent with the results of our isolation procedures, for only a small binding energy would be required to account for the removal of most of these peptides with the DNA on ultracentrifugation or lanthanum precipitation. On the other hand, from the amino acid analyses, these peptides appear to belong to the class of arginine-rich histones, and from the results of Phillips & Simson (1962), discussed above, probably have molecular weights of at least 5000. On extraction with increasing concentrations of salt, the arginine-rich histones are the last to come off and therefore appear to be the most firmly bound (Crampton, Stein & Moore, 1957; Akinrimisi *et al.* 1965).

To resolve this apparent difference in strength of binding, we suggest that the peptides in TNH-T are held in place primarily by hydrogen bonds, London forces and hydrophobic interactions rather than by salt linkages, and they would not be expected to be much affected by increased ionic strength, nor would they compete with streptomycin binding.

The smaller radius of gyration of the TNH-T molecule, which results in a considerably lower viscosity when compared with DNA, may be the result of a disturbance in the structure of organized water about the DNA caused by binding of protein. If 2g. of water is tightly associated with 1g. of purified DNA, as measured by Hearst & Vinograd (1961), in dilute aqueous salt solutions, this might increase the rigidity of the DNA molecule and also affect the distance of closest approach of chain segments, leading to an expansion of the coil. It is conceivable that the associated protein could interfere with the packing of water about the helix, resulting in a decrease in the molecular domain.

Therefore our current model for TNH-T is that of a weakly associated complex molecule consisting of one piece of DNA together with an approximately equimolar amount (amino acid residues/phosphate) of arginine-rich histone of 5000 or greater molecular weight. This histone could be spread out along the length of the DNA, perhaps in one of the grooves, leaving the other groove and the 'backbone'

phosphate groups free for complex-formation with the lysine-rich histones (Wilkins, 1956; Tsuboi, Matsuo & Ts'o, 1966).

In summary, the molecule we have produced by tryptic digestion is probably unlike the original nucleoprotein, though some parts of the original structure may remain. Nevertheless the trypsin treatment appears to be useful in affording a soluble product that has many of the properties of the purified DNA. It may be of practical value in the study of the size of undegraded DNA molecules obtained from chromosomes of eukaryotic nuclei.

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