Studies on the Accessibility of Deoxyribonucleic Acid in Deoxyribonucleoprotein to Cationic Molecules

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The binding of deoxyribonucleoprotein to Toluidine Blue, to cetylpyridinium chloride and to polylysine of various molecular weights was studied to determine the percentage of free DNA phosphate groups in deoxyribonucleoprotein. Binding was measured by addition of these reagents to deoxyribonucleoprotein at a range of concentrations such that complete precipitation of the deoxyribonucleoprotein occurred. With Toluidine Blue the binding corresponded to about 48% of the DNA phosphates in deoxyribonucleoprotein. The dye did not cause appreciable displacement of protein from the DNA. With cetylpyridinium chloride the binding corresponded to about 41% of the DNA phosphates. With polylysine preparations of molecular weight 1250 and 7790 the binding values for deoxyribonucleoprotein were 46 and 38% respectively. The results suggest that the free phosphates lie in stretches sufficiently long to accommodate most of each polylysine molecule. With polylysine of molecular weight 62000 cross-linking of free stretches of DNA on different deoxyribonucleoprotein molecules probably occurs. It is concluded that although most of the free phosphates are probably 'hidden' beneath covering histone, corresponding perhaps to runs of non-basic residues in the latter, they are surprisingly accessible to very large molecules. The relevance of this finding to the problem of gene repression is discussed.

It is generally assumed that in chromatin, proteins must be absent or displaced from the DNA at certain sites to enable transcription to occur. However, on the basis of model-building, Johns (1969) has pointed out that the super-helicity of the DNA rather than the presence of proteins may present a steric hindrance to RNA polymerase; also, the repression of transcription by proteins added to DNA *in vitro* may be related to the insolubility of the deoxyribonucleoprotein in the medium used rather than to any innately lower priming capacity of the DNA (Johns & Hoare, 1970).

An approach to finding the role of the histones is to determine just what proportion of the DNA in deoxyribonucleoprotein they actually 'cover', by estimating the percentage of free DNA phosphates. Previous dye-binding studies on deoxyribonucleoprotein indicated that about 50% of the total phosphates was free (Klein & Szirmai, 1963; Miura & Ohba, 1967); this seemed surprising in view of the approximate numerical equality of the DNA phosphate and the histone basic groups. In the present work, the binding of the DNA phosphates

of deoxyribonucleoprotein to Toluidine Blue and to the cationic detergent cetylpyridinium chloride was studied. Further, to enable an estimate to be made of the size of zones of free phosphates in deoxyribonucleoprotein, poly-L-lysine hydrobromide of various molecular weights was used. In the case of the dye, checks were made to find if there was any binding to the protein of the deoxyribonucleoprotein and if the dye caused displacement of the protein from the DNA. Parallel studies were carried out on the binding of Toluidine Blue, cetylpyridinium chloride and polylysine to free DNA.

The deoxyribonucleoprotein used here was prepared by a very mild procedure in the hope of obtaining material native in configuration and in composition. Previous studies had shown that the properties of the deoxyribonucleoprotein were reproducible, that its content of histone and non-histone proteins was fairly constant, and that by the available criteria, it was native (Itzhaki, 1966*a,b,c*, 1967, Itzhaki & Rowe, 1969). It thus seemed satisfactory for use in binding studies *in vitro* which might relate to the accessibility of the DNA of chromatin *in vivo* to RNA polymerase. The

results show that a surprisingly large amount of the DNA in deoxyribonucleoprotein is, in fact, accessible to large molecules.

EXPERIMENTAL

Materials. Deoxyribonucleoprotein was prepared from rat thymus glands as described by Itzhaki (1966a). The final gel product usually contained about 37% of nucleic acid, 49% of histone protein and 14% of non-histone protein. The gel was diluted to concentrations between 0.0007 and 0.05% DNA usually by using 0.7 mM-sodium phosphate buffer, pH 6.9 (buffer A). Calf thymus DNA was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and was dissolved in buffer A to concentrations between 0.002 and 0.05%.

Toluidine Blue was obtained from Gurr Ltd., London N.W.9, U.K., and was dissolved in buffer A to concentrations of 20–500 μM . Cetylpyridinium chloride was recrystallized from water and was dissolved in buffer A or in water to a concentration of 0.02%. Samples of poly-L-lysine hydrobromide of average molecular weight 1250 (polylysine 1), 7790 (polylysine 2) and 62 000 (polylysine 3) were obtained from Miles-Seravac Ltd., Maidenhead, Berks., U.K. Polylysine was dissolved in buffer A to concentrations of 2–4 mM in terms of lysyl residues. Methyl Orange was recrystallized from water and was dissolved in buffer A to a concentration of 1 mM. Deoxyribonuclease I (EC 3.1.4.5) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

DNA was determined from the value of E_{260} , $E_{1\text{cm}}^{1\%}$ at 260 nm being taken as 210. Protein was determined by a micro-biuret method (Itzhaki & Gill, 1964).

General procedure. All manipulations were done at 0–4°C and all glassware used in the dye studies was Pyrex.

The binding of Toluidine Blue and polylysine was measured by addition to deoxyribonucleoprotein or DNA solutions at a range of concentrations such that the latter were completely precipitated; the difference between the amount of Toluidine Blue or polylysine added and that remaining in excess gave the amount bound. Addition of cetylpyridinium chloride to deoxyribonucleoprotein or DNA caused complete precipitation of the latter only at a very limited range of concentrations and binding was measured simply by finding the cetylpyridinium chloride/DNA ratio that gave maximal precipitation.

Experiments with Toluidine Blue. Toluidine Blue was determined from the E_{635} , $E_{1\text{cm}}^{1\%}$ at 635 nm being taken as 33 200 (Michaelis & Granick, 1945). Values of E_{635} varied linearly with the concentration of Toluidine Blue up to about 22 μM so samples were always diluted to below this value before measurement. Mixtures of deoxyribonucleoprotein and Toluidine Blue gave fibrous precipitates at final Toluidine Blue concentrations of 26–500 μM , for dye/DNA phosphate ratios from 0.1:1 up to at least 3.0:1; at a ratio of 0.05:1, with Toluidine Blue at 26 μM , a gelatinous precipitate formed.

Comparison of supernatants from mixtures kept for 0.5 h, shaken for 0.5 h and shaken for 2 h before centrifugation showed that E_{635} of the shaken samples was the same but that of the unshaken sample was higher. Mixtures centrifuged at 400g for 15 min or 30 min gave supernatants with similar E_{635} values.

The final procedure consisted of addition of 2 ml of

0.05–1 mM-Toluidine Blue to 2 ml samples of deoxyribonucleoprotein (usually 0.0014–0.028% DNA). The mixtures were shaken for 30 min and were then centrifuged at 400g for 15 min. To find if any dye was trapped in the precipitate, the latter was resuspended and shaken in 4 ml of buffer A and was then centrifuged. This procedure was repeated twice. The visible spectra of all supernatants were read and also the u.v.-absorption spectra. The E_{260}/E_{290} ratio increased in the presence of excess of soluble DNA above the value of 0.5 for Toluidine Blue alone and so was used as a test for DNA in the supernatant. The general procedure for DNA was the same as for deoxyribonucleoprotein.

Experiments with cetylpyridinium chloride. Mixtures of cetylpyridinium chloride and deoxyribonucleoprotein near the point of charge neutralization formed fibrous suspensions; when excess of deoxyribonucleoprotein was present a gelatinous suspension formed. Mixtures of cetylpyridinium chloride and DNA formed fine suspensions that became coarser on shaking.

Supernatants from cetylpyridinium chloride–deoxyribonucleoprotein mixtures kept overnight before centrifugation and from those centrifuged immediately after 30 min shaking had similar E_{260} values.

In the case of deoxyribonucleoprotein–cetylpyridinium chloride mixtures, it was necessary to centrifuge at 3000g for 15 min to produce a clear supernatant. Since blanks of deoxyribonucleoprotein alone in buffer A occasionally gave gelatinous suspensions on centrifugation thus, but not deoxyribonucleoprotein in water, studies were made with deoxyribonucleoprotein and cetylpyridinium chloride in water. In the case of DNA, both reagents were dissolved in buffer A.

The eventual procedure was addition of cetylpyridinium chloride at a final concentration of 142 μM to deoxyribonucleoprotein at final DNA concentrations of 0.007–0.014%. DNA was used at similar concentrations, with cetylpyridinium chloride at 284 μM . The mixtures were shaken for 30 min and were then centrifuged at 3000g for 15 min. The u.v. spectra of the supernatants were then read.

Experiments with polylysine. (a) Determination of polylysine. Polylysine was determined by addition of excess of the anionic dye Methyl Orange (R. F. Itzhaki, unpublished work). This dye reacts stoichiometrically with and precipitates polylysine. The amount of unreacted Methyl Orange was found by measurement of E_{465} of the supernatant. A standard calibration curve relating E_{465} to the amount of polylysine added was obtained by preparing serial dilutions of polylysine from a stock solution containing a known weight of the material. Samples (2 ml) of the dilutions were each mixed with 2 ml of 1 mM-Methyl Orange. The mixtures were shaken for 30 min during which time suspensions developed. After centrifugation for 15 min at 400g, the E_{465} of the supernatants, containing excess of Methyl Orange, was measured after dilution in water. In the case of polylysine 1 and polylysine 2, the curve of E_{465} against polylysine concentrations decreased linearly up to about 250 μM -polylysine. In the case of polylysine 3, the curve decreased linearly up to about 300 μM -polylysine. In all cases the curves eventually became concave upwards; extrapolation of the linear part to zero E_{465} gave a value of polylysine concentration of 500 μM , corresponding to 500 μM -Methyl

Orange. In subsequent work polylysine samples were diluted to a concentration such that after reaction with Methyl Orange, E_{465} of the excess of Methyl Orange was within the linear part of the calibration curve. The amount of polylysine remaining unbound after reaction with deoxyribonucleoprotein or DNA was thus obtained and the amount bound was calculated by subtraction from the initial amount added. The error in the method corresponded to about $\pm 5\%$ of the amount of polylysine bound.

(b) Reaction of polylysine with deoxyribonucleoprotein and DNA. Mixtures of deoxyribonucleoprotein or DNA and polylysine formed white fibrous precipitates. Near the charge-neutralization point, neither time of mixing nor centrifugation speed was critical above the minimum of 30 min shaking and speed of 1500g tried. However, with excess of either reagent, the percentage of DNA in the supernatant decreased with increasing centrifugation speed.

In the deoxyribonucleoprotein-polylysine or DNA-polylysine mixtures, an excess of lysyl residues could be present either as wholly unbound polylysine molecules or as unused 'wasted' lysyl residues in polylysine molecules part of which were bound to deoxyribonucleoprotein or DNA. The former value was determined by adding Methyl Orange to the deoxyribonucleoprotein-polylysine or DNA-polylysine supernatant and the total excess of polylysine by adding Methyl Orange to an equivalent, but uncentrifuged, deoxyribonucleoprotein-polylysine or DNA-polylysine mixture.

The final procedure consisted of addition of 1 ml of polylysine at about 2 mM to paired 3 ml samples of deoxyribonucleoprotein at a suitable range of concentrations. Also, paired blank polylysine samples containing no deoxyribonucleoprotein were prepared. The mixtures were shaken for 30 min and then one of each pair was centrifuged for 15 min at 1500g. The supernatant was removed and E_{260} read to check that the deoxyribonucleoprotein had been completely precipitated. A sample (usually 2 ml) was mixed with 1 mM-Methyl Orange (usually 2 ml). Also, the uncentrifuged paired sample was mixed with Methyl Orange (usually 4 ml of each). The mixtures were shaken and then centrifuged at 400g. The E_{465} values of the supernatants due to excess of Methyl Orange were then read. The procedure was the same for DNA except that the initial polylysine concentration was usually about 4 mM and one of each pair was centrifuged for 15 min at 12000g.

RESULTS

Addition of Toluidine Blue to deoxyribonucleoprotein

Metachromasia. At final Toluidine Blue concentrations in the range 30–500 μM , all the DNA precipitated at dye/DNA phosphate ratios above about 0.67:1 and the peak wavelength of the supernatants remained at 635 nm. At ratios between about 0.1:1 and 0.67:1 a very flat peak at about 595 nm was seen. At ratios below 0.1:1, a second peak was found at about 640 nm. A similar 'blue shift', found in soluble complexes of dyes with synthetic polynucleotides, has been attributed to

Fig. 1. Binding of Toluidine Blue (0.33 μmol) to deoxyribonucleoprotein. $\circ$, Control deoxyribonucleoprotein; $\bullet$, formaldehyde-treated deoxyribonucleoprotein. For experimental details see the text.

'stacking' of dye molecules on adjacent phosphate groups (Steiner & Beers, 1961) whereas the 'red shift' has been attributed (Lerman, 1961) to intercalation of dye molecules between the base pairs of DNA.

Binding curves. At a final dye concentration of 20–30 μM , the E_{635} of the supernatant decreased with increasing DNA phosphate concentration up to about 40 μM but the curve was concave upwards. At a final dye concentration in the range 80–500 μM , linear curves were obtained for dye/phosphate ratios down to about 0.67:1. Fig. 1 curve A shows a typical result. Extrapolation to zero E_{635} gives a value of 0.60 μmol of DNA phosphate, corresponding to the binding of 0.33 μmol of Toluidine Blue. Thus the phosphates in deoxyribonucleoprotein available for binding amount, apparently, to 55%. The increase in E_{635} shown at higher deoxyribonucleoprotein concentrations is accompanied by a blue-peak shift and an increase in the E_{260}/E_{290} ratio, presumably due to the presence of the soluble deoxyribonucleoprotein-dye complex.

In four other experiments, with addition of Toluidine Blue to a final concentration of 80–500 μM to deoxyribonucleoprotein, the apparent values of available phosphates ranged from 59 to 72% with an over-all mean of 63%. By washing the precipitates dye was extracted but not DNA, indicating that the dye released was previously 'trapped' rather than bound to deoxyribonucleoprotein; the amount released was usually about 10% of that originally present in the precipitate. Thus, correction for trapped dye gives an apparent value of free phosphates in deoxyribonucleoprotein of about 57%.

The surprisingly high binding value for deoxyribonucleoprotein could result from two possible

artifacts, dye-induced dissociation of the protein from the DNA and binding of dye to deoxyribonucleoprotein-protein acidic groups; both would lead to a spuriously high value of percentage of free DNA phosphate groups. These possible artifacts were examined as described below.

Dissociation of DNA from protein. Attempts were made to detect protein by the micro biuret method in supernatants from Toluidine Blue-deoxyribonucleoprotein mixtures in which the DNA component was known to be completely precipitated. Interference due to the dye decreased the accuracy of determination but indicated that no substantial amount was present. As an alternative test, deoxyribonucleoprotein was mixed with formaldehyde (final concentration 1%) 24 h before addition of dye. This treatment 'fixes' the protein on to the DNA. In fact about 37% of the protein of the formaldehyde-treated deoxynucleoprotein was soluble in 0.2M-hydrochloric acid compared with 73% of the protein in untreated deoxyribonucleoprotein. It was found that no precipitation of deoxyribonucleoprotein occurred if the formaldehyde was added in buffer (0.7mM-sodium phosphate) at pH 7.7 rather than at 6.9. The deoxyribonucleoprotein-formaldehyde mixtures were not dialysed to remove excess of formaldehyde as untreated deoxyribonucleoprotein precipitated on dialysis and so could not be used as a control.

If formaldehyde prevented dissociation of protein from DNA, less dye should be bound by formaldehyde-treated deoxyribonucleoprotein. Fig. 1 curve B shows that on the contrary more dye is bound to formaldehyde-treated deoxyribonucleoprotein than to control deoxyribonucleoprotein at high dye/phosphate ratios; at lower ratios, the reverse is true. Thus the result, though not conclusive, argues against dye-induced dissociation in control deoxyribonucleoprotein.

Binding of Toluidine Blue to protein. Binding of Toluidine Blue to histone had been examined by searching for metachromasia in mixtures (which are soluble) of Toluidine Blue and histone (Miura & Ohba, 1967). However, metachromasia would occur only if sufficient dye molecules are 'stacked' on the protein. Since the acidic residues in histones are irregularly spaced, stacking is unlikely to occur. As an alternative way of examining possible binding, attempts were made to obtain a dye-

protein complex in insoluble form so that binding could be assessed as with deoxyribonucleoprotein, by estimating the excess of unbound dye.

Studies had shown that precipitation of protein occurs on extensive degradation of deoxyribonucleoprotein by deoxyribonuclease (R. F. Itzhaki, unpublished work). Deoxyribonucleoprotein was, therefore, highly degraded by deoxyribonuclease at 2 µg/ml, incubating for 17 h at 37°C. The mixture was centrifuged at 3000g for 15 min and the precipitate was resuspended in buffer A. Toluidine Blue was added to the suspension and to a sample of undegraded deoxyribonucleoprotein (final dye concentration 500 µM) and the mixtures were shaken and centrifuged as usual. Excess of dye was then measured in the supernatants. Table 1 shows the amount of dye bound and gives protein and DNA analyses of the original deoxyribonuclease-produced precipitates. The results suggest that 12-20% of the dye bound to deoxyribonucleoprotein may in fact be bound to the protein acidic groups; correction for this gives a value of 46-50% free DNA phosphates in deoxyribonucleoprotein. Calculation of the maximum possible binding to the protein acidic residues of the original nucleohistone, based on the present histone/DNA mass ratio of 1.3:1 and taking an average of 9 acidic residues/histone molecule, gives a value corresponding to about 40% of the total dye bound to deoxyribonucleoprotein.

Addition of Toluidine Blue to DNA

It was found that at a given Toluidine Blue concentration, DNA was completely precipitated over a concentration range smaller than that of deoxyribonucleoprotein. With Toluidine Blue at final concentrations of 50 and 100 µM, the DNA was completely precipitated only at dye/DNA phosphate ratios above about 2:1. At ratios of 0.5:1-2:1, the supernatants were metachromatic, showing a shift towards 550 nm; at a ratio of about 0.3:1, the main peak wavelength of the supernatant appeared again at about 635 nm. With Toluidine Blue at a final concentration of 450 µM, the DNA was completely precipitated at dye/phosphate ratios above 1.2:1; at lower ratios the peak wavelength of the supernatant showed the blue shift.

With Toluidine Blue at final concentrations of

Table 1. *Binding of Toluidine Blue by protein precipitate from deoxyribonuclease-treated deoxyribonucleoprotein*

Expt.	Dye bound by ppt.	Protein in ppt.	DNA in ppt.
	Dye bound by original deoxyribonucleoprotein	Protein in original deoxyribonucleoprotein	DNA in original deoxyribonucleoprotein
1	20	87	18
2	12	91	21

50 and 100 μM , the E_{635} of the supernatant decreased linearly with increasing DNA concentration for dye/phosphate ratios down to 1.6:1. However, estimates of binding were made only at the higher Toluidine Blue concentration of 450 μM as the range of ratios producing complete precipitation of DNA was much greater. Fig. 2 shows that E_{635} decreases linearly for Toluidine Blue/DNA phosphate ratios down to about 1.2:1. Extrapolation to zero E_{635} gives a value of 1.65 μmol of DNA phosphate, corresponding to the binding of 2.0 μmol of Toluidine Blue. The apparent percentage of free phosphates is thus 121%; on correcting for trapped dye by the washing method used for Toluidine Blue-deoxyribonucleoprotein precipitates, the value becomes 110%. In another experiment, the corrected value was 109%. These values suggest that either trapped dye is not totally released by the washing procedure or that some 10% of the dye molecules may be bound to groups other than phosphates.

Addition of cetylpyridinium chloride deoxyribonucleoprotein or DNA

Figs. 3(a) and 3(b) show the results for deoxyribonucleoprotein and DNA respectively, supernatant values being expressed as percentages of the total E_{260} of the original mixture ($E_{1\text{cm}}^{1\%}$ for cetylpyridinium chloride at 260nm being taken as 122). The minimum of the curve is much broader for deoxyribonucleoprotein than for DNA and so the end point of the 'titration' is more uncertain. At the minimum, the cetylpyridinium chloride/DNA phosphate ratio corresponds to about 39% for deoxyribonucleoprotein and 97% for DNA. In two other experiments, values for deoxyribonucleoprotein were 41 and 43%, giving an over-all mean of 41%, and for DNA 106 and 99%, giving a mean of 101%.

Addition of polylysine to deoxynucleoprotein or DNA

Table 2 lists the range of concentrations of deoxyribonucleoprotein or DNA over which polylysine at a given concentration precipitated more than 95% of the DNA. It is clear that the range increases with decreasing molecular weight of polylysine.

Fig. 4 shows the decrease in amount of unused polylysine 1 with increasing deoxyribonucleoprotein concentration. The suspension values decrease linearly and the curve extrapolates to 3.9 μmol of DNA phosphate, corresponding to 46% free phosphates. Similar results were obtained in another experiment, extrapolation giving again 46% free phosphates. The supernatant values were lower than the suspension values, showing that there is some 'wastage' of lysyl residues.

Fig. 2. Binding of Toluidine Blue (2 μmol) to DNA. For experimental details see the text.

Fig. 3. Binding of cetylpyridinium chloride to (a) deoxyribonucleoprotein (b) DNA. Amount of cetylpyridinium chloride: 0.56 μmol in (a); 1.15 μmol in (b). For experimental details see the text.

Table 2. *Precipitation of deoxyribonucleoprotein and of DNA by polylysine*

The usable concentration of DNA phosphate is that at which complete precipitation of deoxyribonucleoprotein or DNA occurred.

(a) Deoxyribonucleoprotein		Usable range of concn. of DNA phosphate	Polylysine/DNA phosphate in usable range
Mol.wt. of polylysine	Concn. of polylysine (μM -lysyl residues)		
1250	400	160-1000	0.4 -2.5
7790	446-482	480-1200	0.4*-1.0
62000	540	1000-1300	0.4*-0.6
(b) DNA			
Mol.wt. of polylysine	Concn. of polylysine (μM -lysyl residues)		
1250	870	185- 929	0.9*-4.7
7790	940-1200	500-1000	1.0 -2.0
62000	1000-1100	700-1000	1.0 -1.5

* Minimum ratio tried.

Fig. 4. Binding of polylysine (mol.wt. 1250) to deoxyribonucleoprotein. Δ , Unused polylysine in deoxyribonucleoprotein-polylysine suspensions; $\circ$, unused polylysine in deoxyribonucleoprotein-polylysine supernatants. For experimental details see the text.

Fig. 5. Binding of polylysine (mol.wt. 1250) to DNA. ∇ , Unused polylysine in DNA-polylysine suspensions; $\square$, unused polylysine in DNA-polylysine supernatants. For experimental details see the text.

In three experiments with polylysine 1 and DNA (one shown in Fig. 5), the curve through the suspension values was slightly concave upwards, at least at the lower DNA concentrations; extrapolation through the points near the charge-neutralization region gave values of 108, 93 and 100% of free phosphates respectively. Corresponding values for polylysine 2 were 37% for deoxyribonucleoprotein and 101% for DNA (Itzhaki, 1970). Tables 3 and 4 show results obtained on addition of polylysine 3 to deoxyribonucleoprotein and DNA respectively. The mean value of free phosphates is about 44% for deoxyribonucleoprotein and 104% for DNA.

Table 5 shows that 'wastage' increases progressively with increasing molecular weight of polylysine. At least for polylysine 2, where the

most numerous and accurate results have been obtained, 'wastage' is much greater for deoxyribonucleoprotein than for DNA. In all cases, 'wastage' decreases to zero near the charge-neutralization point.

DISCUSSION

The present study suggests that the use of cetylpyridinium chloride and of polylysine for measuring free DNA phosphates is preferable to that of Toluidine Blue as binding to DNA at sites other than free phosphates presumably cannot occur; further, use of polylysine enables determination of the size of 'free' DNA zones in deoxyribonucleoprotein to be made. The disadvantage of cetylpyridinium

Table 3. *Binding of polylysine (mol.wt. 62000) by deoxyribonucleoprotein*

Polylysine was added to paired deoxyribonucleoprotein samples and the lysyl residues remaining unbound were estimated both in the total mixtures (suspensions) and in supernatants obtained by centrifuging one of each pair, as described in the Experimental section. Different experiments refer to different preparations of deoxyribonucleoprotein.

Expt.	Amount of deoxyribonucleoprotein (μ mol of DNA phosphate)	Amount of unused polylysine (μ mol of lysyl residues)		Amount of polylysine bound (μ mol of lysyl residues)		Polylysine bound/DNA phosphate	
		Supernatant	Suspension	Supernatant	Suspension	Supernatant	Suspension
1	0	0.95					
	2.33	0	0	0.95	0.95	≥ 0.41	≥ 0.41
2	0	2.17					
	3.92		~ 0.21		~ 1.96		~ 0.50
3	4.67		~ 0.17		~ 2.00		~ 0.43
	0	2.24					
4	3.68		0.61		1.63		0.44
	4.60		~ 0.25		1.99		0.43
5	5.51		~ 0.13		2.11		~ 0.38
	0	2.15	2.41				
6	4.54	~ 0.11	0.24	2.04	2.17	0.45	0.48
	5.44	0	0	~ 2.15	~ 2.41	≥ 0.39	≥ 0.44

Table 4. *Binding of polylysine (mol.wt. 62000) by DNA*

Polylysine was added to paired DNA samples and the lysyl residues remaining unbound were estimated both in the total mixtures (suspension) and in supernatants obtained by centrifuging one of each pair, as described in the Experimental section. Different experiments refer to different samples of DNA.

Expt.	Amount of DNA phosphate (μ mol)	Amount of unused polylysine (μ mol of lysyl residues)		Amount of polylysine bound (μ mol of lysyl residues)		Polylysine bound/DNA phosphate	
		Supernatant	Suspension	Supernatant	Suspension	Supernatant	Suspension
1	0	4.0	4.1				
	2.81	0.58	1.42	3.42	2.68	1.22	0.95
	3.15	0.42	1.04	3.58	3.06	1.14	0.97
	3.51	0.18	0.64	3.82	3.46	1.09	0.99
	3.86	~ 0.08	~ 0.08	~ 3.92	~ 4.02	1.01	~ 1.04
2	0	4.48	4.48				
	2.92	0.48	1.16	4.0	3.32	1.37	1.14
	3.65	~ 0.20	~ 0.36	~ 4.28	~ 4.12	1.17	1.13

chloride is that in its reaction with deoxyribonucleoprotein, the end-point is uncertain. Scott (1960) suggests that this is a sign of molecular heterogeneity. The fact that the endpoint is much less sharp for deoxyribonucleoprotein than for DNA, although both are presumably heterogeneous with respect to molecular size, suggest that the binding sites in deoxyribonucleoprotein are heterogeneous; this could relate to differences in distribution of histones on the individual DNA molecules. Cetylpyridinium chloride, like Toluidine Blue, suffers also from the disadvantage that much of the molecule is hydrophobic; this might affect the choice of binding sites.

The present dye-binding results resemble those obtained by Klein & Szirmai (1963) who used a similar method for measuring the binding of Azure

A to deoxyribonucleoprotein and DNA. Miura & Ohba (1967) used a filtration method for estimating the binding of Toluidine Blue to deoxyribonucleoprotein and DNA and obtained a correction for intercalated dye by measuring the thermal denaturation of the dye-DNA complex. In both cases, values of about 50% free phosphates in deoxyribonucleoprotein were obtained, no correction being made for protein-binding. The present value (corrected for trapped, but not for protein-bound dye) is slightly higher but this may be related to differences in composition of the deoxyribonucleoprotein used here from those preparations used previously.

In the polylysine study, the question arises as to whether all the free phosphates lie in stretches sufficiently long to accommodate the whole of each

Table 5. 'Wastage' of lysyl residues in polylysine on binding to deoxyribonucleoprotein and DNA

(a) Deoxyribonucleoprotein					
Expt.	Mol.wt. of polylysine	Polylysine added (μ mol)	DNA phosphate (μ mol)	Polylysine added DNA phosphate	Polylysine bound Polylysine 'wasted' + bound
1	1250	1.84	1.74	1.06	0.92
			2.60	0.71	0.95
			3.48	0.53	0.98
			4.36	0.42	1.0
2	7990	1.92	2.48	0.77	0.72
			3.32	0.58	0.82
			4.18	0.46	0.99
				0.39	1.0
3	7990	1.79	2.33	0.77	0.74
			3.12	0.57	0.81
			3.92	0.46	0.84
			4.96	0.38	0.99
(b) DNA					
1	1250	3.49	0.74	4.71	0.79
			1.48	2.36	0.85
			2.22	1.57	0.97
			2.97	1.18	0.99
2	7990	3.92	3.71	0.94	1.0
			2.07	1.90	0.87
			2.76	1.42	0.97
			3.44	1.14	0.98
3	7990	3.08	2.03	1.52	0.91
			2.70	1.14	0.85
4	62000	4.00	2.81	1.42	0.76
			3.16	1.27	0.82
			3.51	1.14	0.87
			3.86	1.04	1.0
5	62000	4.49	2.91	1.54	0.83
			3.65	1.23	0.96

polylysine molecule or whether polylysine links several smaller stretches on the same or on different deoxyribonucleoprotein molecules. With polylysine 1 this is very unlikely as it is too short; assuming that under the present conditions polylysine is in a random configuration in free solution, the length of polylysine would be 24 Å. Polylysine 2 is sufficiently long (150 Å) but at the point of charge neutralization the error in measurement is such that if there were 'wasted' lysyl residues in regions linking free stretches, values above 5% would have been detected, contrary to the results obtained. Undetectable 'wastage' of less than 5% corresponds to only one or two residues, a number unlikely to be adequate for cross-linkage. With polylysine 3 the limited range of usable values decreases the accuracy, including that of estimation of 'wastage'. However, 5% of 300 residues is 15 residues, a number more than sufficient for cross-linkage of the deoxyribonucleoprotein.

'Wastage' of lysyl residues at polylysine/deoxyribonucleoprotein ratios remote from the neutralization point is probably due to competition between excess of polylysine molecules for the limited

available sites. This would lead to only part of each molecule being bound. Near the point of charge neutralization, the absence of 'wastage' suggests that the competition decreases and that the whole of each molecule, at least of polylysine 1 and polylysine 2, is accommodated. If, instead, the free zones were smaller than the length of the polylysine 1 and polylysine 2 molecules, in the absence of cross-linkage the whole of each molecule could never be accommodated and 'wastage' would occur at all polylysine/deoxyribonucleoprotein ratios, contrary to the results obtained. The finding that mixtures of DNA and polylysine 1 and polylysine 2 and also of ribosomal ribonucleoprotein and polylysine (R. F. Itzhaki & R. A. Garrett, unpublished work) show very little 'wastage' of lysyl residues even when excess of polylysine is present, presumably relates to the high percentage of free phosphates that would preclude competition by the polylysine molecules.

The decrease in range of usable concentrations of deoxyribonucleoprotein and of DNA with increasing molecular weight of polylysine may be related to the percentage of lysyl residues on a given molecule

remaining unbound when polylysine is present in excess. This percentage would presumably increase with increasing size of polylysine; the mutual repulsion of the many unbound groups on each polylysine molecule partly attached to deoxyribonucleoprotein or DNA would tend to keep the complex in solution.

The 'titration' values for deoxyribonucleoprotein obtained with polylysine could be affected by the following possible artifacts: (1) displacement of protein from DNA in deoxyribonucleoprotein by polylysine; (2) binding of polylysine to protein anionic groups; (3) displacement of previously bound lysyl residues from DNA by Methyl Orange in the suspensions; (4) binding of Methyl Orange to protein basic groups in the suspensions. The first artifact is unlikely as the solutions are mixed under non-dissociating conditions; further, R. A. Garrett (personal communication) has found that in polylysine-deoxyribonucleoprotein mixtures at the much higher salt concentration of 0.1M, only the F1 histone is displaced. This fraction is thought to bind only to a small percentage of the DNA phosphates. If localized displacement of the protein cationic groups occurred, their number would equal that of the polylysine lysyl residues replacing them and they would be assayed as free 'lysyl residues' by Methyl Orange (which reacts with arginyl residues as well as lysyl residues). Thus the 'titration' value for deoxyribonucleoprotein would still be valid.

As to the second artifact, the histone/DNA mass ratio here is such that on the average each histone must be attached very compactly to the DNA and those stretches containing the bulk of the acidic residues (Delange, Fambrough, Smith & Bonner, 1969) must presumably loop away from the DNA. Thus, if polylysine were to bind to most of the protein anionic groups there would be appreciable 'wastage' of lysyl residues in the regions bridging the DNA and the acidic residues. However, the absence of 'wastage' near the point of charge neutralization argues against this.

The third artifact is unlikely in view of the value of 100% free phosphates obtained for DNA; if displacement occurred, the value would be lower.

The fourth artifact may occur but the sole estimation of unbound basic residues in the deoxyribonucleoprotein-histones amounted to only 20% of the total (Walker, 1965). If Methyl Orange does, in fact, bind to any histone basic residues, the present titration values would be lower than the true values.

Although several studies have been made on the binding of polylysine to DNA, the actual binding site is unknown. Tsuboi, Matsuo & Tso (1966) suggested that polylysine is wound in the large groove whereas D. Carroll (personal communica-

tion) proposed that it lies in the small groove. Olins, Olins & von Hippel (1967) found by using models that it could fit into either groove.

COMMENTS AND CONCLUSION

The fact that an appreciable proportion of the DNA in deoxyribonucleoprotein is accessible to molecules of a range of sizes appears at first to conflict with studies on the enzymic degradation of deoxyribonucleoprotein which indicate that not more than 10% of the DNA in deoxyribonucleoprotein is present as extensive truly free zones (R. F. Itzhaki, unpublished work). However, this can be explained on consideration of the probable arrangement of proteins on the DNA. The deoxyribonucleoprotein used here contains non-histone proteins as well as histones; however, the enzyme studies suggest that the former 'covers' little if any DNA. Discussion will, therefore, be limited to the histones. The distribution of basic residues in F2a1 histone, the sequence of which has been fully determined (and in the other fractions), is very irregular (Delange *et al.* 1969). Model-building studies by Richards & Pardon (1970) have shown that not more than 60% of the DNA phosphates lying beneath the F2a1 fraction could be neutralized. Thus, there may be many free but 'hidden' phosphates in deoxyribonucleoprotein which would not have been assessed as free by the enzyme method. The present results show that in deoxyribonucleoprotein about 48% of the phosphates are available for binding singly charged Toluidine Blue of length about 12 Å, about 41% for binding singly charged cetylpyridinium chloride of length about 19 Å, 46% for binding polylysine 1 with six cationic groups and a length in free solution of 24 Å and 37% for binding polylysine 2 with 37 cationic groups and a length in free solution of 150 Å. Allowing for the fact that the polylysine method would not reveal if one or two phosphates in a run of 37 were neutralized already by the histones, and that the odd lysyl residue may bind to a protein acidic group, fairly long stretches of 'free' phosphates may be present in deoxyribonucleoprotein, presumably corresponding to and 'hidden' by stretches of non-basic residues in the accompanying histone molecule.

The present study indicates that despite the coverage by protein, the DNA phosphates in deoxyribonucleoprotein are surprisingly accessible to large molecules. In fact the percentage of free phosphates may be an underestimate of accessible DNA as RNA polymerase may require merely space on one of the DNA grooves for binding, not free phosphates also, and space would be available if histones were bound only in one groove. The question then arises as to why transcription is lower

from deoxyribonucleoprotein than from DNA. Possibly, the accessibility of deoxyribonucleoprotein to RNA polymerase is in fact lower than that to polylysine. X-ray diffraction studies on deoxyribonucleoprotein-polylysine complexes suggest that polylysine destroys the DNA super-helix (B. M. Richards & J. F. Pardon, personal communication) so unless RNA polymerase does the same, it may bind to deoxyribonucleoprotein only at limited pre-existing, non-superhelical sites. Alternatively, factors other than binding capacity may limit transcription. Shih & Bonner (1970) have reported that RNA polymerase binds equally to DNA and to deoxyribonucleoprotein provided the latter is in a non-aggregated form; they suggest that histones may prevent the separation of the DNA strands thought to be a pre-requisite for transcription. This finding supports the present results in their implication that the role of the histones is other than that of simple gene repressors acting by 'covering' the DNA, thus preventing access of RNA polymerase.

Note added in proof. Clark & Felsenfeld (1971) have very recently described experiments somewhat similar to the polylysine studies here and to the enzyme studies (R. F. Itzhaki, unpublished work) referred to in the Comments and Conclusion section. Their results suggest that about half the DNA in deoxyribonucleoprotein is completely free of protein, whereas the present studies indicate that most of the DNA is covered by, even if not bound to, associated protein. However, their main conclusion confirms the present one that the DNA in deoxyribonucleoprotein is remarkably accessible to large molecules.

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