Chemical Probes of Chromatin Structure†

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ABSTRACT: The accessibility of the DNA of calf thymus chromatin to a variety of chemical probes has been studied. About half of the DNA is digestible by the enzyme staphylococcal nuclease and titratable by purified histone fractions or Mn^{2+}. Kinetics of the digestion reaction are not consistent with random attack, but indicate that there is a class of sites on DNA which is relatively sensitive to nuclease action. The results show that there is a set of regions with weight average size of about 100 base pairs comprising half of the DNA of chromatin, that is protected against reaction with a variety of chemical probes. The contrast with the much more limited accessibility of chromatin DNA to reaction with RNA polymerase provides some information about the role of proteins in restriction of transcription.

It has recently been shown (Cedar and Felsenfeld, 1973) that the number of binding sites for *Escherichia coli* RNA polymerase on calf thymus chromatin is about $1/10$ to $1/30$ the number on thymus DNA; this explains the well-known observation (Bonner and Huang, 1963) that the rate of *in vitro* RNA synthesis is slower when chromatin is used as a template than when an equivalent amount of the homologous DNA is used.

Many investigators have attempted to explain the observed restriction in template activity in terms of chromatin structure. It is evident that the proteins of chromatin alter the biochemical activity of the DNA to which they are bound, and it has often been supposed that those genes that are capable of being transcribed must be completely free of protein, while inactive genes are completely covered. Given our present understanding of the mechanism of messenger RNA synthesis in microorganisms, there is no longer any *a priori* reason to require, in a model of chromatin structure, that each inactive gene be covered completely by protein. We now know from work on bacterial systems that blocking a very small part of the operon can be sufficient to inhibit synthesis of the corresponding messenger RNA. This means that we cannot deduce the arrangement of chromatin proteins on DNA from studies of template activity alone. When used for *in vitro* template activity assays, the RNA polymerase molecule must be viewed only as a kind of chemical probe sensitive to the accessibility of polymerase binding sites.

For this reason, it is important to make use of other probes of chromatin structure. In an earlier paper (Clark and Felsenfeld, 1971) we presented evidence that about half the DNA of chromatin is accessible to digestion by staphylococcal nuclease, and to titration by polylysine. In this paper, we examine the nuclease reaction in greater detail. Both the kinetics of the reaction and the nature of the final products confirm that a distinct set of regions comprising about half the DNA of chromatin are susceptible to nuclease attack. We have also studied the effect of adding purified histone fractions to chromatin. We find that this results in a decrease in the number of sites accessible to nuclease attack. Addition of excess histones also reduces the number of phosphodiester groups on chromatin accessible to titration with Mn^{2+}. The reduction in these sites parallels the decrease in nuclease susceptibility.

Methods

Chromatin was prepared from calf thymus tissue by methods described previously (Clark and Flsenfeld, 1971; Axel *et al.*, 1973). In one preparation (sample 37) the nuclei were treated with sodium bisulfite during preparation (Bartley and Chalkley, 1970); in another (sample 38) the nuclei were washed with 0.25 M NaCl before being exposed to decreasing concentrations of buffer. All samples were tested for restriction of *E. coli* RNA polymerase binding sites (Cedar and Felsenfeld, 1973) and found to have between $1/10$ and $1/25$ the activity of an equivalent amount of calf DNA. Samples prepared by washing of nuclei with 0.25 M NaCl showed negligible loss of f1 histone by proteolysis even after storage at 4° for several days, as judged by disc gel electrophoresis in acetic acid–urea gels (Panyim and Chalkley, 1969). Chromatin was exposed to high concentrations of EDTA during washing procedures involved in preparation, which has the effect of eliminating competing divalent ions in experiments involving the binding of Mn^{2+}. EDTA was present during all steps in the preparation of DNA, including ethanol precipitation procedures. Water used in equilibrium dialysis studies had been passed over deionizing columns. Tris buffer used in these studies was Schwarz/Mann UltraPure grade.

Staphylococcal nuclease was purified by the methods of Moravek *et al.* (1969), and had an activity of 102 units/mg using native calf thymus DNA as substrate. Unfractionated histones from calf thymus were isolated by extraction with cold 0.2 N H_2SO_4 for 10 min, followed by centrifugation (1200*g*, 15 min), and dialysis of the supernate against water. Purified histone fractions were prepared by the method of Johns (1964).

Procedures for digesting chromatin with staphylococcal nuclease have been described previously (Clark and Felsenfeld, 1971). Protected DNA fragments were isolated by centrifuging the limit digest (27,000*g*, 15 min). The pellet was suspended in 2.5 M NaCl–1 mM NaEDTA (pH 7) and incubated with Pronase (0.2 mg/ml) at 37° for 30 min or longer. The solution was applied to a 1 × 1 cm hydroxylapatite column, which was then washed with 50 ml of 2.5 M NaCl–0.05 M sodium phosphate buffer (pH 6.7). The DNA was eluted with about 1.5 ml of 0.5 M phosphate buffer (pH 6.2) and dialyzed against the desired solvent. A somewhat different procedure was used in some cases where optical density methods were needed to measure total DNA in the protected DNA fraction. In a typical experiment, the digest from 50 optical density (OD) units of chromatin was centrifuged, and the pellet suspended in 0.1 ml of 0.1 M EDTA (pH 7) and 6 ml of 2 M NaCl–5 M urea. The dissolved

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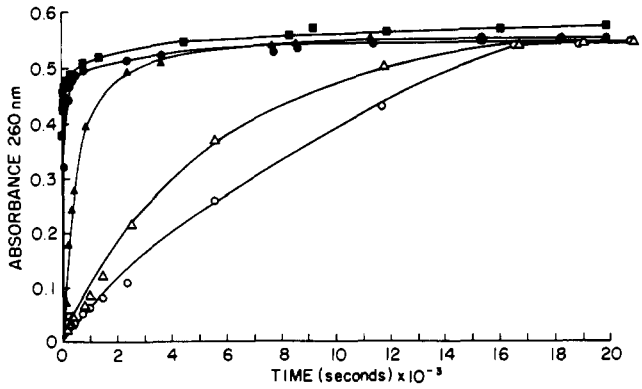

FIGURE 1: Kinetics of digestion of calf thymus chromatin by staphylococcal nuclease. Release of perchloric acid soluble products as a function of time, using a variety of enzyme concentrations. Increasing rates of digestion correspond to 0.5, 1, 2, 10, and 100 μg/ml of enzyme. Solvent contained 5 mM sodium phosphate buffer (pH 6.7) and either 25 μM $CaCl_2$ (data at 100, 10, and 2 μg/ml of enzyme) or 11 μM $CaCl_2$ (data at 1 and 0.5 μg/ml of enzyme).

material was passed over a 1 × 1 cm hydroxylapatite column and washed with 10 ml of 2 M NaCl–5 M urea and with 30 ml of 2.5 M NaCl–10 mM sodium phosphate buffer (pH 6.7). The DNA was eluted with 0.5 M sodium phosphate buffer (pH 6.2) in which had been dissolved 146 g/l. of NaCl.

Selective digestion of single-strand DNA was carried out with staphylococcal nuclease under conditions that are specific for single-strand degradation (Kacian and Spiegelman, 1974). The reaction mixture contained 0.4 M NaCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.9), 0.25 mg of DNA, and 10 μg of enzyme in 0.5 ml total volume.

The oligomer products of staphylococcal nuclease action on double-stranded DNA were obtained by precipitation of the reaction mixture with 0.5 M perchloric acid–0.5 M NaCl and centrifugation. To determine the amount of monomer in the acid-soluble fraction, the supernate was neutralized with 2 M Tris and 2 ml was passed over a 2.5 × 17 cm Bio-Gel P4 column (Bio-Rad Corp.). A mixture of trace amounts of ^{3}H-labeled nucleotides (dAMP, dCMP, dGMP, TMP) was added to the sample before it was placed on the column. The sample was eluted with solvent containing 0.5 M NaCl, 0.5 M $HClO_4$, 0.57 M Tris, and 0.01 M EDTA, and both optical density and radioactivity were measured in effluent fractions. The upper limit to the amount of monomer in the hydrolysate was determined from the amount of absorbing material appearing in fractions which also contained measurable radioactivity.

Results

The kinetics of digestion of calf thymus chromatin by staphylococcal nuclease are shown in Figure 1. The reaction is followed in this experiment by measuring the release of digestion products soluble in perchloric acid. The digestion is carried out in 5 mM sodium phosphate buffer (pH 6.7) and with the addition of small amounts of $CaCl_2$ necessary for the action of the enzyme. Although the initial reaction rate increases with enzyme concentration, the limiting amount of solubilized DNA is essentially identical in all cases, corresponding to about 50% of the total chromatin DNA originally present (Clark and Felsenfeld, 1971). Beyond this point, there appears to be a negligible increase in the amount of acid-soluble product even after quite long digestion times. The 50% digestion point for the chromatin concentration used in Figure 1 would correspond to an A_{260} value of 0.55. The limiting absorbance values observed in this experiment are between 0.54 and 0.56 absorbance units. Similar results have been obtained with chromatin prepared by a variety of methods from different sources (Clark and Felsenfeld, 1971; unpublished data).

TABLE I: Size of Protected DNA in Nuclease Digests.[a]

Nuclease Concn (μg/ml)	Wt Av Length (Base Pairs)
2	96
5	99
10	104
50	97
100	111

[a] Molecular weights were determined at 4° by short column equilibrium centrifugation in the Beckman Model E centrifuge. The solvent was 1 M NaCl–2.5 mM sodium phosphate buffer (pH 6.7).

As the extent of reaction approaches its limiting value, the solution becomes turbid, and at the end of the reaction the suspended material can be isolated by centrifugation (27,000*g*, 10 min). The supernate contains no acid precipitable oligonucleotides. The pellet can be dissolved in 2 M NaCl–5 M urea–1.7 mM NaEDTA (pH 7) and passed over a hydroxylapatite column to separate the proteins from the DNA (see Methods section).

In a typical experiment, 93% of the DNA in the redissolved pellet can be recovered. Of this 96% is precipitable by perchloric acid. If this DNA is now incubated with staphylococcal nuclease under conditions specific for digestion of single-stranded DNA (Methods section), less than 1% of the DNA is made acid soluble. We conclude that the DNA in the pellet is almost entirely two stranded, a result that we reported earlier on the basis of spectral data (Clark and Felsenfeld, 1971).

The weight average molecular weight of the protected DNA isolated from hydroxylapatite is not a function of the enzyme concentration used (Table I). The weight average size of the protected regions is about 100–110 base pairs. This number is smaller than the value of 160 base pairs reported in an earlier paper (Clark and Felsenfeld, 1971); we find some variation in mean size of protected DNA among chromatin samples.

Protein Binding to the Protected DNA. In an earlier paper (Clark and Felsenfeld, 1971) we measured the amount of protein exchange during the digestive process. This was done by adding radioactively labeled calf thymus DNA to the chromatin before nuclease digestion, and measuring the amount of radioactivity in the protected DNA ultimately isolated. We found that very little of the protected DNA arises from an exchange process. The kinetics of nuclease digestion of the added labeled DNA exactly parallel the digestion kinetics of the chromatin DNA (data not shown).

Nearly all of the chromatin proteins are found in the pellet that also contains the protected DNA. Very little protein (Table II) is found in the supernatant. Although it might seem reasonable to conclude that the proteins are bound to the protected DNA, Itzhaki (1971) has pointed out that they might be free but aggregated under the reaction conditions. Almost all the proteins of these calf thymus chromatin preparations are histones, and under certain conditions histones form insoluble aggregates. To test whether this can occur under the conditions of the digestion procedure, we have made a total chromatin histone preparation by acid extraction of chromatin (Methods section). Under the salt and protein concentration conditions typical of our experiments, none of the histones in this mixture

TABLE II: Solubility of Nuclease Digestion Products of Chromatin.[a]

Sample	% Protein in Supernate	% Protein Extracted with EDTA	% Protein Extracted with Urea	% Original DNA Extracted with Urea	Protein/DNA (g/g) in Urea Extract
CHR. 37	2		19	11	2.3
CHR. 38	0		33	24	1.8
CHR. 37	6	2	21	9	3.1

[a] Chromatin was digested as described (Clark and Felsenfeld, 1971) and centrifuged. The four right-hand columns refer to extraction of the digest pellet with 2 mM NaEDTA (pH 7) or with 6 M urea–2 mM NaEDTA (pH 7). Per cent protein refers to the per cent of the total chromatin protein found in each fraction.

TABLE III: Binding of Mn^{2+} to Calf Thymus DNA and Chromatin.[a]

[DNA] (mM)	[Free Mn^{2+}] (mM)	[Bound Mn^{2+}] (mM)	2[Bound Mn^{2+}]/[DNA]
	(a) Binding to DNA		
0.232	0.1	0.0707	0.61
0.245	0.2	0.0762	0.62
0.259	0.3	0.0815	0.63
	(b) Binding to Chromatin		
0.335	0.1	0.0480	0.29
0.312	0.2	0.0520	0.34
0.318	0.3	0.0580	0.36
0.245	0.1	0.0398	0.32
0.252	0.3	0.0504	0.40
0.522	0.1	0.0997	0.38
0.553	0.2	0.1109	0.40
0.553	0.3	0.1134	0.41

[a] All experiments were performed in the presence of 5 mM Tris-HCl (pH 8.0). DNA or chromatin was diluted into solvent containing the appropriate Mn^{2+} concentration and dialyzed against a 100-fold excess volume of the same solvent for 24–48 hr. Achievement of equilibrium was demonstrated by approach from both sides of the equilibrium. All solvents contained trace quantities of $^{54}Mn^{2+}$ ($\sim 10^4$ cpm/ml). At equilibrium, aliquots were taken from both external and internal solutions; DNA concentrations were determined by optical density measurements, and Mn^{2+} by counting. No correction is made here for the Donnan equilibrium, which is estimated to contribute less than 10% to the binding in the worst case.

aggregates, as judged by the fact that there is no measurable loss of protein from solution if it is centrifuged at 15,000*g* for 15 min. We obtain similar results when this experiment is carried out in the presence of a limit nuclease digest of DNA, using a concentration of nucleotides similar to that present at the end of a chromatin digestion.

Another way of testing for the presence of free proteins is to extract the chromatin digest pellet with urea solutions which are known to solubilize aggregated histones. As shown in Table II some protein is made soluble by extraction with 6 M urea–2 mM NaEDTA (pH 7). However, some DNA is also solubilized at the same time. The protein–DNA ratio (grams/gram) of the extracted material is 1.8–3.1, compared to the mean of about 2.7 in the pellet. Extraction with 2 mM EDTA alone does not dissolve significant quantities of protein or DNA. The effect of the urea is to partially solubilize the complex. Preliminary studies of the sedimentation properties of the DNA and protein extracted by urea, carried out in the urea solvent, suggest that the DNA and protein are complexed to one another in solution. In any case, there is no evidence for the presence in the pellet of aggregated histones not bound to DNA.

Binding of Polylysine and Mn^{2+} to Chromatin. We have reported that it is possible to titrate chromatin with polylysine, and that 0.5 lysine residue per nucleotide can be bound without displacement of histone from the DNA (Clark and Felsenfeld, 1971). It has been suggested that the mode of binding of polylysine may differ from that of histones (Carroll and Botchan, 1972; Li *et al.*, 1972), and that these results do not, therefore, measure histone-free sites on chromatin DNA. Recently, Paul and More (1972) have reported that addition of excess histones to chromatin does not reduce the number of phosphodiester groups available for toluidine blue binding. They suggest that the added histones are not binding to free DNA, and conclude that there is no DNA in chromatin available for reaction with excess histone.

To study this problem, we have made use of the observation of Schmidt *et al.* (1972) that about half the phosphodiester groups of chromatin are able to bind Mg^{2+}. We have repeated this experiment using $^{54}Mn^{2+}$, which can be expected to have binding properties rather similar to Mg^{2+}, but which is more convenient to use because of its radioactivity. Using dialysis equilibrium methods, we find that about 0.36 equiv of Mn^{2+} per nucleotide is bound to chromatin under our conditions (Table III). Under the same ionic conditions, protein-free DNA binds 0.62 equiv of Mn^{2+}/mol of nucleotide. Thus, chromatin has about 58% as many binding sites for Mn^{2+} as does the corresponding amount of protein-free DNA.

We have studied the effect of adding histones to chromatin on the binding of Mn^{2+}. If sufficient quantities of histone are added, all of the chromatin DNA is precipitated. The precipitated chromatin is isolated by centrifugation and redissolved, and the amount of bound Mn^{2+} determined. As shown in Tables IVa and IVb, excess histone reduces the amount of bound Mn^{2+}. Continued addition of histone to precipitated chromatin results in further Mn^{2+} displacement. If sufficient histone is added, only small amounts of Mn^{2+} remain bound to chromatin. We have repeated this experiment with Mg^{2+}, using less sensitive titrimetric methods to determine the cation concentration. The results are similar (data not shown).

Adding excess histone to chromatin not only reduces the number of sites on chromatin DNA accessible to Mn^{2+}; it also reduces the amount of DNA accessible to nuclease. The fraction of DNA digested by nuclease is about the same as that which binds Mn^{2+} (Table IV). It should be noted that the insolubility of the histone–chromatin complex does not in itself make the DNA inaccessible to the nuclease.

Early Time Kinetics of Digestion. Although the nuclease is able to digest DNA in insoluble complexes, it is important to show that the apparent protection of half the DNA of chromatin is not in some way related to the insolubility of the limit digest. For this reason, we have studied the digestion process at very early times in the reaction, when no insoluble material is present.

TABLE IV: Histone and Mn^{2+} Binding to Chromatin.[a]

(a) Displacement of Mn^{2+} by Histone I or III

Histone Added (mg)	Net Increase Bound Protein (μg)	Mn^{2+} Bound (μmol)	2[Bound Mn^{2+}]/[DNA]	Fraction Open DNA
0.5 (I)	169	11.8	0.026	0.04
1.0 (I)	177	25.2	0.055	0.09
0.5 (III)	256	7.8	0.017	0.03
1.0 (III)	304	6.0	0.013	0.02
2.0 (III)	302	6.4	0.014	0.02

(b) Correlation of Mn^{2+} Binding and Nuclease Digestion

Histone Added (mg)	2[Bound Mn^{2+}]/[DNA]	Fraction Open DNA	Fraction Digested DNA
1.0 (I)	0.12	0.19	0.15
0.4 (III)	0.26	0.42	0.33
1.0 (III)	0.07	0.11	0.12

[a] Histones were dissolved in 5 mM Tris-HCl (pH 8)–0.2 mM $MnCl_2$, containing a trace of $^{54}Mn^{2+}$. Histone concentrations varied between 60 and 400 μg/ml. In part a above, the total amount of histone I or III shown was added with vigorous stirring to 2.3 ml of calf thymus chromatin at a concentration of 0.4 mM (nucleotides). After centrifugation (27,000g, 15 min) 93% or more of the DNA was in the pellet, which was drained carefully and redissolved in 2 ml of 0.9 M NaCl–0.09 mM EDTA (pH 7). Aliquots of this solution were counted and used for protein determinations (Lowry *et al.*, 1951). The net increase in bound protein (column 2) was calculated using the measured chromatin protein content (380 μg total) and protein assay calibration factors appropriate to each of the added histones. In part b above, the amounts of histone shown were added to 4.4 ml of 0.43 mM chromatin under conditions similar to those described in part a, and the Mn^{2+} bound to the precipitated chromatin was determined. In a parallel set of experiments, the precipitated chromatin was not redissolved after centrifugation, but was resuspended in 4 ml of 1 mM Tris (pH 8)–0.2 mM $CaCl_2$, 40 μg of staphylococcal nuclease were added, and the mixture was incubated for 1 hr at 37°. The extent of digestion was determined by acid precipitation of the mixture of 0.5 M $HClO_4$–0.5 M NaCl and measurement of A_{260} after centrifugation. The ratio of 2[bound Mn^{2+}]/[DNA] is the concentration of bound Mn^{2+} in equivalents/liter divided by the chromatin DNA concentration in moles/liter. Fraction Open DNA is this ratio computed for the chromatin sample in question, divided by the value of this ratio for free DNA, taken as 0.62 from the data of Table IIIa.

Under the conditions used to digest chromatin, the enzyme acts entirely as an endonuclease during the early part of the reaction. If protein-free calf thymus DNA is digested until 15% is acid soluble, less than 5% of the acid soluble product (0.75% of the total DNA) is present as nucleotide, as judged by gel chromatography on Bio-Gel P-4 (Methods section). Similar results are obtained when chromatin is digested. Although staphylococcal nuclease is capable of exonuclease action, this process is much slower than the action as an endonuclease.

We have measured the weight average molecular weight of samples of calf thymus DNA as a function of time of digestion. The molecular weights are plotted in Figure 2 as a function of

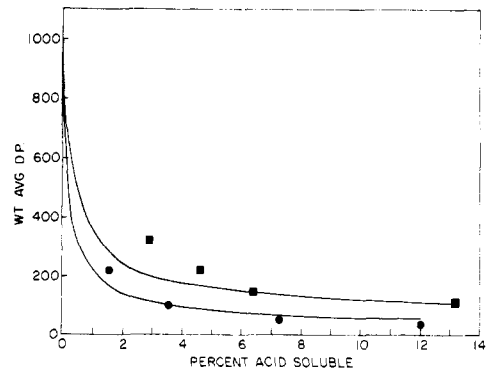

FIGURE 2: Effects of limited digestion by staphylococcal nuclease. DNA or chromatin samples were digested for varying periods of time. Aliquots were taken to determine the amount of acid precipitable DNA (see Methods section). Another set of aliquots was diluted to give a final salt concentration of 1 M NaCl–1 mM sodium phosphate (pH 6.7), and the molecular weight of the entire mixture was determined by short column equilibrium sedimentation, using the absorption optics set at 265 nm. When DNA from chromatin digests was prepared in this way, the measured molecular weight was unchanged by incubation with Pronase. Before digestion, chromatin preparations were sonicated for 15 sec to assure complete dispersal. The weight average degree of polymerization is plotted as a function of the per cent DNA made acid soluble: (●) data points for calf thymus; (■) data points for chromatin. The lines represent theoretical curves for digestion of DNA (lower curve) and a blocked DNA model (upper curve). See Appendix for a description of the calculation.

the amount of acid soluble material produced. Also shown in Figure 2 is the corresponding theoretical curve for random endonucleolytic cleavage of DNA across both strands. The calculation of this curve (Appendix) makes use of the known relationship between oligonucleotide chain length and acid solubility (Cleaver and Boyer, 1972). There is good agreement between the predictions of the random cleavage theory and the data. Direct measurement of the size distribution of single-strand DNA in alkali (Studier, 1965) at very early times in the digestion shows that the double-strand molecular weight falls at the same rate as the single-strand molecular weight, confirming the double-strand cleavage mechanism.

We now ask whether the attack of nuclease on chromatin at early times is random, or whether there are uniquely susceptible regions. If the attack is random, a curve of molecular weight *vs.* per cent acid soluble DNA identical with that for DNA itself should be obtained. The data for chromatin digestion do not fit such a curve. At every point in the digestion (Figure 2) the weight average molecular weight of the digest falls above that for DNA at the same per cent of acid soluble material. A theoretical curve is shown in Figure 2 for a "blocked target" model (Appendix) in which half the DNA is accessible, and the other half inaccessible, to nuclease action, with sets of blocked and open regions each 110 base pairs long. This curve agrees rather well with the data for chromatin digestion, though it should be emphasized that related models in which the blocked regions have a range of sizes averaging 110 base pairs would probably be equally satisfactory.

Discussion

The simplest interpretation of the staphylococcal nuclease digestion results shown in Figure 1 is that about half the DNA of calf thymus chromatin is relatively accessible to the enzyme. This result is not, as Mirsky (1971) has suggested, an accident of the choice of enzyme concentration or Ca^{2+} concentration. It is not dependent upon pH, since we have obtained similar results in 1 mM Tris buffer at pH 8.0. It is not a function of the

method of preparation of chromatin, so far as we have been able to determine by the use of a variety of different preparative techniques (Methods section).

Recently, Schmidt *et al.* (1972) have reported quite similar experiments carried out with the enzyme pancreatic DNase in the presence of Mg^{2+}. They report that under a variety of conditions the digestion occurs in two stages. The first, relatively rapid, proceeds until about half the DNA is digested and most of the binding sites for Mg^{2+} are abolished. The second stage is slower, but ultimately results in complete digestion for the DNA. Using somewhat different conditions, Mirsky (1971) reports that pancreatic DNase digests chromatin DNA completely, and finds that the fraction of DNA digested in the first, rapid, phase is a function of the enzyme concentration. We have not attempted to resolve the discrepancy between these two sets of results. In any case, almost all of our earlier studies, and all of the present ones, make use of staphylococcal nuclease which behaves differently from pancreatic DNase, with a distinct and stable cessation of activity at the end of the first phase of digestion, resulting in a DNA product which is two stranded, and of size independent of the enzyme concentration used. It should be pointed out that neither Mirsky nor Schmidt and his collaborators has used this enzyme.

The staphylococcal nuclease results clearly are not explainable as an accident arising from the choice of conditions. There are two possible interpretations of the experiment. (1) DNA is digested at random, but when half of the dna is digested, the remaining protein–DNA complex becomes resistant to further attack. (2) A distinct set of regions in the DNA of chromatin, comprising about half the total, is intrinsically more susceptible to digestion. The experiments reported here suggest that the second explanation is correct.

The kinetics of molecular weight decrease (Figure 2) provide evidence that there is a distinct set of digestible sequences. The data are not compatible with any simple model in which protein uniformly covers the DNA, and attack is random. To account for the data in terms of such a model, it might be supposed that as soon as the enzyme begins to act it displaces protein from the vicinity of the damaged DNA, resulting in a nonuniform distribution of bound protein. The displaced protein could not be liberated into solution, since none is detected there, nor could it bind to other DNA, since such a process would result in "exchanged" protein, of which there is little (Clark and Felsenfeld, 1971). The protein would have to rearrange on the surface of the DNA molecule to which it was originally bound. It is difficult to imagine how the small number of breaks in the backbone present during the early stages of digestion could cause the rearrangement of half the protein. Nonetheless, the digestion experiments alone do not preclude this possibility. Furthermore, the results of Paul and More (1972) led them to suggest that excess histone might bind to the surface of chromatin through protein–protein interaction. Extending this phenomenon to the digestion process, we might suppose that protein rearrangement does occur, and results in displacement of histones from their original DNA binding sites onto the surface of adjacent histones still bound to DNA.

To investigate this possibility, we have repeated the experiments of Paul and More, with certain important modifications. Paul and More used toluidine blue binding to measure the number of free phosphodiester groups in chromatin, while we used Mn^{2+} binding for the same purpose. Paul and More add excess histone to chromatin after first precipitating the chromatin in 0.15 M NaCl (Phillips, 1968), while we carry out the addition of low ionic strength. Our results (Table IV) show that excess histones reduce the number of Mn^{2+} binding sites on chromatin, and that if sufficient histone is added nearly all the Mn^{2+} is displaced. This result appears to differ from the observation of Paul and More. There are two possible explanations for the discrepancy. The first is that toluidine blue titrations are difficult to carry out quantitatively. There are two classes of binding sites, and determination of the number of phosphodiester sites requires subtraction of the second class of sites, which must be determined by a separate titration at high temperature (Miura and Ohba, 1967). The second possible explanation for the disagreement is that histones may bind to chromatin in a different manner when the chromatin is precipitated at relatively high ionic strength. We believe that the low ionic strength conditions we use to form the complexes are closer to the digestion conditions, and, therefore, more appropriate for the study of that process. It should be recalled that at early times during the digestion no insoluble DNA–protein complex is observed.

In any case, our results show clearly that about 58% of the phosphodiester groups on the DNA of chromatin are capable of reacting with divalent ion, and are also available for reaction with purified histone fractions. Furthermore, addition of histones reduces the amount of DNA digestible by nuclease. There is a correlation between digestibility and Mn^{2+} binding (Table IV). We reported in an earlier paper (Clark and Felsenfeld, 1971) that about 0.5 equiv of polylysine/nucleotide could be bound to chromatin without displacing histone, and that the addition of increasing amounts of polylysine resulted in a corresponding reduction in the amount of digestible DNA. In recent experiments with duck reticulocyte chromatin, we find that only small amounts of polylysine are bound beyond the 50% point. A number of authors (Carroll and Botchan, 1972; Li *et al.*, 1972) have suggested that this should not be interpreted as reflecting the presence of accessible regions on the DNA, since polylysine may bind in the small groove of DNA, while histones are thought to bind principally in the large groove. The titration results show that there are two classes of sites for polylysine binding on chromatin: those that bind polylysine freely and those that do not. The results do not provide information which could lead to a physical model of the mode of binding. Whatever the nature of the noncompetitive site for polylysine, it is unlikely that the results can be accounted for simply in terms of an available narrow groove in DNA, since the amount of polylysine bound, when wound in the narrow groove even in fully extended form, cannot stretch the entire length of the DNA.

All of our results show that the DNA of chromatin can be divided into two classes: a set of regions comprising half the DNA that is chemically reactive to a series of probes and a less reactive set of regions comprising the remainder of the DNA. The probes we have used are the enzyme staphylococcal nuclease, purified histone fractions, the synthetic polypeptide polylysine, and the divalent cation Mn^{2+}. The probes are mutually competitive, suggesting that all of them are reacting with the same sites.

Why is half the DNA of chromatin accessible to these probes, and the other half inaccessible? It is reasonable to suppose that the chromatin proteins are not distributed uniformly along the DNA, and therefore do not provide equal protection everywhere. The unprotected regions might be completely devoid of protein, or they might be covered by polypeptide sequences that are relatively easily displaced from the DNA surface by all of the probes. If such sequences exist, they are not likely to constitute separate molecules, since no protein displacement into solution is detected during nuclease digestion or polylysine titration. It is possible that such weakly bound poly-

peptide sequences are covalently linked to other tightly bound sequences. Histone molecules contain highly asymmetric distributions of acidic and basic amino acid sequences (DeLange and Smith, 1971). It is possible that the protected DNA of chromatin is covered by the basic protein sequences, while the acidic regions are only loosely bound to the DNA, giving rise to the observed pattern of reactivity. Recently, Li *et al.* (1973) have studied the effect of polylysine binding upon thermal denaturation of chromatin. They conclude that polylysine stabilizes the denaturation both of protein-free regions and of regions that are covered by the less basic halves of histone molecules, and they suggest that in the latter case the polylysine may bind to DNA without physically displacing the acidic portion of the histone.

Our chemical probe studies do not permit us to distinguish a model in which reactive regions are loosely covered by protein from one in which they are completely free of protein. Clearly, the extent of the protection afforded by bound protein is a function of the reactivity of the probe that is used. A probe that readily displaces proteins will not be useful in determining their distribution on DNA. Dye molecules, which bind to DNA by intercalation and alter the secondary structure of the DNA over a distance of several base pairs, may bind with a different affinity to regions of protein-free DNA that are surrounded by protein-covered regions. The series of probes we have employed in this study are all sensitive to the same chemical features of chromatin. Half the DNA of chromatin is reactive to all of these probes. The reactive regions are on the average rather short and interspersed with unreactive regions. Kinetic studies of chromatin digestion by staphylococcal nuclease show that the regions are different; that is, they are not created at random along the chain by the action of the probe. These chemical properties must be taken into account in any hypothesis about chromatin structure, and the relation of structure to function. The number of binding sites for *E. coli* RNA polymerase on calf thymus chromatin is between $1/10$ and $1/30$ the number of sites on protein-free DNA (Cedar and Felsenfeld, 1973). The chemical probe studies suggest that there is no straightforward relationship between restriction of template activity and the amount of DNA that is chemically inaccessible because of bound protein.

Appendix

Random Degradation of Partially Blocked DNA. We consider the action of a nuclease that makes simultaneous cuts across both strands of a DNA molecule, so that the degradation is formally like that of a single-stranded polymer. If we start with a very long polymer, and make random cuts, then the weight fraction W_x of material of chain length x is given by

$$W_x = x(1 - p)^2 p^{x-1} \tag{1}$$

where p is the probability that a given bond remains unbroken, and $1 - p$ is therefore the fraction of all bonds broken (Tanford, 1961). The weight average degree of polymerization is given by

$$\langle x \rangle_w = \sum_{x=1}^{\infty} x W_x \tag{2}$$

Cleaver and Boyer (1972) have shown that deoxyribooligomers are soluble in perchloric acid for $x \leq 16$. The fraction of material that is acid soluble at any given value of p is given by the sum of all values of W_x for $1 < x \leq 16$. Using eq 2, we can relate the weight average degree of polymerization to the fraction of DNA solubilized (Figure 2).

We now consider the consequences of blocking a portion of the DNA in such a way as to make it inaccessible to nuclease attack. Suppose that there is a series of protein molecules randomly distributed along the DNA, that each molecule protects a sequence of s nucleotide pairs from digestion, and that $s/(s + \nu)$ of all the DNA is protected in this way. Suppose also that the regions of DNA not covered by protein are subject to random cleavage by enzyme. We wish to calculate the size distribution of DNA as a function of the fraction of all bonds cleaved.

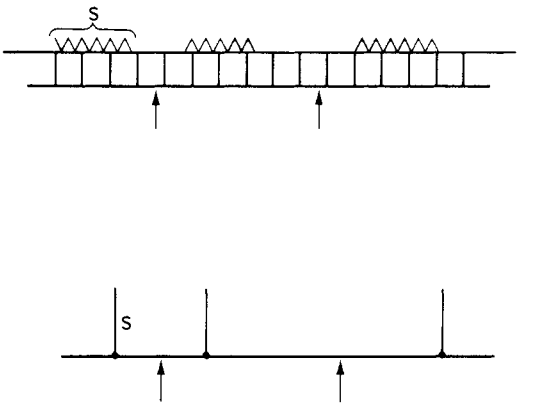

FIGURE 3: Schematic drawing (upper figure) of a blocked DNA molecule in which protein-covered regions of length s are randomly distributed on DNA, and prevent nuclease attack (arrows). The lower figure shows the formally equivalent model used for calculation (Appendix).

The problem is formally equivalent to the following one. Consider a polymer chain with a series of randomly placed side chains of length s attached to it (Figure 3). We consider the side chains to be impervious to cleavage, and the main chain susceptible. What is the size distribution of fragments as a function of the fraction of bonds cleaved? Assume that there is one side chain for every ν main chain elements. If $\xi = 1/\nu$, the probability of finding a total of λ side chains attached to a main chain segment of length x is

$$\sigma_\lambda(x) = \xi^\lambda (1 - \xi)^{x-\lambda} \frac{x!}{\lambda!(x - \lambda)!} \tag{3}$$

During degradation, molecules will be found containing varying lengths of main chain and varying numbers of side chains. A molecule containing λ side chains and a main chain of ϵ elements will have a total number of elements $x = \epsilon + \lambda s$. The probability of finding a chain of size x is therefore the probability of finding an interrupted main chain of that length with no side chains attached, plus the sum of the probabilities of finding main chains of size $(x - \lambda s)$ with λ side chains attached, for all possible values of λ. The weight fraction of main chain of length $x - \lambda s$ is given by $W_{x-\lambda s}$ (see eq 1). The weight fraction of all main chain which is of length $x - \lambda s$ and total size x is given by $W_{x-\lambda s}\sigma_\lambda(x - \lambda s)$. The total contribution to the mass made by such a molecule is given by this product multiplied by $x/(x - \lambda s)$, which takes into account the mass contribution of the attached side chains. The weight fraction of material of size x is thus given by $W_x = F_x/\Sigma_{x=1}^{\infty} F_x$, where

$$F_x = \sum_{\lambda=0}^{L} x[1 - p]^2 p^{x-\lambda s-1} \sigma_\lambda(x - \lambda s) \tag{4}$$

L is the largest value of λ for which $x - \lambda s - 1 \geq 0$. The weight average degree of polymerization can therefore be calculated using eq 2. The relationship between degree of polymerization and the fraction of DNA solubilized is calculated exactly as in the case of degradation of pure DNA. The results shown in Figure 2 were obtained by evaluating the above sums with a digital computer. A sufficient number of terms

was taken to make $\Sigma_{x=1}{}^{x}F_x$ approach 99.5% of its theoretical limit.

References

Axel, R., Cedar, H., and Felsenfeld, G. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2029.

Bartley, J., and Chalkley, R. (1970), *J. Biol. Chem. 245,* 4286.

Bonner, J., and Huang, R. C. (1963), *J. Mol. Biol. 6,* 169–174.

Carroll, D., and Botchan, M. R. (1972), *Biochem. Biophys. Res. Commun. 46,* 1681.

Cedar, H., and Felsenfeld, G. (1973), *J. Mol. Biol. 77,* 237.

Clark, R. J., and Felsenfeld, G. (1971), *Nature (London), New Biol. 229,* 101.

Cleaver, J. E., and Boyer, H. W. (1972), *Biochim. Biophys. Acta 262,* 116.

DeLange, R. J., and Smith, E. L. (1971), *Annu. Rev. Biochem. 40,* 279.

Itzhaki, R. W. (1971), *Biochem. J. 125,* 221.

Johns, E. W. (1964), *Biochem. J. 92,* 55.

Kacian, D., and Spiegelman, S. (1974), *Anal. Biochem.* (in press).

Li, H. J., Chang, C., and Weiskopf, M. (1972), *Biochem. Biophys. Res. Commun. 47,* 883.

Li, H. J., Chang, C., and Weiskopf, M. (1973), *Biochemistry 12,* 1763.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mirsky, A. E. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2945.

Miura, A., and Ohba, Y. (1967), *Biochim. Biophys. Acta 145,* 436.

Moravek, L., Anfinsen, C. B., Cone, J. L., and Taniuchi, H. (1969), *J. Biol. Chem. 244,* 497.

Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337.

Paul, J., and More, I. R. (1972), *Nature (London) 239,* 134.

Phillips, D. M. P. (1968), *Experientia 24,* 668.

Schmidt, G., Cashion, P. J., Suzuki, S., Joseph, J. P., Demarco, P., and Cohen, M. B. (1972), *Arch. Biochem. Biophys. 149,* 513.

Studier, F. W. (1965), *J. Mol. Biol. 11,* 373.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.

Properties of 5′-Nucleotidase from *Bacillus cereus* Obtained by Washing Intact Cells with Water†

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ABSTRACT: 5′-Nucleotidase activity can be detected in intact cells of *Bacillus cereus* and is released simply by washing with aqueous media; the release is partly dependent upon storage of cells at −20°, but is scarcely affected by the solute composition of the washing medium. The enzyme preparation obtained by washing cells catalyzes the dephosphorylation of various 5′-mononucleotides: IMP (K_m = 40 μM) is hydrolyzed 5 times faster than AMP (K_m = 4 μM); dAMP (K_m = 7.5 μM) is also dephosphorylated at a higher rate than AMP. The pyrophosphate linkage of ADP-glucose and NAD^+ is also hydrolyzed. ADP and ATP, which are not attacked by *B. cereus* 5′-nucleotidase, exert a strong inhibition on AMP and dAMP hydrolysis (K_i = 2 and 5 μM, respectively); other nucleoside triphosphates are also inhibitory. AMP hydrolysis catalyzed by intact cells of *B. subtilis* is also sensitive to ATP and ADP inhibition. The optimal pH for adenine nucleotide hydrolysis is around 8.4; divalent cations, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}, activate 5′-nucleotidase below pH 8, thus shifting the optimal pH to less alkaline values. Activation by Mg^{2+} and Ca^{2+} at pH 7.2 is abolished in the presence of ATP, whose inhibitory effect is strengthened by the divalent cation; on the other hand, ADP inhibition and divalent cation activation behave as independent effects at pH 7.2. The apparent surface location of the enzyme and its kinetic properties are interpreted as indicating its potential role in the uptake of extracellular nucleic acid material by chemical conversion into compounds which can penetrate the cell membrane, without affecting the cytoplasmic nucleotide pool.

During an investigation on the cell location of 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity in *Bacillus cereus* vegetative cells, we became aware of the fact that when harvested cells were repeatedly washed before use an appreciable loss of enzyme activity in cell homogenates occurred. The presence of 5′-nucleotidase activity in wash fluids was first observed in an attempt to apply the "osmotic shock" technique of Nossal and Heppel (1966); since *B. cereus* vegetative cells are insensitive to lysozyme (McDonald *et al.*, 1963; Felkner and Wyss, 1968), this method was originally thought to be the best to obtain information on the enzyme location.

The present work describes the variety of conditions under which 5′-nucleotidase is released from *B. cereus* cells upon washing in aqueous media, an almost unique property of this species. The kinetic properties of the enzyme preparation thus obtained are also reported. These include strong inhibition by ADP and ATP and activation by divalent cations. The kinetic

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