The model outlined above and in Figure 7, while necessarily incomplete, can account for 50% nucleolytic attack, for the poly(lysine) titration data and for the toluidine blue binding results, since it does not require an exclusive one-to-one charge binding. Furthermore, it is consistent with the production of positively charged insoluble fragments after DNase digestion (see Figure 7, panels c and d) without assuming the need for a total dissociation and subsequent reassociation of histone, which is precluded by the data of Clark and Felsenfeld (1971). Finally, we note that such a model may give a hint as to the vexing problem of histone deposition during cell replication, which in certain cell lines can continue for a time in the absence of DNA synthesis (Balhorn *et al.*, 1974) without apparently harming the cell. We would not be surprised if a part of the newly synthesized histone were deposited as a prereplicative event, perhaps dictated in some way by the preexisting histone-DNA organization.

In summary, we have proposed a model for nucleohistone structure which encompasses the results of Clark and Felsenfeld (1971), of Itzhaki (1971a), and those described in this paper. We envisage that histones interact asymmetrically with both strands of DNA along the entire length of the molecule, so that seemingly about half of the DNA of calf thymus chromatin is unprotected by proteins and is available for hydrolysis by nucleases or for combination with poly(lysine) but that in fact very few of the DNA-phosphate groups actually exist with free negative charges.

We emphasize that this concept of nucleohistone structure applies to the primary structure. The manner in which interactions among primary structural elements generate secondary structure, such as that recently described by Olins and Olins (1974), remains an area of future research.

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A Predictable Modification of Enzyme Specificity. Selective Alteration of DNA Bases by Metal Ions to Promote Cleavage Specificity by Deoxyribonuclease[†]

P. Clark and G. L. Eichhorn*

ABSTRACT: The specificity of an enzyme that acts on a macromolecule can be altered in a predictable manner. Copper(II), which preferentially binds to guanine sites on DNA, extensively protects guanine sites from action by bovine pancreatic deoxyribonuclease I. Mercury(II), which preferentially binds thymine sites on DNA, extensively protects thymine sites from enzyme action.

Enzymes that act on macromolecules differ from those that act on low molecular weight substrates in that the specificity of the former bears little relation to the dimensions of the substrate. Such enzymes must in fact be able to act on substrates of many sizes. Thus bovine pancreatic deoxyribonuclease I (EC 3.1.4.5) can act on DNA molecules of molecular weights in the millions as well as on tetradeoxynucleotides (Ralph *et al.*, 1962). Obviously this enzyme does not exhibit specificity for the dimensions of the polynucleotide. It is nevertheless sensitive to subtle localized chemical differences; thus the presence of a 2′-OH group on the ribose ring renders the substrate inactive (McCarty, 1946).

It therefore appears that this enzyme recognizes the chemistry and geometry of individual nucleotides even though it is not sensitive to the structure of the polynucleotide. The question arises whether it is possible chemically to

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modify some nucleotides and cause them to resist enzymatic attack while leaving other nucleotides intact so that the enzyme remains free to act on them.

The reaction of metal ions with the bases of DNA provides the opportunity for examining whether such selective modification of DNA results in the selective inhibition of the enzyme. Different metals have different relative affinities for the nucleotide bases (Izatt *et al.*, 1971; Eichhorn, 1973a). We have selected two metal ions—copper(II) and mercury(II)—on the basis that they have very different affinities for the bases. Copper binds most strongly to guanine (Fiskin and Beer, 1965; Ropars and Viovy, 1965; Frieden and Alles, 1958), while mercury has the highest affinity for thymine (Katz, 1963; Ferreira *et al.*, 1961). If binding strength for a base determines the degree of inhibition of the enzyme at that base, then it could be anticipated that Cu^{2+} would inhibit mostly guanine sites and that Hg^{2+} would inhibit mostly thymine sites. Both of these metals would thus considerably alter the specificity of the enzyme, but in quite different and predictable ways. This expectation is indeed borne out by the experiments to be described.

Specificity in enzymatic cleavage implies preference for one kind of bond over another. The first bonds to be cleaved are the preferred ones, but as the reaction proceeds to equilibrium the less readily cleaved bonds will eventually react, after those that are more readily cleaved have already reacted. Thus optimal conditions for specificity are achieved in the early stages of hydrolysis. We have studied the enzymatic hydrolysis at a point at which approximately 15% of the substrate has been rendered acid soluble.

Deoxyribonuclease I is activated by divalent metal ions (McCarty, 1946; Fischer *et al.*, 1941; Laskowski and Seidel, 1945; Laskowski, 1967; Eichhorn *et al.*, 1969; Miyaji and Greenstein, 1951; Wiberg, 1950; Erkama and Suntarinen, 1959; Shack and Bynum, 1964; Desreux *et al.*, 1962; Laskowski, 1972; Junowicz and Spencer, 1973a). Mg^{2+} ions are frequently used for the activation, although other metal ions may be employed. It was previously shown that copper ions in low concentrations give rise to a very low degree of activity, and no other metal ions are required (Eichhorn *et al.*, 1969). The reaction does not proceed in the presence of mercury ions (Zahn *et al.*, 1966) unless other metal ions are present.

Experiments were designed to compare the products of the DNase reaction in the presence of Mg^{2+}, Cu^{2+}, and Hg^{2+}. The terminal nucleotide bases of the solubilized fragments were determined by successive reactions with alkaline phosphatase and venom phosphodiesterase, and subsequent analysis of the nucleoside and nucleotide fragments.

Materials and Methods

The DNase reaction mixture contained 5×10^{-4} M(P) calf thymus DNA, 3.3×10^{-3} M $NaNO_3$, 0.1 M acetate buffer (pH 5), and 0.2×10^{-6} g/ml of DNase, once crystallized and lyophillized (Kunitz, 1950), from Sigma Chemical Co., St. Louis. The reaction was conducted at a volume of 180 ml, at 37°; it was stopped by adding 60 ml of cold 1.5 M $HClO_4$ and chilling in an ice bath. The solution was immediately centrifuged at 9000 rpm for 10 min at 4° in a Lourdes B-fuge. The supernatant was collected and neutralized with 1.5 N KOH at pH ~7, chilled in ice-water for 30 min, then separated from the precipitated $KClO_4$, and desalted by a modified procedure of Rushizky and Sober (1962). The resulting solution was diluted to <0.01 M salt and added dropwise to a freshly prepared DEAE column containing 1.33 mequiv of DEAE/mequiv of salts in the sample. The salt was removed with one or two 8-l. portions of 0.005 M $(NH_4)_2CO_3$ (pH 8.6) and the hydrolysis products were removed with 2 M $(NH_4)_2CO_3$ (pH 8.6). Elution was monitored at 260 nm using a flow cell connected to a Beckman DB spectrophotometer, and the chromatogram was recorded on a Photovolt Model 43 recorder. (This procedure removes Cu^{2+} and Mg^{2+} but not Hg^{2+}. To remove Hg^{2+} the eluted solution was evaporated to 5 ml and brought to pH 3 with 3 M acetic acid and extracted repeatedly with 3 mg/100 ml of dithizone in CCl_4.) The eluent was evaporated in a Buchler flash evaporator until all the $(NH_4)_2CO_3$ had decomposed. The per cent reaction was determined spectrophotometrically on the salt free residue in 5.0 ml of sterilized distilled water (Fredericq *et al.*, 1961).

For the *alkaline phosphatase* (from *Escherichia coli*. EC 3.1.3.1) reaction 0.5 ml of a 15-μg/ml alkaline phosphatase solution in 0.01 M Tris (pH 8.0) was added to 4.1×10^{-3} mequiv of the salt-free DNase hydrolysis products (Tomlinson and Tener, 1963). After heating overnight at 37° an aliquot was removed and analyzed for inorganic phosphate (P_i), using ammonium molybdate and ascorbic acid, according to a modified procedure of Chen *et al.* (1956).

The alkaline phosphatase was inactivated in the bulk of the sample by heating 1 hr in boiling water,[1] and the sample was reacted with 1.0 mg of *venom phosphodiesterase* (from *Crotalus adamanteus*, EC 3.1.4.1, obtained from Sigma and Worthington), 0.05 ml of 0.3 M magnesium acetate, and 0.2 ml of 0.1 M Tris (pH 8.9) for 18 hr at 45°; 0.5 ml of 0.1 M EDTA (pH 7) was then added and the sample allowed to stand 30 min before chromatographing on a Sephadex G-10 column, 35 × 1 cm, and eluting with water. The solutions corresponding to the nucleoside peaks were evaporated and spotted on cellulose MN 300 tlc plates 0.5-mm thick. The plates were developed in (1) 75 parts of 95% ethyl alcohol, 30 parts of 1 M ammonium acetate (pH 7.5) (Palladini and Leloir, 1952; Bergkvist, 1957) and then in (2) 80 parts of saturated ammonium sulfate, 18 parts of water, and 2 parts of isopropyl alcohol. The spots were eluted from the plate with HCl (pH 2). Concentrations of nucleosides were determined spectrophotometrically, and used individually for the results in Table II, and in summation for the per cent nucleoside removed in Table I.

Results and Discussion

Reaction with Enzyme, and End Group Determination. The reaction of DNase in the presence of Cu^{2+} or Hg^{2+} was compared to the commonly employed Mg^{2+} activated reaction. DNA was reacted with DNase for a sufficient period of time to cause approximately 15% of the DNA to become soluble in $HClO_4$. (With Mg^{2+} the reaction extent does not appear to affect the composition of cleavage sites; the results from the more extensive cleavage are similar to

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[1] This technique has been previously used to inactivate alkaline phosphatase (Tomlinson and Tener, 1963), and is in accord with the inactivation studies of Garen and Levinthal (1960). We have carried out control experiments to determine whether inactivation does in fact occur under our reaction conditions. The deactivated enzyme was incubated with 5′-dAMP and all the ingredients present in the venom phosphodiesterase reaction mixture. No phosphate could be detected after 19 hr. This test was repeated many times. The control experiments over a time span greater than that used in the venom reaction were required since Heppel *et al.* (1962) had shown that alkaline phosphatase heated at lower temperature for a shorter time span can renature if allowed to stand.

TABLE I: Characteristics of DNase Reaction Mixtures.

Metal Ion	DNA Conformation	Extent of Reaction	P_i (Inorganic Phosphate)	N (% Nucleoside Recovered)
Mg^{2+a}	Native	10.9	10.8	11.1
		10.9	14.7	11.8
		36.5	12.4	11.7
		20.8		9.0
		20.8		14.0
Cu^{2+b}	Native	18.6	10.3	10.0
		18.6	12.1	8.1
	Denatured	13.25	10.0	9.0
		13.25		10.0
Hg^{2+c}	Native	17.6	14.7	15.2
		17.6	14.7	12.2
		16.8	13.2	11.4

[a] 10^{-2} M. [b] 2.5×10^{-4} M. [c] 10^{-4} M (+ 10^{-2} M Mg^{2+}).

those from the initial reaction.) The time required for this degree of reaction varies with the nature of the metal: 15 min for Mg^{2+}, 2.5 hr for Cu^{2+}, and 2 hr for Hg^{2+}.

The enzymatic reaction is stopped by addition of perchloric acid, which solubilizes the smaller DNA fragments. To determine the specificity of the reaction these fragments are then analyzed for base content of the terminal nucleotide (Hurst and Becking, 1963). The analysis begins with an alkaline phosphatase reaction (Heppel *et al.*, 1962), which cleaves the 5′ terminal phosphate groups. The resulting

$$pN(pN)_x \xrightarrow[\text{phosphatase}]{\text{alkaline}} P_i + N(pN)_x \qquad (1)$$

oligonucleotides are then treated with venom phosphodiesterase (Razzell and Khorana, 1958, 1959; Singer *et al.*, 1958), which breaks all the phosphodiester linkages, producing mononucleo<u>t</u>ides generally but nucleo<u>s</u>ides for the bases at the 5′ termini of the original DNA fragments produced from the DNase action (eq 2). Base analysis of the

$$N(pN)_x \xrightarrow[\text{phosphodiesterase}]{\text{venom}} N + x(pN) \qquad (2)$$

nucleosides is then a direct measure of the bases in the 5′ terminal position of the fragments produced by DNase, and therefore a measure of the specificity of the enzyme.

The stoichiometry of the overall process for the reactions used for terminal base analysis follows from the summation of eq 1 and 2. The concentration of inorganic phosphate

$$pN(pN)_x \longrightarrow P_i + N + x(pN) \qquad (3)$$

(P_i) produced in reaction 1 should therefore equal the total concentration of nucleoside (N) in reaction 2. Table I contains a comparison of these quantities for the DNase reactions activated by a variety of metals. It can be seen that the two quantities are stoichiometric, within the limits of error of experiments that involve numerous steps, three of which are enzymatic reactions. The average error in the values of N, as compared with those of P_i, is 12%, leading to an average ratio of the two quantities of 1.12. Tomlinson and Tener (1963) reported a similar error, which is of no consequence whatever for the data of Table II.

TABLE II: End Group Analysis of DNase Produced Soluble Fragments in the Presence of Various Metal Ions.[a]

Metal Ion	End Groups (% of Total Nucleoside) A	T	C	G
Cu^{2+}	18	55	18	9
Mg^{2+}	19	40	18	23
Hg^{2+} (+Mg^{2+})	52	16	10	21

[a] Boxes show the major decreases in base content of end groups in the reaction with copper and mercury. Standard deviations of the mean are as follows: Cu^{2+}, A, 1.0; T, 1.4; C, 1.5; G, 1.8; Mg^{2+}, A, 0.5; T, 1.9; C, 1.8; G, 1.1; Hg^{2+}, A, 2.5; T, 0.3; C, 3.4; G, 3.1.

Variation of Specificity with Nature of Activating Metal Ion. The base composition of the terminal nucleotides of the DNA fragments is listed in Table II. It can be observed that the reaction with magnesium is not random, but that cleavage at the phosphodiester bonds adjacent to T sites occurs more frequently than expected from the base composition (G = C = 22; A = T = 28), as previously reported (Weiss *et al.*, 1968). Since Mg^{2+} ions bind to the phosphate groups of DNA, but have very little affinity for the bases (Shack and Bynum, 1959; Eichhorn and Shin, 1968; Eichhorn, 1973b), this deviation from randomness is not attributed to any direct effect of the metal ions on the bases, but rather either to an intrinsic relative weakness of the T bonds, or to an effect that depends on the structure of the enzyme. The reaction with Mg^{2+} ions is taken as the standard of comparison with other metal ions.

The participation of Cu^{2+} or Hg^{2+} ions in the enzymatic reaction leads to dramatic changes in the specificity of the enzyme.

The Effect of Copper Ions. The most significant effect of the copper is to decrease the amount of G at the termini of the DNA fragments. Thus the introduction of copper ions serves to inhibit cleavage at those phosphodiester bonds that are adjacent to G bases. The decreased activity at G is accompanied by increased cleavage at T sites.

The very low susceptibility of G sites to cleavage by DNase in the presence of copper is readily explained by the fact that copper binds more strongly to G than to any other base. If an imaginary base sequence contained all guanines and no other bases complexed to copper, then all the bases except guanine would be in their natural conditions. The DNase enzyme, which is presumably tailored to act upon the bases in their natural state, would "recognize" the nonguanine bases and perform its function on the bonds adjacent to them, but the modified guanines would look different to the enzyme, which would then not act on the bonds adjacent to guanine.

This picture is an idealized version based on the assumption that all guanines are complexed to copper, and no other bases are complexed at all. In reality, the specificity of the process is not that clear-cut. The copper does not bind 100% to guanine, and 0% to the rest; rather it binds the bases in the order of affinity G > A > C > T (Fiskin and Beer, 1965; Ropars and Viovy, 1965; Frieden and Alles, 1958). It is therefore anticipated that all nucleotide bonds will be inhibited by copper, but that the guanine sites would be inhibited the most, and the thymine sites the least, with adenine and cytosine intermediate. The results agree with these expectations; the percentage of terminal T's in the DNA fragments from the copper-catalyzed reaction substantially exceeds the terminal T's in the reaction with magnesium.

The Effect of Mercury Ions. The relative binding tendency of mercury to the bases is very different from that of copper; whereas thymine is least strongly bound to copper, it is most avidly bound to mercury (Katz, 1963; Ferreira *et al.*, 1961). Consequently it is to be expected that mercury ions will attach themselves preferentially to T, with the consequence that the phosphodiester bonds next to T are least readily cleaved. In line with this expectation, Table II reveals that the reaction with mercury produces a very considerable decrease in the per cent T at the cleavage sites. The decrease in T is compensated by a large increase in A. The site of major activity cannot be correlated with low complexing ability of the base as in the copper case, because the relative affinities of the bases other than thymine is in doubt. (A variety of results have been obtained which are not altogether in agreement (Izatt *et al.*, 1971; Ferreira *et al.*, 1961; Simpson, 1964; Kan and Li, 1970).

Conclusions

Previous studies have already demonstrated that the products of DNase I digestion can vary with the nature of the activating metal (Hurst and Becking, 1963; Becking and Hurst, 1963; Bollum, 1965; Laskowski, 1972; Junowicz and Spencer, 1973b), but these studies were carried out with metals that, like Mg^{2+}, bind predominantly to phosphate. The differences in the products obtained in these instances cannot be predicted from the chemistries of the metal ions.

The results of Table II support the hypothesis that metal ions can selectively change the susceptibility of the phosphodiester linkage in DNA to nuclease action by virtue of the affinity of the metals to the bases adjacent to the linkages to be cleaved. Not only do these metal ions induce dramatic modifications in the specificity of DNase, these modifications are quite different when the metal is copper and when it is mercury, and the differences are predictable from the relative base affinities of the metals.

These results suggest that modifications of the specificity of enzymes that act on macromolecules can be achieved by selective interaction of the macromolecular components with metal ions. It is reasonable to expect that enzymes other than DNase I can be modified in this manner. The changes in DNase I specificity could prove useful in the determination of base sequence, because cleavages can be produced at different linkages in the presence of different metal ions, and sequence analysis by cleavage depends upon the correlation of fragments obtained by cleavage at different positions. The usefulness of this phenomenon to sequencing is unfortunately limited by the fact that metal ions protect the sites to which they are bound only partially from enzymatic attack. We do not know whether this phenomenon will find application in sequencing. We believe that the major significance of this work lies in the demonstration that an enzymatic reaction can be modified in a rational manner by metal ions to produce a *predictable* change in enzyme specificity.

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The Site of Covalent Attachment in the Crystalline Osmium-tRNA^{fMet} Isomorphous Derivative[†]

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ABSTRACT: The site of osmium attachment in the crystalline isomorphous derivative of yeast tRNA^{fMet} is shown to be cytidine-38, the first hydrogen-bonded base to the 3′ side of the anticodon loop. The site of modification was determined chromatographically from nuclease digests of dissolved crystals of ^{185,191}Os-tRNA^{fMet}. A new and potentially useful column packing, Al-Pellionex-WAX, was employed in this analysis. The osmium adduct includes two pyridine molecules, is unusually stable, and does not interfere with enzymatic aminoacylation. Having established the attachment site, the osmium atom becomes a heavy-atom marker of a specific residue to aid in the interpretation of the electron-density map of yeast tRNA^{fMet}.

An osmium derivative of crystalline formylmethionine transfer RNA from yeast (yeast tRNA^{fMet})[1] has previously been characterized crystallographically (Schevitz *et al.*, 1972) and was shown to have a single dominant osmium site. We report here that the osmium is bound to the cytidine at position 38 in the sequence, the first hydrogen-bonded residue of the anticodon loop (Simsek and RajBhandary, 1972). These experiments were carried out primarily to facilitate the molecular structure determination of yeast tRNA^{fMet} by correlating the position of the osmium in the electron-density map as determined crystallographically, with the position of a specific residue in the sequence, thereby providing a guide point for tracing the polynucleotide chain. Evidence is also presented demonstrating that the addition of osmium to the molecule does not alter the ability of the yeast initiator tRNA to be aminoacylated by methionine:tRNA ligase, indicating that the site of osmium attachment does not interfere with the formation of a productive tRNA-ligase complex.

The original work of Criegee demonstrating that nitrogen heterocycles enhance the stability of osmate esters (Criegee *et al.*, 1942) has been utilized by a number of workers seeking to attach heavy atoms to nucleic acids for structural studies (Beer *et al.*, 1966; Burton and Riley, 1966). Recently, detailed investigations by Behrman and coworkers (Subbaraman *et al.*, 1971) have shown that relatively stable osmium adducts of pyrimidines may be formed in the presence of pyridine. We show here that exposure of crystals of yeast tRNA^{fMet} to a mother liquor containing Os(VI) and pyridine produced a derivative containing approximately 1 atom of osmium and two molecules of pyridine for each molecule of tRNA. The adduct is stable and cannot be reversed by conditions known to disrupt the secondary and tertiary structure of the tRNA molecule. Although the osmium and pyridines are also irreversibly bound to a dodecamer released by ribonuclease T$_1$, a certain degree of three-dimensional structural integrity is apparently required, since complete digestion of this fragment disrupts the adduct.

We also present two new and convenient techniques developed in the course of this work to analyze and isolate nuclease-digest products of subnanomolar quantities of tRNA.

Materials and Methods

Yeast tRNA^{fMet}. tRNA^{fMet} was purified from bulk yeast tRNA prepared according to the method of Holley (1963), using the purification scheme of Johnson *et al.* (1970) as summarized by Pasek *et al.* (1973). Crystals were grown as described by Johnson *et al.* (1970) from preparations of tRNA^{fMet} which accepted 1.8 nmol of methionine/A_{258} unit. Acceptance levels as low as 1.4 nmol/A_{258} unit were encountered but only in crystals which had been irradiated.

Preparation of Os-tRNA^{fMet}. Crystals of yeast tRNA^{fMet} were soaked in a stabilizing supernatant solution containing potassium osmate and pyridine as described by Schevitz *et al.* (1972). This "soak solution" was obtained by a 50-fold dilution of a 1 M $K_2OsO_2(OH)_4$, 3.1 M pyridine "stock solution," prepared by adding an equal volume of a 1:1 (vol.) mixture of pyridine and 1 N HCl to a 0.2 M aqueous suspension of $K_2OsO_2(OH)_4$ (Alfa Inorganics). This "stock

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[1] Abbreviations used are: yeast tRNA^{fMet}, formylatable methionine tRNA from yeast; A_{258} unit, that amount of tRNA when dissolved in 1 ml of H_2O produces an absorbance of 1 at 258 nm with a 1-cm light path.