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Action of Micrococcal Nuclease on Chemically Modified Deoxyribonucleic Acid†

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ABSTRACT: The exposure of alkali-denatured DNA to the reagent *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide (Cmc) *p*-toluenesulfonate results in the substantial blocking of the thymidine and deoxyguanosine moieties of the polynucleotide chains with Cmc groups. The hydrolysis of this modified DNA with micrococcal nuclease followed by the removal of the blocking groups produces a series of oligonucleotides, whereas, under the same enzymatic conditions, unmodified DNA is reduced to mono- and dinucleotides. The oligonucleotides in various size groups have been analyzed by degradation with alkaline phosphatase together with snake venom phosphodiesterase or spleen phosphodiesterase to determine their chain lengths and the identity of their 5'- and 3'-terminal nucleotides. In addition, the tetranucleotides, pdA-dT-dA-dT and pdT-dA-dT-dA, and their di-Cmc derivatives have been digested with the nuclease and the resulting products have been identified. From these results a specific pattern for the action of the nuclease on the modified DNA can be derived: (i) a preferential endonucleolytic cleavage takes place at the -Np-dA- bonds (where N is a modified or unmodified deoxyribonucleoside); (ii) each oligonucleotide, so formed, can then undergo the normal exonucleolytic degradation at its 3' terminal depending on whether it contains a modified or unmodified nucleotide at its 3' terminal; (iii) those oligonucleotides that contain a blocked dT or dG at their 3' terminals tend to be resistant to this exonucleolytic action while the rest of the oligonucleotide species are subject to the stepwise removal of unblocked nucleotides until a blocked dT or dG is located at their 3' terminals. This restricted activity of micrococcal nuclease constitutes a method for the specific cleavage of polydeoxyribonucleotides that is expected to be of some value in future studies on the sequence analysis of DNA.

In comparison with the sequence analysis of RNA molecules the direct determination of the primary structure of DNA chains has presented a somewhat more difficult problem. While a number of sequences of some small sections of DNA molecules have already been assigned it has become apparent that one of the main difficulties in this work arises from the relative lack of enzymatic methods for the cleavage of polydeoxyribonucleotides into specific smaller fragments for subsequent analysis. In the case of the work on RNA, the relative ease with which sequence information can be obtained depends, in large part, on the availability of base-specific endonucleases such as the ribonucleases A, T_1, and U_2. In addition, the reversible chemical modification of certain nucleotides within an RNA chain can be used to induce even greater specificity on the action of these ribonucleases. There are no known counterparts of these ribonucleases that

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could be used for the specific cleavage of DNA molecules, and the present work was begun with the expectation that prior chemical modification of DNA might result in the induction of base specificity in the cleavage mechanisms of some of the common deoxyribonucleases.

Uridine, thymidine, guanosine, and deoxyguanosine and their 5′-phosphates react readily with the cation of the reagent *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate (Cmc *p*-toluenesulfonate),[1] at pH 8–9, and the resulting derivatives can be converted back to the corresponding nucleosides and nucleotides by exposure to dilute ammonia (Gilham, 1962; Ho and Gilham, 1967). The conditions necessary for the chemical modification of these nucleoside moieties within a polynucleotide strand, as well as the conditions required for the subsequent removal of the blocking groups, are of a sufficiently mild nature that the structural integrity of the polynucleotide chain can be preserved. Thus, it has been possible to use this type of chemical modification to restrict, in a specific way, the normal nucleolytic activity of ribonucleases A, T_1, and U_2 toward RNA (Lee *et al.*, 1965; Naylor *et al.*, 1965; Ho and Gilham, 1967; Ho *et al.*, 1969), and these techniques have been used in studies on the nucleotide sequences in a number of RNA molecules. Similarly, denatured DNA has been derivatized with the carbodiimide reagent (Augusti-Tocco and Brown, 1965), and Drevitch *et al.* (1966) have observed that such derivatization of DNA decreases the number of bonds that would normally be subject to cleavage by pancreatic deoxyribonuclease.

Micrococcal nuclease, a phosphodiesterase that is capable of hydrolyzing either DNA or RNA to nucleoside 3′-phosphates, has been extensively studied. The results of the work on its cleavage specificity have been reviewed by Anfinsen *et al.* (1971) and, on the basis of evidence from a number of laboratories, they have concluded that, in native DNA, the -Np-dT- and -Np-dA- bonds are preferentially attacked while, in denatured DNA, the order of cleavage appears to be nearly random. However, a recent study in this laboratory involving a detailed analysis of the terminal nucleosides appearing in polynucleotide fragments during digestion with this nuclease confirms that the preferential endonucleolytic cleavage of -Np-dT- and -Np-dA- bonds occurs also with the denatured form of DNA (Roberts *et al.*, 1962). In addition, the enzyme is known to possess exonucleolytic activity in that, subsequent to its endonucleolytic action, the nuclease is capable of removing nucleoside 3′-phosphate moieties from the newly formed 3′ terminals of the polynucleotide fragments until the entire nucleic acid is reduced to a mixture of mono- and dinucleotides (Reddi, 1960; Sulkowski and Laskowski, 1962). Thus, in the absence of extra cleavage specificity that might be induced by chemical modification of the substrate, it would seem that the enzyme could have very little use in the sequence analysis of DNA.

Salmon sperm DNA was used for the present studies and a number of experimental methods for its derivatization with the Cmc reagent were investigated. The preferred method of derivatization involves the alkaline denaturation of the DNA followed by exposure of the product to a high concentration of the reagent at room temperature for 20 hr. A more rapid derivatization results from a similar treatment of the DNA at higher temperatures but it is accompanied by some cleavage of the polynucleotide chains. Due to the resistance of Cmc-modified nucleic acids to the action of the various nucleases it is not possible to obtain accurate values for the percentages of thymidine and deoxyguanosine moieties that have been modified by the reagent. Rough estimates of the degrees of modification can be achieved by degrading the Cmc-DNA with a mixture of the enzymes, deoxyribonuclease I, micrococcal nuclease, snake venom phosphodiesterase, spleen phosphodiesterase, and alkaline phosphatase. The combined action of these enzymes would be expected to convert all the unmodified nucleotide moieties in the DNA to nucleosides with the possible exception of some of the nucleotides that are connected to the 5′ positions of modified nucleotides within the polynucleotide chain (Naylor *et al.*, 1965). The mixed enzyme hydrolysis of Cmc-DNA prepared at room temperature resulted in the conversion of about 70% of the material to nucleosides and modified nucleosides with the remainder of the products appearing as highly modified oligonucleotides. An analysis of these products indicated that more than 70% of the deoxyguanosine and more than 80% of the thymidine moieties in the DNA had been modified by the reagent.

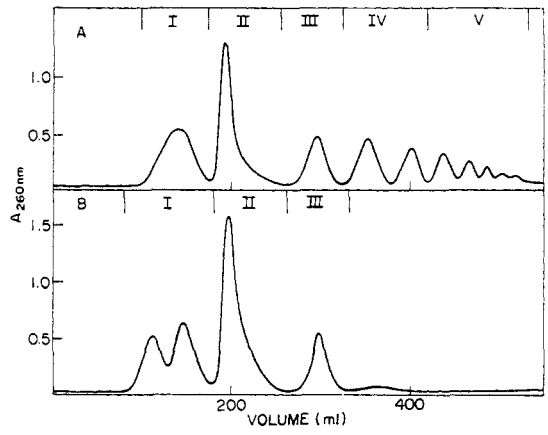

FIGURE 1: Elution patterns from the chromatography of micrococcal nuclease digest on a column (50 × 0.8 cm) of DEAE-Sephadex. Elution was effected with 1000 ml of 0.005 M Tris-Cl–7 M urea (pH 8) containing a linear gradient of 0–1.0 M sodium chloride at a flow rate of 10 ml/hr: (A) Cmc-DNA digested with 100 units of micrococcal nuclease at 37° for 1 hr under the conditions described in the Experimental Section; (B) heat-denatured DNA digested under the same conditions.

Cmc-DNA was treated with micrococcal nuclease and the blocking groups were subsequently removed with alkali. Heat-denatured DNA was also treated with the enzyme under the same conditions and the two sets of products were then separated according to chain length by ion-exchange chromatography with solvents containing 7 M urea (Tomlinson and Tener, 1963). The elution patterns (Figure 1) indicate that, compared with heat-denatured DNA, the Cmc-DNA contains far fewer phosphodiester bonds that are susceptible to the nuclease action. The products from each digestion were combined into fractions as indicated in the elution patterns. These fractions were then rendered free of salt and urea and the oligonucleotide components of each fraction were analyzed with respect to their base compositions, average chain lengths, and their 3′- and 5′-terminal nucleosides. The method of analysis (Ho and Gilham, 1973) consists of (i) the treatment of the fraction with alkaline phosphatase, (ii) the destruction of the enzyme *in situ* by exposure to alkali, (iii) the digestion of the products with snake venom phosphodiesterase or spleen phosphodiesterase, and (iv) the separation of the resulting nucleosides and nucleotides by ion-exchange chromatography. From the results corresponding to elution pattern A (Table I) it can be concluded that most of the larger oligonucleotides

[1] Abbreviation used is: Cmc, *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide.

TABLE I: Analysis of Terminal Nucleosides of Oligonucleotide Fractions Corresponding to Elution Patterns in Figure 1.

Elution Pattern	Fraction	Av Chain Length	% 5′-Terminal Nucleoside				% 3′-Terminal Nucleoside			
			dC	dA	dT	dG	dC	dA	dT	dG
A	I	1.0	32	53	8	7	30	53	12	5
A	II	2.0	11	78	10	<1	22	4	20	54
A	III	3.0	18	75	6	<1	16	<1	42	41
A	IV	4.5	18	80	<1	<1	13	<1	52	34
A	V	8.4	20	78	<1	<1	12	<1	52	35
B	I	1.0	21	34	34	11	21	34	34	11
B	II	2.0	3	49	46	2	27	14	10	49
B	III	3.0	6	41	50	3	15	6	2	77

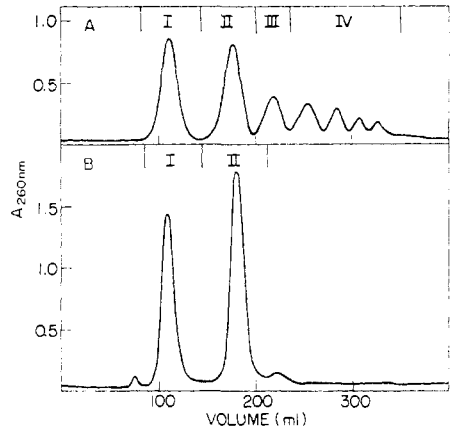

FIGURE 2: Elution patterns from the chromatography of micrococcal nuclease digests on a column (50 × 0.4 cm) of DEAE-Sephadex. Elution was effected with 1000 ml of 0.005 M Tris-Cl–7 M urea (pH 8) containing a linear gradient of 0–1.0 M sodium chloride at a flow rate of 10 ml/hr: (A) Cmc-DNA digested with 600 units of micrococcal nuclease at 37° for 5 hr under the conditions described in the Experimental Section; (B) heat-denatured DNA digested under the same conditions.

deriving from the Cmc-DNA contain dA at their 5′ terminals with essentially no dG or dT, while the predominant nucleoside located at the 3′ terminals of these molecules is dT or dG. In addition, over 80% of the mononucleotides appearing in the digestion of Cmc-DNA can be accounted for as deoxyadenosine and deoxycytidine 3′-phosphates. On the other hand, in agreement with the results obtained by Rushizky *et al.* (1960), the trinucleotide fraction from the denatured DNA digestion consists of products possessing dA or dT at their 5′ terminals and predominantly dG at their 3′ terminals, while the mononucleotide fraction consists of all four nucleotides (Table I, elution pattern B). The amount of dC located at the 3′ terminals of the products in the trinucleotide fraction is about the same as that observed for the corresponding fraction from the Cmc-DNA digestion. In the endonucleolytic attack of the enzyme on modified DNA there is a substantial decrease in the susceptibility of -Np-dT- bonds to cleavage and the analyses listed in Table I are consistent with a pattern of hydrolysis involving a preferential endonucleolytic cleavage of the DNA chain at -Np-dA- bonds followed by a preferential exonucleolytic degradation of those species of the resulting oligonucleotides that possess unmodified nucleotides at their 3′ terminals. The small amount of dT and dG mononucleotides formed in the digestion could have arisen from some of the unblocked dT and dG moieties in the polynucleotide chain.

In another set of experiments Cmc-DNA and heat-denatured DNA were each treated with six times as much enzyme and incubated for five times as long as in the digestions described above. The products were separated and analyzed as before (Figure 2, Table II) and the results indicate that there is no change in the pattern of cleavage of Cmc-DNA upon extended exposure to the nuclease, except that, for the fractions of larger oligonucleotides, the 3′ terminals of the chains consist totally of dT or dG. The marked reduction of the dC content at these terminals compared with the corresponding analyses listed in Table I is presumed to have arisen by more extensive exonucleolytic removal of this nucleotide under the more vigorous digestion conditions. This conclusion is consistent with the results of a third set of experiments in which Cmc-DNA was treated with a smaller quantity of enzyme (about 20 times less than that used in the first set of experiments). The products in this case consisted of a higher proportion of longer oligonucleotides and, on analysis, the oligonucleotides present in the fraction corresponding to an average chain length of 11 were shown to contain dA at 80% of their 5′ terminals together with a percentage distribution of 26, 8, 32, and 34 for dC, dA, dT, and dG, respectively, at their 3′

TABLE II: Analysis of Terminal Nucleosides of Oligonucleotide Fractions Corresponding to Elution Patterns in Figure 2.

Elution Pattern	Fraction	Av Chain Length	% 5′-Terminal Nucleoside				% 3′-Terminal Nucleoside			
			dC	dA	dT	dG	dC	dA	dT	dG
A	I	1.0	14	70	4	12	14	70	4	12
A	II	2.1	12	79	8	<1	19	5	31	45
A	III	3.2	15	83	<1	<1	3	<1	57	39
A	IV	5.3	20	78	<1	<1	<1	<1	52	46
B	I	1.0	21	30	27	22	21	30	27	22
B	II	2.0	3	43	53	1	29	11	11	49

terminals. The marked reduction of dC content at the 3′ terminals under more vigorous digestion conditions is not matched, as might be expected, by an increased proportion of dC in the mononucleotide fraction. The expected increase in dC mononucleotide may, however, be masked by the substantial increase in dA mononucleotide, an increase that could arise from the more extensive cleavage of small dA-rich oligonucleotides caused by extended exposure to the enzyme.

A more definitive indication of this restricted pattern of cleavage can be obtained through the study of the products arising from the nuclease digestion of oligonucleotides of defined sequence. For this work, the tetranucleotides, pdA-dT-dA-dT and pdT-dA-dT-dA, were treated with the carbodiimide reagent and the products, pdA-d$\bar{T}$-dA-d$\bar{T}$ and pd$\bar{T}$-dA-d$\bar{T}$-dA (where d$\bar{T}$ is the chemically modified thymidine moiety), were isolated and purified by paper chromatography. On exposure to the nuclease, the oligonucleotide, pdA-d$\bar{T}$-dA-dT, was converted to pdA-d$\bar{T}$p and dA-d$\bar{T}$ while the unmodified polymer, pdA-dT-dA-dT, gave, under the same conditions, pdAp, dTp, and dA-dT together with small amounts of dAp and dT. The modified sequence isomer, pd$\bar{T}$-dA-d$\bar{T}$-dA, was hydrolyzed to pd$\bar{T}$p, dA-d$\bar{T}$p, and dA, whereas the unmodified form, pdT-dA-dT-dA, was degraded to a mixture of dAp, dTp, pdTp, pdT-dAp, dT-dA, and dA.

These results together with those obtained in the analysis of the degradation products from Cmc-DNA are indicative of a cleavage mechanism that may have sufficient specificity for use in studies on nucleotide sequences in DNA. The general nature of the mechanism appears to involve initially a preferential endonucleolytic attack by the nuclease on -Np-dA- bonds (and to a lesser extent -Np-dC- bonds) where N is a modified or unmodified nucleoside. Depending on the nature of N the initial cleavage can then be followed by an exonucleolytic removal of nucleotides from the 3′ terminals of the oligonucleotides so formed. Those oligonucleotides in which the terminal nucleoside, N, is a modified dT or dG tend to be resistant to this exonucleolytic action, whereas the chains containing dA or dC in their terminal positions are subject to attack. The extent of the exonuclease action on each member of the latter class of molecules is then dependent on the base sequence near its 3′ terminal, in that the stepwise removal of dA and dC nucleotides occurs until a modified dT or dG is located at the 3′ terminal, at which point the molecule is somewhat resistant to further exonucleolytic degradation. Further characterization of the fine details of the action of micrococcal nuclease on Cmc-DNA will result from an analysis of products arising from the cleavage of longer polynucleotides of defined sequence and these studies are in progress.

Experimental Section

Materials. *N*-Cyclohexyl-*N*′-β-(4-methylmorpholinium)-ethylcarbodiimide *p*-toluenesulfonate was purchased from Pierce Chemical Co., Rockford, Ill., and salmon sperm DNA was obtained from Calbiochem, Los Angeles, Calif. The tetranucleotides, pdA-dT-dA-dT and pdT-dA-dT-dA, were the products of Collaborative Research, Inc., Waltham, Mass. *Escherichia coli* alkaline phosphatase (Grade BAPF), micrococcal nuclease (Grade NFCP), bovine pancreatic deoxyribonuclease (Grade DPFF), snake venom phosphodiesterase (Grade VPH), and spleen phosphodiesterase (Grade SPH) were obtained from Worthington Biochemical Corporation, Freehold, N.J. The alkaline phosphatase was dialyzed against 0.005 M Tris-Cl (pH 8) prior to use, and the unit of activity is defined as the quantity required to liberate 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per min at 25° (Garen and Levinthal, 1960). The unit of activity of micrococcal nuclease is equivalent to the amount required to release acid-soluble oligonucleotides corresponding to a change in $A_{260\ nm}$ of 1.0 under the assay conditions of Heins *et al.* (1966). The unit of activity of deoxyribonuclease is defined as that amount of enzyme which causes an increase of $A_{260\ nm}$ of 1.0 in 4 min under the assay conditions of Kalnitsky *et al.* (1959). The snake venom phosphodiesterase was pretreated according to the procedure of Sulkowski and Laskowski (1971) to remove any 5′-nucleotidase activity. Heat-denatured DNA was prepared by heating a solution of it in water (1 mg/ml) at 100° for 10 min and then cooling it rapidly to 0°. DEAE-Sephadex A-25 (Pharmacia) and DEAE-cellulose DE-23 (Whatman) were pretreated according to the directions of the respective manufacturers.

Preparation of Cmc-DNA. Salmon sperm DNA (50 mg) was dissolved in 0.01 M sodium borate (pH 8.5) (50 ml) and the solution was brought to pH 12.5 by the addition of 1 M sodium hydroxide. The solution was kept at this pH for 10 min at room temperature and was then adjusted to pH 10 with 1 M hydrochloric acid. *N*-Cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate (6 g) was added and after 30 min at room temperature, the solution was treated with sodium borate to give a final concentration of 0.1 M and the pH of the solution was brought to 8.5 with hydrochloric acid. After a further 20 hr at room temperature the solution was adjusted to pH 7 with hydrochloric acid. The product was precipitated by the addition of 1 M sodium acetate (pH 7) (10 ml) followed by ethanol (120 ml). The precipitate was collected by centrifugation and dried *in vacuo*. The product was redissolved in water (25 ml) and precipitated by the addition of 1 M sodium acetate (pH 7) (7 ml) followed by ethanol (60 ml). The precipitate was collected by centrifugation and dried *in vacuo*.

Analysis of Cmc-DNA. The derivatized DNA (40 optical density units (ODU_{260nm})) was dissolved in 1 ml of 0.1 M Tris-Cl–0.02 M magnesium chloride (pH 7.5 at 20°) containing a mixture of deoxyribonuclease I (2500 units), micrococcal nuclease (5000 units), snake venom phosphodiesterase (0.5 mg), spleen phosphodiesterase (0.5 mg), and alkaline phosphatase (1.5 units). After incubation at 37° for 20 hr, the digest was applied to Whatman No. 3MM chromatographic paper. Chromatography with ethanol–1 M ammonium acetate (pH 7) (7:3, v/v) gave three bands of products: I, R_F 0.86; II, R_F 0.79; III, R_F 0.62. The products consisted of modified and unmodified nucleosides together with highly modified oligonucleotides. The latter could be detected by treatment with ammonia and rechromatography to yield the corresponding unblocked species with much lower R_F values. Band I, whose R_F value was identical with that of Cmc-dT, was treated with ammonium hydroxide (7 M, 2 hr, 25°) to remove the Cmc group. Paper chromatography of this product in the above solvent system gave thymidine (R_F 0.79). Band II, which had an R_F value similar to that of dT and Cmc-dG, was also treated with ammonia and rechromatographed in the same system to yield dT (R_F 0.79) and dG (R_F 0.58). Band III contained unmodified dG together with dC, dI (deriving from dA through the deaminase activity of the spleen phosphodiesterase preparation), and some highly modified oligonucleotides. Paper chromatography with a solution of ammonium sulfate (40 g) in water (100 ml) at pH 7 gave dG, dI, and dC (with R_F values relative to dC of 0.58, 0.78, and 1.0, respectively). The relative amounts of dT, Cmc-dT, dG, and Cmc-dG in the digest were determined spectrophotometrically.

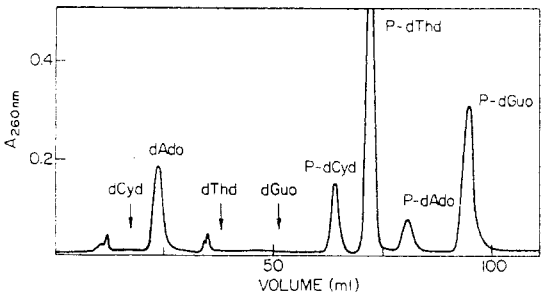

FIGURE 3: Elution pattern from the separation of the products from the alkaline phosphatase–snake venom phosphodiesterase digestion of the oligonucleotides corresponding to fraction V, Figure 1A. The column (100 × 0.2 cm) of AG 1-X4 (400 mesh) ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.) was prewashed with 10% ethanol before the following additions were made by means of a sample injection loop: 0.4 ml of 1 M NaOH, 2 × 0.5 ml of 10% ethanol, the enzyme digest in 0.7 ml of water, and 2 × 0.5 ml of 10% ethanol. The separation was effected by elution at 10 ml/hr, under pressure, with 200 ml of 10% ethanol containing a linear gradient of 0–0.5 M NH_4Cl which had been brought to pH 9.0 with ammonia.

Digestion of Cmc-DNA and Heat-Denatured DNA with Micrococcal Nuclease. Cmc-DNA or heat-denatured DNA (40–60 ODU_{260nm}) was dissolved in 0.05 M Tris-Cl–0.01 M calcium chloride (pH 7.5) at 20° (6 ml) and treated with 100 units of micrococcal nuclease at 37° for 1 hr or with 600 units of the enzyme at 37° for 5 hr. The enzyme was inactivated and the blocking groups were removed by treating the digestion mixture with 2 M sodium hydroxide (0.7 ml) and 1 M EDTA, tetrasodium salt (0.15 ml), and allowing the solution to stand at 37° for 2 hr. The mixture was then diluted with water (33 ml) and the pH of the solution was adjusted to 8 with concentrated hydrochloric acid. Urea (24 g) was then added and the mixture was applied to a column (50 × 0.8 cm) of DEAE-Sephadex; fractionation was effected at 8 ml/hr with 1000 ml of 0.005 M Tris-Cl–7 M urea (pH 8) containing a linear gradient of 0–1.0 M sodium chloride. The elution profiles are shown in Figures 1 and 2. The fractions obtained from these separations were rendered free of salt and urea by dilution and readsorption on a column (20 × 1 cm) of DEAE-cellulose. The salt and urea were removed by elution with 0.03 M triethylammonium bicarbonate and the oligonucleotides were then recovered by elution with 1 M triethylammonium bicarbonate followed by removal of the volatile salt by evaporation *in vacuo*. In the case of the mononucleotide fractions the salt and urea were removed by adsorption of the nucleotides to carbon using the procedure described by Mandeles and Kammen (1966).

Analysis of Oligonucleotides Produced by Micrococcal Nuclease Digestion. The oligonucleotide fractions (as indicated in Figures 1 and 2) from the fractionation of the micrococcal nuclease digestions were analyzed in quantities of 1–5 ODU_{260nm} by the method of Ho and Gilham (1973). Each fraction was treated with alkaline phosphatase and then with sodium hydroxide to destroy the enzyme. The dephosphorylated oligonucleotides were then treated directly with snake venom phosphodiesterase or spleen phosphodiesterase and the products were separated by chromatography on Dowex 1-X4 ion-exchange resin. After spectrophotometric estimation of the amounts of nucleotides and nucleosides produced in these digestions the method allows for the determination of the identity of the 3′- and 5′-terminal nucleosides as well as the average chain length of the components of the oligonucleotide fraction. The elution pattern from one of these analyses is shown in Figure 3. This pattern corresponds to the analysis of the degradation with phosphatase and snake venom phosphodiesterase of the oligonucleotide fraction V, elution pattern A, Figure 1. The results of the analyses on the various oligonucleotide fractions are listed in Tables I and II.

Preparation of Cmc-Tetranucleotides. Either pdT-dA-dT-dA or pdA-dT-dA-dT (20 ODU_{260nm}) was dissolved in 0.05 M sodium borate (pH 8) (0.4 ml) and treated with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate (20 mg) and the solution was allowed to stand at room temperature for 20 hr. The mixture was adjusted to pH 7 and applied as an 8-cm band to Whatman No. 3MM chromatographic paper. Chromatographic elution with ethanol–1 M ammonium acetate (pH 7) (7:3, v/v) indicated that each tetranucleotide had been converted completely to a mixture of mono- and disubstituted derivatives with R_F values of 0.14 and 0.33, respectively (the unsubstituted tetranucleotides have an R_F value of 0.03 in this system). The tetranucleotide derivatives were eluted with water and rendered salt-free by repeated evaporation of their aqueous solutions *in vacuo*. The yield of the disubstituted derivative in each case was about 60%.

Micrococcal Nuclease Digestion of pdA-dT-dA-dT and pdA-dT̄-dA-dT̄. The disubstituted derivative, pdA-dT̄-dA-dT̄ (4 ODU_{260nm}), was dissolved in 0.2 ml of 0.05 M Tris-Cl–0.01 M calcium chloride (pH 7.5) and treated with micrococcal nuclease (500 units) at 37° for 20 hr. The digestion was applied to a small area of Whatman No. 3MM chromatographic paper and elution with ethanol–1 M ammonium acetate (pH 7) (7:3, v/v) gave the two products dA-dT̄ and pdA-dT̄p with R_F values of 0.74 and 0.16, respectively. Both products had λ_{max} 262 nm at pH 7 and the structure of dA-dT̄ was confirmed by the comparison of its R_F with that of an authentic derivative prepared directly from dA-dT. In addition, the product, dA-dT, was rendered salt-free by repeated evaporation of its aqueous solution and then treated with 7 M ammonia for 2 hr at room temperature to remove the blocking group. Chromatographic analyses as described above showed that the dA-dT had been completely converted to dA-dT (R_F 0.55). The structure of this dinucleoside phosphate was confirmed by degradation with snake venom phosphodiesterase. Analysis by the method used above (Ho and Gilham, 1973) showed that the products consisted of equal amounts of dA and pdT. The other product of the nuclease digestion, pdA-dTp, was also exposed to ammonia and then treated with alkaline phosphatase (0.6 unit) in 0.02 M Tris-Cl–0.01 M magnesium chloride (pH 8) (0.2 ml) for 3 hr at 37°. Chromatography, as described above, showed that the dinucleotide was completely converted to dA-dT, and this product was also degraded to equal amounts of dA and pdT on exposure to snake venom phosphodiesterase.

The unmodified tetranucleotide, pdA-dT-dA-dT (6 ODU_{260nm}), was treated with micrococcal nuclease under identical conditions and the products were separated as described above. The products were dA-dT (R_F 0.55), dTp (R_F 0.43), and pdAp (R_F 0.05), together with smaller amounts of dT (R_F 0.78) and dAp (R_F 0.35). The structures of the products were confirmed by the comparison of their ultraviolet spectra and R_F values with those of authentic compounds.

Micrococcal Nuclease Digestion of pdT-dA-dT-dA and dpT-dA-dT̄-dA. The substituted tetranucleotide, pdT̄-dA-dT-dA (5 ODU_{260nm}), was dissolved in 0.05 M Tris-Cl–0.01 M calcium chloride (pH 7.5) (0.2 ml) and treated with micrococcal nuclease (500 units) for 20 hr at 37°. The products, which were separated as described for the isomeric tetranucleotide above, consisted of pdT̄p (R_F 0.55), dA-dT̄p (R_F, 0.50), and dA (R_F 0.68). The products, dA-dT̄p and pdT̄p, were eluted from the

paper and treated with 7 M ammonia for 2 hr at room temperature. The deblocked products were then analyzed by degradation with alkaline phosphatase and snake venom phosphodiesterase followed by ion-exchange chromatography (Ho and Gilham, 1973). With this procedure the dA-dTp produced equal quantities of dA and pdT while the pdTp gave thymidine as the only product.

The unmodified tetranucleotide, pdT-dA-dT-dA (6 ODU_{260nm}), was treated with micrococcal nuclease under identical conditions and the products were separated by paper chromatography as described above. The products, dA (R_F 0.68), dT-dA (R_F 0.55), dTp (R_F 0.43), dAp (R_F 0.35), and pdTp (R_F 0.12), were identified by the comparison of their ultraviolet spectra and R_F values with those of authentic compounds.

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Studies on the Relationship between Deoxyribonucleic Acid Polymerase Activity and Intracisternal A-Type Particles in Mouse Myeloma†

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ABSTRACT: Previous studies have demonstrated a DNA polymerase activity in preparations of mouse intracisternal A particles. In the current study the relationship between this DNA polymerase activity and A particles in mouse myeloma was investigated. Reaction conditions were adjusted for specific measurement of the type of DNA polymerase activity that was found in isolated A particles. This type of DNA polymerase activity was detected in several A-particle containing tissues but not in tissues devoid of or containing very low numbers of A particles. Similarly, four enzymes isolated from myeloma that appear to correspond to mouse cellular DNA polymerases were not active under the reaction conditions used for measurement of the A particle associated DNA polymerase. During subcellular fractionation, the enzyme activity behaved as a particulate cytoplasmic component and was concentrated 30-fold in purified A particles relative to the crude homogenate. A very similar subcellular distribution was observed for an antigen associated with the main A-particle structural protein. The DNA polymerase activity cosedimented with A particles in isopycnic sucrose gradients and was not solubilized by treatment with 1 M KCl or several surfactants. Implications of these findings are discussed.

Mammalian cells contain a number of DNA polymerase activities distinguished on the basis of physical properties, intracellular localization, template specificity, and requirements for maximal activity. Biochemical characterization of these DNA polymerases and elucidation of their physiological roles in DNA replication and repair are matters of current investigation.

In a previous study (Wilson and Kuff, 1972), DNA polymerase activity was detected in preparations of intracisternal A-type particles (Bernhard, 1960; Provisional Committee for Nomenclature of Viruses, 1966) from several mouse tumors. The enzyme possessed an unusual biochemical property in that it was active with poly(riboadenylate) as template, but virtually inactive with either natural or synthetic DNA templates. The suggestion was therefore made that the DNA polymerase activity was not due to contamination by cellular DNA polymerase and was A-particle specific. However, discoveries of normal cellular DNA polymerase activities that copy

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