Characterization of DNA Strand Breakage in Vitro by the Antitumor Protein Neocarzinostatin†

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ABSTRACT: The antitumor protein antibiotic neocarzinostatin causes strand scission of DNA in vitro in the presence of a sulfhydryl compound. The breaks are single stranded in nature and bear 5′-phosphoryl termini. All four deoxymononucleotides are recoverable at the 5′-ends of the cleavage sites, although a higher proportion of dGMP and TMP are consistently found. The lesions are not repairable with polynucleotide ligase from *Escherichia coli*. A quantitative assay was developed to determine the pH profile and time course of the reaction. Data from protection experiments with synthetic and natural DNAs indicate the requirement for thymidylic acid and deoxyadenylic acid in the DNA for cutting. In DNA–RNA hybrids, riboadenylic acid can substitute for deoxyadenylic acid, whereas ribouridylic acid cannot substitute for thymidylic acid. Release of thymine is detected, and the amount of release correlates well with the number of strand scissions.

Neocarzinostatin (NCS[1]), an antibiotic isolated from the culture filtrates of *Streptomyces carzinostaticus* (Ishida et al., 1965), is a single-chain polypeptide with a molecular weight of 10 700 (Meienhofer et al., 1972). The protein has been purified to homogeneity, as judged by polyacrylamide gel electrophoresis at various pH's, and its amino acid sequence has been determined by Meienhofer et al. (1972). NCS is highly acidic in nature (Samy and Meienhofer, 1974) and contains all of the commonly occurring amino acids, including four half-cystines, but lacks histidine and methionine. The molecule is highly resistant to the action of trypsin and is chemically reduced only under severe conditions.

NCS is cytotoxic to gram-positive bacteria (Ishida et al., 1965) and HeLa cells in culture (Ono et al., 1966). It primarily inhibits DNA synthesis, while RNA and protein synthesis are not significantly affected (Homma et al., 1970). As a result of the incubation of HeLa cells with NCS, the cellular DNA has a reduced molecular weight, as analyzed by alkaline sucrose gradient centrifugation (Beerman and Goldberg, 1974; Ohtsuki and Ishida, 1975). This result indicates that NCS directly or indirectly causes strand scission of the genetic material.

The cutting reaction with NCS can be reproduced in vitro in the presence of a reducing agent and is specific for DNA (Beerman and Goldberg, 1974). Native double-stranded or Me_2SO-denatured reovirus RNAs are not substrates for the antibiotic (Beerman et al., 1974). With superhelical simian virus 40 DNA as a model substrate, it was shown that this protein makes single-stranded breaks in the DNA double helix (Beerman and Goldberg, 1974).

In a previous communication from this laboratory (Beerman et al., 1976), it was found that the phenomena of DNA breakage and inhibition of DNA synthesis resulting from the incubation of HeLa cells with NCS were related, and that the subcellular target of action of this antibiotic is DNA. In another report (Beerman et al., 1977), evidence is presented to substantiate that the in vivo and in vitro activities of NCS are due to the same protein molecule and not due to a contaminating endonuclease. In the present study, we undertake to examine the nature and specificity of the strand scission caused by NCS in vitro, using DNA from the bacteriophage λ as the model substrate. We hope that by understanding the chemical nature of the cleavage products and by defining the requirements in the DNA for cutting, we will gain insight into the mechanism of action of the antibiotic.

Experimental Procedure

Materials. Purified NCS (Samy et al., 1977) was a generous gift of Dr. T. S. A. Samy of the Sidney Farber Cancer Institute. Synthetic DNAs were obtained from P-L Biochemicals and Collaborative Research. DNase I, bacterial alkaline phosphatase, snake venom phosphodiesterase, and *Clostridium perfringen* DNA were purchased from Worthington Biochemicals. *Micrococcus luteus* DNA was from Miles Laboratory. Radioactive biochemicals, such as [γ-^{32}P]ATP, [*methyl*-^{3}H]thymidine, and $H_3{}^{32}PO_4$ were purchased from New England Nuclear. Polygram Cel 300 PEI-cellulose thin-layer plates were from Brinkmann Instruments. Bio-Gel P-6 was purchased from Bio-Rad Laboratory. Polynucleotide ligase from *E. coli* was the gift of Dr. J. Lebowitz of the University of Alabama, and polynucleotide kinase from bacteriophage T4-infected *E. coli* was kindly donated by Dr. C. C. Richardson of the Harvard Medical School.

Preparation of λ DNA. The procedures for the radioactive labeling and purification of phage λ from a λ ($C_{I857}S_7$) lysogen of *E. coli* and the isolation of the phage DNA have been previously described (Hedgpeth et al., 1972).

NCS Reaction Condition. Unless otherwise specified, the reaction volume of 50 μl contained 10 mM Tris (pH 7.5), 10 mM 2-mercaptoethanol, 1 μg of λ [^{3}H]DNA (specific activity at 5–50 × 10^3 cpm/μg) and various concentrations of drug. The mixture was incubated for 30 min at 37 °C.

Gradient Analysis. The extent of cutting of DNA was

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[1] Abbreviations used are: PEI, polyethylenimine; NCS, neocarzinostatin; DNase, deoxyribonuclease; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

usually determined by analysis on either alkaline (0.7 M NaCl, 0.3 M NaOH, 0.01 M EDTA) or neutral (0.7 M NaCl, 0.01 M EDTA, 0.01 M Tris, pH 7.6) sucrose gradients (5–20%), spun in a SW 50.1 rotor for 2 h at 20 °C in a Beckman L2-65B ultracentrifuge. At the end of the run, 50% sucrose was pumped through the bottom of the tube and the gradient was collected from the top at five drops per fraction. For neutral gradients, 1 ml of water was added to each fraction prior to the addition of 10 ml of Bray's solution for liquid scintillation counting. For alkaline gradients, 1 ml of 0.06 N HCl was used to neutralize the base, as well as to dilute the sucrose before Bray's solution was added.

Phosphatase-Kinase Assay. The method is basically that of Weiss et al. (1968). At the end of the incubation of λ [^{3}H]DNA with NCS as described, 6 μl of 1 N NaOH was added for 10 min to denature the DNA and inactivate the antibiotic. The mixture was then adjusted to pH 8.0 with 6 μl of 1.1 N HCl–0.2 M Tris. Alkaline phosphatase (0.2 μg) was added and the reaction was further incubated for 30 min at 37 °C. Following incubation, 60 μl of the following mixture was added: 0.02 M $MgCl_2$, 0.02 M 2-mercaptoethanol, 3 mM potassium phosphate (pH 7.0), 1–2 μCi of [γ-^{32}P]ATP (specific activity at 20–50 Ci/mmol), and 1 to 2 units of polynucleotide kinase. The second incubation was carried out at 37 °C for 30 min. Cold 5% trichloroacetic acid was added, and the mixture was allowed to stand in ice for 10 min before the precipitated DNA was filtered on a GF/C glass-fiber filter, which was then dried and counted in a toluene–PPO–POPOP scintillation cocktail.

Analysis of 5′-Mononucleotides. The GF/C glass-fiber filters, containing labeled λ DNA as described in the previous section, were removed from the scintillation cocktail, washed twice with toluene for 10 min each, and then rinsed in 95% ethanol for an additional 10 min before drying in air. The extraction of the 5′-^{32}P labeled DNA fragments, their enzymatic conversion to mononucleotides by the combined action of DNase I and snake venom phosphodiesterase, and the separation and identification of the [5′-^{32}P]mononucleotides have been described by Kelly and Smith (1970).

Protection Experiments. One microgram of λ [^{3}H]DNA was reacted with 5 μg/ml of NCS and 10 mM 2-mercaptoethanol in the presence of various concentrations of the heterologous DNAs. The analysis of the extent of cutting of labeled λ DNA was done by alkaline sucrose gradient centrifugations. The percent protection is calculated by the following equation:

$$\% \text{ protection} = \left(\frac{N - N_P}{N}\right) \times 100\%$$

where N = number of breaks per strand of λ DNA and N_P = number of breaks per strand of λ DNA in the presence of heterologous DNA. The size of the synthetic polynucleotide ranged from 2.5 to 15 S. In experiments involving annealing of synthetic DNA–DNA and DNA–RNA hybrids, an equimolar amount of each polynucleotide was preincubated in 0.1 M NaCl for 10 min at 37 °C before NCS and λ DNA were added.

Results

Single-Stranded vs. Double-Stranded Breaks. It has been reported previously that NCS makes single-stranded breaks in superhelical SV-40 DNA in vitro in the presence of 2-mercaptoethanol (Beerman and Goldberg, 1974). We have performed similar experiments using the double-stranded, linear DNA from bacteriophage λ. The products of NCS digestion

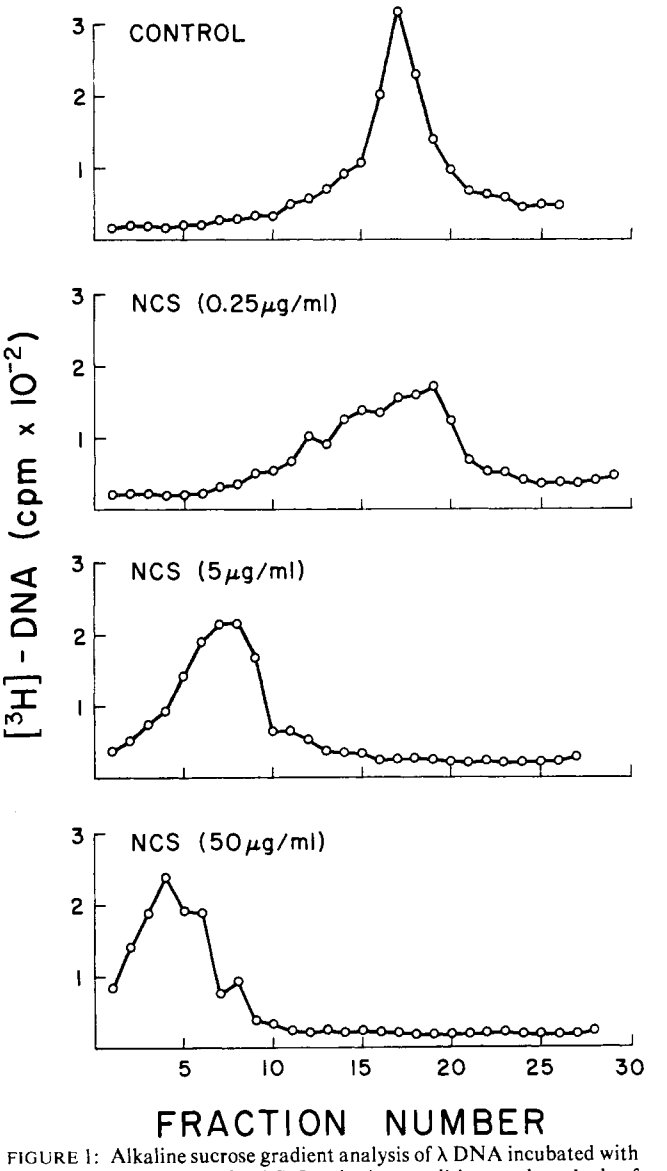

FIGURE 1: Alkaline sucrose gradient analysis of λ DNA incubated with varying concentrations of NCS. Incubation conditions and methods of analysis are as described under Experimental Procedures. Sedimentation is from left to right.

were subjected to centrifugation in sucrose gradients under both alkaline and neutral conditions to determine whether single- or double-stranded breaks were induced by the antibiotic. Figures 1 and 2 show the sedimentation profile of DNA after incubation with various concentrations of NCS. With as low as 0.25 μg/ml of NCS, there is an unmistakable shift in sedimentation of the DNA on alkaline sucrose gradients (Figure 1). The DNA fragments decrease in sedimentation rate as a function of increasing antibiotic concentration. On the other hand, on neutral sucrose gradients (Figure 2), the breakage of DNA is only discernable at the higher drug concentrations. The breaks that are found under neutral gradient conditions have likely been caused by the placement of single-stranded breaks in the same vicinity (within a few base pairs of one another) on the opposite strands of the DNA, thereby destabilizing the short double-stranded region in-between the two nicks. The breaks, however, are not alkali induced, since heat denaturation or treatment with Me_2SO of the DNA after reaction with NCS also results in strand breakage. An attempt was made to estimate the fragment size of the reaction products by determination of their sedimen-

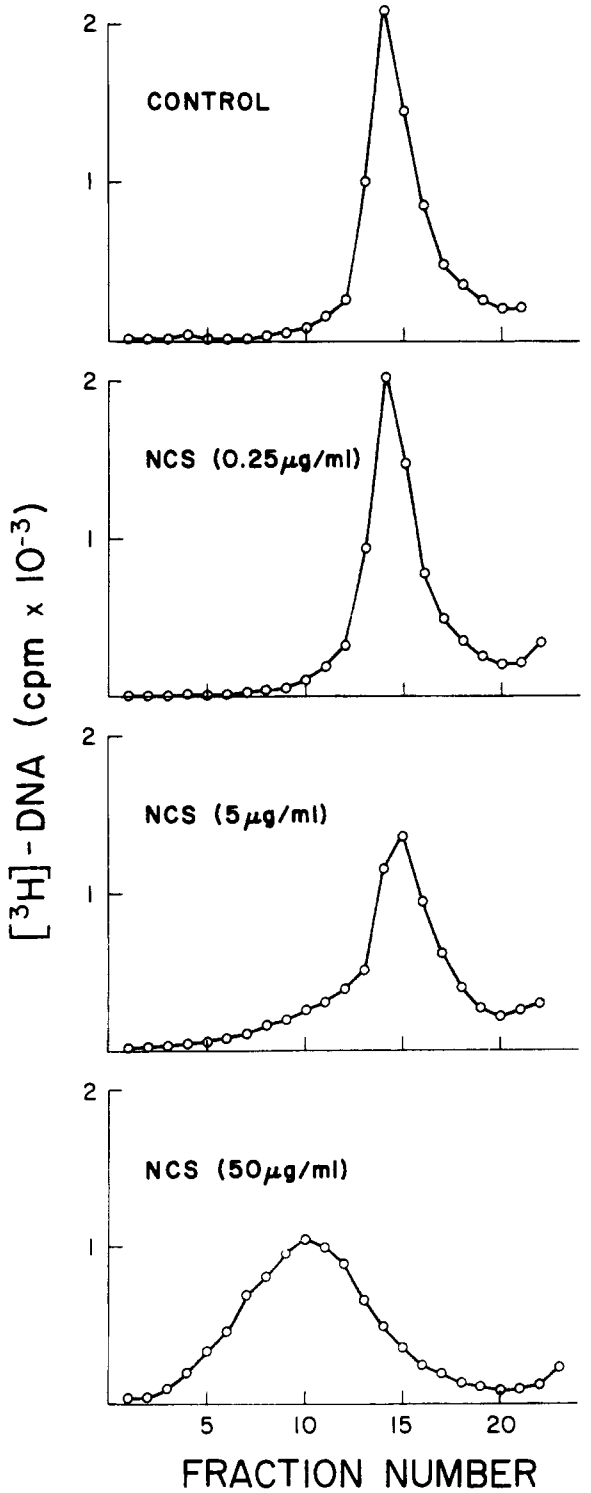

FIGURE 2: Neutral sucrose gradient analysis of λ DNA incubated with varying concentrations of NCS. Incubation conditions and methods of analysis are as described under Experimental Procedure. Sedimentation is from left to right.

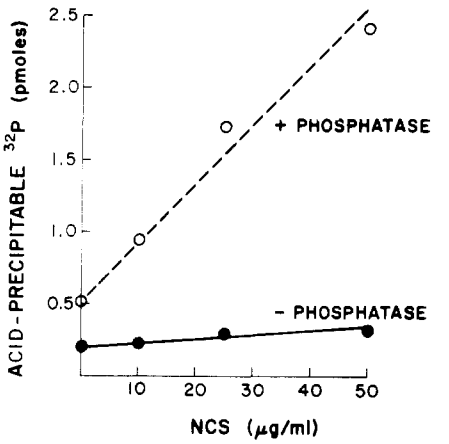

FIGURE 3: Position of the phosphate group at the site of NCS cleavage. λ DNA was treated with various concentrations of NCS and the samples were divided into two equal aliquots, one of which was then treated with alkaline phosphatase. Both sets were subsequently incubated with polynucleotide kinase and [γ-^{32}P]ATP, and the trichloroacetic acid precipitable radioactivity was determined at the end of the reaction, as described under Experimental Procedure.

TABLE I: Determination of the Number of Breaks by the End-Labeling Method.[a]

	Experiment		
	1	2	3
Amount of NCS (μg/ml)	10	25	50
^{32}P incorp into DNA (pmol)[b]	0.414	1.195	1.888
DNA added (nmol)	3	3	3
Av chain length	7386	2558	1619
No. of breaks/strand[c]	6.5	19	30

[a] The data for these calculations are from Figure 3. [b] These values have been corrected for the blank in which NCS has been omitted. [c] Each strand of λ DNA consists of 4.8×10^4 nucleotides.

tation relative to that of intact λ DNA under alkaline gradient conditions. Using the equation of Abelson and Thomas (1966), it was calculated that at 50 μg/ml of NCS about 35–40 breaks on the average were introduced into each strand of the λ DNA molecule.

Generation of 5′-Phosphoryl Ends. In order to elucidate the chemical structure of the site after cleavage, we first determined the position of the phosphate group after the NCS reaction. It is well established that polynucleotide kinase from bacteriophage T4-infected *E. coli* can only phosphorylate the 5′-hydroxyl termini of nucleic acids (Richardson, 1965). Thus, an experiment was designed to test whether the DNA fragments resulting from NCS cleavage can be phosphorylated with [γ-^{32}P]ATP in the absence of prior treatment with alkaline phosphatase (Figure 3). It can be seen that the removal of the phosphate group is a necessary prerequisite for the subsequent phosphorylation of the DNA fragments. This result demonstrates that the DNA fragments generated by NCS cleavage bear 5′-phosphoryl ends.

Quantitation of the Number of Breaks. Effect of Drug Concentration, pH, and Length of Incubation. The data in Figure 3 also show that the strand-breaking action of NCS is linear with increasing drug concentration. Based on the known specific activities of the [γ-^{32}P]ATP and the λ [^{3}H]DNA, the number of moles of phosphate enzymatically attached to the fragmented λ DNA can be calculated and, thus, the fragment size and, consequently, the number of breaks induced by NCS can be determined (Table I). By the nature of the method used for these determinations, the number of breaks calculated are minimum estimations, since both enzymes involved in the assay, alkaline phosphatase and polynucleotide kinase, would have to be 100% efficient for the estimated value to be equivalent to the actual number of breaks. The minimum value of 30 breaks thus obtained is consistent with the average value

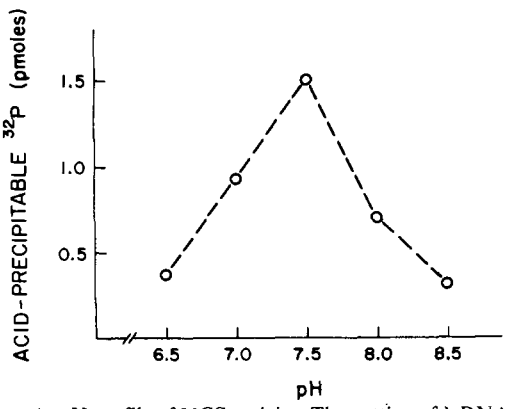

FIGURE 4: pH profile of NCS activity. The cutting of λ DNA was performed with 20 μg/ml of NCS at the indicated pHs, and the extent of strand breakage was measured by determining the amount of acid-precipitable radioactivity as in Figure 3.

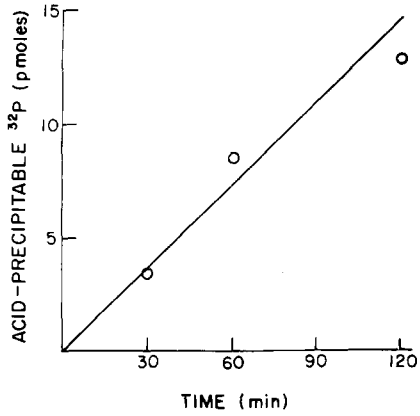

FIGURE 5: Time course of NCS activity. The cutting of λ DNA was performed with 100 μg/ml of NCS at 24 °C for various lengths of time. The extent of strand breakage was measured by determining the amount of acid-precipitable radioactivity as in Figure 3.

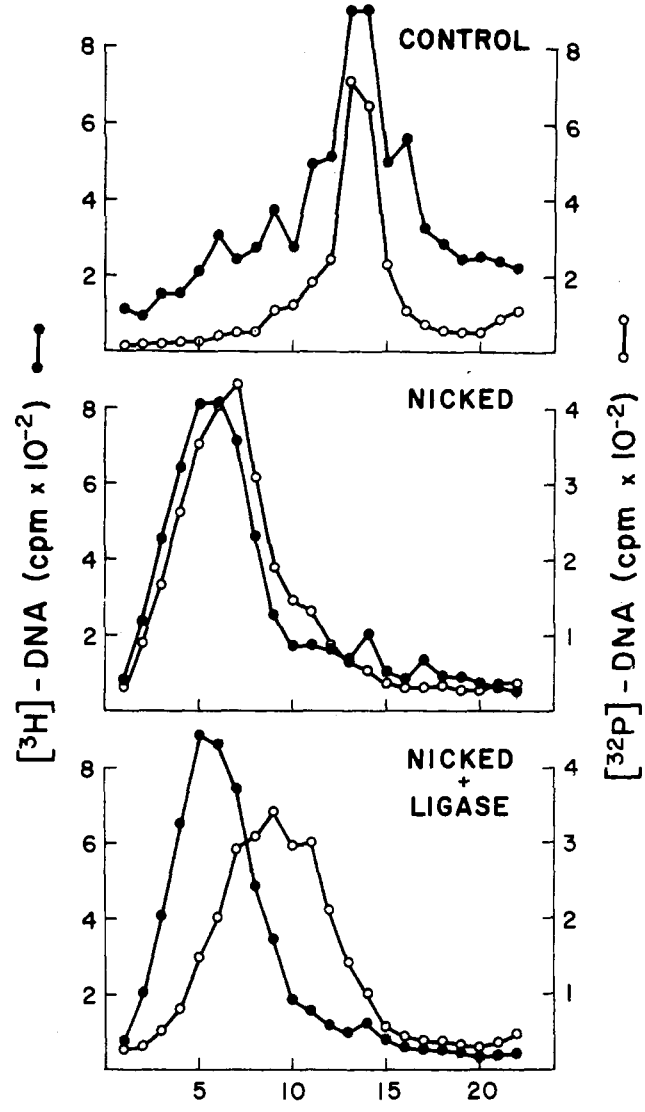

FIGURE 6: Repairability of NCS-induced lesions by polynucleotide ligase. Ten micrograms of λ [^{3}H]DNA was nicked with 4 μg/ml of NCS in 500-μl volume under standard conditions. Ten micrograms of λ [^{32}P]DNA was incubated with 0.2 ng of DNase I in 500 μl volume containing 0.01 M Tris (pH 7.5) and 5 mM $MgCl_2$. The reactions were kept at 30 °C for 30 min. The mixtures were extracted twice with equal volumes of neutralized phenol and the aqueous phases were then extensively dialyzed against 0.01 M Tris (pH 8.0), 0.1 mM EDTA. Equimolar amounts of nicked ^{3}H- and ^{32}P-labeled DNAs were mixed and incubated with polynucleotide ligase under the conditions described by Olivera and Lehman (1967a,b). The analysis of the size of DNAs at the various stages were performed as described under Experimental Procedure, except that the fractions collected from the gradients were precipitated with 5% trichloroacetic acid and filtered on GF/C glass-fiber filters. Sedimentation is from left to right. Calculations reveal that NCS produced seven breaks per strand and none were repaired by polynucleotide ligase; DNase I produced six breaks per strand and four were repaired by the enzymes.

of 35–40 breaks calculated from alkaline sucrose gradient analysis at the 50 μg/ml drug dosage.

With the availability of the end-labeling technique as a quantitative assay for NCS activity, one can easily characterize certain parameters of the drug–DNA reaction. Figure 4 shows the hydrogen ion dependency of NCS activity in vitro. Deviation of one pH unit from the optimal pH of about 7.5 reduces the activity by over 70%. As a function of time, the cutting activity appears to be linear for at least up to 2 h (Figure 5). Therefore, there does not seem to be an appreciable amount of inactivation of the protein antibiotic under the incubation conditions.

Nonrepairability of the Break by Polynucleotide Ligase. So far, it has been established that, after NCS treatment, the double-stranded λ DNA molecule remains intact (at least when incubated with lower concentrations of drug) and that the phosphoryl groups are at the 5′ ends. If the phosphodiester bond is the only site of damage, then the lesion should be repairable by *E. coli* polynucleotide ligase (Gellert, 1967; Olivera and Lehman, 1967a). DNase I treated DNA, which is known to have single-stranded nicks and to possess 5′-phosphoryl and 3′-hydroxyl termini, and, therefore, to be a substrate for the repair enzyme, was used as a control. ^{3}H- and ^{32}P-labeled λ DNA were separately incubated with 4 μg/ml of NCS and an amount of DNase I previously determined to produce an equivalent number of single-stranded breaks, respectively. The nicked DNAs were purified and then mixed in equimolar amounts for the reaction with polynucleotide ligase. Alkaline sucrose gradient analyses of the nicked DNAs before and after resealing with polynucleotide ligase are shown in Figure 6. It can be seen that, under the conditions used, the DNA fragments resulting from drug or nuclease treatment are similar in size, about 6–7 breaks per original strand of λ DNA (as determined by the method of Abelson and Thomas, 1966). After reaction with polynucleotide ligase, the sedimentation

TABLE II: Identification of the Mononucleotides at the 5′ Ends of the DNA Fragments Resulting from NCS Cleavage.[a]

Deoxyribonucleoside Monophosphate Examined	Total Radioactivity for 10 μ/ml of NCS		50 μg/ml of NCS	
	cpm	%	cmp	%
dGMP	101	36	421	40
dTMP	90	32	351	34
dCMP	47	17	162	15
dAMP	44	16	112	11

[a] The details are described under Experimental Procedure. The radioactivities of the mononucleotide at the 5′ ends of λ DNA which had not been reacted with NCS were subtracted to give the above values.

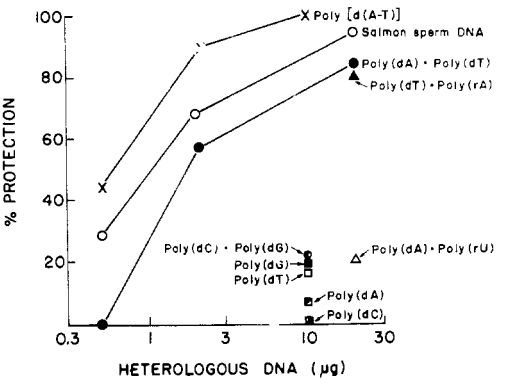

FIGURE 7: Protection of λ [^{3}H]DNA from NCS-cutting by natural and synthetic DNAs. Reaction conditions are described in Experimental Procedure. The amount of protecting DNA in 50 μl of reaction was varied as indicated. Each reaction contained 1 μg of λ [^{3}H]DNA.

of the NCS-treated DNA fragments remained unchanged, indicating that the drug-induced break is not a substrate for the polynucleotide ligase. The nuclease-treated DNA, however, has a faster sedimentation rate after reaction with polynucleotide ligase. Calculations reveal that about two-thirds of the breaks caused by DNase I have been repaired.

Base Specificity at the Site of Strand Breakage. A simple and rapid way of determining whether base sequence specificity exists at the site of cutting is to examine the mononucleotides at the 5′ ends of the DNA fragments resulting from NCS cleavage. After labeling the 5′ termini with ^{32}P by the combined use of alkaline phosphatase–polynucleotide kinase, the DNA was digested with DNase I and snake venom phosphodiesterase, and the resultant mixture of mononucleotides was separated by PEI-cellulose thin-layer chromatography (Randerath and Randerath, 1967). The result is shown in Table II. Although all four mononucleotides are found to contain ^{32}P, the majority of the radioactivity (approximately 70%) is recovered in 5′-TMP and 5′-dGMP.

Role of Base Sequence and Secondary Structure of the DNA in Determining Drug Interaction. To determine whether NCS recognizes a specific base sequence(s) in the DNA for purposes of initial binding and/or cutting, various DNAs, synthetic or natural, differing in base composition and/or sequence were tested for their abilities to protect the radioactive λ DNA from being cut at limiting drug concentrations. The underlying rationale is that the DNA containing more of the preferred base sequence(s) would provide the best protection. It is appreciated that such studies do not distinguish base sequence specificity for initial binding from that at the point of strand breakage, should they be separate and different.

In these studies, the amount of cutting of λ [^{3}H]DNA was assayed by alkaline sucrose gradients and the degree of protection offered by a particular DNA was calculated as described under Experimental Procedure. As can be seen in Figure 7, salmon sperm DNA, which has a base composition similar to that of λ DNA (Felix et al., 1956; Meyer et al., 1961), protects on an equimolar basis (1 μg provides 50% protection). The alternating copolymer poly[d(A-T)] affords greater protection (0.6 μg provides 50% protection) than salmon sperm DNA, whereas the equimolar mixture of homopolymers poly(dA)·poly(dT) is a less preferred substrate (1.8 μg provides 50% protection). In experiments where two different size classes of poly(dT) (2.5 and 10 S) were annealed to poly(dA) (10 S), equivalent degrees of protection were found. However, poly(dC)·poly(dG) (and also the alternating copolymer poly[d(G-C)], data not shown), even at a concentration tenfold higher than the λ DNA, gave virtually no protection. The same is true for all four of the homodeoxypolymers. To differentiate the relative importance of deoxyadenylic and thymidylic acids in the substrate recognition process, we have tested synthetic DNA–RNA hybrids, such as poly(dT)·poly(rA) and poly(dA)·poly(rU) for their potencies in the protection assay. Figure 7 shows that the presence of thymidylic acid is critical, whereas riboadenylic acid can substitute for deoxyadenylic acid with retention of protecting activity. Nevertheless, since poly(dT) alone does not protect at all, it appears that the presence of thymidylic acid is necessary but not sufficient and that the secondary structure, such as exists in double-stranded DNA, is probably also required. This notion finds support in the observation (data not shown) that the random copolymer poly[d(A,T)], which lacks ordered secondary structure, does not protect against λ DNA cutting by NCS. It should be noted that the "single-stranded" DNAs from bacteriophages φX174 and fd do get cut by NCS (Beerman et al., 1976), but recent evidence suggests that these DNAs contain highly ordered base-paired regions (Blakesley and Wells, 1975). We have also found that heat-denatured salmon sperm DNA protects much less effectively than native DNA.

It is known that synthetic DNAs have unusual secondary structures (Chamberlain, 1965); therefore, it appeared warranted to verify the above results with natural DNAs with differing base compositions. Accordingly, we have used *Micrococcus luteus* and *Clostridium perfringens* DNAs, which have low- and high-dAT contents, respectively. It is clear from a series of protection experiments that the degree of protection of λ DNA from NCS cutting is a linear function of the thymidylic–deoxyadenylic acid content of the various DNAs, except, again, for the synthetic poly(dA)·poly(dT) (Figure 8). It should be noted that the alternating copolymer poly[d(A-T)] not only protects against λ DNA cutting, but can itself be cut (data not shown).

Formation of Acid-Soluble Material and Release of Thymine. Recently, it was shown that NCS at very high levels degrades DNA to some acid-soluble products, and the release of thymine was detected (Ishida and Takahashi, 1976). We have also monitored for base release in our system and have attempted to correlate the amount of base released with the number of strand breaks. Figure 9 shows the release of acid-soluble radioactivity as a function of NCS concentration, [^{3}H]thymidine-labeled λ DNA as the substrate. The acid-soluble material produced by 250 μg/ml of NCS (5% of the

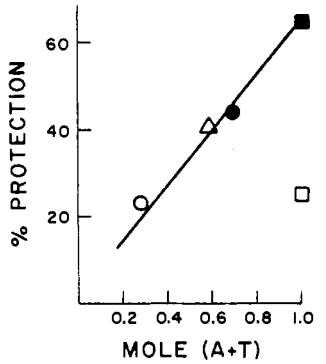

FIGURE 8: Relationship between protection and mole d(A + T) composition of the DNA. Conditions were the same as in Figure 7 except that 1 μg of each heterologous DNAs was used for protection. ○, *M. luteus* DNA; ●, *C. perfringens* DNA; △, salmon sperm DNA; ■, poly[d(A-T)]; □, poly(dA)·poly(dT).

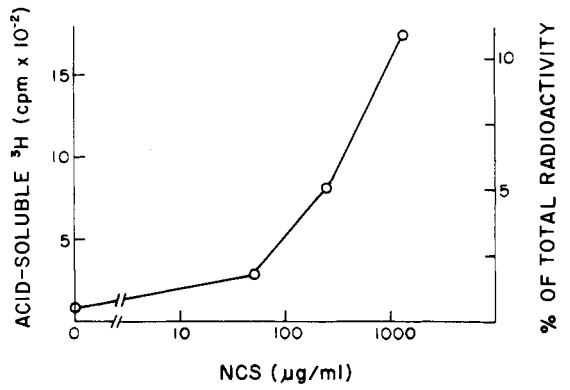

FIGURE 9: Release of acid-soluble radioactivity by NCS. [^{3}H]Thymidine-labeled λ DNA was incubated with various concentrations of NCS under standard conditions. Fifty microliters of 0.34 mg/ml of salmon sperm DNA and 400 μl of cold 5% trichloroacetic acid were added. After 10 min at 4 °C, the samples were centrifuged at 5000 rpm for 15 min. Aliquots of the supernatant fraction were counted in Bray's solution.

total radioactivity) was neutralized and passed through a Bio-Gel P-6 column with nonradioactive thymine as marker (Figure 10). Calculations show that 33% of the acid-soluble radioactivity and, therefore, 1.7% (based on the data in Figure 9) of the total λ DNA radioactivity cochromatographed with the thymine marker. In this system, thymine can be distinguished from thymidine and thymidylic acid. Since the λ DNA is radioactive only in the thymine moiety, 1.7% of radioactivity would correspond to about 0.4% of the total bases. If every thymine released is equivalent to a strand break, this would correspond to slightly less than 200 breaks/strand of λ DNA at the 250 μg/ml drug level. Extrapolating from data derived from Figure 1, which showed that at 50 μg/ml the drug induced 35–40 breaks/strand of λ DNA, one can compute that at 250 μg/ml of NCS, approximately 175–200 breaks/strand would result. This value is in close agreement with the number obtained from thymine release, which was based on the assumption that every thymine released corresponds to a break in the phosphodiester linkage.

We have performed similar experiments using [^{3}H]deoxyadenosine-labeled λ DNA in an effort to detect adenine release by NCS. From the chromatographic position on Bio-Gel P-6 column and PEI-cellulose thin-layer plates, we have identified adenine in the acid-soluble material. However, the amount of adenine released is only about 15% of that of thymine at a comparable antibiotic dosage.

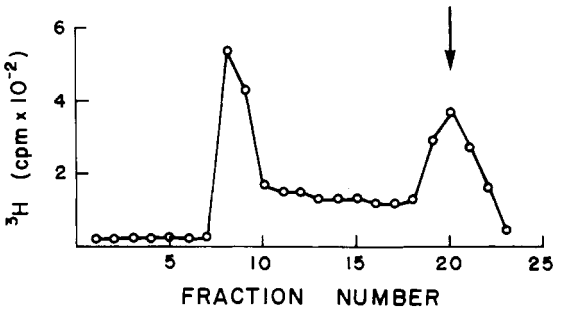

FIGURE 10: Thymine release by NCS. The supernatant fraction obtained in Figure 9 at 250 μg/ml of NCS was neutralized with 1 N NaOH. The sample was dried down in a 45 °C water bath under a stream of N_2, and resuspended in 50 μl of water containing 50 μg of thymine and 5% sucrose. The material was chromatographed on a Bio-Gel P-6 column (0.6 × 20 cm) and 300-μl fractions were collected. Absorbance at 260 nm and radioactivity were determined. Arrow indicates position of elution of the thymine marker.

Discussion

We have shown that NCS creates single-stranded interruptions in the λ DNA molecule, and that the newly created ends bear 5′-phosphates. *E. coli* polynucleotide ligase is unable to repair these nicks, indicating that the reaction involves more than a simple splitting of the phosphodiester bond. Since the mononucleotides at the 5′ ends were recovered undamaged (Table II), it is likely that damage had taken place at the 3′ end of the break to account for the inability of the polynucleotide ligase to seal the break. Whether this is solely a consequence of base release (usually thymine) at this position or whether other changes, such as modification of the sugar moiety or the presence of a phosphate on the 3′ position, also occur remains to be determined. This proposal is supported by the finding that the sites generated by NCS-induced strand scission do not act as primer for *E. coli* DNA polymerase I, although the enzyme does bind to the nick (Kappen and Goldberg, 1977). That base release may be accompanied by β-elimination reactions producing 3′- as well as 5′-phosphate ended polynucleotide fragments is suggested by preliminary experiments in which the activity of the NCS-treated DNA for DNA polymerase I was markedly increased by prior treatment with nuclease-free alkaline phosphatase.

An initial attempt to determine possible sequence specificity of the DNA-cutting reaction by identifying the 5′-mononucleotide at the nicked site showed the presence of all four bases. However, the reproducibility of the recovery of similar proportions of the mononucleotides from different experiments suggests that NCS may not be cutting the nucleic acid on a random basis. The reproducibility of the labeling pattern at the 5′ end may reflect the existence of a specific sequence at the 3′ end for recognition of the antibiotic. Such a possibility is further supported by the fact that the observed labeling pattern differs from the nearest-neighbor analysis for thymidylic acid in λ DNA (Subak-Sharpe et al., 1966). It is of interest that the glycopeptide antibiotic bleomycin also cleaves DNA in the presence of a reducing compound and has a much greater but not absolute preference for cleavage on the 3′ side of TpGpT in lactose operator DNA (Gilbert, W., personal communication). An analogous situation may apply to NCS. In fact, preliminary electrophoretic analysis on polyacrylamide-agarose gels showed that, with bacteriophage fd DNA as the substrate, NCS produces specific bands at a low drug concentration, but the bands become smeared at higher anti-

biotic concentrations where less preferred sites may be cleaved as well.

In preliminary data published earlier (Beerman et al., 1976), it was suggested that NCS made only a limited number of breaks (about 30/strand) in the λ DNA molecule. However, subsequent experiments with other batches of NCS have revealed that strand breakage can be much more extensive (e.g., in Figure 5, 100 μg/ml of NCS produces 13 pmol of acid-precipitable ^{32}P or almost 190 breaks/strand in 2 h). Low activity and perhaps instability of some batches of NCS may account for the previous results. This may also account for initial failure to detect thymine release.

The other approach used to elicit a possible base sequence preference by NCS was the testing of various synthetic and natural DNAs for their abilities to protect radioactive λ DNA from being cut. The conclusion from this series of experiments was that the extent of strand scissions induce by NCS was a function of the mole ratio of deoxyadenylic and thymidylic acids in the DNA. Although thymidylic acid seems to be more essential, it alone is not sufficient in providing protection. Both base sequence and secondary structural considerations are clearly involved. The basis of the requirement for thymidylic acid in the DNA became obvious when it was demonstrated that the antibiotic also caused the release of thymine. The amount of thymine release correlated quantitatively with the number of strand scissions, although their functional relationship remains to be established. The small amount of adenine release may appear to be in conflict with the protection data. However, under conditions of a high ratio of DNA to antibiotic (as in the case of the protection experiments), the antibiotic may interact only with the preferred sequence, whereas under conditions of limiting DNA and excess NCS (in the base-release experiments), the preferred, as well as less preferred, bases are released. The much smaller release of adenine, which by itself cannot account for the number of strand breaks, indicates that deoxyadenylic acid may be at a site of breakage which is less preferred by the antibiotic.

The functionally similar glycopeptide antibiotic bleomycin has been shown to release only thymine, and base release precedes DNA strand scission (Muller et al., 1972). However, Haidle et al. (1972) reported the appearance of all four bases after reaction of DNA with very high concentrations of bleomycin (mg/ml). Protection experiments similar to those presented in Figure 7 were performed with bleomycin using SV-40 DNA as the radioactive substrate (Asakura et al., 1975). It was found that both poly[d(G-C)] and poly[d(A-T)] provided protection, whereas transfer RNA poly(dA) and poly(dT) did not. While there are certain similarities between the functional properties of these two antibiotics, there are also distinct differences, such as optimal reaction pH and temperature, activation by oxidizing agents, inhibition of DNA polymerase, etc. (Umezawa, 1974).

Certain aspects of NCS action resemble that of the restriction-modification enzyme from *E. coli* B, which makes a limited number of breaks on unmodified DNA (Linn et al., 1974). The restricted fragments bear 5′-phosphoryl (and 3′-hydroxyl) termini, and the nicks cannot be sealed by polynucleotide ligase. The site of cleavage in the DNA has been shown to be separate from the recognition (modification) site. The nuclease activity of the enzyme appears to be stoichiometric rather than catalytic. To date, we have been unable to obtain quantitative data implicating a catalytic mechanism in the in vitro cutting of DNA by NCS, although it is possible that a small "activated" fraction of the antibiotic, not yet identified, is responsible for the strand scission. It is tempting to speculate that the DNA-nicking activity of the antibiotic protein may have been involved in the genetic recombination of the host organism at some point in its evolutionary history.

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References

Abelson, J. N., and Thomas, C. A. (1966), *J. Mol. Biol. 18,* 262.

Asakura, H., Hori, M., and Umezawa, H. (1975), *J. Antibiot. 28,* 537.

Beerman, T. A., and Goldberg, I. H. (1974), *Biochem. Biophys. Res. Commun. 59,* 1254.

Beerman, T. A., Goldberg, I. H., Kappen, L. S., Poon, R., and Suzuki, H. (1976), *Adv. Enzyme Regul. 14,* 207.

Beerman, T. A., Goldberg, I. H., and Poon, R. (1974), *Excerpta Med. Int. Congr. Ser., No. 353, 5,* 347.

Beerman, T. A., Poon, R., and Goldberg, I. H. (1977), *Biochim. Biophys. Acta* (in press).

Blakesley, R. W., and Wells, R. D. (1975), *Nature (London) 257,* 421.

Chamberlain, M. J. (1965), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24,* 1446.

Ebina, T., Ohtsuki, K., Seto, M., and Ishida, N. (1975), *Eur. J. Cancer 11,* 155.

Felix, K., Jieke, I., and Zahn, R. K. (1956), *Hoppe-Seyler's Z. Physiol. Chem. 303,* 140.

Gellert, M. (1967), *Proc. Natl. Acad. Sci. U.S.A. 57,* 148.

Haidle, C. W., Weiss, K. K., and Kuo, M. T. (1972), *Mol. Pharmacol. 8,* 531.

Hedgpeth, J., Goodman, H. M., and Boyer, H. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3448.

Homma, M., Koida, T., Saito-Koida, T., Kamo, I., Seto, M., Kumagai, K., and Ishida, N. (1970), *Progr. Antimicrob. Anticancer Chemother., Proc. Int. Congr. Chemother., 6th, 1969 2,* 410.

Ishida, N., Miyazaki, K., Kumagai, K., and Rikimaru, M. (1965), *J. Antibiot. 18,* 68.

Ishida, R., and Takahashi, T. (1976), *Biochem. Biophys. Res. Commun. 68,* 256.

Kappen, L. S., and Goldberg, I. H. (1977), *Biochemistry 15* (preceding paper in this issue).

Kelly, T. J., and Smith, H. O. (1970), *J. Mol. Biol. 51,* 393.

Linn, S., Lautenberger, J. A., Eskin, B., and Lackey, D. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1128.

Meienhofer, J., Maeda, H., Glaser, C. B., Czombos, J., and Kuromizu, K. (1972), *Science 178,* 875.

Meyer, F., Mackel, R. P., Tao, M., and Evans, E. A. (1961), *J. Biol. Chem. 236,* 1141.

Muller, W. E. G., Yamazaki, Z., Breter, H. J., and Zahn, R. K. (1972), *Eur. J. Biochem. 31,* 518.

Ohtsuki, K., and Ishida, N. (1975), *J. Antibiot. 28,* 143.

Olivera, B. M., and Lehman, I. R. (1967a), *Proc. Natl. Acad. Sci. U.S.A. 57,* 1426.

Olivera, B. M., and Lehman, I. R. (1967b), *Proc. Natl. Acad. Sci. U.S.A. 57,* 1700.

Ono, Y., Watanabe, Y., and Ishida, N. (1966), *Biochim. Biophys. Acta 119,* 46.

Randerath, K., and Randerath, E. (1967), *Methods Enzymol.*

12, 323.
Richardson, C. C. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 158.
Samy, T. S. A., Hu, J.-M., Meienhofer, J., Lazarus, H., and Johnson, R. K. (1977), *J. Natl. Cancer Inst.* (in press).
Samy, T. S. A., and Meienhofer, J. (1974), *Proc. Int. Workshop on Horm. Proteins, 1973,* 143.
Subak-Sharpe, H., Burk, R. R., Crawford, L. V., Morrison, J. M., Hay, J., and Keir, H. M. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31,* 737.
Umezawa, H. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 2296.
Weiss, B., Live, T. R., and Richardson, C. C. (1968), *J. Biol. Chem. 243,* 4530.

Synthesis and Template-Directed Polymerization of Adenylyl(3′–5′)adenosine Cyclic 2′,3′-Phosphate†

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ABSTRACT: Adenylyl(3′–5′)adenosine cyclic 2′,3′-phosphate (A-A>p) was synthesized and its polymerization was attempted under various conditions in the presence of poly(uridylic acid) and 1,3-propanediamine. Reaction at −20 °C for 16 days gave polymerized products (up to the 8-mer) in 15% yield and was proved to be dependent on the template. Reaction at 0 °C for 16 days gave more extensive (up to the 10-mer) and more efficient (35%) polymerization. The newly formed phosphodiester linkage was exclusively 2′–5′. These results are discussed in comparison with the monomer-condensation reaction.

It is very difficult to synthesize a long oligonucleotide with chain length higher than 10 by purely chemical methods. This is a particularly serious problem in the case of ribooligonucleotides. A reasonable approach for the preparation of a very long oligonucleotide is to synthesize relatively short oligonucleotides first by purely chemical methods and then to join them by another method. One of the possible ways of accomplishing the second step is to use a complementary template for holding the two reacting termini of the short oligonucleotide chains close to each other and join them chemically. This approach was first tried by Naylor and Gilham in 1966. They used poly(A)[1] as a template, oligo(thymidylic acid) with a 5′-terminal phosphate group as a fragment, and a water-soluble carbodiimide as a condensing agent in aqueous solution and obtained the dimerized product, $p(dT)_{12}$, in 5% yield. Only a few papers have been published on template-directed chemical condensation of oligonucleotides since this paper (Shabarova and Prokofiev, 1970; Uesugi and Ts'o, 1974). Uesugi and Ts'o succeeded in polymerizing oligo(2′-*O*-methylinosinic acid) with a 3′-terminal phosphate group using poly(C) and a water-soluble carbodiimide in practical yields (40–70%) for the first time. On the other hand, Orgel, Lohrmann, and their co-workers have reported detailed studies on condensation and polymerization reactions of mononucleotides in the presence of a complementary polynucleotide template (see the review by Orgel and Lohrmann, 1974). In one of these papers, Renz et al. (1971) reported that adenosine cyclic 2′,3′-phosphate (A>p) could be dimerized in the presence of poly(U) and polybasic amines and the best yield (23%) of condensation was obtained in a frozen solution.

As we have been working on the synthesis of ribooligonucleotides in our laboratory, we are interested in the chemical joining of ribooligonucleotides on appropriate templates. In order to obtain basic data on such systems, we examined the polymerization reaction of di(adenylic acid) with a terminal 2′,3′-cyclic phosphate group (A-A>p) in the presence of poly(U) and 1,3-propanediamine. We wish to report here the results and discuss the difference between the dimer polymerization and the monomer dimerization reaction. After completion of this work, a preliminary report on a template-directed polymerization reaction of hexa(adenylic acid) in a similar system was published by Usher and McHale (1976).

Experimental Procedure

General Methods. Paper chromatography was carried out by the descending technique using Toyo filter paper no. 51A in the following solvent systems: A, 2-propanol–concentrated ammonia–water (7:1:2, v/v); B, 1-propanol–concentrated ammonia–water (55:10:35, v/v); C, ethanol–1 M ammonium acetate (pH 7.5) (7:3, v/v); D, ammonium sulfate (20 g)–0.1 M NaH_2PO_4 (pH 7.0) (100 ml). Paper electrophoresis was carried out on the same paper at 35 V/cm in 0.05 M $Et_3NH_2CO_3$ buffer (pH 7.5). UV absorption spectra were obtained on a Hitachi EPS-3T or Hitachi 124 spectrophotometer. For t_m measurements, a Hitachi 124 spectrophotometer, equipped with a Komatsu Solidate SPD-H-124 thermostated cell, was used. The temperature within the cell was measured by a Shibaura MGB-III thermistor. High-pressure liquid chromatography was carried out on a Varian LCS-1000 system using a column (1 mm × 300 cm) of pellicular anion-exchange resin and gradient elution, 0.01 M KH_2PO_4 (pH 3.55) to 1.0 M KH_2PO_4 (pH 4.0) at 70 °C and 2100–2300 psi. The molar extinction coefficients (ϵ) deter-

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[1] Abbreviations used are: >p, 2′,3′-cyclic phosphate group; RNase M, ribonuclease M (*Aspergillus saitoi,* EC 2.7.7.17); Ap-urea, *N*-(adenylyl)-*N,N′*-dicyclohexylurea; Bz, benzoyl; A.A, A(2′–5′)A; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); DEAE, diethylaminoethyl; UV, ultraviolet.