a neutral compound, both the sphingosine base and fatty acid are ionizable, *i.e.*, the hydrogen ion concentration must be added to the equation. Also, the concentration of water was taken as "unit activity" and its concentration was therefore not introduced into the formula employed to calculate the equilibrium constant. The possibility must be considered that only a fraction of the water concentration should be employed in calculating the equilibrium constant. This fraction includes only those water molecules actually participating in the reaction. They are "compartmentalized" at the point of interaction of the enzyme with the substrate micelles. Their concentration might be very small, *i.e.*, of the order of magnitude of the "activity" of the substrate. Introducing this low value into the denominator of the equilibrium equation might raise the constant to a higher value. The standard free energy corresponding to this value might more reasonably fit a reversible reaction.

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Studies on Nuclear Exoribonucleases. III. Isolation and Properties of the Enzyme from Normal and Malignant Tissues of the Mouse*

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ABSTRACT: An exoribonuclease, which degrades single-stranded ribonucleic acid to nucleoside 5′-monophosphates, has been isolated from the nuclei of mouse liver, kidney, embryo, mammary tumor, and Ehrlich ascites tumor. The enzyme is inactivated by heating at 50° and treatment with 3.2 M urea. The enzyme is specific for polyribonucleotides; it will not hydrolyze deoxyribonucleic acid, pTpT, or thymidine 5′-*p*-nitrophenylphosphate. Studies on the mechanism of attack on polyribonucleotides show that: (1) the enzyme (which degrades from the 3′-OH end) catalyzes attack on the 5′-phosphorus atom of the termina nucleotide, with resultant P–O splitting to yield a nucleoside 5′-phosphate; (2) a single polynucleotide molecule stays bound to the enzyme until that polynucleotide molecule is degraded to completion, in a manner similar to the mechanism for degradation of polynucleotides by bacterial polynucleotide phosphorylase and ribonuclease II; and (3) the enzyme is strongly inhibited by the presence of terminal 3′-phosphate or 2′,3′-cyclic phosphate groups on a potential substrate molecule.

The control of nuclear RNA metabolism is one of the central problems in cell biology. It is now apparent that gene function in the nucleus may be regulated by the control of degradation of nuclear RNA (Harris, 1963; Georgiev, 1967; Shearer and McCarthy, 1967; Church and McCarthy, 1967; Soerio *et al.*, 1968; Stewart and Farber, 1968) as well as by control of synthesis of nuclear RNA. It is thus important to define the various nuclear enzymes which degrade RNA and to establish their functional role in the cell.

The present series of papers deals with an exoribonuclease found in a wide variety of cell nuclei; this enzyme degrades single-stranded RNA to nucleoside 5′-monophosphates. Two previous papers in this series (Lazarus and Sporn, 1967; Lazarus *et al.*, 1968) have described the isolation of the enzyme from Ehrlich ascites tumor cell nuclei and a kinetic model for degradation of polynucleotides by the enzyme. The present paper and a following one (H. M. Lazarus and M. B. Sporn, in preparation) will describe the

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isolation and properties of the enzyme from several different types of mouse tissues, as well as detailed studies on the mechanism and kinetics of attack on polynucleotides by the enzyme.

Experimental Procedure

Materials. Sources of reagents were as follows: poly A, poly U, and [^{18}O]H_2O, Miles Laboratories, Elkhart, Ind.; Triton N-101, Rohm and Haas, Philadelphia, Pa.; DEAE-cellulose (microgranular), H. Reeve Angel, Clifton, N. J.; [^{3}H]CTP, Schwarz BioResearch, Orangeburg, N. Y.; *Crotalus adamanteus* venom, Sigma Chemical, St. Louis, Mo.; Micrococcal nuclease, spleen phosphodiesterase, and *Escherichia coli* alkaline phosphatase, Worthington Biochemical, Freehold, N. J.; thymidine 5′-*p*-nitrophenylphosphate and thymidine 3′,5′-diphosphate, Calbiochem, Los Angeles, Calif. The following compounds were generous gifts: the dinucleotide, pTpT (Drs. P. T. Gilham, Purdue University and J. G. Moffatt, Syntex Institute of Molecular Biology); 5-fluorodeoxyuridine 3′,5′-disulfate (Dr. P. Wigler, University of Tennessee); 5-iododeoxycytidine 3′,5′-disulfate and 5-iododeoxyuridine 3′,5′-disulfate (Drs. L. Sciarini and J. Cramer, Yale University). Thymidine 3′,5′-disulfate and uridine 2′,3′,5′-trisulfate were prepared by treatment of the respective nucleosides with pyridine–sulfur trioxide by methods similar to those described by Wigler and Choi (1964) and Chang *et al.* (1967); these compounds were synthesized for the National Cancer Institute by Ash Stevens Inc., Detroit, Mich., under Contract PH 43-66-929. The dinucleotide, pApA, was prepared by degradation of poly A with an endonuclease (similar to the one described by Heppel *et al.*, 1956) from mouse liver nuclei, followed by chromatography of the digest on DEAE-cellulose (Stevens and Hilmoe, 1960).

Isolation of Nuclei; Separation of Endoribonuclease and Exoribonuclease Activity in Nuclear Extracts. Nuclei were isolated from Ehrlich ascites tumor cells and mouse liver with the nonionic detergent, Triton N-101, as described previously (Lazarus and Sporn, 1967). The method used for preparation of mouse liver nuclei was also used for preparation of kidney, embryo, and mammary tumor nuclei. Embryos were whole 11–14-day fetuses from C3H/He mice. Mammary tumors were subcutaneous transplants, carried in C3H/He mice, of the spontaneous mammary adenocarcinoma which occurs with high incidence in female C3H/He mice. The tumor is known to be caused by an RNA virus (Lyons and Moore, 1965). Tumors were harvested between 2 and 3 weeks after transplantation and were free of necrosis.

Separation of endoribonuclease from exoribonuclease activity in saline extracts of all five types of nuclei was performed as described previously (Lazarus and Sporn, 1967). Nuclei were extracted with saline; the portion of the saline extracts precipitating between 30 and 50% saturation of ammonium sulfate was saved and then applied to DEAE-cellulose columns which were eluted as shown in Figure 1. The standard nuclease assay

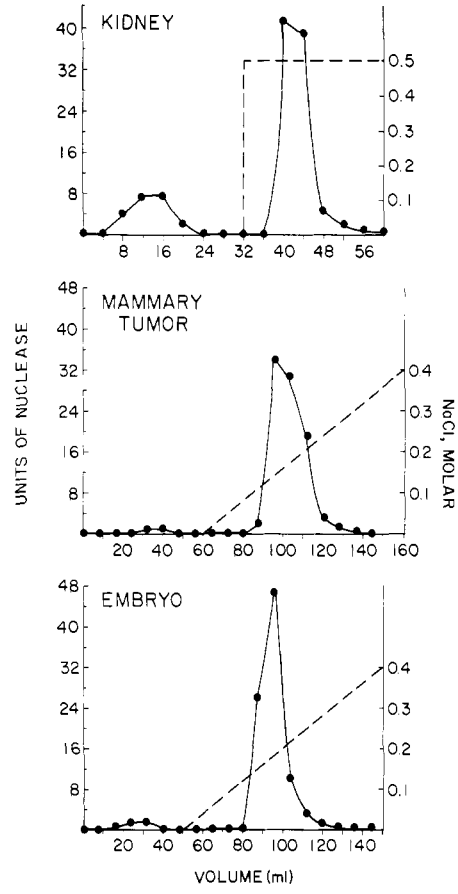

FIGURE 1: Separation, by chromatography on DEAE-cellulose, of endoribonuclease and exoribonuclease activities from different mouse tissues. Nuclear extracts were prepared as described under Methods and applied to DEAE-cellulose columns, which were eluted with 0.05 M Tris-Cl (pH 8.0) containing 0.001 M dithiothreitol, plus a NaCl gradient as shown (in the case of the kidney preparation, stepwise elution with 0.5 M NaCl was begun at the point marked by the dashed line). The amounts of starting material, total units of nuclease applied to the column, and column sizes (diameter × length) for each tissue were as follows: kidney, 40 g, 70 units, 1 × 8 cm; embryo, 40 g, 200 units, 1 × 15 cm; and mammary tumor, 40 g, 800 units, 1 × 15 cm. The units of nuclease recovered from the columns have been normalized to 100%; actual recoveries in all instances were greater than 90%.

(which was used both for whole nuclei and purified enzyme fractions) and the further purification of the exoribonuclease from Ehrlich ascites tumor cell nuclei have been reported previously (Lazarus and Sporn, 1967). A unit of enzyme is defined as the amount which forms 1 μmole of AMP residues/hr from poly A under standard reaction conditions.

Degradation of Poly U in the Presence of [^{18}O]H_2O. The incubation mixture contained the following, in a final volume of 1 ml: poly U, 10 mg (dissolved in 0.5 ml of [^{18}O]H_2O, 93.4 atom %, ^{18}O); Tris-Cl, pH 8.6, 100 μmoles; dialyzed bovine serum albumin, 200 μg; and Ehrlich exoribonuclease, 36 units. Digestion was allowed to proceed for 8 hr at 37°, at which time all poly U had been converted into uridine 5′-phosphate. Then 200 μg of *E. coli* alkaline phosphatase was

TABLE I: Degradation of Polyadenylic Acid by Isolated Nuclei.[a]

Tissue	μmoles of Adenylic Acid Residues Liberated/ 15 min per mg of DNA
Liver	4.3
Kidney	1.2
Embryo	1.3
Mammary tumor	4.2
Ehrlich tumor	3.5

[a] Nuclei were prepared and assayed as described under Methods.

added and the mixture incubated for an additional hr at 37° to convert UMP quantitatively into uridine. Perchloric acid was added at 0° to give a final concentration of 0.2 M, protein was removed by centrifugation, and uridine in the supernatant was adsorbed to charcoal at 0°. The charcoal was washed three times with ice-cold 0.1 M HCl, and the washings were discarded. The uridine was then eluted quantitatively from the charcoal with a 1:1 ethanol–2 M aqueous NH_4OH mixture at 50°, and crystallized from ethanol. Mass spectra were recorded on an AE1 Model MS-9 mass spectrometer, with the sample on a probe. The mass spectrum obtained with commercial reagent grade uridine was essentially similar to that reported by Biemann and McCloskey (1962).

Preparation of RNA Labeled at 3'-OH End and Degradation of this RNA by Exoribonuclease. The procedure used to prepare tRNA labeled at the 3'-OH(-pCpCpA) end was the one described by Weiss (1960), with the following exceptions: 0.05 μmole/ml of [^{3}H]CTP (specific activity 1 mCi/μmole) was used to label the tRNA, and carrier tRNA was not added to the preparation. The labeled tRNA had a specific activity of 4.0×10^5 cpm/mg.

The labeled tRNA was used as a substrate for Ehrlich exoribonuclease, and the rate of conversion of both radioactivity and total polynucleotide into acid-soluble form was measured. The incubation mix (3 ml) was as follows: Tris-Cl, pH 7.4, 0.01 M; potassium phosphate, pH 7.4, 0.012 M; dithiothreitol, 0.0002 M; $MgCl_2$, 0.0005 M; urea, 2.1 M; tRNA, 66 μg/ml; and Ehrlich exoribonuclease, 2 units/ml. The relatively low magnesium concentration and the presence of 2 M urea both lessen the degree of secondary structure of tRNA (Monier and Grunberg-Manago, 1962; Littauer and Daniel, 1962) and make it more susceptible to degradation by exoribonuclease. The reaction mixture was incubated at 37°; at various time intervals, 0.5-ml aliquots were removed and chilled to 0°. Dialyzed bovine serum albumin (2 mg) and ice-cold perchloric acid (final concentration 0.4 M) were then added; the mixture was centrifuged for 30 min at 1900*g*. Both absorbancy at 260 mμ and radioactivity were measured on aliquots of the clear supernatant fluid.

Snake venom digestion of the labeled tRNA was performed to validate that the label on tRNA was in fact terminal; the digestion mixtures were identical with those used with exoribonuclease, except for the substitution of *Crotalus adamanteus* venom (0.1 mg/ml) for exoribonuclease, and the omission of urea.

Other Methods. The solvent systems used for thin-layer chromatography on cellulose were as follows: solvent 1, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent 2, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v). Hydrolysis of thymidine 5'-*p*-nitrophenylphosphate was measured by the method of Razzell (1963). DNA was determined by the method of Burton (1956).

Results

Degradation of Polyadenylic Acid by Isolated Nuclei and Purified Nuclear Extracts. Nuclei purified by Triton N-101 from mouse liver, kidney, embryo, mammary tumor, and Ehrlich ascites tumor all degrade poly A to acid-soluble products, as shown in Table I. The assay of degradation of poly A by whole nuclei measures the combined activities of at least two enzymes, as was shown by salt extraction of the nuclei and DEAE-cellulose chromatography of the nuclear extracts. This procedure was performed on all five types of nuclei; the DEAE-cellulose chromatograms of the extracts from kidney, embryo, and mammary tumor are shown in Figure 1. Chromatograms of liver and Ehrlich ascites tumor extracts have been published previously (Lazarus and Sporn, 1967). In the case of liver and kidney, two major peaks of activity were found; the first peak is easily eluted from the column with 0.05 M Tris-Cl (pH 8.0) (with no additional salt), while the second peak requires stronger salt for elution. In the case of the embryo, mammary tumor, and Ehrlich ascites tumor, there is a barely detectable first peak, while the second peak comprises almost all of the activity in the saline extract.

The first peak in the chromatograms from mouse liver, kidney, and Ehrlich ascites tumor nuclei has been identified as an endonuclease (first described by Heppel *et al.*, 1956) which hydrolyzes poly A to oligonucleotides with 5'-phosphate and 3'-hydroxyl end groups. Poly A was degraded by enzyme from the first peak under standard assay conditions and the digest analyzed by thin-layer chormatography. The products migrate more slowly in solvents 1 and 2 than does 5'-AMP; moreover, the products are resistant to calf spleen phosphodiesterase (indicating the presence of a 5'-phosphate end group), but quantitatively converted by *Crotalus adamanteus* venom to adenosine (indicating the absence of a 3'-phosphate end group; Heppel, 1961).

The second peak in the chromatograms from all five tissues has been identified as an exoribonuclease which produces adenosine 5'-monophosphate from poly A. Analysis of both early and late digestion mixtures by thin-layer chromatography shows that 5'-AMP is the sole product of poly A degradation; at no time are

oligonucleotides, ADP, 3′-AMP, or adenosine seen in digestion mixtures. The ability of the exoribonuclease (purified by chromatography on DEAE-cellulose) from all five tissues to hydrolyze the *p*-nitrophenyl ester of thymidine 5′-phosphate was also measured; this substrate is hydrolyzed very rapidly both by snake venom phosphodiesterase and microsomal phosphodiesterase from liver and kidney (Razzell, 1963). Exoribonuclease from Ehrlich ascites tumor, embryo, and liver had no detectable ability to liberate *p*-nitrophenol from thymidine 5′-*p*-nitrophenylphosphate (less than 1% of activity toward this substrate, as compared with activity toward poly A, would have been detected), while exoribonuclease from mammary tumor and kidney hydrolyzed thymidine 5′-p-nitrophenylphosphate at rates that were 2.5 and 1.6%, respectively, found for hydrolysis of poly A. This small amount of nonspecific phosphodiesterase activity most likely reflects the presence of a trace amount of contaminating enzyme. Thus, the exoribonuclease from all five tissues presently studied strongly resembles the leukemia cell phosphodiesterase studied by Anderson and Heppel (1960) in that it requires a nucleoside residue to be present on each side of the phosphodiester linkage being hydrolyzed. The remainder of this paper will deal with the properties of the exoribonuclease, particularly that from Ehrlich ascites tumor cell nuclei, since the enzyme from this course has been most highly purified and characterized (Lazarus and Sporn, 1967).

Thermal Stability of Exoribonuclease; Effect of Urea on Activity. Preincubation of purified exoribonuclease from Ehrlich ascites tumor, mammary tumor, embryo, and liver at 50° in 0.1 M Tris-Cl (pH 8.0), for 15 min resulted in loss of 20–30% of activity, while heating at 50° for 60 min resulted in loss of 40–60% of activity. The enzyme is thus clearly different from the heat-stable pancreatic endoribonucleases (Anfinsen and White, 1961). Moreover, the Ehrlich and embryo exoribonucleases retain only 60% activity in the presence of 1.6 M urea, 40% activity in the presence of 2.4 M urea, and 25% activity in the presence of 3.2 M urea, in contrast to pancreatic endoribonuclease, which retains full activity in 8 M urea (Anfinsen, 1956).

The stability of all five exoribonucleases upon prolonged storage at low temperature was also measured. Tubes of enzyme, in solution in the same buffer used for purification on DEAE-cellulose (Figure 1), were stored at −70°. More than a year later all five enzyme preparations retained over half of their activity.

Specificity of Exoribonuclease. The selective action of Ehrlich exoribonuclease on single-stranded, nonhelical RNA, as well as the resistance of native and heat-denatured DNA to the enzyme, has been reported previously (Lazarus and Sporn, 1967). The specificity of the enzyme for polyribonucleotides has been confirmed by comparing the degradation of the dinucleotides, pApA and pTpT, by the enzyme. Each of these two dinucleotides (0.6 μmole) were incubated for 23 hr at 37° in a 0.5-ml reaction mixture containing 0.1 M Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 25 mM potassium phosphate (pH 7.4), and 12.5 units of Ehrlich exonuclease. Thin-layer chromatography of the reaction mixtures, using solvent 1, showed total conversion of pApA into pA, while there was no detectable hydrolysis of pTpT. The following R_f values were observed for the mono- and dinucleotides: pA, 0.06; pApA, 0.03; pT, 0.06; pTpT, 0.02. Although the above compounds do not migrate rapidly in solvent 1, the spots are extremely compact and the mononucleotides are well resolved from the dinucleotides.

The role of the 2′-hydroxyl group of the substrate during enzymatic catalysis is not understood at present. However, it appears that this group is not required for the substrate to bind to the enzyme, since thymidine nucleotides are potent inhibitors of the enzyme. Thus, thymidine 3′,5′-diphosphate, at 4×10^{-5} M, inhibits degradation of poly A by 60%, while pTpT, at 5×10^{-4} M, inhibits degradation of poly A by 30%.

Absence of Endonuclease Activity in Purified Enzyme. Although no oligonucleotides are seen in thin-layer chromatograms of digests of poly A by Ehrlich exoribonuclease, it was desired to use a more sensitive technique to ascertain whether any endonuclease activity could be detected in purified enzyme. Gel filtration on Sephadex G-100 (Birnboim, 1966) offers a sensitive assay for distinguishing between endonucleolytic and exonucleolytic types of degradation.

The degradation of poly A by Ehrlich exonuclease was stopped at various stages of digestion by addition of equal volumes of 1 M NaCl–0.01 M Na_3EDTA to aliquots from reaction mixtures. Analysis of the digests on Sephadex G-100 failed to reveal any endonucleolytic cleavage when poly A was degraded by highly purified Ehrlich exonuclease (Figure 2A–C). Thus, even when greater than 50% of the original poly A in a digestion mixture had been degraded to 5′-AMP (Figure 2C), there was no indication of the formation of products of intermediate molecular weight. In contrast (Figure 2D), crude enzyme preparations, which have not been purified by DEAE-cellulose chromatography (and which thus contain endonuclease) do yield products which are of molecular weight between poly A and 5′-AMP.

Cleavage Point during Hydrolysis of Polynucleotides. The products of hydrolysis of polyribonucleotides are exclusively nucleoside 5′-phosphates; hydrolysis begins from the free 3′-OH end of the polynucleotide (Lazarus and Sporn, 1967). Two possible mechanisms can be suggested (Figure 3) for the cleavage of the phosphodiester linkage by the enzyme, namely, (1) an attack on the 5′-phosphorus atom of the terminal nucleotide or (2) an attack on the 3′-carbon atom of the penultimate nucleotide. Previous work by Hilmoe *et al.* (1961) has shown that calf spleen phosphodiesterase and bovine pancreatic ribonuclease (both of which produce nucleoside 3′-phosphates) catalyze attack on the phosphorus atom during phosphodiester cleavage. In contrast, it had previously been shown by Cohn (1949) that when the phosphomonoester glucose 1-phosphate is cleaved by muscle phosphorylase or sucrose phosphorylase, rupture of the bond occurs between carbon and oxygen of glucose 1-phosphate. In order to distinguish whether mechanism 1 or 2 is the method of attack by Ehrlich exoribonuclease,

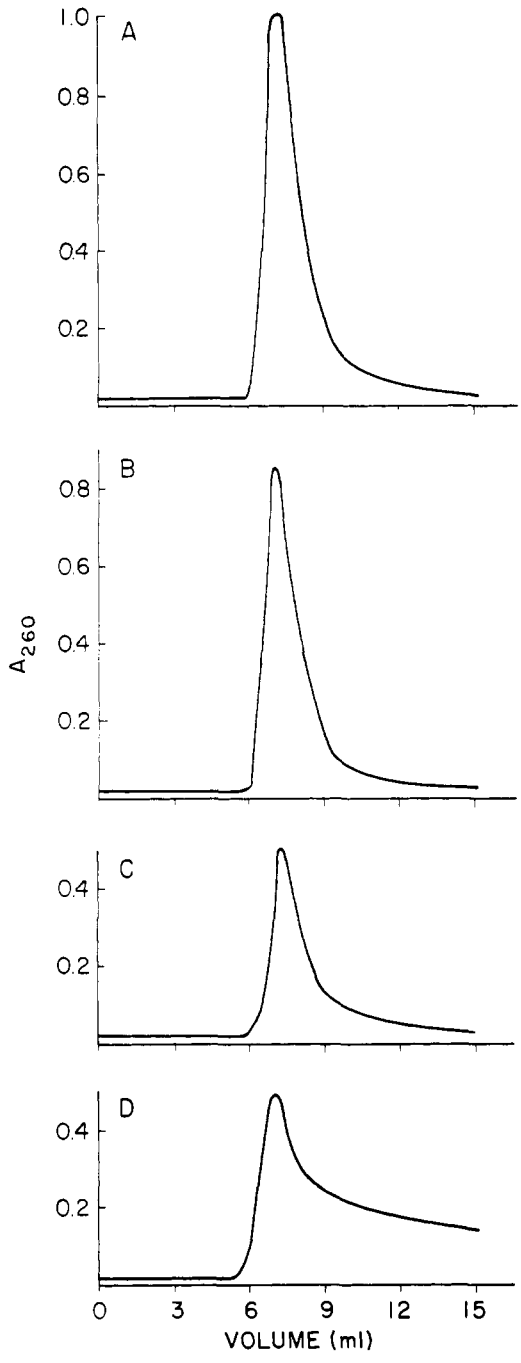

FIGURE 2: Chromatography on Sephadex G-100 of poly A, during different stages of its degradation by Ehrlich exoribonuclease. Poly A was degraded in a standard 2.0-ml reaction mixture containing 0.9 unit of Ehrlich exoribonuclease/ml of (B–D) or no enzyme (A). At different times, 0.5-ml aliquots of the digestion mixture were removed and the reaction stopped by addition of an equal volume of a solution containing 1 M NaCl and 0.01 M Na_3EDTA. Aliquots (0.2 ml) of these samples were then applied to Sephadex G-100 columns, 1.5-cm diameter × 10 cm long. The columns were eluted at constant flow rate (1.2 ml/min) with 0.5 M NaCl, 0.002 M Na_3EDTA, and the absorbance of the effluent at 260 mμ measured by a Beckman DB spectrophotometer equipped with a flow cell. (A) Poly A, undegraded by exoribonuclease (160-min incubation without enzyme). (B) Poly A after 80-min digestion with highly purified exoribonuclease. (C) Poly A after 160-min digestion with highly purified exoribonuclease. (D) Poly A after 10-min digestion with crude exoribonuclease contaminated with a small amount of endonuclease (saline extract of Ehrlich ascites tumor nuclei); note presence of large amount of material eluting between 9 and 15 ml. In expt B–D, a large peak of absorbancy at an effluent volume of 21 ml, corresponding to 5′-AMP, was found.

the following experiment was performed: poly U was degraded by enzyme in the presence of $[^{18}O]H_2O$ (46.7 atom %, ^{18}O); the resulting uridine 5′-phosphate was then converted into uridine by *E. coli* alkaline phosphatase and the molecular weight of the uridine was determined in a mass spectrometer. If the attack is on phosphorus (mechanism 1), the uridine obtained should be of normal mass, but if the attack is on carbon (mechanism 2), the uridine should contain ^{18}O in its 3′-OH group. When mass spectra were taken of uridine obtained from poly U which was hydrolyzed in the presence of exoribonuclease and $[^{18}O]H_2O$ (and then dephosphorylated), they could not be differentiated from spectra of commercial reagent grade uridine. A definite peak at mass 133, representing the ribose fragment, was seen for both uridine samples, while no peak at mass 135 (which would have been present if attack had been on the 3′-carbon) was found for either sample.

The data thus suggest that Ehrlich exoribonuclease catalyzes attack on the 5′-phosphorus atom of the terminal nucleotide, with resultant P–O splitting to yield a nucleoside 5′-phosphate.

Processive Degradation of Polynucleotides. It has been demonstrated that three bacterial exonucleases, namely *E. coli* exoribonuclease II (Nossal and Singer, 1968), *E. coli* polynucleotide phosphorylase (Thang *et al.*, 1967), and *M. lysodeikticus* polynucleotide phosphorylase (Klee and Singer, 1968) remain complexed with their polynucleotide substrates during degradation (rather than dissociating from the substrate after each phosphodiester bond cleavage) until an individual polynucleotide molecule is almost completely degraded to mononucleotides; this mode of attack has been termed processive degradation (Nossal and Singer, 1968).

The Ehrlich ascites tumor cell exoribonuclease also degrades polynucleotides by a continuous, processive mechanism. The experimental method used to validate this conclusion was very similar to that used for the bacterial enzymes. RNA was isolated with phenol from a rat liver supernatant fraction and then labeled at the 3′-OH (-pCpCpA) end with ^{3}[H]CTP and supernatant pH 5 enzyme (Hecht *et al.*, 1958; Weiss, 1960). The labeled RNA was then reisolated with phenol and used as a substrate for the Ehrlich exoribonuclease. The results are shown in Figure 4, which also shows the effect of *Crotalus adamanteus* venom on the terminally labeled RNA. It has previously been reported (Preiss *et al.*, 1961; Weiss, 1960) that snake venom phosphodiesterase digestion of tRNA results in essentially complete loss of acceptor activity or terminal -pCpCpA

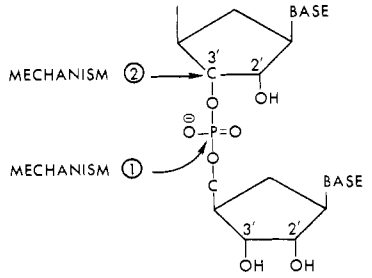

FIGURE 3: Diagrammatic representation of two possible mechanisms for cleavage of internucleotide linkage to yield nucleoside 5'-monophosphates. Mechanism 1 requires P–O bond cleavage; mechanism 2 requires C–O bond cleavage.

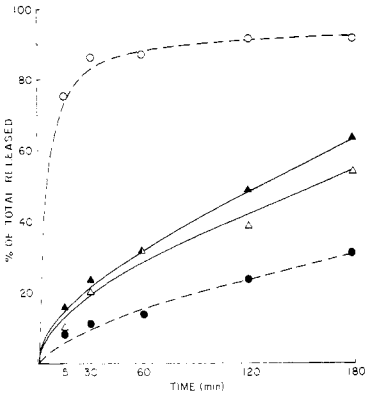

FIGURE 4: Degradation of tRNA labeled at 3'-OH(-pCpCpA) end. Labeled tRNA was prepared and degraded by Ehrlich tumor exoribonuclease and *Crotalus adamanteus* venom as described under Methods. △ = release of radioactivity, and ▲ = release of total polynucleotide, by Ehrlich tumor exoribonuclease. ○ = release of radioactivity, and ● = release of total polynucleotide, by venom.

label when approximately 5% of the total RNA has been degraded. Similar results were obtained (Figure 4, open and closed circles) with the terminally labeled tRNA prepared in our laboratory. In contrast, when this material was degraded by Ehrlich exoribonuclease, (Figure 4, open and closed triangles), we found that the enzyme converted essentially equal amounts of radioactivity and total polynucleotide into acid-soluble form at any given time during the period of degradation. The data are very similar to those reported by Thang *et al.* (1967) for phosphorolysis of terminally labeled tRNA by polynucleotide phosphorylase; these investigators found a good correlation between the percentage of terminal label liberated and the extent of total phosphorolysis of the tRNA. Likewise, Nossal and Singer (1968) found that when *E. coli* exoribonuclease II degraded polynucleotides labeled at the 3'-hydroxyl end, the extent of hydrolysis of the labeled ends and the extent of hydrolysis of the molecules as a whole were equivalent. Thus, the results obtained with Ehrlich exoribonuclease, polynucleotide phosphorylase, and *E. coli* ribonuclease II are in marked contrast to those obtained with snake venom exonuclease: the former enzymes remain complexed with an individual polynucleotide molecule while degrading it to completion, while the snake venom exonuclease attacks available ends at random (Razzell and Khorana, 1958; Lehman, 1960; Nossal and Singer, 1968; Klee and Singer, 1968).

Resistance of Polyadenylic Acid with 3'-Phosphate End Groups to Degradation. The nature of the end groups of polynucleotides is of critical importance in determining whether or not they are susceptible to exonucleolytic degradation. If a potential polynucleotide substrate molecule has a terminal phosphate group on the end from which an exonuclease begins attack, that polynucleotide will be resistant to degradation by many exonucleases. These exonucleases include snake venom exonuclease (Privat de Garilhe *et al.*, 1957; Koerner and Sinsheimer, 1957; Turner and Khorana, 1959; Felix *et al.*, 1960), calf spleen exonuclease (Heppel *et al.*, 1956; Gilham and Khorana, 1958; Razzell and Khorana, 1961), and *E. coli* exonuclease I (Lehman, 1963).

In contrast, *E. coli* exoribonuclease II, which attacks from the 3'-OH end, will degrade the heptanucleotide $(Ap)_7$, which is phosphorylated at the 3'-terminus, at the same rate as the dephosphorylated heptamer (Nossal and Singer, 1968). It was thus of interest to determine whether a terminal 3'-phosphate group on polyadenylic acid would confer resistance to attack by Ehrlich exoribonuclease, which also attacks from the 3'-OH end.

As shown in Table II, Ehrlich exoribonuclease will not hydrolyze poly A if there is a terminal 3'-phosphate group on this substrate. Poly A (which is ordinarily rapidly degraded by Ehrlich exoribonuclease) was pretreated for a short time with small amounts of micrococcal nuclease, to yield a product that was still almost totally acid insoluble, but with 3'-phosphate end groups (Privat de Garilhe *et al.*, 1957; Alexander *et al.*, 1961). Treatment of poly A for 15 min with 0.01 μg of micrococcal nuclease converted it into a form that was hydrolyzed very slowly by Ehrlich exoribonuclease while treatment with 0.1 μg converted it into a form that was totally resistant to Ehrlich exoribonuclease. In contrast, brief treatment of poly A with small amounts of mouse liver endoribonuclease I, (which introduces additional 3'-OH and 5'-phosphate groups into poly A) increased the susceptibility of poly A to degradation.

The inability of Ehrlich exoribonuclease to hydrolyze poly A terminated with 3'(2')-phosphate (or 2',3'-cyclic phosphate) was also confirmed by measuring the activity of the enzyme on poly A that had been briefly treated at 100° at neutral pH in aqueous solution. Treatment of poly A for either 10 or 30 min at 100° in 0.1 M Tris-Cl (pH 8.0) (pH measured at 37°) converted the substrate into a form that was hydrolyzed only 46 or 14% (respectively) as rapidly as the original material; under these heating conditions less than one-

TABLE II: Effect of 3′-Phosphate and 3′-Hydroxyl End Groups on Degradation of Poly A by Exoribonuclease.[a]

Experiment	mμmoles of AMP Residues Liberated/hr
1. Exonuclease + poly A, untreated	184
2. Exonuclease + poly A, pretreated with 0.01 μg of micrococcal nuclease[b]	11
3. Exonuclease + poly A, pretreated with 0.1 μg of micrococcal nuclease[c]	0
4. Exonuclease + poly A, pretreated with mouse liver endoribonuclease[d]	268

[a] The final reaction mixture was the standard assay system, except that the concentration of poly A was .001 M (adenine equivalent). In each experiment, the same amount of exoribonuclease (0.18 unit) was used. In expt 2 and 3, poly A (5 mg/ml) in 0.05 M Tris-Cl (pH 8.8) was pretreated with micrococcal nuclease at 37° for 15 min in the presence of 5×10^{-4} M $CaCl_2$. In expt 4, poly A (5 mg/ml) in 0.05 M Tris-Cl (pH 8.0), was pretreated with mouse liver nuclear endonuclease at 37° for 15 min in the presence of 5×10^{-4} M $MgCl_2$. [b] Essentially none (less than 0.2%) of the original poly A was converted to acid-soluble form by this treatment. [c] Approximately 2% of the original poly A was converted into acid-soluble form by this treatment. [d] The amount of endoribonuclease used to pretreat the poly A converted essentially none (less than 0.2%) of this material into acid-soluble form during 15 min at 37°.

half of one per cent of the original poly A was converted into acid-soluble material. In the above experiments, using poly A that had been heated at neutral pH, the nature of the new end groups introduced into poly A by heating was not determined; they probably include both 2′,3′-cyclic phosphate groups that are the initial products of hydrolysis by either H^+ or OH^-, as well as 3′- (or 2′-) phosphate groups formed by subsequent attack of H^+ or OH^- on the cyclic phosphate structure (Brown and Todd, 1952; Markham and Smith, 1952; Witzel, 1963).

Comparison of Nucleoside 3′,5′-Diphosphates and Nucleoside 3′,-5′-Disulfates as Inhibitors. Since thymidine 3′,5′-diphosphate is an excellent inhibitor of Ehrlich exoribonuclease (60% inhibition at 4×10^{-5} M), it was desired to know if nucleoside 3′,5′-disulfates would also inhibit. The following nucleoside sulfates were therefore tested at concentrations between 1×10^{-3} and 1×10^{-4} M: thymidine 3′,5′-disulfate, uridine 2′,3′,5′-trisulfate, 5-fluorodeoxyuridine 3′,5′-disulfate, and 5-iododeoxyuridine 3′,5′-disulfate; none gave any appreciable inhibition. Inhibition is not only lost when the phosphate anions of a nucleoside 3′,5′-diphosphate are replaced by sulfate;

TABLE III: Inhibition of Exoribonucleases by 2′-TPN and Equilibrium Mixture of 2′- and 3′-TPN.[a]

Tissue Source of Exoribonuclease	% Inhibn by 2′-TPN	% Inhibn by 2′- and 3′-TPN
Ehrlich tumor	3	64
Embryo	5	52
Mammary tumor	0	59

[a] Figures are per cent inhibition of enzyme obtained with 1×10^{-3} M *total* TPN, using standard exoribonuclease assay.

it is also lost if the phosphate anions are located in the 2′ and 5′ position. This is shown in the results of Table III, which compares the inhibition of exoribonuclease by the naturally occurring triphosphopyridine nucleotide (which contains a 2′- and 5′-phosphate group) and an equilibrium mixture of TPN and its 3′-phosphate isomer (3′-TPN). TPN was isomerized in dilute HCl (Shuster and Kaplan, 1955) to an equilibrium mixture of approximately 50% 2′-TPN and 50% 3′-TPN. The acid was then neutralized to pH 7.4 with Tris buffer and the effect on exoribonuclease measured. The naturally occurring 2′-TPN did not inhibit exoribonuclease, while the 3′-isomer did.

Discussion

The data presented in this and two previous papers show the presence of an exoribonuclease with unique properties for degrading single-stranded RNA in several different types of nuclei from mouse tissues. Moreover, the occurrence of this enzyme in nucleate cells is not limited to the tissues of mice; it has also been identified in nuclei of hamster tumors induced by SV40 virus (Lazarus *et al.*, 1967) and in nuclei of human HeLa cells grown in suspension culture (D. M. Berkowitz and M. B. Sporn, unpublished experiments). While RNA with a high degree of helical structure is resistant to degradation, the rapidly labeled, newly synthesized RNA of the nucleus is readily degraded by this particular enzyme (Lazarus and Sporn, 1967).

Since it has now been established that there are many species of nuclear RNA which are not represented in the cytoplasm (Sporn and Dingman, 1963; Shearer and McCarthy, 1967; Church and McCarthy, 1967; Dingman and Peacock, 1968) and that a great deal of the rapidly labeled, newly synthesized RNA of the nucleus is degraded within the nucleus itself (Harris, 1963; Roberts, 1965; Attardi *et al.*, 1966; Scherrer *et al.*, 1966; Soerio *et al.*, 1968), it is reasonable to postulate that the presently described exoribonuclease plays an important role in nuclear function. It is of definite interest that it has recently been shown that bacterial mRNA is degraded exonucleolytically from the 3′-hydroxyl end (Baker and Yanofsky, 1968), presumably by enzymes whose kinetics of attack on polynucleotides are very similar to those of the

presently described mammalian nuclear exoribonuclease (Thang *et al.*, 1967; Nossal and Singer, 1968; Klee and Singer, 1968).

It has been suggested that the protection of newly synthesized RNA from degradation may be a key step in the control of gene action (Harris, 1968). Thus, further elucidation of mechanisms which either directly modify the activity of the nuclear exoribonuclease (by converting the enzyme into a more or less active form) or indirectly modify the activity of the nuclear exoribonuclease (by converting a potential RNA substrate molecule into a resistant form) would appear to be badly needed. Some of the allosteric and isosteric mechanisms which modify the activity of nuclear exoribonuclease will be discussed in a following paper (H. M. Lazarus and M. B. Sporn, in preparation). In contrast, the nuclear mechanisms for protection of newly synthesized RNA from exonucleolytic degradation in the living cell are almost totally unknown at present.

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The Role of Transport Phenomena in Ion Binding Studies of Serum Albumin*

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ABSTRACT: Two hypotheses previously suggested to explain the apparent dependency on protein concentration of equilibrium dialysis measured binding of long-chain ionic ligands to bovine serum albumin have been tested and disproved. Of greater importance, it is shown that the apparent protein concentration effect was an experimental artifact which originated in the anomalously slow approach to equilibrium with these ligands under the conditions of the earlier experiments. The effects of protein concentration, ligand size, supporting electrolyte, and temperature on the approach to dialysis equilibrium is examined. Hitherto unobserved and only partially understood kinetic anomalies occur when ionic ligands of high affinity are dialyzed *into* protein solutions which are appreciably more concentrated than 0.1%.

It has been reported from this laboratory (Ray *et al.*, 1966) that the binding isotherms of two long-chain ligands, dodecyl sulfate and dodecanol, to bovine serum albumin depend upon protein concentration. For example, the mole binding ratio, $\bar{\nu}$, in the range $\bar{\nu} < 10$ as measured by equilibrium dialysis with $\geq 1\%$ bovine serum albumin was considerably lower than that measured with 0.1% protein. This result and the observation by Klotz and Urquhart (1949) of a similar but much smaller effect in the binding of methyl orange to albumin are the only published references to such an effect.

It was suggested by Ray *et al.* (1966) that dimerization of serum albumin through a bifunctional ligand could account for the observed decrease in $\bar{\nu}$ at high protein concentration. This explanation was particularly attractive since the concentration effect was not observed with ligands containing less than 12 carbon atoms. Alternatively it was deemed possible that the ligand contained a small amount of low-affinity impurity which was not distinguishable from the ligand itself by the method of assay. Such an impurity would masquerade as a protein concentration effect.

We report tests of these two suggestions, the first by osmotic pressure measurements, and the second by measuring the binding by a triple-dialysis procedure which will eliminate any effect of ambiguous low-affinity impurities. Both hypotheses are shown to be invalid.

Of more importance the experiments reported here demonstrate that the large concentration effect observed by Ray *et al.* (1966) was an experimental artifact originating in their failure to recognize the extremely long times required for attainment of equilibrium under the conditions of their experiments. Data are presented to show that with certain ligands, and certain combinations of temperature, protein concentrations, and supporting electrolyte the approach to thermodynamic equilibrium is unexpectedly slow.

Experimental Section[1]

Materials. Crystalline BSA (Nutritional Biochemicals Corp., lot no. 5776, 7799, and 9385) was dissolved in distilled water and deionized by passage through a mixed-bed ion-exchange column (Ag 501-X8, Bio-Rad Laboratory) containing a small portion of Dowex 50W-X8 at the bottom. The protein solutions were refrigerated and used within 2 weeks.

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[1] "Certain commercial equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards."