Ca^{2+}-Stimulated Ribonucleases from Rat Mammary Gland and R3230AC Mammary Adenocarcinoma Nuclei†

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ABSTRACT: Ribonuclease activity in the nuclei of lactating rat mammary gland and R3230AC mammary tumor is several times higher with Ca^{2+} (5 mM optimum) in the assay than with Mg^{2+} or no added divalent cations. The Ca^{2+}-stimulated RNase (Ca^{2+} RNase) activities from the nuclei of both mammary gland and R3230AC tumor are found primarily in the nucleoli-free nuclear sonicate. High-speed centrifugation of the sonicate yields Ca^{2+} RNase activity in both supernatant (Sap I) and pellet fractions. DEAE-cellulose chromatography of Sap I revealed a single peak of activity (Ca^{2+} RNase I) that was eluted at 0.15 M NaCl. Similar chromatography of the 1 M NaCl extract of the pellet (Sap II) yielded a peak (Ca^{2+} RNase IIa) that appeared prior to the salt gradient and a major peak (Ca^{2+} RNase IIb) that appeared at 0.08 M NaCl. The molecular weights of Ca^{2+} RNases I and IIb are approximately 52 500 and 32 400, respectively, as determined by Sephadex G-100 gel filtration. Ca^{2+} RNases I and IIb are both endonucleases as judged by sucrose density gradient analysis of ^3H-labeled 45S RNA reaction products. Ca^{2+} RNases I and IIb catalyze the degradation of ^3H-labeled poly(U), but are less active against poly(C). The mammary gland enzymes are inactive against poly(G) and poly(A); tumor Ca^{2+} RNase I is inactive against poly(G) but is slightly active against poly(A). The potential substrates, poly(dT), DNA-RNA hybrids, ribosomal RNA, and DNA, were not degraded into ethanol-soluble products by tumor Ca^{2+} RNase I. The enzymes are inhibited by polyamines, heparin, polydextran sulfate, NaF, and sodium pyrophosphate. PEI-cellulose thin-layer chromatography of labeled poly(U) and poly(C) degradation products catalyzed by Ca^{2+} RNases I and IIb revealed the presence of 2′,3′-cyclic mononucleotides and oligonucleotides with 3′-phosphoryl and 5′-hydroxyl termini. In summary, nuclei from lactating rat mammary gland and R3230AC mammary tumor contain Ca^{2+}-dependent, pyrimidine-specific, 3′-phosphate-forming RNA endonucleases.

Nuclear RNases[1] have been implicated in the processing of precursor RNA to mature cytoplasmic forms as well as in the degradation of nontransported RNAs (Liau et al., 1968; Darnell, 1968; Burdon, 1971; Kelley and Perry, 1971; Hurlbert et al., 1973; Perry and Kelley, 1972; Mirault and Scherrer, 1972; Winicov and Perry, 1974; Perry, 1976). A number of nuclear RNases have been identified and isolated from animal cells (Lazarus and Sporn, 1967; Sporn et al., 1969; Kelley and Perry, 1971; Prestayko et al., 1972; Perry and Kelley, 1972; Winicov and Perry, 1974; Kwan et al., 1974; Boctor et al., 1974), but the precise cellular function of these enzymes is not clear, partly because the natural RNA substrates are not known.

The potentially important role of RNases in the qualitative and quantitative changes in rat mammary gland RNA during pregnancy and lactation have led us to begin studies on the RNases of rat mammary gland and mammary tumor (Liu et al., 1975, 1976). It is known that RNA/DNA ratios in mammary gland increase gradually during pregnancy and more sharply after parturition in preparation for the synthesis and secretion of milk (Munford, 1964). These increases in mammary gland RNA are due in part to increased rates of synthesis (Wang and Greenbaum, 1962; Banerjee and Banerjee, 1973) but could also be due to decreased rates of degradation as suggested by large increases in the cytoplasmic RNase inhibitor (Liu et al., 1975).

In this report we present evidence for the existence of several Ca^{2+} RNases in nuclei isolated from lactating rat mammary gland and from the R3230AC transplantable rat mammary adenocarcinoma. We found that the RNases of the R3230AC mammary tumor were qualitatively similar to the RNases of the mammary gland.

Materials and Methods

Animals. Female Fischer rats were maintained in air conditioned animal quarters at 24 ± 1 °C with 12 h of artificial light from 7 a.m. to 7 p.m. Animals were provided with Wayne Chow (Allied Mills, Inc., Chicago, Ill.) and tap water ad libitum. The stage of lactation was counted beginning with the day of parturition as day 1. Only females with litters of six or more pups were used. Lactating females (160–200 g) were kept with their litters until sacrificed. The R3230AC mammary tumor was provided to us originally by Dr. Russell Hilf of the University of Rochester. Tumor transplantation was carried out as previously described (Liu et al., 1975).

Materials. Blue dextran 2000, cytochrome *c*, hemoglobin, DEAE-cellulose, yeast RNA, bovine serum albumin, calf-thymus DNA, heparin, spermine, spermidine, and putrescine were all from Sigma Chemical Co. Chymotrypsinogen was from Worthington Biochemical Corp. Snake venom and spleen phosphodiesterases were from Boehringer Mannheim Biochemicals. Dithiothreitol was from Calbiochem. Poly(U),

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[1] Abbreviations used are: RNase, ribonuclease; Ca^{2+} RNase, Ca^{2+}-stimulated RNase; Mg^{2+} RNase, Mg^{2+}-stimulated RNase; $Mg(OAc)_2$, magnesium acetate; solution STD, 0.34 M sucrose–50 mM Tris-HCl (pH 7.3)–0.6 mM dithiothreitol; NaOAc, sodium acetate; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; PEI, phosphoethylenimine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)-aminomethane.

poly(A), poly(C), poly(G), [5-^{3}H]poly(U) (19 μCi/μmol of P, 7.3 S), [8-^{3}H]poly(A) (94 μCi/μmol of P, 3 S), [^{3}H]poly(C) (49 μCi/μmol of P, 4.9 S), [^{3}H]poly(G) (52.8 μCi/μmol of P, 10 S), and [^{3}H]poly(dT) (50.3 μCi/μmol of P) were purchased from Miles Laboratories. [5-^{3}H]Orotic acid (15 Ci/mmol), ammonium sulfate (special enzyme grade), and sucrose (RNase free) were from Schwarz/Mann and 2,5-diphenyloxazole was from New England Nuclear Corp. PEI-cellulose TLC plates were purchased from Arthur H. Thomas Co. ^{3}H-Labeled 45S RNA was prepared according to Smith et al. (1974). The yeast RNA solution was prepared as previously described (Liu et al., 1975). Phosphoglycerate kinase was prepared from rat skeletal muscle (Hass et al., 1974).

Assay of RNase Activity. The standard reaction mixture contained in a final volume of 200 μL: 100 mM Tris-HCl (pH 7.4 to 25 °C), 0.3 mM dithiothreitol, 0.24 nmol of phosphate of [^{3}H]poly(U) (19 μCi/μmol of P), 5 mM $CaCl_2$ or $Mg(OAc)_2$, and 50 μL of enzyme solution. The reaction was started by adding the enzyme solution and was maintained at 37 °C for 15 min in a Dubnoff metabolic shaking bath. The blank, to determine background precipitation, contained 50 μL of solution STD[1] in place of the enzyme solution. The reaction was stopped by adding 0.6 mL of ice-cold 95% ethanol–10 mM $Mg(OAc)_2$ (Razzell, 1963). Yeast RNA (60 μg in 60 μL of 0.58 M NaOAc, pH 4.8) was added as a carrier to aid in precipitation. After storing at −20 °C for at least 60 min or overnight, the tubes were centrifuged in an International table top centrifuge at 1860g for 15 min. The supernatant was mixed with a toluene-based scintillation mixture (4.5 mL of 0.7% 2,5-diphenyloxazole in scintillation grade toluene, 1.0 mL of Beckman Bio-Solv-3, and 0.5 mL of water) and counted in a Searle Isocap/300 liquid scintillation spectrophotometer. The counting efficiency was approximately 30% based upon measurement of a ^3H_2O standard under the same conditions of sample counting. A unit of enzyme activity represents the amount of enzyme which converts 0.1 of the poly(U) substrate (0.024 nmol of P) into ethanol-soluble products in 15 min under the standard assay conditions. If more than one-half of the substrate was converted into ethanol-soluble products during the course of the assay, the enzyme solution was diluted with solution STD before assay in order to maintain first-order kinetics during the assay.

In preliminary experiments, total nuclear RNase was measured using yeast RNA as substrate (Liu et al., 1975). When ^{3}H-labeled 45S RNA was used as substrate, the reaction mixture contained in a final volume of 200 μL: 100 mM Tris-HCl (pH 7.8), 0.3 mM dithiothreitol, either Ca^{2+} or Mg^{2+} (0.55 mM) or no added ion, 20 μg of ^{3}H-labeled 45S RNA containing 1000 cpm, and nuclear sample containing 60–140 μg of DNA. The reaction was carried out at 37 °C for 30 min and stopped by adding 2.5 vol of ice-cold 5% $HClO_4$. After centrifugation the acid-soluble radioactivity in the supernatant was measured as above.

Protein Determination. In order to eliminate the effect of dithiothreitol and other interfering materials, protein was measured by a modified Lowry method (Ross and Schatz, 1973). For solutions with very dilute protein concentration, a method using amido black was used (Schaffner and Weissmann, 1973). Bovine serum albumin was used as the standard in both methods.

DNA Determination. DNA was extracted as described by Munro and Fleck (1966) and was measured by the diphenylamine reaction (Burton, 1956).

Isolation of Nuclei. The animals were killed by decapitation. Mammary gland and mammary tumors were excised while the carcass was chilled on ice. In the case of liver nuclei, the liver was removed from the same lactating rat immediately after the animals were bled. Tissues were placed separately in ice-cold 0.25 M sucrose. All the following steps were carried out at 4 °C. Lymph nodes, major blood vessels, and surface connective tissue were removed from the mammary gland. Milk was removed by rinsing the gland with 0.25 M sucrose until the washing was clear. Membranous connective tissue on the outside and necrotic tissue in the interior of the tumor were removed and the tumor was washed with 0.25 M sucrose until the washing was clear. Nuclei were isolated essentially by the method described by Busch (1967). Tissues were blotted dry, weighed, minced finely with scissors, and homogenized in 10.5 vol of 2.4 M sucrose–3.3 mM $Mg(OAc)_2$ with four up-and-down strokes in a Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle (clearance about 0.5 mm). The homogenate was filtered through four layers of gauze and centrifuged at 40 000g for 1 h in a Sorvall RC2-B centrifuge. The supernatant minus the floating layer of cell debris and lipid was saved for preparation of the 105 000g supernatant. Nuclear pellets were removed with a stainless steel spatula and combined by suspending in 0.34 M sucrose–1 mM $Mg(OAc)_2$–0.5% Triton X-100. The suspension was centrifuged in a swinging bucket rotor at 2000g for 15 min in an International centrifuge. Liver and mammary gland nuclei isolated by this procedure were free of cytoplasmic contamination as verified by phase-contrast microscopy. Mammary tumor nuclei contained a minimal amount of dense fibrous particles but were essentially free of endoplasmic reticulum. In some experiments, as indicated, nuclei were prepared by the method of Blobel and Potter (1964).

Separation of Nucleoli-Free Nuclear Sonicate and Nucleoli. The Triton X-100 washed nuclear pellet was suspended in 0.34 M sucrose (nuclei from 4–5 g of tissue per mL) and sonicated in an ice bath using a Branson sonifier with four bursts of 5 s each, separated by 30-s intervals between bursts. The sonication was usually carried out in 6-mL portions. No intact nuclei were detected when the sonicate was examined under the microscope. The sonicate was made up to 15 mL with 0.34 M sucrose and underlayered with an equal volume of 0.88 M sucrose. The samples were then centrifuged at 2000g for 15 min in an International centrifuge attached with swinging buckets. As verified by phase-contrast microscopy, the pellet contained pure nucleoli; the supernatant contained no nucleoli and was designated nucleoli-free nuclear sonicate.

Separation of RNases from Nucleoli-Free Nuclear Sonicate. Procedures for the isolation of RNases from the nucleoli-free nuclear sonicate are outlined in Figure 1. The nuclear sap was adjusted to 0.34 M sucrose by adding ice-cold distilled water and was then centrifuged at 94 000g for 2 h using a 30 rotor in a Spinco L65 ultracentrifuge. Following centrifugation, the supernatant designated as Sap I was adjusted with 1 M Tris-HCl (pH 7.3) and 100 mM dithiothreitol to make final concentrations of 50 and 0.6 mM, respectively. Sap I was kept frozen at −20 °C until used in the next step, usually within 2 days. The pellet was suspended in half of the original volume of solution STD by homogenizing gently by hand in a loose-fitting Teflon-pestled Potter Elvehjem tissue grinder. To this suspension, ice-cold 5 M NaCl was added to a final concentration of 1 M. After standing at 4 °C for 3 h with occasional stirring, the suspension was centrifuged as above at 94 000g for 2 h. The pellet, which contained minimal RNase activity by our assay method, was discarded and the supernatant was dialyzed overnight against solution STD with two changes of the same buffer. The dialyzed sample was centrifuged at

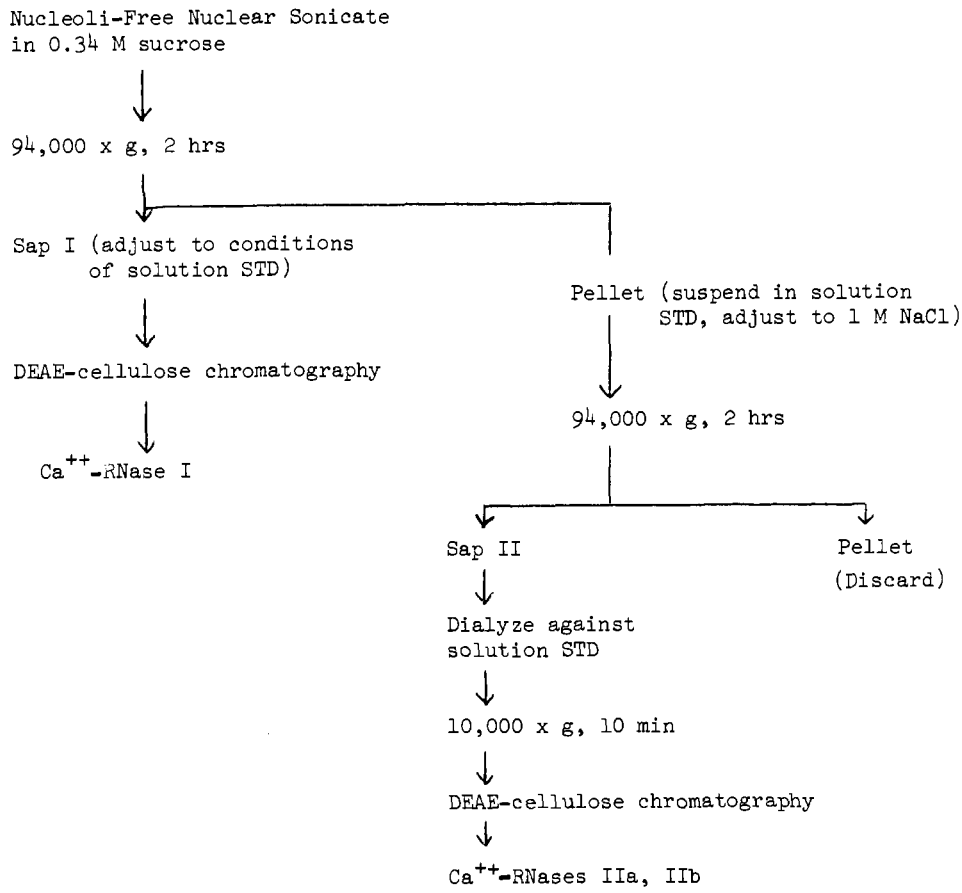

FIGURE 1: Extraction of Ca^{2+} RNases from rat mammary gland nucleoli-free nuclear sonicate. All steps were carried out at 4 °C. See text for details.

10 000*g* for 10 min to remove the precipitate, and the supernatant, designated as Sap II, was kept at −20 °C.

DEAE-Cellulose Chromatography of Saps I and II. After thawing, Sap II was diluted twofold with solution STD to lower the NaCl concentration. Saps I and II were separately applied to DEAE-cellulose columns (1.1 × 6.0 cm) equilibrated with solution STD and the columns were washed with 5 mL of solution STD. RNases were eluted with a linear gradient of 0–0.40 M NaCl in 50 mL of solution STD. Sodium chloride concentrations in the eluates were determined as Na^+ by flame spectrophotometry (Flame Photometer Model 143, Instrumentation Laboratory Inc.).

Enzymic Reaction Product Analysis. PEI-Cellulose TLC. The reaction mixture contained in a final volume of 100 μL: 0.1 M Tris-HCl (pH 7.4), 0.6 mM dithiothreitol, 5 mM $CaCl_2$, 12.6 μmol of nucleotide phosphate of [^{3}H]poly(C) (92 500 cpm), and 10 μL of mammary gland Ca^{2+} RNase I containing 0.4 μg of protein. Aliquots (10 μL) were removed at times 0, 15, 30, 60, and 90 min and spotted on PEI-cellulose TLC plates along with 3′-CMP, 5′-CMP, and 2′,3′-cyclic CMP. The plates were developed and nucleotides were analyzed by the procedures described by Randerath and Randerath (1964).

DEAE-Cellulose in 7 M Urea. The reaction mixture contained 0.1 M Tris-HCl (pH 7.4), 0.6 mM dithiothreitol, 5 mM Ca^{2+}, ^{3}H-labeled 45S RNA (22 700 cpm), and 1 μg of Ca^{2+} RNase I. The mixtures were incubated at 37 °C. At the end of incubation, 2 vol of ethanol was added and stored at −20 °C overnight. The ethanol-soluble products were analyzed by a DEAE-cellulose column (1 × 32 cm) equilibrated with 0.05 M Tris-HCl (pH 8), 0.025 M NaCl, and 7 M urea as described by Tomlinson and Tener (1963).

Molecular Weight Determination. The Sephadex G-100 column (0.5 × 108 cm) was equilibrated with solution STD containing 0.5 M KCl. The gel column elution volume (V_0) of 10.2 mL was measured using Blue dextran 2000. Proteins of known molecular weight used for calibration were: cytochrome *c* (15 200), α-chymotrypsinogen (23 000), phosphoglycerate kinase (43 000), and hemoglobin (62 000). Ca^{2+} RNase samples were those of post-DEAE-cellulose chromatography. Ca^{2+} RNases and standard proteins were applied to the column in less than 1 mL of solution STD and 1-mL fractions were collected. The elution volume (V_e) was the volume measured at the fraction with maximum A_{280} absorbance or enzyme activity.

Results

Presence of Ca^{2+} RNase Activity in Rat Mammary Gland and R3230AC Mammary Tumor. Early studies included liver in order to compare the purity of nuclei and the RNase activity with mammary gland or tumor. Preliminary experiments using yeast RNA as substrate and a divalent cation in the assay (Liu et al., 1975) revealed that the total nuclear RNase activity per milligram of DNA was consistently higher in lactating mammary gland than in the liver from the same animal. Unexpectedly, when the Triton X-100 washed nuclear pellets (Blobel and Potter, 1964) were suspended separately in 0.25 M sucrose containing either 3.3 mM $CaCl_2$ or $MgCl_2$, the mammary gland sample in Ca^{2+}-containing medium had more than 3 times as much RNase activity as the nuclei in Mg^{2+}-containing medium or when no ions were added (Table I). When ^{3}H-labeled 45S RNA was used as substrate, the RNase activity in the presence of Ca^{2+} was also 3 times higher than the ac-

TABLE I: Total Nuclear RNase Activity of Mammary Gland and Liver from Lactating Rats.[a]

Ions	Mammary gland	Liver
Yeast RNA as Substrate (A_{260} (mg of DNA)$^{-1}$ 30 min^{-1})		
None	6.90 ± 1.75 (3)[b]	2.45 ± 0.44 (3)
Mg^{2+}	4.74 ± 2.17 (3)	1.83 ± 0.50 (3)
Ca^{2+}	22.7 ± 4.8 (3)	0.65 ± 0.47 (3)
^{3}H-Labeled 45S RNA as Substrate ($\times 10^{-3}$ cpm (mg of DNA)$^{-1}$ 30 min^{-1})		
None	5.9	4.5
Mg^{2+}	6.5	5.1
Ca^{2+}	17.6	3.5

[a] Nuclei were prepared by the method of Blobel and Potter (1964) from pooled mammary glands and livers of two rats at the sixth day of lactation. The assay conditions are described under Materials and Methods. [b] The numbers in parentheses indicate the number of experiments and the values are means ± standard error of the mean.

tivity measured with Mg^{2+} or with no ion added. However, there was no stimulation by Ca^{2+} of the RNase activity in liver nuclei. This result indicates that rat mammary gland contains Ca^{2+} RNase activity. Such activity has not been previously reported. Ca^{2+} RNase activity was also found in the R3230AC transplantable rat mammary adenocarcinoma.

Subnuclear Distribution of Ca^{2+} RNase Activity. Based upon tissue weight and without correcting for the yield of nucleoli, there were 3 and 4 times more Ca^{2+} RNase in the nucleoli-free nuclear sonicate than in the nucleoli of tumor and mammary gland, respectively (Table II). In the experiment shown, Ca^{2+} RNase activity in lactating mammary gland nucleoli was about 2.4 times greater than Mg^{2+} RNase activity, while in the nuclear sonicate there was about 5 times more Ca^{2+}- than Mg^{2+}-stimulated activity. Ca^{2+} RNase activities in the nucleoli and nuclear sonicate were, respectively, 3.2 and 13.7 times higher in Ca^{2+} than in the absence of added divalent cations. Similar results were obtained using the R3230AC mammary tumor. The tumor nuclear sonicate consistently showed lower Ca^{2+} RNase activity than the samples from lactating gland. The ratios of activity in the presence of Ca^{2+} to that in the presence of Mg^{2+} were about 1.3:1 and 3:1 in the tumor nucleoli and nuclear sonicate, respectively. The Ca^{2+} RNase activity in the nucleoli fraction could be the result of a true distribution of the enzyme or might be due to contamination or redistribution of nucleoplasmic Ca^{2+} RNase during preparation of nucleoli. The liver contained much lower Ca^{2+} RNase activities in both subnuclear fractions. The RNase activity in liver nuclear sonicates, as well as in the nucleolar fraction when assayed in the presence of Ca^{2+}, was lower than when assayed with Mg^{2+} or no added ions (Table II).

Isolation of Ca^{2+} RNases. A typical enzyme preparation began with 22–25 g of mammary tissue from three 6-day lactating rats. In the case of R3230AC tumor, tissue was collected from 6 or more rats 6 to 7 weeks after transplantation. The results are summarized in Table III.

Almost half of the total Ca^{2+} RNase activity from nucleoli-free nuclear sonicates was recovered in the supernatant designated Sap I. Since the pellet contained most of the protein, the specific activity of the enzyme was increased more than threefold by this simple centrifugation step. Ca^{2+} RNase activity remaining in the pellet was extracted by 1 M NaCl in solution STD. This extract (Sap II) contained a greater part of the pellet proteins and a small portion of enzyme activity.

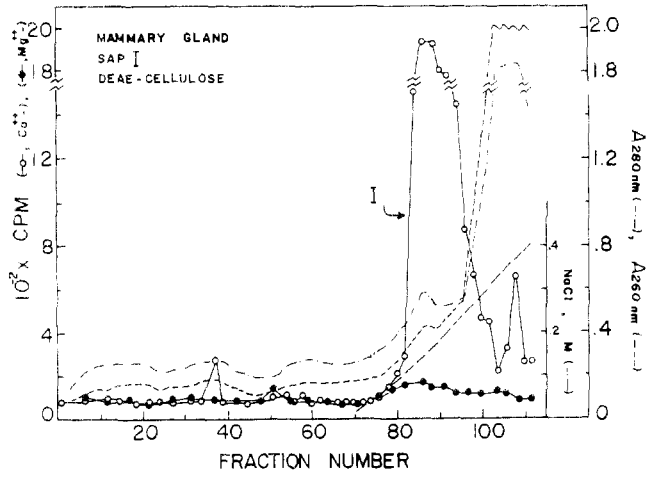

FIGURE 2: DEAE-cellulose chromatography of Sap I from rat mammary gland nucleoli-free nuclear sonicate. See Figure 1 and Materials and Methods for details. The activity was assayed by using [^{3}H]poly(U) in the presence of 5 mM Ca^{2+} or Mg^{2+} ion as described under Materials and Methods. The flow rate was 25 mL/h and 0.4–0.5-mL fractions were collected. Fractions were monitored for 280-nm (·–·) and 260-nm (- - -) absorbance and were assayed for RNase activity as described under Materials and Methods using either Ca^{2+} (○–○) or Mg^{2+} (●–●). Every tenth tube was analyzed for Na^+ (– – –) using a flame photometer (Model 143, Instrument Laboratories).

No RNase activity was detected in the second pellet after NaCl extraction. After dialysis of the NaCl extract, most of the protein precipitated but the Ca^{2+} RNase remained soluble. The combined yield of enzyme activity in Sap I and Sap II was approximately 55%. The intrinsic instability probably accounts for some of the loss, particularly that of Sap II where a prolonged dialysis step is part of the procedure. When Saps I and II were applied separately to DEAE-cellulose columns (1.1 × 6.0 cm), they showed different elution profiles for Ca^{2+} RNase activities. The activity in Sap I eluted as a single large peak with maximum activity at about 0.15 M NaCl and some trailing activity at higher NaCl concentrations (Figure 2). Fractions eluted between 0.12 and 0.24 M NaCl which contained the majority of RNase activity were pooled and concentrated in dialysis bags against dry sucrose. This pooled, concentrated sample was designated Ca^{2+} RNase I. When the fractions were assayed in the presence of 5 mM Mg^{2+}, little RNase activity was found (Figure 2). A large quantity of A_{260} absorbing material (presumably from various nuclear RNAs and some degraded DNAs) was eluted at higher salt concentrations.

DEAE-cellulose chromatography of dialyzed Sap II produced a different elution profile of Ca^{2+} RNase (Figure 3). There was a region prior to the NaCl gradient that contained Ca^{2+} RNase activity (designated Ca^{2+} RNase IIa) as well as a rather sharp peak of Ca^{2+} RNase containing the major portion of activity that eluted with maximum activity at 0.08 M NaCl. Fractions eluted between 0.06 and 0.12 M NaCl were pooled and designated Ca^{2+} RNase IIb. Similar to the situation with Sap I, there was very low Mg^{2+} RNase activity in either Ca^{2+} RNase IIa or IIb and no major Mg^{2+} RNase activity peak was detected in the remaining fractions. There was also a large peak of A_{260} absorbing materials at higher NaCl concentrations indicating that Sap II also contained large quantities of RNA and perhaps DNA. The results indicate that Sap I and Sap II contain different classes of Ca^{2+} RNase which may be separated by centrifugation of the nucleoli-free nuclear sap. Thus, Ca^{2+} RNase I is a soluble form and Ca^{2+} RNases IIa and IIb are probably chromatin-bound forms.

TABLE II: RNase Activity of Nucleoli and Nucleoli-Free Nuclear Sonicate.[a]

Tissue	RNase act. (cpm/30 min) None	Mg^{2+}	Ca^{2+}	Ca^{2+}/none	Ca^{2+}/Mg^{2+}
			Nucleoli		
Mammary gland	144	193	457	3.2	2.4
R3230AC tumor	306	390	505	1.7	1.3
Liver	256	344	173	0.7	0.5
			Nuclear Sonicate		
Mammary gland	153	417	2090	13.7	5.0
R3230AC tumor	304	555	1690	5.6	3.0
Liver	275	473	125	0.5	0.3

[a] Nuclei were isolated by the method of Blobel and Potter (1964) from mammary glands of 4 rats at day 21 of lactation and from rats bearing tumors transplanted 7 weeks earlier. Separation of nucleoli and nucleoli-free nuclear sonicate was as described under Materials and Methods. The RNase activity was assayed by using ^3H-labeled 45S RNA as substrate with or without 0.55 mM Ca^{2+} or Mg^{2+} as described under Materials and Methods. The values represent the soluble nucleotide radioactivity produced by the nucleoli or nuclear sap isolated from 0.5 g of tissue without correcting for the yield of nuclei and nucleoli. The amounts of DNA from 0.5 g of mammary gland, R3230AC tumor, and liver were, respectively, for nucleoli, 2.54, 5.88, and 3.66 μg, and for nuclear sap, 259, 344, and 302 μg. It should be noted that since the yield of nuclei from the tissue and the yield of nucleoli from nuclei varied among tissues, the comparisons among tissues are only first approximation values. The yield of nuclei normally ranged from 50 to 75% for liver, and from 30 to 50% for mammary gland and tumor.

TABLE III: Separation of Ca^{2+} RNases of Nucleoli-Free Nuclear Sonicate.[a]

Purification steps	Total protein (mg)	Activity Specific (units/mg)	Total (units)
Nucleoli-free nuclear sonicate	139	168	23 300
Sap I	2.0	5 440	10 880
DEAE-cellulose			
Ca^{2+} RNase I	0.26	28 600	7 430
Sap II	3.2	569	1 820
DEAE-cellulose			
Ca^{2+} RNase IIa	0.11	1 790	197
Ca^{2+} RNase IIb	0.20	3 560	712

[a] The enzyme purification steps are the same as those shown in Figure 1 and detailed under Materials and Methods. Nuclei were isolated from 22 g of mammary gland pooled from three rats at their sixth day of lactation. The enzyme activity was assayed using [^3H]poly(U) in the presence of 5 mM Ca^{2+}, as described under Materials and Methods.

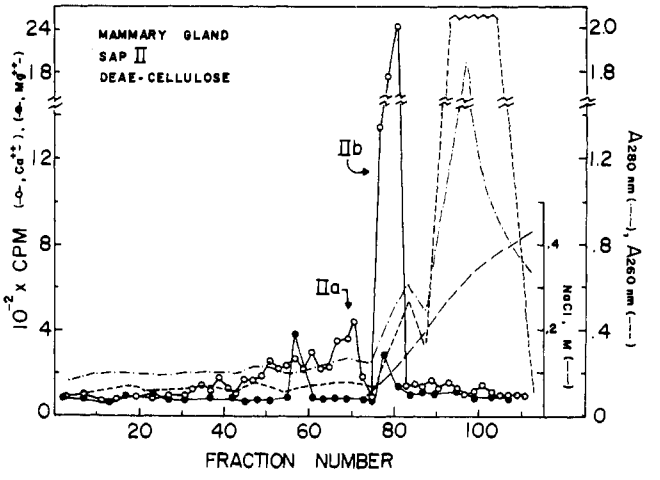

FIGURE 3: DEAE-cellulose chromatography of Sap II from rat mammary gland nucleoli-free nuclear sonicate. See Figure 1 and Materials and Methods for details. The assay condition and the symbols are the same as those in Figure 2.

Molecular Weight of Ca^{2+} RNases. The molecular weights of mammary gland and tumor Ca^{2+} RNases I and of mammary gland Ca^{2+} RNase IIb are approximately 52 500 and 32 400, respectively, as determined by gel filtration on Sephadex G-100.

Optimal Ion Concentration. The optimal Ca^{2+} concentration range for Ca^{2+} RNases I, IIa, and IIb was approximately 5–10 mM (Figure 4). The plateau in activity of Ca^{2+} RNase I between 2 and 20 mM Ca^{2+} was apparently due to a high enzyme to substrate ratio. At Ca^{2+} concentrations above 20 mM, the activity was decreased. The enzymes in the original nuclei or nucleoli-free nuclear sap required a much lower Ca^{2+} concentration (0.5 mM) to achieve optimal activity, probably because of endogenous Ca^{2+}. In the usual assay, using the amount of enzyme needed to maintain linearity, the enzyme activity was very low in 5 mM Mg^{2+} and without Ca^{2+}. However, the activity in 0.55 mM Mg^{2+} was about 2 times as much as that in 5 mM Mg^{2+}.

Stability of Ca^{2+} RNases. The Ca^{2+} RNases in the unfractionated nuclear sap were stable at −20 °C. Exhaustive dialysis of the nuclear sap against solution STD resulted in decreasing amounts of Ca^{2+} RNase activities. When dialysis was followed by DEAE-cellulose chromatography, some Ca^{2+} RNase activity was found in the fractions eluting before the salt gradient. Dialysis of R3230AC tumor Ca^{2+} RNase I against 20 mM Ca^{2+} resulted in the retention of even smaller amounts of activity on the column. This phenomenon was time dependent and was partially reversible when the sample was re-dialyzed in the absence of Ca^{2+} (Liao, 1976).

Partially purified Ca^{2+} RNases I and IIb, which had been concentrated against dry sucrose, were also stable at −20 °C. However, the nonconcentrated solutions of both Ca^{2+} RNases I and IIb in fractions eluted from DEAE-cellulose columns

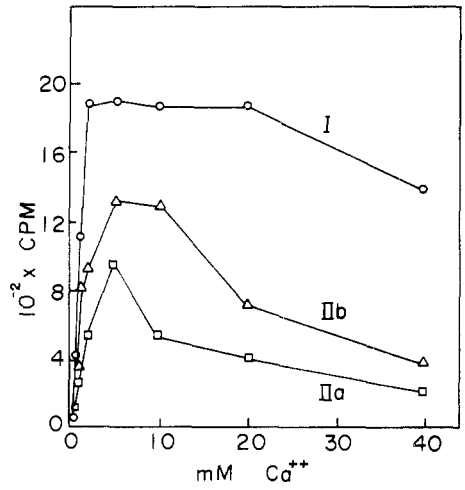

FIGURE 4: Effects of Ca^{2+} concentration on the activity of rat mammary gland and tumor Ca^{2+} RNases. The enzyme samples were post-DEAE-cellulose. The activity was assayed by using [^{3}H]poly(U) as described under Materials and Methods except with different Ca^{2+} concentrations as indicated. The activity represents the amount of soluble nucleotides produced in 15 min. The amounts of protein used in the assays were: Ca^{2+} RNase I, 0.4 μg; Ca^{2+} RNase IIa, 1.34 μg; and Ca^{2+} RNase IIb, 1.75 μg.

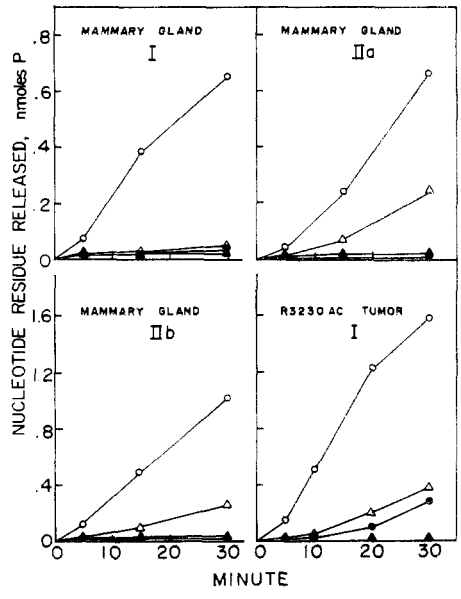

FIGURE 5: Substrate specificity of rat mammary gland and tumor Ca^{2+} RNases. The post-DEAE-cellulose Ca^{2+} RNases I, IIa, and IIb from mammary gland contained, respectively, 0.4, 1.34, and 1.75 μg of protein in the assay. Ca^{2+} RNase I from tumor contained 6.6 μg of protein. Each assay contained in 200 μL: 0.1 M Tris-HCl (pH 7.4), 0.6 mM dithiothreitol, 5 mM $CaCl_2$, 10 μL of enzyme, and 2.24 nmol of nucleotide phosphate of ^{3}H-labeled homoribopolymer: (○) poly(U), 28 400 cpm; (▲) poly(G), 79 000 cpm; (△) poly(C), 73 300 cpm; and (●) poly(A), 67 500 cpm. Activity is expressed as nanomoles of ethanol-soluble nucleotide residues released during the indicated times at 37 °C.

were less stable. Storing dilute solutions of Ca^{2+} RNases I and IIb for 1 week at 4 °C resulted in almost total loss of activity. Ca^{2+} RNase IIa was even more labile; after 2 days at 4 °C, no activity was found. The enzymes were unstable above pH 8. Overnight storage at pH 8 of Ca^{2+} RNases I and IIb at −20 °C resulted in total loss of Ca^{2+} RNase activities. Ca^{2+} RNase I was not stable in H_2SO_4; at 4 °C in 0.1 N H_2SO_4, 95% of the activity was lost. Ca^{2+} RNase I is most active at pH 7 at 37 °C. The activity decreased above 40 °C and more than 95% of the activity was lost at 65 °C in 2 min.

Substrate Specificity of Ca^{2+} RNases I and IIb. Measurements on intact nuclei and nucleoli-free nuclear sap showed that Ca^{2+} RNase could catalyze hydrolysis of both yeast RNA and rat liver ^{3}H-labeled 45S RNA yielding ethanol-soluble nucleotides or oligonucleotides (Tables I and II). The use of [^{3}H]poly(U) as substrate in the assay during the partial purification steps (Figures 2 and 3) indicated that the enzymes were active against uridine nucleotide moieties. In order to compare the relative activity toward nucleotide chains of various bases, we used a fixed amount of enzyme and an amount of the four homopolyribonucleotides (2.24 nmol of nucleotide phosphate) which was ninefold more concentrated than in the standard assay. The results showed that the relative activity of all the Ca^{2+} RNases against the homopolymers was poly(U) > poly(C) > poly(A) > poly(G) (Figure 5). The finding that Ca^{2+} RNase I of the mammary gland was much more active toward poly(U) than toward poly(C), while Ca^{2+} RNases IIa and IIb were fairly active toward [^{3}H]poly(C), confirmed that these were different enzymes. In addition, only tumor Ca^{2+} RNase I had significant activity toward [^{3}H]poly(A), although the activity was less than that toward the two pyrimidine-based homopolymers. None of the enzymes was active toward [^{3}H]poly(G). When tumor Ca^{2+} RNase I was tested against ^{3}H-labeled poly(dT), DNA–RNA hybrids, ribosomal RNA, and DNA (either single or double stranded) no ethanol-soluble radioactivity was found.

Endonuclease Action of Ca^{2+} RNases I and IIb. In order to determine whether the Ca^{2+} RNases cleave polyribonucleotides at termini or at internal sites of the polynucleotide chain, we used sucrose density gradient centrifugation to analyze the products of the enzyme-catalyzed reactions. The size distributions of Ca^{2+} RNase I catalyzed breakdown products of ^{3}H-labeled 45S RNA at 0, 5, 15, and 60 min are shown in Figure 6. The same result was obtained with either mammary gland or tumor Ca^{2+} RNase I. At the end of the 5-min incubation, almost all of the original size molecules had disappeared and shifted to smaller-sized molecules in the gradient, but at this time with less than 3% of total radioactivity rendered ethanol soluble no mononucleotides were observed by separating on DEAE-cellulose in 7 M urea. After 15 and 60 min, while only 7 and 29%, respectively, of the activity was ethanol soluble, almost all the RNA molecules were reduced to sizes less than 18 and 4 S, respectively. The reaction catalyzed by Ca^{2+} RNase IIb produced a similar result. From these results showing the rapid shift to smaller RNA sizes after enzymatic action and that no detectable mononucleotides are produced within 5 min of incubation, we conclude that both Ca^{2+} RNases I and IIb are endoribonucleases.

Product Analysis by PEI-Cellulose Thin-Layer Chromatography. When these enzymes catalyze the depolymerization of [^{3}H]poly(U) and [^{3}H]poly(C), the products of the reaction are oligonucleotides with 3′-phosphate termini and cyclic 2′,3′-phosphate mononucleotides. Evidence to support these conclusions was obtained using PEI-cellulose TLC. It was demonstrated that a time-dependent accumulation of oligonucleotides and 2′,3′-cyclic CMP resulting from Ca^{2+} RNase I action on [^{3}H]poly(C) occurred. Similar results were obtained using [^{3}H]poly(U) as substrate with either mammary gland Ca^{2+} RNase IIb or tumor Ca^{2+} RNase I. When the ethanol-soluble fraction from a reaction mixture was incubated at 37 °C for 1 h in 0.1 M HCl and then neutralized with NaOH before PEI-cellulose TLC, the cyclic nucleotide was converted to 3′-CMP. After separation on DEAE-cellulose in 7 M urea, the dinucleotide products of the enzyme-catalyzed

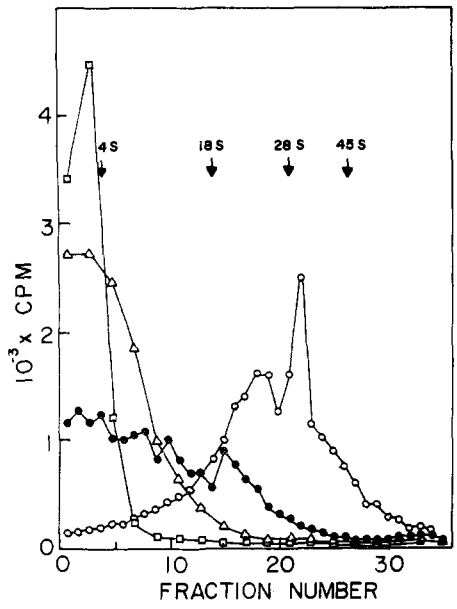

FIGURE 6: Product analysis. Sucrose density gradient centrifugation of ^{3}H-labeled 45S RNA breakdown products catalyzed by rat mammary gland Ca^{2+} RNase I. ^{3}H-Labeled 45S RNA was isolated from rat liver and used as the substrate for Ca^{2+} RNase I. Seventy-five micrograms of ^{3}H-labeled 45S RNA containing 22 700 cpm was reacted with 1 μg of Ca^{2+} RNase I in 0.1 M Tris-HCl (pH 7.4), 0.6 mM dithiothreitol, and 5 mM Ca^{2+}. Incubations were at 37 °C for the indicated times after which the reaction mixture was precipitated overnight with 2.5 vol of ethanol–10 mM $Mg(OAc)_2$ at −20 °C. The ethanol-soluble counts were measured and the results are described in the text. The precipitate was dissolved in a small volume of water and layered on a 10–40% sucrose gradient containing 100 mM NaCl and 10 mM NaOAc (pH 5.1). Centrifugation was for 16 h in an SW27 rotor at 100 000*g*. Fractions (0.5 mL) were collected and the radioactivity was determined by liquid scintillation counting as described under Materials and Methods. RNA standards of 4, 18, 28, and 45 S were isolated from rat liver (Smith et al., 1974) and their positions in the gradients are indicated in the figure. The symbols are: (○) 0-min incubation; (●) 5 min; (△) 15 min; and (□) 30 min. It was noted that the 45S RNA was partially degraded during storage even without incubation.

reaction of [^{3}H]poly(U) were not further broken down by snake venom phosphodiesterase. However, they were converted by spleen phosphodiesterase to 3′-UMP indicating that the dinucleotide existed in the form UpUp. Within short periods of enzymic action the products were predominantly oligonucleotides with varying lengths and a small amount of 2,3′-cyclic mononucleotides, further suggesting that the enzymes acted as endonucleases.

Inhibition of Ca^{2+} RNases by Various Compounds. In order to compare the relative inhibition by various compounds on Ca^{2+} RNases I and IIb, we express data as the concentration of compound which produces 50% inhibition (Table IV). Putrescine was a less potent inhibitor than spermine or spermidine. Compared to Ca^{2+} RNase IIb, Ca^{2+} RNase I was less inhibited by polyamines. At final concentrations of less than 2 mM under standard assay conditions, less than 50% of the original Ca^{2+} RNase IIb activity remained, while more than 2 mM was required to inhibit 50% of Ca^{2+} RNase I activity. Heparin is a potent inhibitor of both Ca^{2+} RNases I and IIb. At 3×10^{-3} mM, less than 10% of Ca^{2+} RNase IIb activity and about 50% of Ca^{2+} RNase I activity remained. Similarly, when polydextran sulfate was incubated with tumor Ca^{2+} RNase I, less than 50% activity remained even at 8×10^{-6} mM. Both Ca^{2+} RNases I and IIb are sensitive to increasing concentration of salts and sulfhydryl blocking agents. Sodium fluoride, sodium pyrophosphate, and *p*-chloromercuribenzenesulfonate at concentrations above 5 mM are strongly inhibitory. Sodium chloride, ammonium sulfate, and potassium phosphate above 100 mM are also strong inhibitors of tumor Ca^{2+} RNase I.

TABLE IV: Inhibition of Ca^{2+} RNases by Various Compounds.[a]

Compound	Concn (mM) which inhibits 50% of act.		
	Mammary gland		Tumor
	I	IIb	I
Spermine	2.5	0.8	2.0
Spermidine	4.0	1.4	9.5
Putrescine	32.0	7.0	38.0
Heparin	3×10^{-3}	1×10^{-3}	n.a.
Polydextran sulfate	n.a.	n.a.	8.2×10^{-6}
p-Chloromercuribenzenesulfonate	0.8	n.a.	4.0
NaF	n.a.	n.a.	2.4
Sodium pyrophosphate	n.a.	n.a.	1.3

[a] The amounts of each Ca^{2+} RNase used in the assay were the same as those used in Figure 5. The standard assay described under Materials and Methods (using [^{3}H]poly(U) in the presence of 5 mM Ca^{2+}) was used except that a series of different amounts of each inhibitor was included in the assay. The activity values were plotted against inhibitor concentrations. The values in the table are the inhibitor concentrations which produce 50% inhibition and were obtained from the inhibition curve. n.a. indicates not assayed.

Discussion

There is an increased requirement for calcium during lactation, not all of which can be accounted for by increased output in milk and urine (Nelson and Evans, 1961). Also there apparently is an increased tissue demand for calcium during lactation, the reasons for which are not entirely understood, so that it is of more than passing interest that the mammary gland contains calcium stimulated ribonucleases that apparently increase in activity during lactation (unpublished work). Of the numerous mammalian ribonucleases that have been studied, many seem to require no divalent cation (Mirault and Scherrer, 1972; Winicov and Perry, 1974; Goodlad and Ma, 1975; Elson and Glitz, 1975; Kuciel and Ostrowski, 1975; Poels, 1976), while many others require Mg^{2+} in the assay (Lazarus and Sporn, 1967; Liau et al., 1968; Sporn et al., 1969; Kelley and Perry, 1971; Boctor et al., 1974; Kwan et al., 1974; Cordis et al., 1975; Rech et al., 1976). It is not clear whether these enzymes have an absolute requirement for Mg^{2+} or whether they are simply stimulated by the cation. At any rate, the discovery of Ca^{2+} RNase in mammary gland and a mammary tumor points the way to future, more extensive, studies on the effects of divalent cations on tissue-specific ribonucleases under various physiological conditions.

The nuclear Ca^{2+} endonucleases of the mammary gland and tumor release 3′-phosphoryl oligonucleotides and show preference for pyrimidine bases. Thus, these enzymes are different from the nuclear endonuclease reported recently by Cordis et al. (1975) which releases 5′-phosphoryl oligonucleotides. The Ca^{2+} RNases reported here are similar to the enzyme from nucleoli which releases 3′-phosphoryl oligonucleotides (Winicov and Perry, 1974). The major breakdown products of nuclear heterogeneous RNA apparently are 5′-phosphoryl nucleotides (Hurlbert et al., 1973); in addition, the majority of newly synthesized polynucleotides never reach the cytoplasm (Weinberg, 1973). Thus, it is attractive to suggest that the RNases which are able to release 5′-phosphoryl nucleotides

are responsible for processing and salvaging of the nucleotides. Since the Ca^{2+} RNases from mammary gland and mammary tumor produce 3′-phosphoryl oligonucleotides as a result of their catabolic action, it might be that the enzymes are associated with disposal of polynucleotide materials. Since the 3′-phosphate ends produced by the endonucleases are resistant to 3′-hydroxyl specific exonuclease activity, it is also possible that the enzymes might have a stabilizing as well as a processing role (Winicov and Perry, 1974).

The two nuclear Ca^{2+} RNases we reported here apparently are distributed extranucleolarly. This conclusion is based upon the greater activity and Ca^{2+}/Mg^{2+} activity ratio found in the nucleoli-free nuclear sonicate than that in the nucleolar fraction. Ca^{2+} RNase I is apparently present in nonbound form in the nucleoplasm while Ca^{2+} RNase IIb is firmly bound either to membranes or to chromatin. The smaller amount of Ca^{2+} RNase present in the nucleolar fraction could be due to redistribution of the enzymes during the separation steps. Extraction of the nucleolar Ca^{2+} RNase and separation on a DEAE-cellulose column revealed that most of the activity was due to Ca^{2+} RNase I (unpublished results). We also found Ca^{2+} RNase activities present in other cellular fractions including mitochondrial, microsomal, and 105 000*g* supernatant fractions. Since we isolated nuclei by a hypertonic sucrose procedure and the isolated nuclei were washed with a medium containing Triton X-100, the isolated nuclei were devoid of outer nuclear membranes and attached pieces of endoplasmic reticulum membrane; thus, it seems unlikely that there would be any major contamination by extranuclear RNases. When the RNase in the 105 000*g* supernatant was separated by DEAE-cellulose chromatography and assayed in the presence of Ca^{2+}, most of the activity was eluted at an NaCl concentration between 0.13 and 0.30 M with a 0.20 M peak which is higher than both nuclear Ca^{2+} RNases I and IIb (unpublished results).

The two major nuclear Ca^{2+} RNases in the nucleoli-free nuclear sonicate could also be extracted at pH 8 in Tris-HCl buffer followed by ammonium sulfate fractionation similar to that of Lazarus and Sporn (1967). The results of DEAE-cellulose chromatography showed one minor and two major enzyme activity peaks occurring at 0, 0.08, and 0.15 M NaCl which correspond to Ca^{2+} RNases IIa, IIb, and I, respectively. Little or no activity was detected in these fractions when assayed in the presence of 5 mM Mg^{2+} and poly(A). Since the enzymes were labile at pH 8, the recovery of enzyme activity was inconsistent from experiment to experiment. The presence of other more labile forms of Ca^{2+} RNase in fractions recovered following DEAE-cellulose chromatography (Figures 2 and 3) emphasizes the complex nature of this class of enzymes and indicates that further detailed studies on their exact cellular distribution are needed.

The nuclear Ca^{2+} RNases are also unstable to sulfhydryl blocking agents, dilute sulfuric acid, and heat, thus distinguishing them from the cytoplasmic alkaline RNase which, like the pancreatic RNase, is resistant to such treatments. Moreover, those enzymes do not require divalent ions for activity (Razzell, 1963). The difference between the Ca^{2+} RNases and the cytoplasmic alkaline RNase or pancreatic RNase apparently extends to their substrate specificity as well. Although the above enzymes produce oligonucleotides possessing 3′-phosphate at termini, the Ca^{2+} RNases were slightly active toward poly(A) and not at all toward yeast phenylalanyl-tRNA while pancreatic RNase readily degraded phenylalanyl-tRNA (unpublished results). We have reported that levels of mammary gland cytoplasmic alkaline RNase do not change greatly during mammary gland development while the cytoplasmic RNase inhibitor increases greatly in the pregnant and lactating gland, and is abundant in the R3230AC tumor (Liu et al., 1975). Preliminary results show that this inhibitor has little effect on Ca^{2+} RNase activity.

In summary, we have detected RNase activities which could be stimulated by Ca^{2+} in mammary gland nuclei of lactating rats and in a transplantable rat mammary tumor. These enzyme activities are very low without Ca^{2+}, and substitution with other divalent ions such as Mg^{2+} or Mn^{2+} does not stimulate the activity. To our knowledge, this is the first demonstration of mammalian RNases which require Ca^{2+} for maximal activity.

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Purification and Some Properties of 4-Hydroxyphenylpyruvate Dioxygenase from *Pseudomonas* sp. P.J. 874†

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ABSTRACT: 4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) has been purified to apparent homogeneity from *Pseudomonas* sp. P.J. 874, induced to grow on tyrosine as the sole source of carbon. The enzyme protein was a tetramer of equally sized subunits, each with a mass of 36 kdaltons and the NH_2-terminal amino acid sequence Ala-Asp-Leu/Ile-Tyr-. It had a molecular mass of 150 kdaltons, a sedimentation coefficient in water at 20 °C of 7.6 S, and a Stokes radius of 4.9 nm. The isoelectric point was around pH 4.8. The enzyme protein showed a broad absorbance in the blue region of the visible spectrum. The purified enzyme protein contained variable amounts of iron, 0.6 to 1.3 mol/mol, and copper, 0.2 to 0.5 mol/mol. ^{59}Fe included in the culture medium followed enzyme activity through the purification. Inhibition experiments with metal chelators indicated an essential role for enzyme-bound Fe^{2+}. An optimized combination of ascorbate (50 mM), catalase (2.4 g/L), and iron (50 μM) activated the enzyme more than a combination of 2,6-dichlorophenolindophenol, glutathione, and catalase. The former system was also more effective than the latter in protecting the enzyme from inactivation at 37 °C. No stimulation by iron was observed when 2,6-dichlorophenolindophenol was the reductant. Reduced 2,6-dichlorophenolindophenol and ascorbate were hyperbolic uncompetitive activators when 4-hydroxyphenylpyruvate was the variable substrate. The enzyme showed a constant ratio of 4-hydroxyphenylpyruvate to phenylpyruvate activity during purification. One optimum around pH 7 was observed for both activities at 37 °C. The apparent Michaelis constant was 30 μM for 4-hydroxyphenylpyruvate and 0.52 mM for phenylpyruvate at pH 7.5 in the presence of 2,6-dichlorophenolindophenol (0.15 mM), glutathione (10 mM), and catalase (0.8 g/L). The corresponding values of the apparent maximal velocity were 900 and 20 nmol min^{-1} (mg of protein)$^{-1}$. The apparent Michaelis constant and maximal velocity were 0.15 mM for 4-hydroxyphenylpyruvate and 20 μmol min^{-1} (mg of protein)$^{-1}$, respectively, in the presence of the optimized combination of ascorbate, catalase, and iron. Substrate inhibition was observed at high concentrations of both 4-hydroxyphenylpyruvate and phenylpyruvate.

4-Hydroxyphenylpyruvate is enzymically converted to homogentisate by 4-hydroxyphenylpyruvate dioxygenase (4-hydroxyphenylpyruvate:oxygen oxidoreductase (hydroxylating, decarboxylating) (EC 1.13.11.27)). Hitherto, only the enzymes from human (Lindblad et al., 1971; Lindblad et al., 1977), chicken (Fellman et al., 1972a), and bovine (Nakai et al., 1975) liver have been obtained in highly purified forms. Both the human and the chicken liver enzymes have been resolved into multiple forms (Lindblad et al., 1972; Wada et al., 1975; Rundgren, 1977a). The proposed reaction mechanisms, which at present are seriously considered (Goodwin and Witkop, 1957; Lindblad et al., 1970; Hamilton, 1971), are closely related to those suggested for the 2-oxoglutarate-dependent oxygenases (Holme et al., 1968; Lindblad et al., 1969; Hamilton, 1971; for a review see Abbott and Udenfriend, 1974). In these mechanisms a transition-metal ion has been implicated for the activation of oxygen. Evidence for the presence of enzyme-bound copper or iron in 4-hydroxyphenylpyruvate dioxygenase has accumulated (Goodwin, 1972; Goodwin and Werner, 1973; Laskowska-Klita and Mochnacka, 1973; Wada et al., 1975; Lindblad et al., 1977). The enzymes from various sources have shown a requirement for catalase and a reductant, reduced 2,6-dichlorophenolindophenol being most effective (Goodwin, 1972; Laskowska-Klita and Mochnacka, 1973; Nakai et al., 1975; Lindblad et al., 1977). The stimulation by the naturally occurring reductant, ascorbate, is reported to decrease with purification (Zannoni, 1962). It has been reported that reductants prevent and reverse both the inactivation by storage and the inhibition by high concentrations of 4-hydroxyphenylpyruvate (Goodwin, 1972). This paper describes the purification of 4-hydroxyphenylpyruvate dioxygenase from a *Pseudomonas* strain, which has been isolated by enrichment culture (Midtvedt et al., unpub-

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