The ribonucleases of bovine skeletal muscle

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Bovine skeletal muscle contains small amounts of at least six heat- and acid-stable RNA-degrading enzymes. Our results are the first evidence for multiple ribonucleases in skeletal muscle. Three of these have been highly purified, and each has been shown to be a pyrimidine-specific endoribonuclease by use of a rapid sequencing technique employing gel electrophoresis. However, synthetic co-polymers containing adenylate or guanylate residues in addition to pyrimidine residues are hydrolysed at higher rates than are the pyrimidine homopolymers. With 0.63 mM yeast RNA as substrate, all three enzymes (ribonucleases I, II and III) are optimally active in alkaline solution (pH 7.5–8.5) containing 0.05–0.15 M univalent salts, do not require bivalent cations, and have molecular weights of 13 000–20 000. The properties of muscle ribonuclease I are very similar to those of bovine pancreatic ribonuclease A. Muscle ribonucleases II and III have characteristics similar to those of ribonucleases found in various other bovine tissues. In common with all previously studied pyrimidine-specific endoribonucleases, the bovine muscle ribonucleases are inhibited by such purine homopoly-nucleotides as polyadenylate. Furthermore, polyamines, present in low concentrations, can reverse or regulate the amount of inhibition of enzyme activity.

Although enzymic activity against RNA has been detected in crude extracts or slightly purified preparations of skeletal muscle from a variety of sources (Tomlinson, 1958, 1959; Tappel *et al.*, 1962; Zalkin *et al.*, 1962; Abdullah & Pennington, 1968; Little & Meyer, 1970; Goldspink & Pennington, 1971; Meyer *et al.*, 1972; Little *et al.*, 1973; Kakulas & Meyer, 1976; Kershner & Meyer, 1976; Kitavtsev *et al.*, 1977), the RNA-degrading enzymes of this tissue have not been studied extensively. To date, only one such enzyme, an alkaline RNAase from human skeletal muscle, has been significantly purified and characterized (Goldspink & Pennington, 1970). The paucity of information on these enzymes is surprising in view of work showing that changes in muscle RNA-degrading activity occur in various diseases as well as after drug treatment. Mice, chickens and humans with congenital muscular dystrophy have increased activities of RNA-degrading enzymes in skeletal muscle (Tappel *et al.*, 1962; Abdullah & Pennington, 1968; Goldspink & Pennington, 1971; Little *et al.*, 1973; Kakulas & Meyer, 1976). Rabbits with vitamin E deficiency have increased activities of muscle lysosomal enzymes, including an activity

against RNA (Zalkin *et al.*, 1962). A decrease in RNA-degrading activity has been found in muscle from glucocorticoid-treated mice (Kershner & Meyer, 1976). Since most of these studies have dealt with crude extracts of muscle, the nature of the enzymic activity is ill-defined, and an attempt to better characterize the ribonucleases of skeletal muscle appears justified. Bovine muscle was selected for study because RNAases from other tissues of this animal have been examined, and it was decided to determine whether similarities exist among the skeletal-muscle enzymes and RNAases derived from these other tissues.

In the present paper we show that bovine skeletal muscle contains multiple heat- and acid-stable RNA-degrading enzymes, of which three could be sufficiently purified for meaningful characterization. The quantities of these enzymes present in the bovine tissue were very low, and purifications beginning with kilogram quantities of muscle yielded only microgram amounts of enzyme. Although limited quantities of each RNAase, in terms of protein, were available after a lengthy purification procedure, the specific activities of the enzymes were high. In addition, results obtained by the use of polynucleotide/polyacrylamide-gel electrophoresis, a technique described in the following paper

Abbreviations used: RNAase, ribonuclease; DNAase, deoxyribonuclease.

(Karpetsky *et al.*, 1980), demonstrated the RNAase homogeneity of each of the purified enzymes.

The use of a 5'-labelled *Escherichia coli* 5S rRNA fragment as substrate permitted rapid determination of specificity by polyacrylamide-gel electrophoresis of the products of enzymic digestion (Donis-Keller *et al.*, 1977). We also examined the purine homopolynucleotide-induced inhibition of the muscle enzymes in further attempts to define the generality of this type of inhibition. Regulation of the amount of inhibition was achieved by varying the quantity and identity of polyamine present. The interaction of a polypurine such as poly(A) with pyrimidine-specific endoribonucleases would appear to delineate one possible function of 3'-terminal poly(A) in mRNA stabilization (Levy, 1975; Levy *et al.*, 1975; Hieter *et al.*, 1976; Karpetsky *et al.*, 1979).

Experimental

Materials and general methods

Synthetic polynucleotides were the products of Miles Laboratories, Elkhart, IN, U.S.A. RNA was prepared from yeast (Crestfield *et al.*, 1955). Coomassie Blue, spermidine trihydrochloride, spermine tetrahydrochloride, calf thymus DNA (type I), electrophoretically pure bovine pancreatic DNAase I and β -alanine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Mononucleotides and agarose-poly(A) were from P-L Biochemicals, Milwaukee, WI, U.S.A. Sephadex products and Ficoll-400 were supplied by Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A., and microgranular CM-cellulose was from Whatman, Maidstone, Kent, U.K. Bovine pancreatic RNAase A was the product of Worthington Biochemical Corp., Freehold, NJ, U.S.A. Protein standards for molecular-weight determinations were from Worthington Biochemical Corp. or Sigma Chemical Co. Dialysis tubing (8000-mol.wt. cutoff, from Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) was treated before use to remove impurities (McPhie, 1971).

Enzyme and protein assays

The standard reaction mixture (1 ml) contained 20 mM-Tris/HCl buffer, pH 7.5, 100 mM-NaCl, 0.63 mM yeast RNA and enzyme. After 15 min at 37°C, the reaction was stopped with 1 ml of cold 12% (v/v) HClO₄ containing 20 mM-La(NO₃)₃. After standing for 10 min at 4°C, the solution was centrifuged at 1000g for 7 min and the supernatant was removed for measurement of absorbance at 260 nm. Under these conditions, 1 unit of enzyme activity is defined as an increase of 1 A_{260} unit in 15 min.

DNAase activity was assayed by the method

described for RNAase, except that the reaction mixtures contained 100 mM-Tris/HCl buffer, pH 7.5, 0.25 mg of calf thymus DNA/ml, 5 mM-MgCl₂, 0.1 mM-CaCl₂ and enzyme. An increase of 1 A_{260} unit in 15 min is defined as 1 unit of enzyme activity.

Phosphodiesterase and phosphomonoesterase activities were measured by using modifications of published procedures (Sinsheimer & Koerner, 1952; Garen & Levinthal, 1960). Reaction mixtures contained, in addition to 100 mM-Tris/HCl buffer, pH 7.5, 100 mM-NaCl and enzyme, either 1.0 mM-sodium bis-*p*-nitrophenyl phosphate or 1.0 mM-disodium *p*-nitrophenyl phosphate and 10 mM-MgCl₂.

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Yeast RNA concentration is expressed as the concentration of RNA P as determined by assay (Carrara & Bernadi, 1968) after ashing the polynucleotide in Mg(NO₃)₂ (Ames & Dubin, 1960). Synthetic polynucleotide concentration is similarly expressed but was calculated from absorption coefficients provided by the manufacturer.

Molecular-weight determinations

Each of the purified RNAases (50–200 units in 1 ml) was chromatographed on a column (2.6 cm × 33 cm) of Sephadex G-100 (superfine grade) by the method of Andrews (1964). Ovalbumin, bovine pancreatic RNAase A, DNAase I, chymotrypsinogen and horse heart cytochrome *c* were used as standards. RNAase III (5–20 units in 0.1 ml) was centrifuged in a linear sucrose gradient by the method of Martin & Ames (1961). The gradient contained 5–20% (w/v) sucrose, 10 mM-Tris/HCl buffer, pH 7.5, and 200 mM-NaCl. By using the approximation $s_1/s_2 = [(M_r \text{ of } 1)/(M_r \text{ of } 2)]^{1/3}$ (Martin & Ames, 1961), the migration of the muscle RNAase was compared with those of lysozyme, ovalbumin, chymotrypsinogen and bovine pancreatic RNAase A at 0.5 mg/ml. The reported molecular weight of RNAase III is the average of the four values thus obtained.

Products of enzymic digestion of poly(U)

A 15 mg sample of poly(U) was digested with 160 units of bovine muscle RNAase in 10 ml of 100 mM-Tris/HCl buffer, pH 7.5. After 12 h at 37°C, the digest was deproteinated with chloroform/octanol (9:1, v/v) and extracted six to eight times with diethyl ether. Residual ether was evaporated with a stream of air, and a portion of the digest (10 μ l) was applied to Whatman no. 3MM paper for ascending chromatography in ethanol/1.0 M-ammonium acetate (pH 7.5) (7:3, v/v) (P-L Biochemicals, 1956). Mononucleotides were located by comparison with standards (5 μ g of cyclic 3':5'-UMP and cyclic 2':3'-UMP) run in parallel. The identity of each mononucleotide was confirmed in two ways: (1)

rechromatography in the above solvent as well as in 0.1 M-sodium phosphate buffer (pH 6.8)/(NH₄)₂SO₄/propanol-1-ol (50:30:1, v/w/v) (P-L Biochemicals, 1956); (2) chromatography after hydrolysis of cyclic mononucleotides by acidification to pH 1 with HCl (30 min at 37°C).

The remainder of the RNAase-catalysed digest of poly(U) from the above experiment was adjusted to pH 1 with HCl, left for 30 min at 37°C, neutralized with NaOH, diluted 10-fold with water, and applied to a column (1.5 cm × 30 cm) of DEAE-Sephadex A-25 (carbonate form in water). The column was washed with several volumes of 0.02 M-(NH₄)₂CO₃, and the digestion products were eluted with a linear gradient (1800 ml) of 0.02–1.0 M-(NH₄)₂CO₃. Fractions of volume 10 ml were collected and the absorbances of each fraction was measured at 260 nm. Oligonucleotide chain length was determined by the position in the elution profile as well as by the ratio of total to terminal P (Carrara & Bernardi, 1968).

Polyacrylamide-gel electrophoresis

The purified RNAases were concentrated about 50-fold in dialysis tubing surrounded by Ficoll-400 and analysed in polyacrylamide gels (10% acrylamide) in the acidic buffer system of Reisfeld *et al.* (1962) with the sample gel omitted. Gels were stained with Coomassie Blue (Weber & Osborn, 1969) or cut into 28 equal segments that were each soaked overnight in 500 mM-Tris/HCl buffer, pH 7.5. The standard assay procedure was utilized to measure RNAase activity.

Enzyme purification

All steps except the heat and acid treatment were done at 4°C. Except where noted, 20 mM-Tris/HCl, pH 7.5, was utilized as buffer. CM-Sephadex C-50 and CM-cellulose columns were prepared in this buffer containing 40 mM-NaCl. All gradients were linear. Poly(A)-agarose was used as described previously for poly(G)-agarose (Levy *et al.*, 1973), except that elution was by gradient rather than by steps. For regeneration, poly(A)-agarose was removed from the column, washed with 1.0 M-NaCl and re-equilibrated with buffer.

Step 1: crude extract. Fresh bovine skeletal muscle (thigh or cheek) was obtained from a slaughterhouse. A 3 kg portion of muscle was cut into small pieces and homogenized for 45 s in 12 litres of 2% (w/v) KCl in a Waring blender at medium speed. The homogenate was centrifuged at 25 000 g for 20 min, and the supernatant solution was poured through several layers of cheesecloth to remove floating particles of fat.

Step 2: (NH₄)₂SO₄ fractionation. The pH of the crude extract obtained above was adjusted to 7.5 with 12 M-NaOH, and solid (NH₄)₂SO₄ was added to

give a final concentration of 1.0 M. The mixture was left to stand for 6 h and was then centrifuged at 25 000 g for 15 min. The precipitate was discarded, and additional (NH₄)₂SO₄ was added to the supernatant solution to give a final concentration of 2.6 M. After standing overnight, the mixture was centrifuged, and the precipitate was thoroughly mixed with 1.0 litre of water to give a muddy suspension.

Step 3: heat and acid treatment. The pH of the suspension was adjusted to 5.0 by the addition of a few millilitres of 17 M-acetic acid with vigorous stirring. The material was heated (at 60°C for 15 min) and the resulting voluminous precipitate was removed by centrifugation at 25 000 g for 30 min. The supernatant solution was then adjusted to pH 4.0 with a few millilitres of 17 M-acetic acid, heated at 75°C for 20 min and centrifuged as before. This supernatant solution was adjusted to pH 7.5 with 12 M-NaOH, dialysed for 12 h against four changes (10 litres each) of 40 mM-NaCl in buffer, and filtered under suction through Whatman no. 42 paper to give a clear yellow solution.

Step 4: first CM-Sephadex column. The heat- and acid-treated material was applied to a column (2.6 cm × 33 cm) of CM-Sephadex, and, on washing with several volumes of 40 mM-NaCl in buffer, all but a few percent of the RNAase activity was retained by the column. Enzyme was eluted with a linear gradient (1600 ml) of 0.04–0.5 M-NaCl in buffer. Fractions of volume 8 ml were collected and assayed for activity against yeast RNA and poly(C) to locate the three major RNAases (Fig. 1). RNAase I was always well separated from RNAases II and III; however, the resolution of RNAases II and III shown in Fig. 1 was not consistently obtained at this step, and these two enzymes were usually combined. Fractions containing RNAase I were pooled and dialysed for 8 h against three changes (4 litres each) of 40 mM-NaCl in buffer. Fractions containing RNAases II and III were pooled and similarly dialysed against 0.1 M-NaCl in buffer.

Step 5: second CM-Sephadex column. (a) RNAase I was applied to a column (1.5 cm × 25 cm) of CM-Sephadex and eluted with a linear gradient (800 ml) of 0.04–0.4 M-NaCl in buffer. Fractions of volume 5 ml were collected, and active fractions were combined. (b) Combined RNAases II and III were applied to a column (1.5 cm × 25 cm) of CM-Sephadex and eluted with a linear gradient (1200 ml) of 0.1–0.8 M-NaCl in buffer. Fractions of volume 6 ml were collected, and those containing RNAase II were pooled, as were those containing RNAase III. Fractions containing both of these enzymes were excluded.

Step 6: third CM-Sephadex column (RNAases II and III only). Pooled fractions of either RNAase II or III were diluted with 2 vol. of buffer, applied to columns (1.5 cm × 25 cm) of CM-Sephadex and

Fig. 1. *Elution of bovine muscle RNAases from the first CM-Sephadex column (step 4 of the purification procedure)* Heat- and acid-treated enzyme was applied to a column (2.6 cm $\times$ 33 cm) of CM-Sephadex. After washing of the column with 20 mM-Tris/HCl buffer, pH 7.5, containing 0.04 M-NaCl (160 ml), the enzymes were eluted by using a linear gradient (1600 ml) of 0.04–0.50 M-NaCl in 20 mM-Tris/HCl buffer, pH 7.5 (Δ). Fractions of volume were collected and RNAase activity was measured by using the standard assay procedure except that individual reaction mixtures contained 630 μ mol of either yeast RNA ($\bullet$) or poly(C) ($\square$). Experimental details are given in the text.

Fig. 2. *Poly(A)-agarose chromatography of bovine muscle RNAase III*

The enzyme, purified through to step 7 of the isolation procedure (see the Results section for details), was applied to a column (0.9 cm $\times$ 12 cm) of poly(A)-agarose and eluted with a gradient of 0.1–0.8 M-NaCl in 20 mM-Tris/HCl buffer, pH 7.5. The concentration of NaCl (Δ) is depicted in the region of enzyme activity, which was measured with yeast RNA as substrate ($\bullet$). The absorbance at 280 nm of individual fractions (O) is also shown. Experimental details are given in the text.

eluted with linear gradients (1000ml) of 0.1–0.8M-NaCl in buffer. Fractions of volume 5 ml were collected.

Step 7: Sephadex-gel filtration. Columns of Sephadex G-75 (for RNAase I) and G-100 (for RNAase II or III), 5cm × 100cm in size, were equilibrated with 0.1M-NaCl in buffer. Each RNAase preparation was concentrated to less than 75 ml in dialysis tubing surrounded by Ficoll-400. Each of the three enzymes was chromatographed on the appropriate column with an elution rate of 60–80 ml/h. Fractions of volume 10 ml were collected.

Step 8: CM-cellulose column (RNAase II only). Pooled fractions containing RNAase II were diluted with 1 vol. of buffer, applied to a column (1.6cm × 20 cm) of CM-cellulose and eluted with a linear gradient (600ml) of 0.05–0.35M-NaCl in buffer. Fractions of volume 5 ml were collected.

Step 9: poly(A)-agarose chromatography. (a) RNAase I from the Sephadex G-75 column (step 7) was diluted with 2 vol. of buffer, applied to a column of (0.9cm × 12 cm) poly(A)-agarose and eluted with a linear gradient (300ml) of 0.02–0.4M-NaCl in buffer. Fractions of volume 4 ml were collected, and active fractions were combined to give the final purified enzyme. (b) RNAase II from the CM-cellulose column (step 8) was diluted with 2 vol. of buffer and chromatographed as in (a) except for use of a linear gradient of 0.04–0.5M-NaCl in buffer. (c)

RNAase III from the Sephadex G-100 column (step 7) was applied to the poly(A)-agarose column without dilution. Elution was accomplished with a linear gradient of 0.1–0.8M-NaCl in buffer. The elution profile of this enzyme is shown in Fig. 2.

Minor bovine muscle RNAases

Three additional RNA-degrading enzymes were detected in very small amounts in many preparations, but were not studied further: (a) in step 4, an enzyme active against poly(C) was occasionally noted as a small shoulder on the ascending portion of the activity profile for RNAase I; (b) in step 4 or 5(b), an enzyme that hydrolysed yeast RNA but that was not active against poly(C) was occasionally noted as a small peak eluted just before RNAase II; (c) in steps 4, 5(b) and 6, an enzyme active against poly(C) was seen in the activity profile as a small peak located between RNAases II and III. This minor enzyme tended to contaminate preparations of RNAase II up to step 8. CM-cellulose rather than CM-Sephadex is used in step 8 because the cellulose ion-exchanger was found to separate residual minor enzyme from RNAase II more effectively than the Sephadex product.

Enzyme yield, purity and stability

The purification is summarized in Table 1. Increase in RNAase activity with heat and acid

Table 1. *Purification of bovine muscle RNAases*

Enzyme activity was measured as described in the Experimental section. The procedure of Lowry *et al.* (1951) was used to measure the protein content of samples. Experimental details are given in the text.

Step	Enzyme activity (units)	Specific activity (units/mg)	Recovery (% yield)
1. Crude extract	67 000	0.9	
2. (NH ₄) ₂ SO ₄ fractionation	27 000	0.8	
3. Heat and acid treatment	130 000	160	100
4. 1st CM-Sephadex	55 000		42
RNAase I	18 000	320	
RNAases II and III	37 000	990	
5. 2nd CM-Sephadex	51 000		39
RNAase I	12 000	1100	
RNAase II	16 000	540	
RNAase III	23 000	3800	
6. 3rd CM-Sephadex			
RNAase II	13 000	660	
RNAase III	14 000	4100	
7. Gel filtration	21 000		16
RNAase I	8200	2300	
RNAase II	4000	730	
RNAase III	8500	9200	
8. CM-cellulose			
RNAase II	2100	3600	
9. Poly(A)-agarose	13 000		10
RNAase I	4400	44 000	
RNAase II	990	8300	
RNAase III	8000	50 000	

treatment is probably due to removal of an RNAase inhibitor known to be present in many tissues, including muscle (Little & Meyer, 1970; Goldspink & Pennington, 1970, 1971; Meyer *et al.*, 1972; Little *et al.*, 1973; Kakulas & Meyer, 1976; Kershner & Meyer, 1976). As an approximation, by using a specific activity of 1.5×10^6 units/mg calculated for homogeneous bovine pancreatic RNAase A, about $87 \mu\text{g}$ of total RNAases was obtained from 3 kg of muscle after the heat and acid step, and the final yield of 10% represents only a few micrograms of each enzyme.

Relatively low tissue content and a multiplicity of enzymes have also been difficulties in purification of RNAases from other bovine sources, particularly

liver and spleen (Maver & Greco, 1962). Our purification, however, gave considerable increases in specific activity (more than 40000-fold for RNAases I and III). Furthermore, each RNAase is free of contamination by other RNAases, since each gave a single area of activity after gel electrophoresis or polynucleotide/polyacrylamide-gel electrophoresis [see the Experimental section and the following paper (Karpetsky *et al.*, 1980)] and a single symmetrical peak of activity on sucrose gradients (results not shown).

The purified muscle RNAases were stable for at least 3 months at -20°C or for several weeks at 4°C . They were unstable in solutions containing less than 20–50 mM-NaCl.

Fig. 3. Dependence of activity on pH for RNAases I–III of bovine muscle

The standard assay procedure (see the Experimental section) was utilized with the following exceptions. Individual reaction mixtures contained 1 unit of RNAase I or II or III, as indicated. Sodium acetate (O) or sodium phosphate (●) or Tris/HCl buffer (Δ) (each at 100 mM) was used in the preparation of reaction mixtures. Experimental details are given in the text.

Results

Properties of bovine muscle RNAases

pH-dependence. In 100 mM-Tris/HCl buffer, with 0.63 mM yeast RNA as substrate, all three muscle RNAases have optimal activity at pH 7.5–8.5, but in 100 mM-sodium phosphate buffer RNAase I has optimal activity at pH 6.5 (Fig. 3), similar to that of bovine pancreatic RNAase A.

Effects of salts. All three muscle RNAases required 50–150 mM-Tris/HCl, -NaCl, -KCl or -ammonium acetate for optimal activity, with inhibition occurring at higher salt concentrations. The first transition series bivalent cations, Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} , present in individual reaction mixtures at a concentration of 1.0 mM, decreased the activities of bovine muscle RNAases I, II and III to less than one-third of the values measured in the absence of these metal ions (Table 2). Significant differences were found among the responses of the activities of these enzymes to the presence of Mg^{2+} , Ca^{2+} and Mn^{2+} . Any of these three metal ions caused a loss of more than 59% of the activity of RNAase II. On the other hand, Mg^{2+} and Ca^{2+} stimulated the hydrolysis of substrate mediated by RNAase III, whereas the use of Mn^{2+} resulted in modest loss (27%) of enzyme activity. RNAase I, in the presence of Mg^{2+} , Ca^{2+} or Mn^{2+} , yielded rates of hydrolysis that were intermediate between those of RNAases II and III.

Estimation of molecular weights of RNAases I, II and III. The molecular weights of the bovine muscle

RNAases were measured by using gel filtration on a Sephadex G-100 (superfine grade) column (see the Experimental section for details). For RNAases I and II, the estimated molecular weights were found to be 13 000 and 23 000 respectively. RNAase III had an unreasonably high K_{av} (corresponding to a mol.wt. of 8000) when chromatographed with buffer containing either 0.4 M- or 0.8 M-NaCl. This finding probably results from the weak cation-exchange properties of Sephadex gels as well as the basicity of this RNAase. When subjected to sucrose-density-gradient centrifugation (see the Experimental section), the molecular weight of RNAase III was estimated to be 17 000.

Products of hydrolysis of poly(U). In order to determine the mode of action of the bovine muscle enzymes, poly(U) was subjected to hydrolysis by each of the RNAases for 12 h and the reaction products were separated by DEAE-Sephadex chromatography as described in the Experimental section. The distribution of oligonucleotides produced by RNAase-III-mediated digestion of poly(U) is shown in Fig. 4. Analysis of the size distribution of the oligonucleotides recovered indicated that about 53% averaged between 2 and 3 nucleotides in length, whereas 28% of the fragments were 4–6 nucleotides long. Only 19% of the products were mononucleotides, the product expected if hydrolysis resulted from exonucleolytic attack. Similar results were obtained with RNAases II and III. The formation of oligonucleotides having a range of chain lengths, rather than exclusively mononucleotides, indicates that the three bovine muscle RNAases can be classified as endonucleases.

The sole mononucleotide product of poly(U) digestion by each of the three muscle enzymes was cyclic 2':3'-UMP (see the Experimental section for details of reaction conditions and chromatographic procedure). Cyclic 2':3'-UMP (3.0 mM) was not converted into a detectable amount of 3'-UMP by the RNAases under the same conditions (allowing for slight spontaneous hydrolysis also seen in a control without enzyme). Inadequate amounts of the muscle RNAases were available to test thoroughly for cyclic 2':3'-UMP breakdown, but most, if not all, RNAases that form cyclic phosphates will convert the latter into 3'-mononucleotides if enough enzyme is used (Barnard, 1969).

Hydrolysis of synthetic polynucleotides. No hydrolysis of calf thymus DNA, *p*-nitrophenyl phosphate or bis-*p*-nitrophenyl phosphate was observed in reaction mixtures containing any of the bovine muscle enzymes. These RNAases therefore do not appear to function as DNAases, phosphomonoesterases or phosphodiesterases. A comparison of the amount of hydrolysis of various synthetic polynucleotide substrates with that of yeast RNA by RNAases I, II and III is shown in Table 3.

Table 2. Effects of bivalent cations on bovine muscle RNAase activity

Enzyme activity was measured by the standard assay procedure (see the Experimental section) except that individual reaction mixtures contained 100 mM-Tris/HCl buffer, pH 7.5, 1.0 unit of enzyme and 1 mM of the metal chlorides indicated below. NaCl was not present in these mixtures. To facilitate comparison of one set of activities with another, each activity is expressed as a percentage of the activity obtained in the absence of bivalent cation. 100% activity corresponds to an increase of one A_{260} unit with the standard assay procedure with the exceptions noted. Experimental details are given in the text.

Bivalent cation	Activity (%)		
	RNAase I	RNAase II	RNAase III
None	100	100	100
Mg^{2+}	70	40	110
Ca^{2+}	83	33	120
Mn^{2+}	39	27	73
Co^{2+}	28	18	30
Ni^{2+}	20	5.9	17
Cu^{2+}	15	32	14
Zn^{2+}	0	0	0

Fig. 4. DEAE-Sephadex chromatography of oligonucleotide fragments obtained after digestion of poly(U) by bovine muscle RNAase III

Poly(U) was exhaustively hydrolysed by the ribonuclease as described in the Experimental section. After acidification and neutralization, the dilute reaction mixture was applied to a column (1.5 cm $\times$ 30 cm) of DEAE-Sephadex A-25 (carbonate form in water). After washing of the column with several volumes of 0.02 M-(NH₄)₂CO₃, the digestion products were eluted with a linear gradient (1800 ml) of 0.02–1.0 M-(NH₄)₂CO₃. The absorbance at 260 nm was measured for each 10 ml fraction. Numerals above the peaks represent oligonucleotide chain length as determined from the ratio of total to terminal P (Carrara & Bernardi, 1968). Experimental details are given in the text.

Table 3. Activity of bovine muscle RNAases against synthetic polyribonucleotides

The standard assay procedure (see the Experimental section) was used to measure enzyme activity except that individual reaction mixtures contained 100 mM-Tris/HCl buffer, pH 7.5, and 0.63 mM yeast RNA or synthetic polynucleotides as indicated below. Each activity is expressed as a percentage of the activity obtained with yeast RNA. Experimental details are given in the text.

Substrate	Activity (%)		
	RNAase I	RNAase II	RNAase III
Yeast RNA	100	100	100
Poly(C)	54	330	0
Poly(U)	8.0	83	13
Poly(A,C)	630	9300	7.4
Poly(A,U)	240	130	670
Poly(C,U)	3.9	3.8	0
Poly(G,U)	30	52	68
Poly(A,G,U)	260	280	420

The homopolypyrimidines poly(C) and poly(U) are generally degraded at lower rates than yeast RNA. It is noteworthy that the presence of both cytidylate and uridylate residues in the same polymer strand, as in poly(C,U), results in lower rates of hydrolysis than if either poly(C) or poly(U) is utilized. Certain co-polymers, however, are excellent substrates for the muscle enzymes. Co-polymers containing adenylate residues, for example, in addition to either cytidylate or uridylate residues, are hydrolysed 1.3 to 93 times faster than yeast RNA (Table 3), with one exception. This occurs with RNAase III and poly(A,C), which is degraded at 7.4% of the rate of yeast RNA.

Changing the identity of the purine portion of the co-polymer from adenylate to guanylate yields such co-polymers as poly(G,U). Use of this substrate resulted in large decreases (60–90%) in hydrolysis relative to the degradation of poly(A,U). Thus, with the hydrolysis of yeast RNA as a reference, poly(A,U) is a superior substrate for RNAases I, II and III, whereas the hydrolysis of poly(G,U) by these enzymes occurs at a lower rate than for the naturally occurring substrate. The addition, however, of adenylate residues to poly(G,U) results in a substrate, namely poly(A,G,U), that is hydrolysed at least 2.6 times as rapidly as yeast RNA by the muscle enzymes. This occurs even though two-thirds of the total number of polynucleotide residues are purine moieties. Although at least 5.4 times more poly(A,G,U) than poly(G,U) is hydrolysed by RNAases I, II and III under the assay conditions, different results are obtained on comparing the hydrolysis of poly(A,G,U) with that of poly(A,U). With RNAase I, these two co-polymers were hydrolysed at similar rates. Changing substrate identity from poly(A,U) to poly(A,G,U) resulted in a 37% decrease in polynucleotide degradation mediated by RNAase III and a 220% increase in substrate hydrolysis by RNAase II.

All the above results suggest that the presence of adenylate residues in co-polymers containing pyrimidines enhances the rate of enzyme-catalysed breakdown of the polymer even though such homopolymers as poly(A) or poly(G) do not function as substrates for these enzymes under our conditions. Although much less effective than adenylate moieties, the addition of randomly placed guanylate residues to poly(U) resulted in large increases in the hydrolysis of this synthetic polynucleotide (RNAases I and III).

The specificities of bovine muscle RNAases I, II and III were determined with a technique by which terminally labelled RNA molecules of known sequence are subjected to limited enzymic hydrolysis. The products of degradation are then examined with denaturing polyacrylamide slab gels. These gels were prepared and electrophoresed

essentially as described by Donis-Keller *et al.* (1977). It was found that each enzyme cleaved a 5'-labelled fragment of *E. coli* 5S rRNA at cytidylate and uridylylate residues, and was similar to bovine pancreatic RNAase A in this respect.

Inhibition by homopolynucleotides

Homopolypurines have been found to inhibit the activities of a number of endonucleases from microbial (Levy *et al.*, 1973, 1975; Frank *et al.*, 1975; Hieter *et al.*, 1976) as well as mammalian and human (Schmukler *et al.*, 1975; Frank & Levy, 1976; Hieter *et al.*, 1976; Neuwelt *et al.*, 1976; Karpetsky *et al.*, 1977) sources. In common with these enzymes, the bovine muscle RNAases are all strongly inhibited by poly(A) or poly(G) (Table 4). Comparison of K_i values obtained with poly(A) with those determined in the presence of poly(G) reveals that these two polynucleotides have similar efficacies of inhibition for RNAases II and III. Considering RNAase I and bovine pancreatic RNAase A, however, the ratios of $K_{i, \text{poly(A)}}/K_{i, \text{poly(G)}}$ for these two enzymes were 117 and 126 respectively, indicating that poly(G) is a much better inhibitor of these enzyme activities than poly(A). Furthermore, on comparison of K_i values for all the enzymes tested, it is noteworthy that poly(G) is a superior inhibitor of RNAase I and RNAase A, compared with RNAases II and III. The K_i values for the former enzymes are no more than 7% of the values of the inhibitor constants for RNAases II and III. Exactly the opposite situation pertains on consideration of poly(A) as an inhibitor. $K_{i, \text{poly(A)}}$ for RNAase I or RNAase A is at least 421% of the value for this constant measured with RNAases II and III. Therefore poly(A) is a weaker inhibitor and poly(G) a stronger inhibitor of both RNAases I and A, compared with both RNAases II and III. The

finding that the values of $K_{i, \text{poly(A)}}$ and $K_{i, \text{poly(G)}}$ are very similar for RNAase I and RNAase A, as well as the fact that the values of the $K_{i, \text{poly(A)}}/K_{i, \text{poly(G)}}$ ratios are virtually identical for both enzymes, strongly suggests a close similarity between bovine

Table 5. *Effects of ionic strength or pH on poly(G)-induced inhibition of RNAase III activity*

The effects of varying ionic strength on inhibited enzyme activity were examined by using reaction mixtures containing 50 mM-Tris/HCl buffer, pH 7.5, 0.63 mM yeast RNA, 1 unit of bovine muscle RNAase III, 27 μ M-poly(G) and the NaCl concentrations indicated below. Over the range of NaCl concentrations employed, the pH of reaction mixtures varied by less than 0.1 pH unit. Changes in RNAase III inhibition mediated by pH were studied by using reaction mixtures containing 0.63 mM yeast RNA, 1 unit of muscle RNAase III, 1.4 μ M-poly(G) and a buffer of different pH values and constant ionic strength. This buffer consisted of 44.0 mM-imidazole, and acetic acid to give the desired pH, as well as NaCl to give an ionic strength of 0.044. Activity is expressed as a percentage of the activity obtained in the absence of inhibitor. Experimental details are given in the text.

Concn. of NaCl (mM)	Activity (%)
0	0
30	9.4
60	15
120	45
180	72
pH	Activity (%)
6	55
6.5	63
7	68
7.5	85
8	83

Table 4. *Inhibition of bovine muscle RNAases by polynucleotides*

Enzyme activity was measured in reaction mixtures containing 10 mM-Tris/HCl buffer, pH 7.5, 0.13–0.95 mM yeast RNA and one of the homopolynucleotide inhibitors indicated below. Concentrations of homopolynucleotide inhibitors were chosen to produce a maximum of 85% inhibition. With the exception that each reaction mixture was incubated for 5 min at 37°C, the assay procedure was as described in the Experimental section. Enzyme activity was linear for at least 5 min at the highest and lowest substrate concentrations. Kinetic constants were determined from double-reciprocal plots as reported previously (Igarashi *et al.*, 1977). The values of K_m were as follows (μ M): RNAase I, 80; RNAase II, 77; RNAase III, 100; bovine pancreatic RNAase A, 140. Experimental details are given in the text.

Homopolynucleotide inhibitor	K_i (μ M)			
	RNAase I	RNAase II	RNAase III	Bovine pancreatic RNAase A
Poly(A)	2.4	0.57	0.55	2.7
Poly(C)*	—	—	13	—
Poly(G)	0.019	0.37	0.59	0.023

* RNAases I and II, as well as bovine pancreatic RNAase A, rapidly hydrolyse poly(C).

Table 6. *Reversal of poly(A) and poly(G) inhibition of bovine muscle RNAases by the addition of polyamines*

Enzyme activity was measured in individual reaction mixtures containing 10mM-Tris/HCl buffer, pH 7.5, 0.32mM yeast RNA, 1 unit of bovine muscle RNAase I and the indicated concentrations of polyamine and homopolypurine inhibitor. Each polyamine was used at a concentration determined previously to give optimal stimulation of RNAase activity in the absence of inhibitor. With the exception that each reaction mixture was incubated for 10min at 37°C, the assay procedure was as described in the Experimental section. Experimental details are given in the text.

Polyamine	RNAase I activity in the presence or absence of homopolypurine inhibitors (units/ml)		
	No inhibitor	50µM-Poly(A)	0.81µM-Poly(G)
None	2.5	0.38	0.23
0.1 mM-Spermine	6.6	6.9	6.5
0.2 mM-Spermidine	6.1	5.6	6.1
1.0mM-Putrescine	7.2	5.6	7.1

muscle RNAase I and bovine pancreatic RNAase A.

As noted in the preceding section, poly(C) is not hydrolysed by muscle RNAase III. This polypyrimidine functions, however, as an inhibitor of the enzyme. Poly(C) is not as efficient an inhibitor as either poly(A) or poly(G), since $K_{i, \text{poly(C)}}$ is about 23 times the value of either $K_{i, \text{poly(A)}}$ or $K_{i, \text{poly(G)}}$ (Table 4).

Regulation of polypurine-induced inhibition of RNAase activity may be achieved by altering either the ionic strength or the pH of the reaction mixture (Table 5). If the inhibition of RNAase III by poly(G) is considered, an increase in the NaCl concentration of individual reaction mixtures results in a recovery of enzyme activity. As the NaCl concentration is raised from 30 to 120mM, for example, enzyme activity increases from 9.4 to 45% of the values obtained in the absence of poly(G). It is important to note that, even at high concentrations of salt (0.18M), total enzyme activity is not restored (Table 5), attesting to the potency of poly(G)-induced inhibition. In the presence of fixed ionic strength, enzyme inhibition may also be regulated by alterations in pH (Table 5). Although increasing the pH from 6.0 to 7.5 in the presence of poly(G) causes a 30% increase in enzyme activity relative to uninhibited controls, this change in the rate of substrate hydrolysis is not as dramatic as those achieved by changes in total ionic strength.

The addition of a polyamine, such as spermine, to reaction mixtures containing RNAase I inhibited with either poly(A) or poly(G) restores essentially all enzyme activity (Table 6). Similar results were obtained with muscle RNAases II and III and bovine pancreatic RNAase A. The concentrations of low-molecular-weight organic cations employed in these studies do not significantly influence the overall ionic strength. As noted for various other inhibited RNAases (Levy *et al.*, 1973, 1974; Levy, 1975; Schmukler *et al.*, 1975; Frank *et al.*, 1976; Hieter *et*

al., 1976; Igarashi *et al.*, 1977; Kumagai *et al.*, 1977; Karpetsky *et al.*, 1979), therefore, the effects of polyamines are specific and distinct from those generated by alterations in ionic strength. Furthermore, polyamine effectiveness depends on the polyamine molecular charge density. Thus the concentrations of spermine, spermidine and putrescine that must be used to completely reverse poly(G)-induced inhibition of RNAase I are in the proportions 1:2:10 (Table 6).

Discussion

Comparison with other endoribonucleases

Alkaline cyclizing endoribonucleases are present in bovine pancreas (Richards & Wyckoff, 1971), milk (Bingham & Zittle, 1964; Coulson & Stevens, 1964; Bingham & Kalan, 1967), spleen (Kaplan & Heppel, 1956; Maver & Greco, 1962), liver (Maver & Greco, 1962), thymus (Bernardi, 1961), aorta (Gamble *et al.*, 1967; Lewis & Gamble, 1969), seminal fluid or vesicles (D'Alessio *et al.*, 1972, 1975; Floridi *et al.*, 1972; Irie & Tsubota, 1974), serum (Anai *et al.*, 1972; Von Tigerstrom & Manchak, 1976), brain (Niedergang *et al.*, 1974; Elson & Glitz, 1975; Okazaki *et al.*, 1975) and adrenal gland (Miller *et al.*, 1975). We have shown that bovine skeletal muscle has at least three pyrimidine-specific endoribonucleases of this type, and three additional heat- and acid-stable RNA-degrading enzymes that were not studied further.

The properties of muscle RNAases are similar to those of certain known bovine RNAases. Muscle RNAase I, for example, is virtually identical with pancreatic RNAase A in all properties studied. Muscle RNAase II is similar to RNAases from bovine brain cytoplasm (Okazaki *et al.*, 1975) and aorta (Gamble *et al.*, 1967; Lewis & Gamble, 1969). As with RNAase II, the brain enzyme is more basic than pancreatic RNAase A and degrades poly(C) to a far greater extent than it does other homo-

polymers. The brain enzyme has a mol.wt. of 37000 by gel filtration or 25000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Okazaki *et al.*, 1975), compared with a mol.wt. of 23000 for bovine muscle RNAase II. Of two RNAases partially purified from aorta, one degrades poly(C) and, to a smaller extent, poly(U) (Gamble *et al.*, 1967; Lewis & Gamble, 1969). Although a direct comparison of the muscle, brain and aorta enzymes was never done, and reaction conditions for the measurement of enzyme activity were different for each enzyme, nevertheless there is a qualitative similarity among all three enzymes. Muscle RNAase III resembles RNAases described in bovine serum (Von Tigerstrom & Manchak, 1976) and aorta (Lewis & Gamble, 1969). Bovine serum contains an RNAase similar to pancreatic RNAase A in addition to an enzyme that, in common with RNAase III, is much more active against poly(U) than against poly(C) and has a K_{av} higher than that of pancreatic RNAase A when chromatographed on Sephadex G-75 (Von Tigerstrom & Manchak, 1976). Bovine aorta also has a RNAase that is much more active against poly(U) than against poly(C) (Lewis & Gamble, 1969).

Very few RNA-degrading enzymes have been purified from muscle, perhaps as a consequence of the low concentrations of these proteins in that tissue. Lingcod skeletal muscle contains an enzyme that was described as a ribonuclease (Tomlinson, 1958) but was later found to resemble bovine spleen exonuclease in substrate specificity (Tomlinson, 1959). Human deltoid muscle contains an alkaline cyclizing endoribonuclease that has a mol.wt. of about 11500 and digests poly(C) and poly(U) but not poly(A) or poly(G) (Goldspink & Pennington, 1970). This RNAase differs considerably from bovine muscle RNAases, since the human enzyme is not retained on CM-cellulose under conditions where all but a few percent of the bovine RNAase is tightly bound. Skeletal muscle of many animals probably contains endoribonucleases, since crude extracts degrade RNA in the presence of EDTA (Tappel *et al.*, 1962; Zalkin *et al.*, 1962; Abdullah & Pennington, 1968; Little & Meyer, 1970; Goldspink & Pennington, 1971; Meyer *et al.*, 1972; Little *et al.*, 1973; Kakulas & Meyer, 1976; Kershner & Meyer, 1976). The significance of changes in these RNA-degrading activities in muscular dystrophy or other conditions has not been established, and the reason for a multiplicity of endoribonucleases in bovine skeletal muscle is not clear at the present time.

By means of a sensitive sequencing technique, we were able to determine the specificity of each of the three purified bovine muscle RNAases, although only very small amounts of enzymes were available (Donis-Keller *et al.*, 1977). The specificities of the bovine muscle RNAases were found to be similar to,

it not identical with, that of pancreatic RNAase A. These results contrast sharply with the marked differences in rates of hydrolysis of synthetic polyribonucleotides exhibited by RNAases I, II and III. Bovine serum and human pancreatic RNAases also show a lack of correlation between susceptibility to enzymic cleavage at pyrimidine residues in homopolymers and in naturally occurring RNA (Bardon *et al.*, 1976; Von Tigerstrom & Manchak, 1976). As emphasized in a review by Sierakowska & Shugar (1977), conclusions about RNAase specificity based only on studies with homopolynucleotides may not be valid. However, results obtained with synthetic polynucleotides are of value, because they serve to indicate that, although the specificities of the bovine muscle enzymes are similar, the rates at which certain linkages in the synthetic polymers are hydrolysed differ considerably among the three RNAases (Table 3).

Inhibition of bovine muscle RNAases by polynucleotides

Poly(A) and poly(G) have been shown to be potent inhibitors at physiological pH of pyrimidine-specific endoribonucleases from microbial, mammalian or human sources (Schmukler *et al.*, 1972, 1975; Levy *et al.*, 1973, 1974, 1975; Levy, 1975; Hieter *et al.*, 1976; Karpetsky *et al.*, 1979). Our results with bovine muscle RNAases (Tables 4–6) increase the number of pyrimidine-specific endoribonucleases known to be strongly inhibited by purine homopolynucleotides. In common with other studies, the inhibitions of RNAases I, II and III were found to be dependent on ionic strength and to be subject to reversal by polyamines (Levy *et al.*, 1973, 1974, 1975; Levy, 1975; Schmukler *et al.*, 1975; Frank *et al.*, 1976; Hieter *et al.*, 1976; Igarashi *et al.*, 1977). The polycationic polyamines may overcome polynucleotide inhibition of ribonucleases by binding to polyanionic nucleic acids, thereby neutralizing charge (Cohen, 1971). Reversal of poly(G) inhibition of *Citrobacter* and *Enterobacter* ribonucleases, however, is associated with binding of the polyamine to the enzyme as well (Levy *et al.*, 1974; Frank *et al.*, 1976). Because of the influences of changes in ionic strength or alterations in polyamine concentration on inhibited enzyme activity, it would appear that adjustment of these parameters, as well as the amount of poly(A), could result in a very close regulation of the rates of RNAase-mediated hydrolysis of RNA.

Evidence exists for a physiological role for RNAase inhibition by polypurines (Karpetsky *et al.*, 1979). For example, poly(A), containing 150–200 nucleotides, occurs at the 3'-terminus of most eukaryotic mRNA (Brawerman, 1976). The poly(A) segment is known to be necessary for optimal stability of rabbit globin mRNA injected into

Xenopus laevis oocytes or added to a Krebs ascites-cell free extract (Allende *et al.*, 1974; Huez *et al.*, 1974, 1975; Sippel *et al.*, 1974; Soreq *et al.*, 1974; Marbaix *et al.*, 1975; Nudel *et al.*, 1976). Poly(A) of various lengths added on to *E. coli* 5S rRNA protects the rRNA against degradation by several RNAases from various sources and having different specificities (Hieter *et al.*, 1976; Karpetsky *et al.*, 1977, 1979). Furthermore, there is a reasonable correspondence between the length of poly(A) needed for protection in the extracellular model system and the ones *in vivo* (Nudel *et al.*, 1976). Purine homopolynucleotide inhibition of pyrimidine-specific endoribonucleases therefore may be a fairly general effect contributing to mRNA stability.

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