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Alkaline Ribonuclease Associated with Polyribosomes in Fibroblasts of Experimental Granulation Tissue

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Alkaline ribonuclease (RNase) from polyribosomes derived from experimental granulation tissue has been purified 1900-fold through affinity chromatography. The preparation was homogeneous in sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis with an estimated molecular weight of 15 000. Purified RNase was completely inhibited in the presence of divalent ions Mg^{2+}(100 mM) and Ca^{2+}(100 mM) but activated slightly with Na^{+}(50 mM). The enzyme is an endonuclease and the best substrates were poly(U), mixed RNA from yeast, rRNA from granulation tissue and poly(C). The estimated apparent K_m-values were 0.037, 0.064, 0.13 and 0.27 g l^{-1}, respectively. In polyribosomes RNase occurred in both free and *p*-chloromercuribenzoate (pCMB)-liberated forms. The total activity was at the highest but the proportion of the free activity minimal in the granulation tissue during the maximal synthesis of collagen.

Mammalian tissues and body fluids contain a number of different RNA-degrading enzymes.[1–3] Polysomal RNase and its cytoplasmic inhibitor have been implicated in the regulation of polyribosome stability[4–7] and hence, of protein synthesis,[8] *e.g.*, during growth periods.

When polyribosomes from experimental granulation tissue are isolated by the Mg^{2+}-precipitation method,[9] they exhibit "free" and pCMB-liberated "latent" RNase activity. When these same polyribosomes are used for cell-free protein synthesis,[10] the incorporation of ^{14}C-proline is only moderate. In incubated slices of granulation tissue the polyribosomes are labile and degraded in a few hours.[10] Our interest in the collagen-synthesizing polyribosomes encouraged the present work on the purification and characterization of a polysomal RNase from granulation tissue.

EXPERIMENTAL

Preparation of polyribosomes from granulation tissue

Granulation tissue was induced by the subcutaneous implantation of viscose-cellulose sponge (Kongsfoss Fabrikker A/S, Oslo 2, Norway) into 3-month-old female rats (Wistar strain) for three weeks.[11]

The 24 granulomas (wet wt 1.0 – 1.2 g each) from four rats were harvested into ice-cold 0.9 % NaCl, and rinsed. The surrounding capsules were dissected off and the granulomas minced with scissors and immersed into 25 mM tris-buffer, (3 – 4 g/15 ml), pH 7.5, containing also 25 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml heparin (Oy Star Ab, Tampere, Finland) and 2 % Triton X-100.[9] After homogenization in an Ultra-Turrax (Janke & Kunkel, Staufen i. Breisgau, Germany) homogenizer, coupled to a Desoutter Bros. Ltd., London, Type B 511-L drive, 40 000 rpm, twice for 5 s, the preparation was centrifuged at 27 000 *g* for 10 min to remove the remnants of sponge and other debris. The precipitation of polyribosomes was carried out from the supernatant according to Palmiter.[9] The polysome pellet was suspended in 20 ml of 50 mM tris-buffer, pH 7.8, containing 25 mM NaCl and 5 mM $MgAc_2$. Polysome suspensions not to be used immediately were rapidly frozen and stored in liquid nitrogen.

Assays

RNase. The RNase activity was determined by the method of Shortman[12] as modified by Gribnau *et al.*[13] The standard reaction mixture contained 200 µl of yeast RNA-solution (Sigma, Type II, St Louis, Mo., USA; dialyzed for 24 h against 10 mM EDTA and for a further 24 h against water). The concentration of RNA was adjusted to 2 % on the basis of UV absorption (21 A_{260}-units correspond to 1 mg/ml[14]). The assay mixture also contained 200 µl of 0.02 %

bovine serum albumin (Sigma A 4378) and was filled to 1 ml with 0.10 M tris-buffer, pH 7.8, containing 25 mM NaCl and 5 mM $MgAc_2$(350 – 595 µl), and with the solution (5 – 250 µl) containing the RNase activity to be measured. To assay total (free + latent) RNase, the reaction mixture also contained 0.8 mM pCMB (Fluka AG, Buchs, Switzerland), which was added in the bovine serum albumin solution.

After the addition of the enzyme the reaction mixtures were incubated in a Heto thermostat at 37 °C for 15, 30 or 60 min. The reaction was stopped by pipetting the mixture into an equal volume (250 or 500 µl) of ice-cold 75 % (v/v) ethanol in 1 M HCl [12] or of ice-cold 12 % (w/v) $HClO_3$ in 20 mM $La(NO_3)_3$.[15] These mixtures were kept in an ice-bath for 1 h to complete the precipitation of undegraded RNA. The blank assay, in which the precipitation was made after the enzyme was added and just before incubation, was carried out simultaneously but kept in an ice-bath. There was no spontaneous liberation of nucleotides from the yeast RNA. The assay tubes were centrifuged at 2500 *g* for 30 min at 4 °C and the supernatants were diluted with water and the absorbance at 260 nm was measured. The increase of 1 absorbance unit at 260 nm (molar extinction coefficient = 11 000) equals the liberation of 90 µmol of nucleotide residues.[16]

Protein was estimated by the method of Lowry *et al.*[17] using bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

Molecular weight determination of proteins in SDS-polyacrylamide-gels was carried out according to Lehtinen *et al.*[18] in cylindrical gels, length 6.5 cm, diameter 0.4 cm. Acrylamide and *N,N'*-methylenebisacrylamide were purified as described by Loening.[19] The proteins in solutions containing 8 M urea (Schwarz-Mann, 909200, Orangeburg, N.Y. 10962, USA), 2 % (w/v) sodium dodecyl sulfate (SDS, BDH Chemicals, 30176, Poole, England) and 2 % (w/v) mercaptoethanol were subjected to electrophoresis at room temperature in 5 % (w/v) polyacrylamide gels with 4.06 % cross-linking for 10 min at a constant current of 1 mA and then for 2 h at 8 mA. The reference proteins were bovine serum albumin (Sigma), trypsin (Sigma, Type II) and cytochrome *c* (Boehringer, Mannheim, Germany). Proteins were stained with Coomassie Brilliant Blue R (G.T. Gurr, 9120, Searle Diagnostic, High Wycombe, England) and destained in 7.5 % (v/v) acetic acid as described by Lenard.[20] The stained gels were scanned on a Perkin-Elmer UV-VIS 139 spectrophotometer equipped with a home-made adapter and a Servogor RE 514.9 recorder.

Disc electrophoresis of high molecular weight RNA was performed at room temperature in 3 % acrylamide gels containing 0.2 % SDS,[19] with samples dissolved in tris-phosphate buffer containing 0.2 % SDS and 5 % glycerol. The electrophoresis was carried out first for 30 min at 1 mA, and then continued for 65 min at 5 mA. After the run RNA was stained for 10 min in methylene blue solution (2.3 ml of glacial acetic acid, 13.5 g of sodium acetate and 0.5 g of methylene blue in a total volume of 250 ml) and destained overnight in water.

Polyacrylamide gel electrophoresis for native basic proteins and the localization of RNase in the gels. The system comprised a 3 % large-pore spacer gel and a 15 % small-pore separation gel in β-alanine – acetic acid buffer (31.2 g l^{-1} and 8 ml l^{-1}, respectively).[21] Samples (in 10 % glycerol) were run with methylene blue as a tracing dye for 60 – 70 min at a constant current of 6 mA. The proteins were stained as earlier, or else the gels were incubated for 2 h at 37 °C in 0.1 M tris-buffer, pH 7.8, containing 25 mM NaCl, 5 mM $MgAc_2$ and 0.5 % yeast RNA (w/v) and thereafter stained with methylene blue for 2 h. After destaining in water the areas of RNase-split RNA appeared as lighter bands in the gels.[22]

RNase isolation

Step 1. The main steps in the enzyme isolation are shown in Fig. 1. The polysome suspension (20 ml) from 24 granulomas was made 1.5 M in LiCl and 4 M in urea to make the proteins soluble under mild conditions.[23] The solution was kept for 2 – 3 days at 4 °C, at which temperature all the subsequent operations for enzyme purification were carried out. After the LiCl-urea treatment polysome suspension was centrifuged first at 27 000 *g* for 30 min and then at 105 000 *g* for 2 h. The pellet from the 27 000 *g* centrifugation containing the main part of the polysomal RNA (18S and 28S rRNA, $A_{260}/A_{280} = 1.93$) was suspended into 5 ml of water and dialyzed exhaustively against 5 mM EDTA and water, frozen rapidly and stored in liquid nitrogen for later use as an RNA-substrate. The pellet from the 105 000 *g* centrifugation was small and discarded.

Step 2. The salt and urea content of the 105 000 *g* supernatant was changed in a BIO-GEL P-2 (BIO-RAD Laboratories, 1500140, Bromley, England) column (4.2 × 26 cm) at a flow rate of 250 ml/h in 25 mM tris-HCl buffer, pH 7.8, containing 25 mM NaCl, 5 mM $MgAc_2$ and 2 M urea. The elution was monitored at 280 nm with a Perkin-Elmer 139 UV-VIS spectrophotometer and Servogor RE 514.9 recorder. The first 40 ml of the macromolecular fraction, containing 15 mg protein and some RNA was collected.

Step 3. DEAE-cellulose chromatography. Preswollen DEAE-cellulose (Whatman DE-52; Whatman Biochemicals Ltd., Maidstone, England) was treated alternatively with 0.5 M HCl

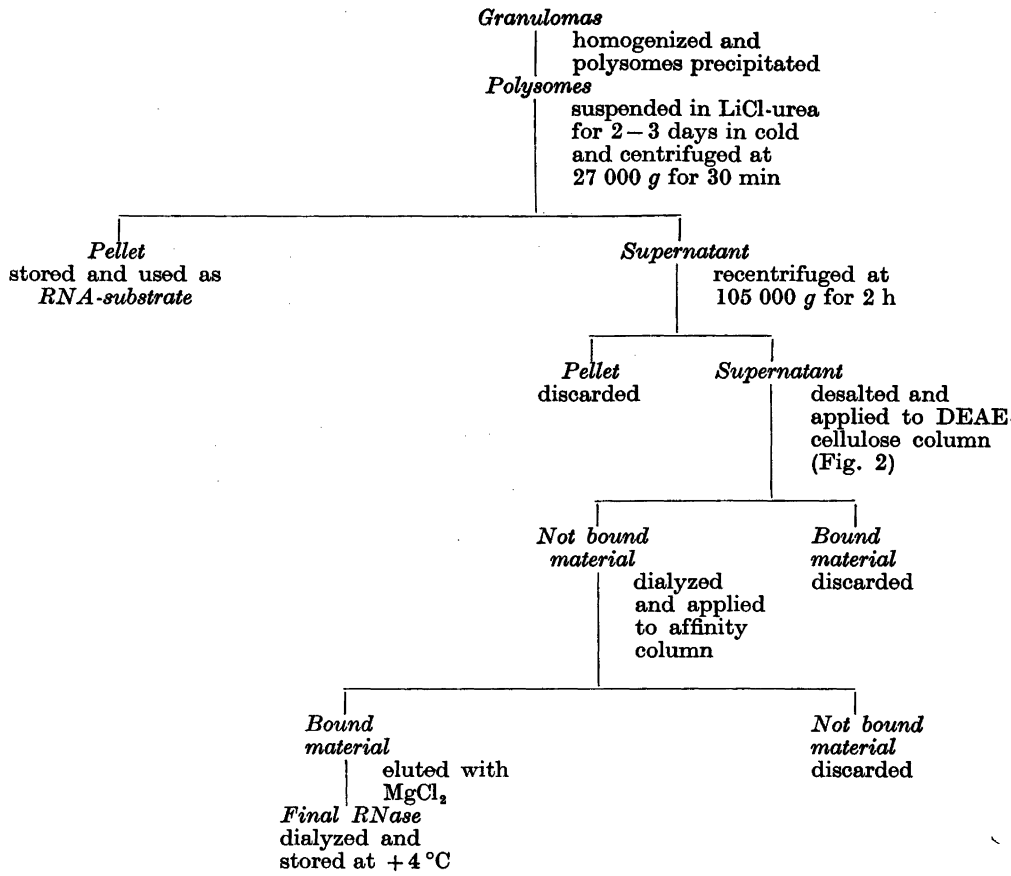

Fig. 1. Flow sheet of enzyme isolation.

and 0.5 M NaOH and packed in a column, which was equilibrated at the flow rate of 35 ml/h with 25 mM tris-HCl buffer, pH 7.8, containing 25 mM NaCl, 5 mM $MgAc_2$ and 2 M urea. After 5 h stabilization the DEAE-cellulose column (2.2 × 18 cm) was loaded with the macromolecular fraction from the BIO-GEL P-2 column and eluted with an NaCl-gradient (200 ml, from 0.025 to 1.0 M). Fractions of about 4.5 ml were collected and monitored at 260 and 280 nm. Those with highest RNase activity were pooled (fractions 10 – 21 in Fig. 2, containing 3 mg proteins) and dialyzed against 50 mM tris-HCl buffer at pH 7.8.

Step 4. Affinity chromatography. Forty A_{260}-units (2 mg) of 5′-(*p*-aminophenylphosphoryl)-guanosine-2′(3′)-monophosphate (Sigma) were coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) which had been pretreated as described by the manufacturer. The procedure of Wilchek and Gorecki [24] was used, except that the buffers were 0.5 M in NaCl. About 80 % of the monophosphate was coupled. The remaining reactive CNBr-groups were removed with 1 M ethanolamine. Finally the resulting NH_2-ppGp-Sepharose 4B was washed with 100 ml of 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl. The column (final size 1.2 × 3.5 cm) was equilibrated with 50 mM tris-HCl buffer, pH 7.8, for 5 h at a flow rate of 20 ml/h.

The pooled and dialyzed RNase fraction from the DEAE-cellulose column was loaded in the affinity column at a flow rate of 10 ml/h. The column was then washed with 10 ml of the 50 mM tris-HCl buffer, pH 7.8, at a flow rate of 20 ml/h. The bound material was eluted in one step with 0.2 M $MgCl_2$. Fractions of 1 ml were collected and absorbances at 280 nm were recorded. The $MgCl_2$-eluted fraction was dialyzed against the 50 mM tris-HCl buffer at pH 7.8 and stored in 1 ml aliquots at 4 °C.

Table 1. Enzyme purification. For experimental details see Fig. 1 and the text.

Sample	Total protein/ mg	Volume/ ml	Total activity/ mmol l^{-1} h^{-1}	Specific activity [b]	Purification coefficient	A_{260}/A_{280} [a]
Polysomal suspension	35	20	5.10	0.146	1	54/33=1.64
BIO-GEL fraction	14.4	40	9.75	0.68	4.7	1.025/0.976=1.05
DEAE-cellulose fractions	3.0	65	9.25	3.08	21	0.075/0.093=0.81
Affinity chromatography fraction	0.33	5	92	278	1904	0.082/0.120=0.68

[a] Absorbance units in 1 ml. [b] Calculated per mg protein.

RESULTS

Enzyme purification

The purification of the RNase is shown in Table 1. The LiCl-urea treatment dissolved all RNase from polyribosomes and the loss of activity was small during the centrifugations. If the 105 000 *g* supernatant was desalted by dialysis in step 2, a white precipitate, containing RNase activity appeared (*cf.* Ref. 8, 25, 26). Gel filtration prevented such a loss of activity. If all urea were removed during this step, RNase and RNA-molecules would reassociate and disturb the fractionation of RNase in the subsequent DEAE-cellulose chromatography.

The desalted 105 000 *g*-supernatant eluted in DEAE-cellulose chromatography in three major peaks (Fig. 2). Most basic proteins including RNase (the first peak) were not retained by the column. The second peak was a mixture of proteins and RNA and the third peak contained only RNA on the basis of the absorbance ratio A_{260} to A_{280}. The RNA peak was not homogenous with regard to size when studied in polyacrylamide-gel electrophoresis (not shown).

The affinity chromatography column was first tested with RNase A (Sigma R-4875) as described by Wilchek and Gorecki.[27] The binding of this RNase A was almost complete at pH 5.7 in the 50 mM phosphate buffer. Un-

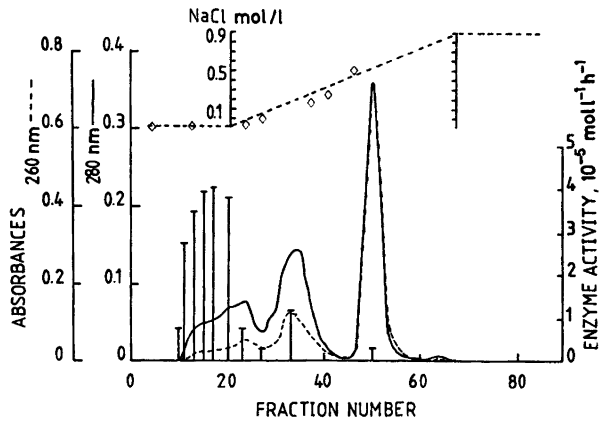

Fig. 2. DEAE-cellulose column chromatography for purification of RNase. The macromolecular fraction from Step 2 (15 mg protein) was applied. For experimental conditions see the text. The NaCl-gradient was followed by conductivity (◇). Absorbance at 280 nm (——) and at 260 (- - -) was monitored and RNase activity (vertical bars) was measured under the standard assay conditions with yeast RNA as substrate.

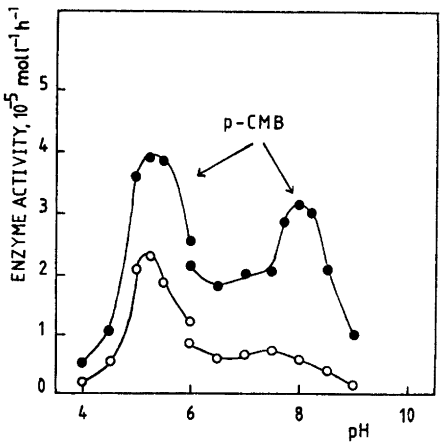

Fig. 3. RNase activity in polysomes of three-week-old granuloma as a function of pH. Under the standard assay conditions pH was varied in succinate-borate and sodium acetate buffers (0.1 M) in the pH-range 4.0 – 6.0 and in phosphate succinate and tris-HCl buffers (0.1 M) in the pH-range 6.0 – 9.5 with (●) and without (○) pCMB. The reaction mixture contained 1.2 mg crude polyribosomes as RNA substrate. No exogeneous RNase was added.

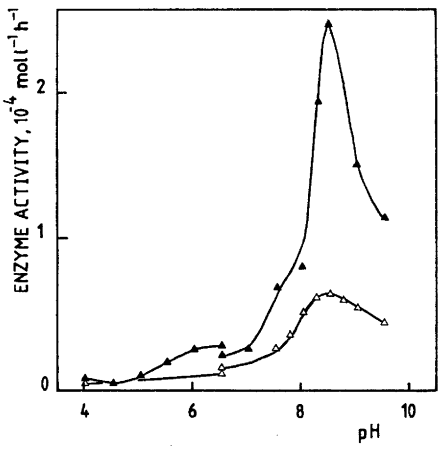

Fig. 4. The effect of pH on purified RNase. The effect was tested at two different enzyme concentrations; 0.3 μg (△) or 1.0 μg (▲) protein from the affinity chromatography fraction was added to the standard reaction mixture. The substrate was yeast RNA and the buffers were as in Fig. 3, except that the buffer was changed at pH 6.5.

specific binding of albumin and cytochrome *c* could not be observed at pH 7.8 in the 50 mM tris-HCl buffer. Initially we eluted the RNase from the column with 0.2 M acetic acid, but because Mg^{2+} was found to inhibit RNase even at low concentrations (see below), we replaced the acetic acid with 0.2 M $MgCl_2$ in later work. The purified RNase was stable and it could be stored at 4 °C, sometimes for months, without any loss of activity.

The purification of the enzyme was continued further by gel filtration, but the material became so diluted that the affinity fraction was preferred for the study of the properties of the purified enzyme. However, in the gel filtration with Sephadex G-100 (column of 1.6 × 55 cm; eluted with 5 mM tris-HCl, pH 8.5, containing 25 mM NaCl and 5 mM $MgAc_2$) we observed one broad peak of activity corresponding to an estimated molecular weight between 25 000 and 12 000, and less activity in macromolecules larger than 25 000. Aggregated forms of RNase, which have been described by several authors,[28–30] could explain this finding.

The characterization of the purified RNase

pH-Optimum. Crude polysomal RNase was active over a broad range of pH values with maximal activities at pH 5.5 and 8.0 (Fig. 3). The purified RNase was active over a narrower pH range (7.5 – 9.5), maximally at pH 8.5 (Fig. 4).

Effects of ions. Na^+ activated RNase up to concentrations of 50 mM but inhibited at higher concentrations (Fig. 5). Both Mg^{2+} and Ca^{2+} inhibited RNase; in 0.1 M solutions the inhibition was complete, and in 50 mM solutions 80 %. At 50 mM EDTA caused a 30 – 40 % inhibition, but at the lowest concentrations (5 mM) a slight activation was seen.

Heat inactivation. Only high temperatures inactivated RNase irreversibly. Heating at 80 °C for 1 h left 30 % of activity and after heating for 2 h the loss of activity was complete. No inactivation was detected at 56 °C after 2 h heating; on the contrary, a slight increase was seen.

Molecular weight. In the SDS-polyacrylamide gel, 10 mg protein of the affinity fraction gave a single band (Fig. 6). The migration rate,

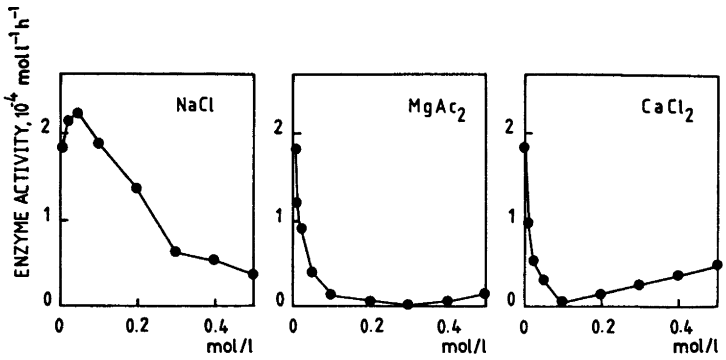

Fig. 5. Effect of some cations on the RNase activity. One μg protein of the affinity chromatography fraction was tested under the standard assay conditions with various Na^+, Mg^{2+} and Ca^{2+} concentrations.

compared with the standards, correspond to a molecular weight of 15 000. As a native protein the mobility of purified RNase was a little slower than the mobility of the major component of the pancreatic RNase A preparation (Sigma R 4875) (Fig. 7), which degraded yeast RNA in many molecular weight areas.

Substrate specificity. Kinetics with various substrates are presented in Fig. 8. The best substrates were mixed yeast RNA, rRNA from granulation tissue (dialyzed pellet from the 27 000 *g* centrifugation; the electrophoretic profile is shown in Fig. 9) and the synthetic polypyrimidines poly(U) and poly(C) (Boehringer). They were split at almost similar rates at low concentrations, but at greater concentrations the synthetic polypyrimidines caused a strong substrate inhibition. Coprecipitation of reaction products at greater concentrations is improbable, because the reaction mixture con-

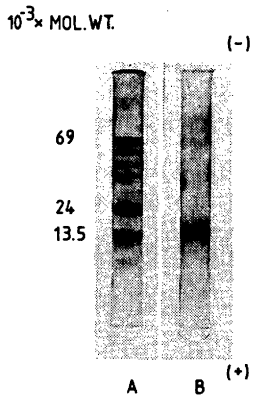

Fig. 6. SDS-polyacrylamide gel electrophoresis of standard proteins and purified RNase. (A) Electrophoresis of standard proteins (10 μg albumin, 20 μg trypsin and 2 μg cytochrome *c*) in 5 % polyacrylamide gel in the presence of 0.1 % SDS at pH 7.0 as described in the text, and stained with Coomassie Blue; (B) Electrophoresis of affinity chromatography fraction with 10 μg protein. Gel and buffer systems as in (A).

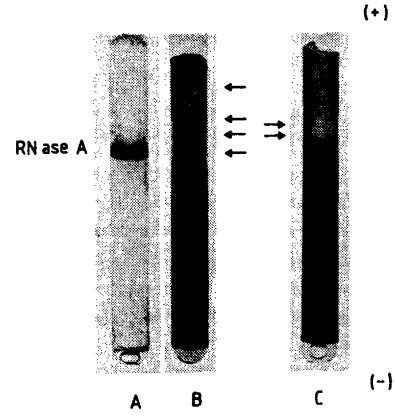

Fig. 7. Electrophoresis of RNase as a native protein in polyacrylamide gel. After electrophoresis in 15 % polyacrylamide at pH 4.0 (see text) gels were treated as follows: (A) 10 μg RNase A stained with Coomassie Blue; (B) 10 μg RNase A stained with yeast RNA and methylene blue, as described in the text; (C) 1 μg protein of the affinity chromatography fraction stained as in (B). No bands were observed with Coomassie Blue. The arrows point to the light bands due to the RNase action.

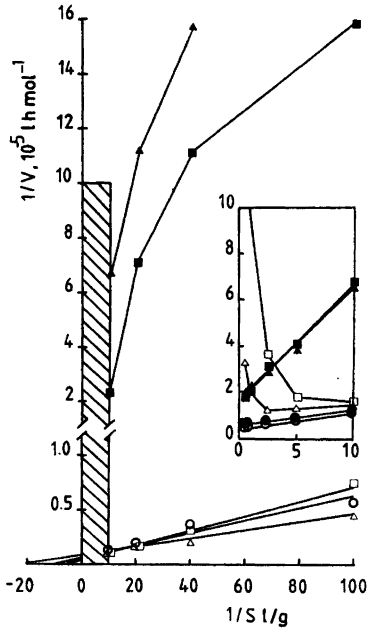

Fig. 8. Lineweaver-Burk plots of the rates of hydrolysis of various polyribonucleotides by purified RNase. Standard assay conditions were used except that the precipitation of undigested substrate was carried out with 20 mM $La(NO_3)_3$ in 12 % $HClO_3$ (w/v). Ribosomal RNA of granuloma (●), yeast mixed RNA (○), poly(A) (■), poly(C) (□), poly (G) (▲) and poly(U) (△) were tested at concentrations of 2 – 0.1 g l^{-1} with 0.1 μg and of 10^{-1} – 10^{-2} g l^{-1} with 0.5 μg protein of the affinity chromatography fraction. The shadowed area is enlarged in the insert. The concentrations of rRNA and yeast mixed RNA were based on the UV-absorption. The other were weighed as sodium salts.

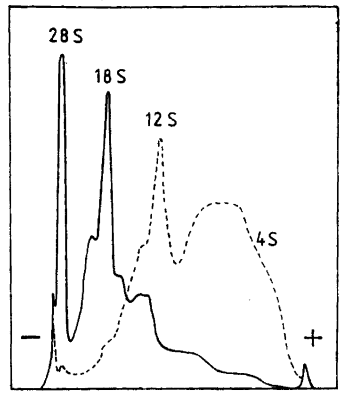

Fig. 9. Endoribonuclease action of purified RNase on ribosomal RNA. 1.2 mg of ribosomal RNA of granuloma (see text) was split by 0.5 μg protein of the affinity chromatography fraction under standard assay conditions. An aliquot of 1.5 A_{260}-units from the reaction mixture was run before incubation (——) and after 10 min of incubation (- - -) in 3 % polyacrylamide gels by the method of Loening.[19] Ribosomal 23S, 16S and 5S RNA of *E. coli* from Miles Laboratories Ltd. (Lausanne, Switzerland) were used as standard.

tained only poly(U) or poly(C) and not RNA. The same inhibition was also observed when poly(U) or poly(C) was added with yeast RNA. K_m-values (g l^{-1}) for poly(U), yeast RNA and poly(C) were 0.037, 0.064 and 0.27, respectively, when measured from concentrations of 10^{-1} – 10^{-2} g l^{-1}, but 0.13 and 0.17 g l^{-1} for rRNA and yeast RNA, respectively, from concentrations of 2 – 0.1 g l^{-1}. The synthetic polypurines poly(A) and poly(G) (Boehringer) could be split by the purified RNase at high concentrations only, when K_m-values for them, from concentrations 2 – 0.1 g l^{-1}, were 0.30 and 0.37 g l^{-1}, respectively.

tRNA extracted by phenol-ethanol[31] from granulation tissue was tested as a substrate at several different concentrations. Measured by the present method, the degradation rate of tRNA was 40 % of the degradation of rRNA and yeast RNA. No cleavage products were found when calf thymus DNA (Sigma, Type I) was tested as a substrate.

Mode of action. The purified enzyme degrades 18S and 28S RNA-molecules very rapidly (Fig. 9). The amount of acid-soluble oligonucleotides appearing is small (about 1/30) compared with the amount of large RNA which disappears. The enzyme was tested in the presence of various concentrations of three different mononucleotides, 2′(3′)-AMP, 2′(3)-UMP and 5′-UMP (Boehringer) using a constant level of yeast RNA. No inhibition of the reaction could be seen, even when the absorbance ratio of mononucleotide to RNA was 1. These results indicate that the enzyme is an endoribonuclease. The enzyme reaction stops spontaneously in 3 – 4 h, and this suggests that the inhibitor at that point must be an oligonucleotide.

Developmental phase and RNase activity

The "crude" polysome suspension described above contained "free" and a pCMB-liberated "latent" or inhibited RNase activities. The ratio of free to total (free+latent) activity at pH 7.8 in crude polysomal fraction varied with the age of granulomas (Fig. 10). The ratio was minimal between the age of three and four weeks, mostly due to the increased "latent" activity at pH 7.5–8.5 (*cf.* Fig. 3). This increase in "latent" activity occurs only in polysome preparations, not in the supernatants from which the polyribosomes of granulation tissue of various age have been precipitated (not shown).

DISCUSSION

The criteria of the purification. The purification coefficient (Table 1) depends not only on the diminished inactive protein in the preparations but also on the increased enzyme activity, especially in the affinity chromatography fraction. Presumably, this increase is due, in part, either to the relaxation of the ribosome structure or to the removal of some inhibitor. Moreover, the assay method used, in which the mononucleotides and oligonucleotides up to hexa- and decamers are acid-soluble, gives in concentrated solutions a proportionately greater specific activity for the endonuclease enzyme. Hence, the degradation of various substrates cannot be accurately measured and compared because the substrates have different three-dimensional structures and the molecular weights vary from ten thousand to more than a million.

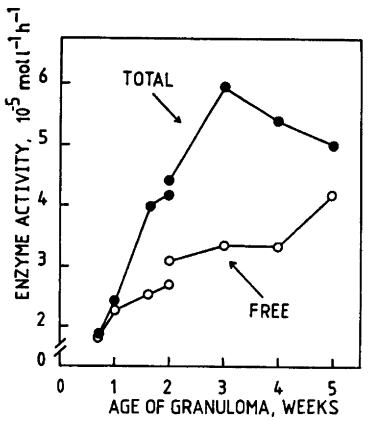

Fig. 10. RNase activity in polyribosomes of developing granuloma. 1.2 mg of crude polysomes were incubated in the presence (●) or absence (○) of pCMB. No exogenous RNase was added. Analyses were performed during two time periods of 5–14 days and 14–35 days and the RNase activities are calculated against the granuloma wet weight.

In disc-electrophoresis we observed only one major denatured protein fraction in the $MgCl_2$-eluate of the affinity chromatography column, but in native form, with the help of RNA in the gels we observed two very close bands. With purified pancreatic RNase A we observed in the same system four distinct bands. The purified enzyme is active over a narrow pH range and because it is completely inhibited by Mg^{2+} and Ca^{2+} there cannot be any exonuclease(s) [1] in the affinity chromatography fraction. These results confirm that in this fraction the purification of RNase is good enough to yield preparations suitable for molecular studies of the enzyme.

The same affinity chromatography method has been used in purification of plant ribonucleases.[32] The affinity chromatography fractions from the poly(G)-Sepharose 4B column have been used in studies of human plasma and liver RNases [28,29] and for their immunological characterization.[33]

Classification of this RNase. Ribosomes of mammalian cells possess ribonuclease activity, which can be observed only after the ribosomal structure is destroyed.[6,8,24] It is not clear, however, whether the "latent" nucleases are structural proteins of mammalian ribosomes or contaminants adsorbed from other parts of the cells, *e.g.*, cytosol, during the isolation procedures. In crude polysome suspension of granuloma cells we found two pH-optima for rRNA degradation, probably due to the presence of two different RNases in this fraction. The alkaline optimum is due to the present purified RNase and the acidic RNase probably originates from lysosomes. In the presence of pCMB, which dissociates the enzyme-inhibitor complex by destroying free SH-groups, the pH-optima did not change; only the activities increased, especially at pH 8.0. This suggests that pCMB reacts with an inhibitory protein that is a part of the ribosome structure.

Alkaline RNase II [1] is widely distributed in animal cells and associated with an endogenous inhibitor,[4,5,7,34] apparently specific, because the affinity of the inhibitor to RNase A was shown to be high and they were bound in the molecular ratio of 1:1.[35,36] Physical and catalytical properties of RNase II are similar to those of pancreatic RNase (RNase A, EC 3.1.4.11). The present RNase has similar properties, *e.g.*, molecular weight and ionic requirements. The pH-optimum of the purified enzyme is a little higher than the pH-optima reported for the alkaline RNase II but it may vary with the assay conditions. The properties of alkaline RNases vary with the tissue source,[8,24,34,37–39] perhaps because of the many functions of RNases, *e.g.*, the processing and degrading of RNA, where such properties of enzyme as ionic requirements may differ with the substrate used.

The granulomas contain blood cells and macrophages in addition to the fibroblasts. The macrophages seem to contain another alkaline RNase different from the RNase purified here (not bound to the guanosine monophosphate-Sepharose 4B column, lower and broader pH-optimum, different substrate specificity; unpublished results). The RNases of prokaryotic cells [40] form a special group of their own.

Ribonuclease and its inhibitor in developing granuloma. The ratio of free to total RNase in the polysomal fraction changed with the age of granuloma (Fig. 10). At the age of 2–4 weeks, when the crude polysomal suspension contained maximally latent RNase and, hence, RNase inhibitor, the protein synthesis, including collagen, of granuloma is maximal.[41,42] Alkaline RNase and its cytoplasmic inhibitor have been implicated in both the regulation of polysome stability [4–7] and the protein synthesis. In granulation-tissue polyribosomes the inhibitor of RNase was located in ribosomes themselves like that described in the membrane-bound ribosomes from rat liver [25,43] and from rabbit reticulocytes.[42] Moreover, it has been demonstrated that the polyribosomal fraction isolated from mouse ascites cells contains a neutral RNase inhibitor, which stabilizes globin mRNA in an *in vitro* translation system.[5] The increased endogenous inhibition of alkaline RNase possibly correlates with increased amount of membrane-bound ribosomes during collagen synthesis and its secretion by the fibroblasts. The membrane-bound ribosomes and possible mRNAs may thus be protected against the alkaline RNase by a pCMB-sensitive component. Hulea and Arnstein [45] have described quantitative and qualitative changes in RNase activity during an erythroid cell development.

The alterations in apparent and total RNase activities during the cell maturation may therefore be closely related to the biochemical and morphological events associated with the development, differentiation and hormonal response of various tissues.

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