Acid Ribonucleases of Lysosomal and Soluble Fractions from Rat Liver*

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SUMMARY

1. Lysosomal acid RNase was partially purified from a rat liver homogenate and a lysosomal fraction. This RNase, of optimum pH 5 to 6, cleaved RNA to form nucleoside 2',3'-cyclic phosphates, which were hydrolyzed to nucleoside monophosphates. This enzyme was heat-labile and had a molecular weight of about 24,000 to 28,000, as estimated by gel filtration with Bio-Gel P-60. This lysosomal acid RNase activity was not found in the supernatant of normal rat liver.

2. The location of this lysosomal RNase in the isolated subcellular fractions derived from normal liver was different from that found in fractions obtained from precancerous liver induced by 4-dimethylaminobenzene. In the latter, the activity was proved to be present in the supernatant fraction, as well as in the lysosomal fraction, as indicated by column chromatography and other enzymological studies.

3. Another acid RNase may be present in the supernatant fraction, although it could not be separated from the alkaline RNase of this fraction. This soluble acid RNase differed from lysosomal acid RNase in molecular weight and in the effects of various compounds on its activity.

4. The effects of ions and other compounds on the two acid RNases and rat liver alkaline RNase were compared. Lysosomal acid RNase was strongly inhibited by ZnCl₂, CuCl₂, and HgCl₂, whereas the other RNases were inhibited only slightly by these ions.

5. Ribosomes from rat liver did not inhibit lysosomal acid RNase, alkaline RNase from rat liver of Escherichia coli RNase I, although E. coli RNase I was inhibited by E. coli ribosomes.

A considerable amount of acid RNase was found by Wattiaux et al., (1) in the soluble fraction of liver. It is not known if this enzyme activity exists in situ in the soluble phase of the cell cytoplasm or if it is released from the lysosomes during the fractionation procedure.

This paper reports the partial purification of lysosomal acid RNase from a rat liver whole homogenate, and from the lysosomal and soluble fractions. The location of this enzyme in subcellular fractions of precancerous liver induced by 4-dimethylaminobenzene differed from that found in normal liver. Another acid RNase, which was distinct from the lysosomal acid RNase, was also isolated from the soluble fraction. These two acid RNases were compared with rat liver alkaline RNase.

EXPERIMENTAL PROCEDURES

Animals—Male Donryu strain rats weighing 150 to 200 g were used. Similar results were obtained with male Wistar strain rats. Animals were killed by decapitation and livers were removed, frozen, and stored for up to 3 months at -15° until used. For the preparation of acid RNase from the soluble or lysosomal fraction, fresh livers of normal or precancerous rats were used immediately after perfusion with 50 ml of ice-cold 0.25 M sucrose.

Cellular Fractionation—All operations were carried out at 4°. One hundred grams of fresh perfused liver from normal or precancerous rats were cut up with scissors, suspended in 350 ml of 0.25 M sucrose, and homogenized in a Waring Blender running at medium speed for 2 min. The supernatant fraction, obtained by centrifugation at 6300 × g for 15 min, was centrifuged at 10,000 × g for 20 min. The precipitate (light mitochondrial fraction) thus obtained was used as the lysosomal fraction, and the supernatant as the soluble fraction, unless otherwise mentioned.

Induction of Malignant Liver with 4-dimethylaminobenzene—Male Donryu strain rats were fed with 4-dimethylaminobenzene (0.06% in standard solid laboratory diet) for 120 days. The basal diet (catalogue no. CF2) containing this carcinogen was obtained from Nihon Clea, Tokyo, Japan. Histochemical observations suggested that almost all the rat livers used were precancerous.

At present there is considerable interest in lysosomal function, but since many lysosomal enzymes have not yet been characterized, further information is needed on the individual enzymes in these particles.

One of the most common enzymatic markers for lysosomes is acid RNase. However, it is still uncertain whether the acid RNase is located exclusively in the lysosomal fraction and whether some conditions such as carcinogenesis may change its location.

* A preliminary account of this study was presented at The Symposium on Enzyme Chemistry, April 9, 1968, at Kanazawa, Japan.

1 At the time of sacrifice, the percentages of animals with livers having carcinomas, adenomas, polymorphic cells, and hyperplastic cells were approximately 10, 60, 90, and 90, respectively. Thus, essentially all the livers used were in the precancerous stage. This pathological study was done by Dr. T. Yokoyama of Department of Pathology, Faculty of Medicine, University of Tokyo.
Substrates—Commercial yeast RNA (Toyo Boseki Company, Japan) was purified using the phenol method described by Kirby (2), and lyophilized after extensive dialysis against distilled water. The apparent s value of the purified yeast RNA was 3.0 s as judged from analytical centrifugation in a Spinco model E ultracentrifuge. Rat liver ribosomal RNA and Escherichia coli soluble and ribosomal RNA were prepared by published methods (2, 3). Polyadenylic acid (about 9 s) was obtained from Miles Chemical Company. Guanosine 2',3'-cyclic phosphate and uridine 2',3'-cyclic phosphate were generous gifts from Professor T. Ukitaka. Carboxymethyl cellulose (0.86 meq per g) and glucose 6-phosphate phosphatase were measured by the published procedures (14-16).

Other Materials—E. coli RNase I was prepared as previously described (17). Carboxymethyl cellulose (0.36 meq per g) and Sephadex G-25 were obtained from Brown Company and Pharmacia, respectively.

Two buffers were used for purification of the enzyme: Buffer A, 0.01 m sodium phosphate buffer (pH 6.5) and Buffer B, 0.01 m sodium phosphate buffer (pH 7.5).

All other reagents used were analytical grade.

RESULTS

1. Purification of Lyosomal and Soluble Acid RNase from Two Acid RNases from Rat Liver

Except when otherwise noted, all procedures were carried out at 0-4°C, and centrifugation was done at 10,000 × g.

1. Purification of Acid RNase from Whole Homogenate

Step 1—Three hundred grams of frozen liver were cut up with scissors and homogenized in 450 ml of 0.10 m KCl in a Waring Blender running at maximum speed for 3 min. This homogenate was passed through nine layers of gauze and centrifuged for 10 min at 4000 × g. The supernatant fraction was used as the crude extract (Table I, Fraction 1).

Step 2—The crude extract was adjusted to 0.25 n H₂SO₄ by dropwise addition of cold 2.0 n H₂SO₄, with vigorous stirring. The supernatant fraction, obtained by centrifugation, was neutralized by dropwise addition of 2.0 n NaOH with vigorous stirring. The supernatant fraction was obtained by centrifugation for 15 min. This step was performed within 30 min (Fraction 2).

Step 3—Fraction 2 was fractionated with ammonium sulfate as follows. Solid ammonium sulfate (123 g) was slowly added to 350 ml of Fraction 2 within 1 hour with constant stirring. The mixture was allowed to stand for 20 min. After removal of m Triu (pH 7.4) containing 1 mM or 10 mM MgCl₂ or 10 mM MgCl₂ and 0.025 mM KCl.

Other procedures—Protein was estimated by the method of Lowry et al. (12) and by its absorption at 280 μg (13) before and after Step 4, respectively. Concentrations of NaCl were determined by titration with AgNO₃ using 1 drop of 10% K₂Cr₂O₇ as an indicator. Cytochrome c oxidase, β-alanine oxidase, and glucose 6-phosphate phosphatase were measured by the published procedures (14-16).

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Two buffers were used for purification of the enzyme: Buffer A, 0.01 m sodium phosphate buffer (pH 6.5) and Buffer B, 0.01 m sodium phosphate buffer (pH 7.5).

All other reagents used were analytical grade.

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the precipitate by centrifugation for 20 min, 121 g of solid ammonium sulfate were slowly added and the mixture was stored over-night with gentle stirring. The resulting precipitate was collected by centrifugation and dissolved in 30 ml of Buffer A (0.01 M sodium phosphate buffer, pH 6.5) and dialyzed against several changes of solid sucrose. The volume was reduced to approximately 5 ml (Fraction 3).

Step 4—Fraction 3 was passed through a column of Sephadex G-25 (2 cm × 80 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 6.5) (Buffer A) and eluted by the same buffer. Fractions containing RNase activity were pooled. Then two-fifths of the enzyme fraction were applied to a carboxymethyl cellulose column (1 cm × 35 cm) equilibrated with Buffer A. The column was washed with a small portion of the same buffer, and then linear gradient elution was performed, starting with 300 ml of 0.4 M NaCl in Buffer A in the reservoir and 300 ml Buffer A in the mixer. Fractions of eluate of 5.5 ml were collected with a fraction collector. The average flow rate was 30 ml per hour. As shown in Fig. 1, RNase activities were obtained in three peaks: nonadsorbed, Peak 1, and Peak 2.

The nonadsorbed fraction was a minor fraction containing less than 10% of the total activity applied. Rechromatography was performed under the same conditions, one with the pooled nonadsorbed fraction or another with the fraction after treatment with 0.25 M H₂SO₄. Almost all activity was again recovered in the same position in both cases. These results suggest that RNase activity in the unadsorbed fraction is not simply an artifact formed during chromatography.

Peak 1 was almost free from alkaline RNase and was eluted in 0.06 M to 0.11 M NaCl. Peak 2, which was eluted in 0.15 M to 0.25 M NaCl, contained both acid and alkaline RNase activities. Both RNase peaks were recovered in the same positions on rechromatography on carboxymethyl cellulose, as shown in Fig. 2.

When acetone fractionation (from 20 to 60% saturation) was applied between Steps 3 and 4, only Peak 2 was obtained. Treatment of Peak 1 with a large excess of acetone did not change the pattern of the chromatogram; Peak 1 activity was separated from Peak 2 activity and then precipitated quantitatively by 90% acetone after rechromatography. The precipitate was dissolved in Buffer A and chromatographed on a carboxymethyl cellulose column under the same conditions described above, the activity being recovered in the same position as Peak 1 (Fig. 2C).

Further studies showed that Peaks 1 and 2 were obtained from the lysosomal and soluble fractions, respectively.

A summary of the results of a typical purification is given in Table I. At the final stage of purification, acid RNase (Peak 1) was approximately 150-fold purer than the crude extract and free from detectable alkaline RNase. Peak 2 contained both alkaline and acid RNase, which were, respectively, 600- and 200-fold purer than in the crude extract. Alkaline RNase free from acid RNase could be obtained by heating the Peak 2 fraction as described below. The preparation at Step 4 was used for further studies.

Contaminating Enzymes—The possible presence of contaminating enzymes in acid RNase Peak 1 and RNase Fraction Peak 2 were examined by essentially the same methods as those described previously (18). Alkaline and acid phosphatases could not be detected after 2 hours of incubation using 1 unit of either enzyme fraction. Moreover, phosphodiesterase activities, which cleave di-p-nitrophenyl phosphate or p-nitrophenyl uridine 5'-monophosphate, were not detected at pH 9.0 or 5.5 in either enzyme fraction under the same conditions as those described above. Deoxyribonuclease activity was also not detected in 1 unit of either fraction after 2 hours of incubation.

2. Studies on Location of Acid RNase Activity Using Purification Procedures

Much acid RNase activity was found in the supernatant fraction (1). It is unknown whether this activity normally exists in the soluble fraction or whether it is derived from lysosomes. Accordingly, studies were made on the location of acid RNase using the purification procedures described above.

Acid RNase from Lysosomal Fraction—The lysosomal and soluble fractions were prepared separately from 102 g of rat liver by the procedures described under “Experimental Procedures.” The nuclear fraction was discarded because it contained only a trace of activity, as shown previously (1). Although alkaline RNase was present in this lysosomal fraction, no latency of this enzyme activity, assayed in 0.5 M sucrose, was observed, suggesting that it was a contaminant of the lysosomal fraction. Approximately 25% of total alkaline RNase activity was recovered in this lysosomal fraction.

Acid RNase of the lysosomal fraction was solubilized by gentle stirring for 30 min in 50 ml of Buffer A. After removal of the precipitate by centrifugation for 10 min, the supernatant fraction was adjusted to 0.25 M H₂SO₄ by dropwise addition of cold 2.0 M H₂SO₄ with vigorous stirring, and the resulting precipitate was discarded by centrifugation. The supernatant solution was neutralized by the dropwise addition of 2.0 N NaOH with vigorous stirring for 30 min in 50 ml of Buffer A. After removal of the precipitate by centrifugation for 10 min, the supernatant fraction was adjusted to 0.25 M H₂SO₄ by dropwise addition of cold 2.0 M H₂SO₄ with vigorous stirring, and the resulting precipitate was discarded by centrifugation. The supernatant solution was neutralized by the dropwise addition of 2.0 N NaOH with vigorous stirring. The supernatant fraction obtained by centrifugation for 15 min was fractionated with ammonium sulfate. The precipitate obtained in the same saturation of ammonium sulfate
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Fig. 2. Rechromatography of RNases on carboxymethyl cellulose. a, rechromatography of Peak 1. b, rechromatography of Peak 2. The fractions in Peak 1 or Peak 2 of the first chromatography (Fig. 1) on carboxymethyl cellulose were pooled and rechromatographed on the same adsorbent after passage through Sephadex G-25. Acid RNase activity (O-O) and alkaline RNase activity (C-C) were measured as described in the text. Concentration of NaCl is shown by the dotted line. c, rechromatography of Peak 1 after acetone treatment. After rechromatography as above and concentration on solid sucrose, Peak 1 was mixed with 9 volumes of acetone with 20 mg of carrier bovine serum albumin at -60°C with gentle stirring. This acetone fraction was dissolved in Buffer A and chromatographed on a carboxymethyl cellulose column under the same conditions as described above. Acid RNase activity (O-O) was measured as described in the text. Concentration of NaCl is shown by the dotted line.
Thus, acid RNase obtained from the 10,000 × g pellet was confirmed to be derived from lysosomes.

Acid RNase Activity from Soluble Fraction—To see whether acid RNase activity in the soluble fraction is the same as that in the lysosomal fraction, the activity in the soluble fraction was studied. The soluble fraction, prepared from 102 g of rat liver by the procedures described under “Experimental Procedures,” was adjusted to 0.25 N H$_2$SO$_4$ by dropwise addition of cold 2.0 N H$_2$SO$_4$ with vigorous stirring. The supernatant fraction obtained after centrifugation was neutralized as Step 2 from whole homogenate and centrifuged for 15 min. The supernatant fraction was fractionated with ammonium sulfate. The precipitate obtained in the same saturation of ammonium sulfate as Step 3 from whole homogenate was applied on carboxymethyl cellulose after gel filtration with Sephadex G-25. For carboxymethyl cellulose chromatography, a linear concentration gradient was applied essentially as described above. As shown in Fig. 3b, most activity was eluted at the position of lysosomal acid RNase, but at a similar position to Peak 2, as shown in Fig. 1, although it could not be separated from alkaline RNase.
TABLE II

Hydrolysis of various RNAs with three RNases

Two milligrams of each RNA were incubated under the standard conditions. See text for details.

<table>
<thead>
<tr>
<th>RNA</th>
<th>RNase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak I (acid RNase)</td>
</tr>
<tr>
<td>Yeast RNA</td>
<td>0.910</td>
</tr>
<tr>
<td>Rat liver ribosomal RNA</td>
<td>0.643</td>
</tr>
<tr>
<td>E. coli soluble RNA</td>
<td>0.660</td>
</tr>
<tr>
<td>Polyadenylic acid</td>
<td>0.615</td>
</tr>
</tbody>
</table>

Recovery and specific activity of acid RNase were 8% and 30 units per mg of protein, respectively. Evidence that this soluble RNase activity at acidic pH values is not that of alkaline RNase, but that of true acid RNase, is described below.

These results suggest that the RNase activity of Peaks 1 and 2 obtained from the whole homogenate can be designated as lysosomal and soluble acid RNase as almost all the activity was recovered in the two fractions. Hence, the terms "lysosomal" acid RNase and "soluble" acid RNase are tentatively used in the following sections.

3. Change of Location of Acid RNase of Precancerous Liver Induced by 4-Dimethylaminoazobenzene

During the process of carcinogenesis induced by 4-dimethylaminoazobenzene increment of acid RNase activity in the supernatant fraction of rat liver was shown by Deckers-Passau, Maisin and deDuve (21) and Reid and Nodes (22). However, this activity cannot be simply attributed to lysosomal acid RNase. Acid RNase activity in the precancerous liver induced by 4-dimethylaminoazobenzene was studied to see whether this increase was due to synthesis de novo of soluble acid RNase or to release of acid RNase from lysosomes.

The soluble and lysosomal fractions from 80 g of liver of rats fed with 4-dimethylaminoazobenzene were treated in the same way as normal rat liver. The chromatographic pattern obtained on carboxymethyl cellulose is shown in Fig. 4. From the lysosomal fraction, most acid RNase activity was obtained at the same concentration of NaCl as Peak 1 (Fig. 4b). Only a little activity was obtained at the position of Peak 2, with contaminating alkaline RNase.

On the other hand, two acid RNase activities were obtained from the soluble fraction (Fig. 4a), unlike the case with normal rat liver (Fig. 3b). These activities corresponded to the soluble and lysosomal acid RNases obtained from normal liver (Fig. 3). Specific activities of the acid RNases in the homogenate fractions from the livers of normal and 4-dimethylaminoazobenzene-fed rat liver were approximately the same level; normal liver, 0.36, and 4 dimethylaminoazobenzene liver, 0.37 unit per mg of protein. Essentially the same result was obtained using soluble fraction of 78,000 X g for 40 min. Thus, approximately 50% of lysosomal acid RNase activity was shifted from lysosomal to soluble fraction as calculated roughly by the results of Fig. 4.

These results together with enzymatic studies shown below suggest that lysosomal acid RNase was obtained from the soluble fraction of precancerous liver induced by 4-dimethylaminoazobenzene.

![Fig. 5. Effect of pH on enzyme activity. The buffers used were sodium acetate (O-O), potassium phosphate (O-O), Tris-chloride (A-A), and glycine-NaOH (△-△). a, effect of pH on acid RNase from Peak 1. b, effect of pH on acid and alkaline RNase from Peak 2. RNase of Peak 2 was heated at 80° for 50 min in Buffer A and activity was measured with various buffers.](http://www.jbc.org/)
TABLE III
Hydrolysis of nucleoside 2',3'-cyclic phosphate

Five hundred millimicromoles of each nucleoside 2',3'-cyclic phosphate was incubated at 37°C for 1 hour in a total volume of 0.20 ml using the same buffers as those in standard conditions. After incubation, 30 μl of each sample were immediately spotted on paper. After overnight development using Solvent 4, the spots corresponding to nucleoside 2',3'-monophosphate and nucleoside 2',3'-cyclic phosphates were cut out and eluted with 3.0 ml of 0.1 N HCl at 37°C overnight. Then absorbance at 260 nm was measured and the percentage of cyclic phosphate hydrolyzed was calculated.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Amount of enzyme</th>
<th>Hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>Uridine 2',3'-cyclic phosphate</td>
</tr>
<tr>
<td>Peak I acid</td>
<td>0.48</td>
<td>78</td>
</tr>
<tr>
<td>Peak II acid</td>
<td>0.60</td>
<td>58</td>
</tr>
<tr>
<td>Peak II alkali</td>
<td>1.40</td>
<td>13</td>
</tr>
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</table>

TABLE IV
Stability of RNases

Each RNase was treated as specified under "Treatment" in Buffer A in the absence or presence of ammonium sulfate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in Buffer A</td>
<td>in the presence of 0.33 M (NH₄)₂SO₄ in Buffer A</td>
</tr>
<tr>
<td>Peak I acid</td>
<td>No</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>40°C, 5 min</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>51°C, 5 min</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>80°C, 5 min</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>37°C, 2 hrs</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>4°C, 12 hrs</td>
<td>37</td>
</tr>
<tr>
<td>Peak II acid</td>
<td>51°C, 5 min</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4°C, 12 hrs</td>
<td>38</td>
</tr>
<tr>
<td>Peak II alkali</td>
<td>51°C, 5 min</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>80°C, 5 min</td>
<td>103</td>
</tr>
</tbody>
</table>

* No tests were done.

II. Properties of Lysosomal Acid RNase and Existence of Soluble Acid RNase

The properties of lysosomal acid RNase and the existence of soluble acid RNase were studied using RNases prepared from the homogenate fraction.

Substrate Specificity—The initial velocities of hydrolysis of various RNAs with the two acid RNases and alkaline RNase were studied (Table II). Although the acid RNase from the soluble fraction could not be separated from alkaline RNase, the activity at pH 5.0 was taken as that of the soluble acid RNase. This assumption was supported by subsequent experiments (Fig. 5, Table IV). Ribosomal and soluble RNAs were digested more slowly than yeast RNA with all three enzymes.

Products of Hydrolysis of RNA Including Cyclic Nucleotides by Two Acid RNases and Alkaline RNase—One milligram of E.

![Figure 6. Gel filtration of RNases with Bio-Gel P-60. Pancreatic RNase from rat (a), rat liver soluble acid RNase (O—O), and alkaline RNase (O—O) (b), and rat liver lysosomal acid RNase (c) were each chromatographed on Bio-Gel P-60 with bovine serum albumin (BSA), ovalbumin (Alb), and cytochrome c (Cyt C) as markers. See details for text.](http://www.jbc.org/)
The effect of pH on the RNase activity of Peak 2 was studied; maximum activity was shown at pH 7.7 to 8.0 and the ratio of the activity at pH 8.0 to that at pH 5.5 was 1.8 (Fig. 5a). RNase activity at pH 5.5 decreased when Peak 2 was heated at 80° for 5 min or stored for 12 hours in Buffer A at 4°. After heat treatment, the ratio of the activity at pH 8.0 to that at pH 5.5 was 8.0 (Fig. 5c). These and other enzymatic properties shown above suggested that Peak 2 from soluble fraction or homogenate consisted of mixtures of the acid and alkaline RNase; the acid RNase was denatured by the treatment as shown above.

**Effect of Heat on Enzyme Activity—**Lysosomal and soluble acid RNases were more labile than alkaline RNase to heat treatment. As shown in Table V, on incubation at pH 6.5 for 5 min at 80°, lysosomal acid RNase lost almost all its activity. When the two acid RNases were stored at 4° for 12 hours, only 24% of the original activity remained. The two acid RNases were rather stable on addition of ammonium sulfate or sodium chloride (Table IV).

**Estimation of Molecular Size of RNases—**The molecular sizes of acid RNase and alkaline RNase were estimated by filtration on Bio-Gel P-60. Fig. 6 shows typical filtration patterns. Lysosomal acid RNase and acid RNase from the homogenate of Peak 1 were estimated to have approximate molecular weights of 24,000 to 28,000. Peak II containing both acid RNase and alkaline RNase activity had a molecular weight of 11,500 to 12,500. Similar results were obtained using elution buffer containing 0.2 M NaCl or on filtration after incubation for 1 hour with 0.1% Triton X-100.

Rat pancreatic RNase, which was purified by the same procedure as rat liver RNases to show a peak similar to Peak 2 on the carboxymethyl cellulose column, was estimated to have a molecular weight of 11,500 to 12,500.

**Comparative Studies of Rat Liver RNases—**The effects of various compounds and metal ions on the activities of the three RNases of rat liver were compared. The results are summarized in Table V. Lysosomal acid RNase activity was strongly inhibited by ZnCl₂, CuCl₂, and HgCl₂, whereas other RNases were inhibited only slightly by these ions. Other metal ions at concentrations of 10⁻³ to 10⁻⁴ M had no marked effects on enzyme activities (Table V). With the addition of NaCl at concentrations of 0.1 to 0.2 M, RNase activities were activated approximately 1.6- to 1.8-fold, as shown in Fig. 7.

Of such compounds as EDTA, ICH₂COONa, KCN, KI, p-chloromercuribenzoate, and urea, only urea had a significant effect on enzyme activity. On the addition of 0.8 M urea acid RNase of Peak 1 and alkaline RNase of Peak 2 became 1.5-fold and acid RNase of Peak 2 1.2-fold than control level.

**Peak 1 Acid RNase in Soluble Fraction of Liver of Rats Fed with 4-Dimethylaminoazobenzene—**Properties of acid RNase of Peak 1, obtained from soluble fraction of the liver of rats fed with 4-dimethylaminoazobenzene were examined. This RNase had an optimal pH of 5.2 and was recovered in the same position as lysosomal acid RNase on rechromatography with carboxymethyl cellulose. Molecular weight of this acid RNase was similar to Peak 1 obtained from lysosomal fraction as estimated by Bio-Gel P-60.

Inhibitory effects of various metal ions on the three RNases of the soluble fraction of 4-dimethylaminoazobenzene liver were compared. Only Peak 1 was sensitive to ZnCl₂, CuCl₂, and HgCl₂. Relative activities on addition of these salts were the same as Peak 1 obtained from lysosomal fraction. Relative activities of Peak 1 obtained from 4-dimethylaminoazobenzene liver on addition of 10⁻⁴ to 10⁻⁸ M of ZnCl₂, CuCl₂, and HgCl₂ were 20, 9, and 7%, respectively; those of Peak 1 of lysosome of normal liver were 29, 3.8, and 13, respectively (Table V). Effects of other metal ions were the same as on lysosomal acid RNase. Effects of these compounds on Peak 2 acid and alkaline RNase prepared from rat fed with 4-dimethylaminoazobenzene were the same as the normal enzymes (Table V).
TABLE VI

Effect of ribosomes on RNase activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Amount of ribosomes as RNA</th>
<th>Increase in absorbance at 260 mμ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>Rat liver lysosomal acid RNase</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td>0.308</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.343</td>
</tr>
<tr>
<td>E. coli ribosomes</td>
<td>0.20</td>
<td>0.318</td>
</tr>
<tr>
<td>Rat liver ribosomes I</td>
<td>0.38</td>
<td>0.968</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td>1.01</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.97</td>
</tr>
<tr>
<td>Rat liver ribosomes II</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Rat liver ribosomes III</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

Effect of Ribosomes on RNase Activity—The effect of ribosomes on the three RNases was studied. The ribosomes were prepared from E. coli, and from rat liver with or without deoxycholate.

As shown in Table VI, E. coli RNase I was inhibited by ribosomes from E. coli but not by ribosomes from rat liver. Rat liver ribosomes had no inhibitory effects on RNases from rat liver, regardless of how they were prepared.

DISCUSSION

Lysosomal acid RNase, which degrades RNA to produce nucleoside 2',3'-cyclic phosphates and oligonucleotides, was purified from rat liver in the present study. Our method of purification of the enzyme from the rat liver homogenate is applicable to the purification of enzyme from the soluble and lysosomal fractions, giving both fractions in approximately the same yield and increase of specific activity. Acid RNase has already been purified from hog spleen by Bernardi and Bernardi (23). However, their enzyme, which was not demonstrated to be derived from lysosomes, differs from the present enzyme in its source and in some enzymological properties. Lysosomal acid RNase was separated from other known nucleolytic enzymes of rat liver. Thus, specific assays for rat liver phosphodiesterase and deoxyribonuclease gave negative results (18).

The presence of another type of acid RNase in the rat liver soluble fraction has been suggested. On carboxymethyl cellulose chromatography of a preparation from the whole liver homogenate, two peaks of activity (Peaks 1 and 2) were obtained (Fig. 1). The activity of Peak 1 was shown to be similar in chromatographic behavior to that of lysosomal acid RNase (Fig. 3).

Peak 2 contained both alkaline and acid RNase derived from the soluble fraction. The presence of acid RNase in Peak 2 is suggested by comparison of the heat stability, specificity, and other properties with those of alkaline and lysosomal acid RNase (Figs. 5 and 6 and Tables II through V). Since only a little Peak 2 was obtained when enzyme was derived from lysosomes and only Peak 2 was obtained when the lysosomes were discarded, the acid RNase activity in Peak 2 can be considered as derived from cytoplasmic fluid (post-lysosomal supernatant) (Fig. 3). As shown by previous workers, a considerable amount of RNase activity was obtained in the soluble fraction after cellular fractionation (1). This activity, as shown by the present studies, is different from lysosomal acid RNase in molecular weight and chromatographic behavior on carboxymethyl cellulose. Thus, the supernatant RNase activity shown by the previous workers cannot simply be said to be released from lysosomes.

Soluble acid RNase could not be separated from alkaline RNase by starch block electrophoresis at pH 7.5, gel filtration with Sephadex G-100, or chromatography with Amberlite XE 64.

Alkaline RNase, which is purified after denaturation of the activity at acidic pH, was independently purified by other workers. Our results essentially agree with theirs.

Studies on lysosomal acid RNase suggest that some compound is bound to this enzyme, although the nature of the compound is unclear. This compound can be separated by acid treatment, which is an essential step in this purification procedure. Since treatment of lysosomal fraction with Triton X-100 also gave the same acid RNase, the compound is not bound to the RNase by covalent bond. The significance of this presumed complex remains to be determined. The effect of the two treatments—acid and Triton X-100—is probably not on the state of aggregation of acid RNase itself, since no aggregation of the purified acid RNase was observed at pH 7.5, 6.5, or 5.5 in 0.01 M sodium phosphate buffer as studied by gel filtration.

Normal supernatant fraction contained only soluble acid RNase (Fig. 3b), whereas in the supernatant fraction from precancerous liver, a peak of acid RNase identical with Peak 1 was obtained together with that of soluble acid RNase (Fig. 4a). This acid RNase activity of Peak 1 is considered to be of lysosomal origin, on the basis of studies involving rechromatography, gel filtration, and effects of metal ions. These results suggest that a significant amount of the enzyme must have changed its location in the precancerous liver. It is certain, however, whether the acid RNase activity was released from lysosomes in vivo, or whether the lysosomes of precancerous liver become fragile and, unlike normal liver, were broken during the fractionation procedures.

There have been many studies on ribonuclease and cancer, as reviewed by Roth (25). It is obvious that many, if not most, of the studies discussed in the above review have dealt with over-all RNase activity of the tissues without fractionation of the acid RNase activities of the cancer cells. The rise of free acid RNase activity in precancerous liver induced by 4-dimethylaminoazobenzene was shown by Deckers-Passau et al. (21) and Reid and Noden (22). Specific activities of the homogenate from normal and precancerous liver, in the present experiments, were, after disruption of lysosome, approximately the same, confirming the findings of previous workers (21). As discussed above, our data have shown that the acid RNase of lysosomes has changed its location to soluble fraction in the case of precancerous liver. Therefore, it is suggested that this rise was not due to synthesis de novo of soluble acid RNase but to change of the location of RNase from lysosomes. It is uncertain whether or not this

change is related to the carcinogenic effect of 4-dimethylaminoazobenzene.

Many histochemical and cell fractionation experiments have suggested that release of lysosomal enzymes occurs in vivo under various conditions, as reviewed by Allison (26). But considering the existence of isoenzymes such as β-glucuronidase in cellular fractions other than the lysosomes (27), it seems important to study the release of lysosomal enzymes from lysosomes and to specify the properties of enzymes in lysosomes, as presented here.

As shown in Table VI, ribosomes from rat liver had no inhibitory effect on the RNases, although ribosomes from E. coli remarkably inhibited the activity of RNase I of E. coli (28, 29). As the ribosomes tested were prepared both with and without use of detergent, ribosomes from rat liver were found to have no inhibitory factor for RNase of this tissue. Thus, the ribosomes of rat liver were degraded in vitro at both acidic and alkaline pH values without lag periods, as shown previously (30).

The basis of the difference in inhibitory activities of ribosomes of E. coli and rat liver remains to be ascertained.

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