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Studies on Extracellular Ribonucleases of *Ustilago sphaerogena*

PURIFICATION AND PROPERTIES

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1. Four ribonucleases were isolated from culture media of *Ustilago sphaerogena*. They were designated ribonucleases U_1, U_2, U_3 and U_4. 2. They were purified about 1600-, 3700-, 1100- and 16-fold respectively. 3. It was shown by gel filtration that ribonucleases U_1, U_2 and U_3 have molecular weights about 10000 like ribonuclease T_1, and that ribonuclease U_4 is much larger. 4. Ribonucleases U_1, U_2 and U_3 are thermostable, but ribonuclease U_4 is not. 5. The pH optimum of ribonucleases U_1 and U_4 is pH 8·0–8·5, and that of ribonucleases U_2 and U_3 is pH 4·5.

In the past 4 years a systematic search for ribonucleases has been carried out in our Laboratory in the hope of finding new enzymes that will be of value in the analysis of nucleotide sequences in RNA.

As a part of the search, four ribonucleases have been isolated from culture media of the smut fungus, *Ustilago sphaerogena*, from which an extracellular ribonuclease [ribonucleate (guanine nucleotide)-2′-transferase (cyclizing), EC 2.7.7.26] was isolated by Glitz & Dekker (1963, 1964*a*,*b*).

The present paper deals with the purification and some of the properties of extracellular ribonucleases of *Ustilago sphaerogena*. The substrate specificity of the enzymes is reported in the following paper (Arima, Uchida & Egami, 1968).

MATERIALS AND METHODS

Materials. The low-molecular-weight yeast RNA used for ribonuclease assay was kindly given by Toyo Spinning Co. (Inuyama, Aichi, Japan). DEAE-cellulose (0·87 m-equiv./g.) was obtained from Brown Co. (New York, N.Y., U.S.A.), and was washed before use with 0·5 N-NaOH; fines were removed by repeated decantation. CM-cellulose (0·52 m-equiv./g.) was obtained from Serva Co. (Heidelberg, Germany) and was washed before use with 0·5 N-HCl. Sephadex G-75 was a product of Pharmacia Co. (Uppsala, Sweden). Before use, gels were washed with 0·01 M-sodium acetate buffer, pH 6·0. *Ustilago sphaerogena* used in this experiment was kindly given by Dr C. A. Dekker.

Assay for ribonuclease. The enzyme activity was determined by measuring E_{260} of the acid-soluble digestion products from yeast RNA with an Ito spectrophotometer (Ito Chotanpa Co., Hakusan, Bunkyo, Tokyo, Japan) with silica cells of 1 cm. light-path, according to the assay for ribonuclease T_1 described by Takahashi (1961), except that 0·2 M-sodium acetate buffer, pH 4·5, was used for ribonucleases U_2 and U_3 instead of 0·2 M-tris–HCl buffer, pH 7·5. The enzyme unit and specific activity were calculated as described by Takahashi (1961).

Assay for non-specific phosphodiesterase and phosphomonoesterase. The 30-fold-concentrated supernatant was tested for enzyme activity as described by Koerner & Sinsheimer (1957) with a slight modification.

The assay mixture for phosphodiesterase contained the following: mM-sodium di-*p*-nitrophenyl phosphate, 1·0 ml.; M-tris–HCl buffer, pH 8·5, or M-ammonium acetate buffer, pH 5·5, 0·2 ml.; enzyme solution, 0·3 ml. After incubation at 37° for 24 hr. 1·5 ml. of N-NH_3 was added to the mixture and E_{410} measured.

The assay mixture for phosphomonoesterase contained the following: 2 mM-disodium *p*-nitrophenyl phosphate, 1·0 ml.; M-tris–HCl buffer, pH 8·5, or M-ammonium acetate buffer, pH 5·5, 0·2 ml.; enzyme solution, 0·3 ml. After incubation at 37° for 60 min. 1·5 ml. of N-NH_3 was added to the mixture and E_{410} measured.

Formation of ribonucleases. The growth medium (standard medium) consisted of glucose (2%), glycine (0·2%), KH_2PO_4 (0·05%), K_2HPO_4 (0·05%), $MgSO_4$ (0·01%), KCl (0·01%) and $CaCl_2$ (0·01%). Portions (250 ml. each) of growth medium in 500 ml. Sakaguchi flasks were kept for 15 min. at 120° for sterilization, cooled to room temperature and then directly inoculated with 1 ml. of preincubated culture. Growth was estimated by measuring the turbidity at 650 mμ. The flasks were incubated for about 60 hr. at 30° on a reciprocal shaker operating at 90 cyc./min. until cell growth reached a maximum (turbidity at 650 mμ reached 10·0).

Determination of protein concentration. The protein concentration was determined by measuring E_{280} of the enzyme solution for ribonucleases U_1, U_2 and U_3, but for the purification of ribonuclease U_4 the method of Lowry, Rosebrough, Farr & Randall (1951) was used.

Existence of ribonucleases. Gel filtration with Sephadex G-75 of the culture medium was carried out as follows to determine how many ribonucleases were present. A column (1·5 cm. × 80 cm.) of Sephadex G-75 was washed with 400 ml.

Table 1. *Purification of ribonuclease U_1*

Ribonuclease activity was measured at pH 7·5.

Purification step	Total vol. of enzyme soln. (ml.)	Total protein (E_{280})	Total activity (units)	Specific activity	Purification	Yield (%)
1. Crude medium	25000	84100	28000	0·033	(1)	(100)
2. Batch-wise treatment with DEAE-cellulose	5000	10350	7500	0·073	2	26·8
3. Batch-wise treatment with CM-cellulose	275	178	2460	1·38	42	8·8
4. First DEAE-cellulose column chromatography	28	33	1810	5·52	165	6·5
5. Second DEAE-cellulose column chromatography	32	22	1310	5·78	173	4·7
6. Sephadex G-75 gel filtration	15	1·6	885	54·4	1640	3·2

Table 2. *Purification of ribonuclease U_2*

Ribonuclease activity was measured at pH 4·5.

Purification step	Total vol. of enzyme soln. (ml.)	Total protein (E_{280})	Total activity (units)	Specific activity	Purification	Yield (%)
1. Crude medium	25000	84100	17250	0·021	(1)	(100)
2. Batch-wise treatment with DEAE-cellulose	5000	10350	13350	0·129	6·3	77·5
3. Batch-wise treatment with CM-cellulose	275	178	5750	3·215	157	33·3
4. First DEAE-cellulose column chromatography	35	19	3720	19·8	965	21·6
5. Second DEAE-cellulose column chromatography of fractions 22–26	20	2·6	1220	47·6	2310	7·1
Second DEAE-cellulose column chromatography of fractions 17–21	20	2·6	740	28·3	1380	4·3
6. Sephadex G-75 gel filtration of fractions 17–21	10	0·6	446	77·0	3750	2·6

Table 3. *Purification of ribonuclease U_3*

Ribonuclease activity was measured at pH 4·5. Steps 1–3 are the same as in the purification of ribonuclease U_2.

Purification step	Total vol. of enzyme soln. (ml.)	Total protein (E_{280})	Total activity (units)	Specific activity	Purification	Yield (%)
1. Crude medium	25000	84100	17250	0·021	(1)	(100)
2. Batch-wise treatment with DEAE-cellulose	5000	10350	13350	0·129	6·3	77·5
3. Batch-wise treatment with CM-cellulose	275	178	5750	3·215	157	33·3
4. DEAE-cellulose column chromatography	28	4·8	146	3·01	147	0·8
5. Sephadex G-75 gel filtration	9	0·37	85	22·8	1110	0·5

of 10mM-sodium acetate buffer, pH 6·0, before applying 2·0ml. of enzyme solution. Elution was begun with 10mM-sodium acetate buffer, pH 6·0. Fractions (3ml.) were collected at a flow rate of 20ml./hr. and ribonuclease activity was measured at pH 7·5 and 4·5.

Purification of ribonucleases U_1, U_2 and U_3. After the culture (standard medium) had been incubated for 60hr. at 30°, the cells were removed by centrifugation at 800 *g* for 15min. and discarded (step 1). All the following steps were carried out at 4°. The supernatant (25l.) was adjusted to

Table 4. *Purification of ribonuclease U_4*

Ribonuclease activity was measured at pH 7·5.

Purification step	Total vol. of enzyme soln. (ml.)	Total protein (mg.)	Total activity (units)	Specific activity	Purification	Yield (%)
1. Crude medium	5000	16500	19450	0·118	(1)	(100)
2. $(NH_4)_2SO_4$ precipitation	20	66	5125	7·720	65	26·3
3. Sephadex G-75 gel filtration	180	18	1395	7·760	66	7·2
4. DEAE-cellulose column chromatography	40	7·4	212	2·860	24	1·1
5. Sephadex G-75 gel filtration	2	4	72	1·800	15·3	0·4

pH 8·5 with N-NaOH and to it was added 100g. of DEAE-cellulose that had been treated with 0·01 M-tris–HCl buffer, pH 8·5. After 1 hr. the suspension was filtered by suction. The filtrate was adjusted again to pH 8·5 with N-HCl, another 50g. of DEAE-cellulose was added and the suspension filtered. Both filter cakes thus obtained were combined and dispersed in 5l. of 0·3 M-NaCl in 10 mM-tris–HCl buffer, pH 8·5. After standing for 30 min. the suspension was filtered (step 2).

The filtrate was freeze-dried and dissolved in a small volume of water. The enzyme solution was extensively dialysed against water and adjusted to pH 4·0 with N-HCl before adding CM-cellulose (100g.) that had been treated with 10 mM-sodium acetate buffer, pH 4·0. After 1 hr. the suspension was filtered by suction and the filter cake thus obtained dispersed in 1l. of 0·2 M-NaCl in 10 mM-sodium acetate buffer, pH 4·0. The suspension was filtered and the filtrate freeze-dried and dissolved in a small volume of water (step 3).

After extensive dialysis against water, insoluble material was centrifuged off and the supernatant adjusted to pH 8·5 with N-NaOH. The solution was applied to a column (1·1 cm. × 20 cm.) of DEAE-cellulose that had been equilibrated with 10 mM-tris–HCl buffer, pH 8·5. The column was washed with 40 ml. of the same buffer and the effluent discarded. The enzyme was eluted with 280 ml. of 10 mM-tris–HCl buffer, pH 8·5, with a linear gradient of NaCl up to 0·33 M. Fractions (4 ml.) were collected at a flow rate of 20 ml./hr. Fractions from the column were subjected to the quantitative assay for ribonuclease activity at pH 7·5 and 4·5. Three peaks of fractions giving a positive ribonuclease test were pooled separately (step 4) and were designated ribonucleases U_1, U_2 and U_3 respectively. After dialysis, ribonuclease U_1 and U_2 fractions were rechromatographed separately under the same conditions as step 4 (step 5).

Ribonucleases U_1 and U_2 after step 5, and ribonuclease U_3 after step 4, were dialysed against distilled water, freeze-dried and dissolved in 1·0 ml. of distilled water. The enzyme solutions were separately applied to columns (1·5 cm. × 60 cm.) of Sephadex G-75 that had been equilibrated with 10 mM-sodium acetate buffer, pH 6·0. The protein was eluted with the same buffer. Fractions (3 ml.) were collected at a flow rate of 20 ml./hr. Fractions from the column were subjected to the quantitative assay for ribonuclease activity and those fractions giving a positive ribonuclease test were pooled (step 6). Data on the purification of enzymes from 25l. of growth medium are summarized in Tables 1, 2 and 3.

Purification of ribonuclease U_4. The RNA medium (see below) was used for the purification of ribonuclease U_4 as it gave a much higher yield than the standard medium. It contained 0·1% RNA instead of phosphates as a sole phosphorus source. A 5l. volume of medium was pooled and the cells were centrifuged off (step 1). The supernatant was freeze-dried, dissolved in 250 ml. of water and then dialysed against water. Insoluble material formed in the dialysis tube was centrifuged off and the supernatant

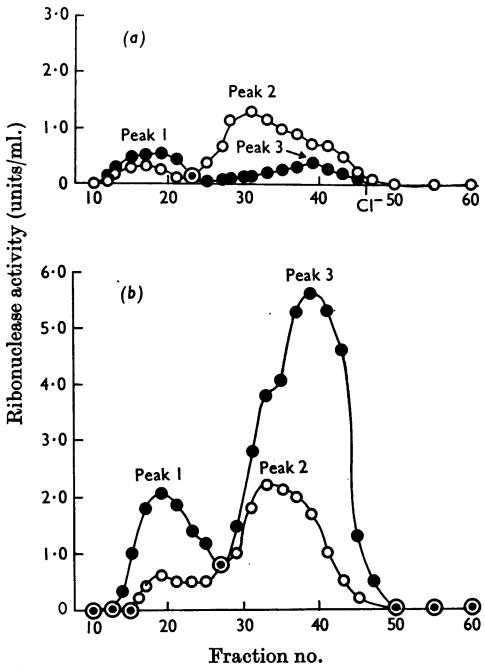

Fig. 1. Existence of ribonuclease in the growth medium. Supernatants of the standard medium (*a*) and the RNA medium (*b*) were concentrated 30-fold and 1 ml. of each was applied separately to a Sephadex G-75 column (1·5 cm. × 80 cm.) for gel filtration. Elution was begun with 10 mM-sodium acetate buffer, pH 6·0. Fractions (3 ml.) were collected at a flow rate of 20 ml./hr. ○, Ribonuclease activity measured at pH 4·5 in 50 mM-sodium acetate buffer; ●, ribonuclease activity measured at pH 7·5 in 50 mM-tris–HCl buffer.

brought to 95% saturation with $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 11000 *g* for 60min. (step 2).

The precipitate was dissolved in a small volume of water and dialysed against water. The contents of the dialysis tube (20ml.) were applied to a column (6cm. × 23cm.) of Sephadex G-75 that had been equilibrated with 10mM-sodium acetate buffer, pH6·0. The protein was eluted with the same buffer and fractions (10ml.) were collected at a flow rate of 20ml./hr. The first 240ml. was discarded and the following 180ml. collected (step 3).

The collected enzyme solution was adjusted to pH8·5 with N-NaOH and applied to a column (1·5cm. × 20cm.) of DEAE-cellulose that had been treated with 10mM-tris–HCl buffer, pH8·5. The enzyme was passed through the column at a flow rate of 10ml./hr. (step 4).

The eluate was freeze-dried, dissolved in 1ml. of distilled water and applied to a column (1·5cm. × 60cm.) of Sephadex G-75 that had been equilibrated with 10mM-sodium acetate buffer, pH6·0. The protein was eluted with the same buffer under the same conditions as the final step of the purification of ribonucleases U_1, U_2 and U_3 (step 5).

Data on the purification of ribonuclease U_4 from 5l. of growth medium are summarized in Table 4.

Inhibition test for the enzyme. Inhibition studies of the enzymes were performed by using the usual quantitative assay for ribonuclease activity, with the addition of a given inhibitor instead of EDTA.

RESULTS

Non-specific phosphodiesterase and phosphomonoesterase activity. Thirtyfold concentrated standard medium and RNA medium have neither phosphodiesterase nor phosphomonoesterase activity.

Existence of ribonucleases in growth medium. Supernatants of the standard medium and the RNA medium were concentrated 30-fold separately, 1ml. of each was applied to Sephadex G-75 and the ribonuclease activity of each fraction determined by the usual ribonuclease assay. As shown in Figs. 1(*a*) and 1(*b*), *Ustilago sphaerogena* produces at least three ribonucleases in the standard medium. The production of two of them (peaks 1 and 3 correspond to ribonucleases U_4 and U_1 respectively; see Fig. 3) was greatly enhanced in RNA medium.

Purification of ribonucleases U_1, U_2 and U_3. The results of the purification of these ribonucleases from 25l. of standard growth medium are given in Tables 1, 2 and 3. In step 2, batch-wise treatment with DEAE-cellulose of the crude medium could be performed with only slight loss in the activity measured at pH4·5.

In step 3, batch-wise treatment with CM-cellulose resulted in a considerable increase in the specific activity.

With DEAE-cellulose column chromatography (step 4), three peaks of ribonuclease activity appeared (Fig. 2). They were designated ribonucleases U_1, U_2 and U_3. Ribonuclease U_1 was more active when measured at pH7·5, and ribonucleases U_2 and U_3 were more active at pH4·5. This step resulted in an increase in the specific

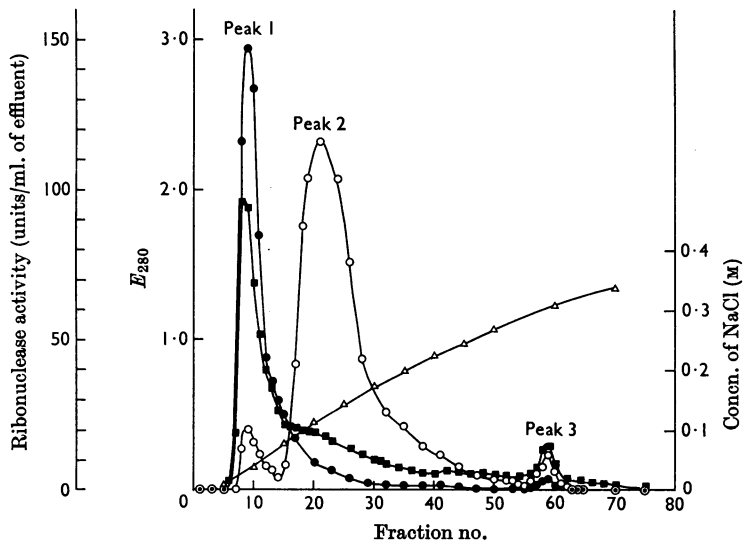

Fig. 2. DEAE-cellulose column chromatogram of the ribonucleases in the standard growth medium. The enzyme solution at step 4 in the purification of ribonucleases U_1, U_2 and U_3 was applied to a DEAE-cellulose column (1·1cm. × 20cm.). The enzyme was eluted with 280ml. of 10mM-tris–HCl buffer, pH8·5, including a linear gradient of 0–0·33M-NaCl. Fractions (4ml.) were collected at a flow rate of 20ml./hr. Ribonuclease activity was measured in the usual assay procedure. ○, Ribonuclease activity at pH4·5; ●, ribonuclease activity at pH7·5; △, NaCl concentration; ■, protein concentration.

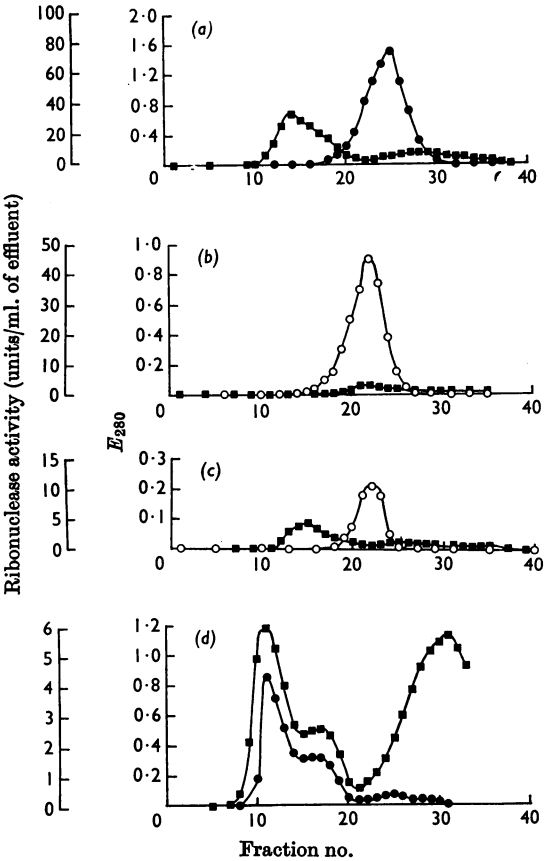

Fig. 3. Sephadex G-75 gel-filtration patterns of ribonucleases U_1 (*a*), U_2 (*b*), U_3 (*c*) and U_4 (*d*). Enzyme solution (1 ml.) of the final step was applied to a Sephadex G-75 column (1·5 cm. × 60 cm.). The enzyme was eluted with 10 mM-sodium acetate buffer, pH 6·0. Fractions (3 ml.) were collected at a flow rate of 20 ml./hr. ○, Ribonuclease activity measured at pH 4·5; ●, ribonuclease activity measured at pH 7·5; ■, protein concentration.

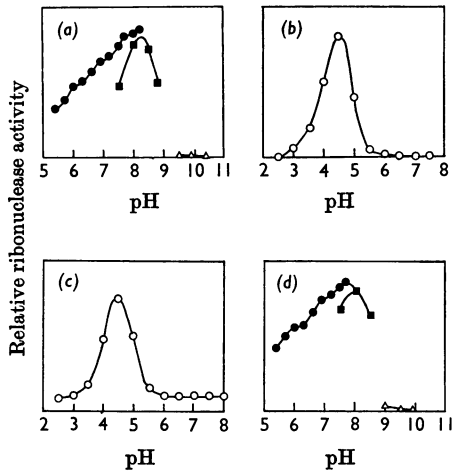

Fig. 4. pH-dependence of the activities of ribonucleases U_1 (*a*), U_2 (*b*), U_3 (*c*) and U_4 (*d*). Ribonuclease activity was measured at various pH values in the usual assay procedure: ●, in 50 mM-sodium–potassium phosphate buffer; ○, in 50 mM-sodium citrate phosphate buffer; ■, in 50 mM-tris–HCl buffer; △, in 50 mM-carbonate–bicarbonate buffer.

activity of ribonucleases U_1 and U_2, but with ribonuclease U_3 resulted in a slight decrease in the specific activity. Rechromatography on DEAE-cellulose could be performed reproducibly and the specific activities of ribonucleases U_1 and U_2 were increased further (step 5).

With Sephadex G-75 gel filtration (step 6), further purification was performed and finally ribonucleases U_1, U_2 and U_3 were purified about 1600-, 3700- and 1100-fold respectively.

In this Sephadex G-75 gel filtration (Figs. 3*a*, 3*b* and 3*c*), it was shown that ribonucleases U_2 and U_3 were eluted in the same fractions, and that ribonuclease U_1 was in later fractions. No ribonuclease activity was found to pass through the column, so it is considered that ribonuclease which should be in this fraction (see Fig. 1) was lost in the purification procedure. This ribonuclease was designated ribonuclease U_4.

Purification of ribonuclease U_4. The results of the purification of ribonuclease U_4 from 5 l. of RNA medium are presented in Table 4. Concentration of the crude medium could be performed with freeze-drying without loss of enzyme activity.

In step 2, ribonuclease activity measured at pH 7·5 could not be precipitated completely. The reason is unknown. In Sephadex G-75 gel filtration (step 3), the fractions that passed through the column were collected.

In step 4, ribonuclease U_4 was not adsorbed on DEAE-cellulose, but contaminating ribonucleases were adsorbed.

The results of the final step, Sephadex G-75 gel filtration, are shown in Fig. 3(*d*). The peak of ribonuclease activity is identical with peak 1 of Fig. 1.

Properties of ribonucleases. pH–activity curves for ribonucleases U_1, U_2, U_3 and U_4 are shown in Fig. 4, measured in the usual assay procedure. None of them needs protective protein for the measurement of enzyme activity even at the final stage of purification.

No metallic cofactor was necessary for the action of the ribonucleases (Table 5). The degree of activation and inhibition for yeast RNA digestion by added compounds is shown in Table 5. Silver nitrate and copper sulphate inhibited the action of

Table 5. *Inhibitors and activators*

After preincubation with reagents at 37° and pH 6·0 for 30 min., ribonuclease activity was measured at the optimum pH in the usual assay procedure.

Reagent	Final concn. (−log M) Ribonuclease ...	Remaining activity (%) U_1	U_2	U_3	U_4
NaCl	2	83	71	85	—
	3	107	104	106	100
$AgNO_3$	2	5	14	28	—
	3	60	60	61	12
$MgSO_4$	2	33	79	70	—
	3	96	110	113	21
$CaCl_2$	2	56	94	113	—
	3	98	119	132	62
$MnCl_2$	2	24	72	83	—
	3	104	158	166	54
$CuSO_4$	2	6	5	10	—
	3	61	53	72	6
$FeSO_4$	3	109	112	112	102
Iodoacetic acid	2	117	55	47	—
	3	108	103	93	65
EDTA	2	115	91	112	—
	3	113	100	102	479
Zinc acetate	2	4	56	72	—
	3	41	102	111	32
p-Chloromercuribenzoate	4	101	95	115	100
None		100	100	100	100

all four ribonucleases on yeast RNA. Zinc acetate inhibited the action of ribonucleases U_1 and U_4 on yeast RNA. Magnesium sulphate and manganous chloride inhibited the action of ribonuclease U_4. These inhibitions, except that of silver nitrate, were reversed by EDTA. Manganous chloride activated ribonucleases U_2 and U_3. Incubation of ribonucleases with mM-iodoacetate at 37° at pH 6·0 for 30 min. caused no loss of ribonuclease activity. Since *p*-chloromercuribenzoate had no effect on these ribonucleases, it appears that they are not thiol enzymes.

Ribonucleases U_1, U_2 and U_3 were as stable proteins as ribonuclease T_1 (Table 6). No loss of activity was observed after heating at 80° for 4 min. in solution (24 μg., 10 μg. and 38 μg. of protein/ml. of 6·6 mM-sodium phosphate buffer, pH 6·9, respectively), but ribonuclease U_4 is as heat-labile as most enzymes. It retained only 12% of its activity after this heat treatment in solution (277 μg. of protein/ml. of the same buffer). On freeze-drying and on freezing and thawing, all the ribonucleases were quite stable. They also could be stored frozen for several months or in solution in the cold-room for several weeks without appreciable loss in activity at neutrality (about pH 6·0).

Table 6. *Thermostability of the ribonucleases*

Ribonuclease activity was measured in the usual assay procedure after heating at 80° for 4 min. in 6·6 mM-sodium phosphate buffer, pH 6·9. The protein concentrations of ribonucleases U_1, U_2, U_3 and U_4 were 24, 10, 38 and 277 μg. of protein/ml. respectively.

Ribonuclease	Remaining activity (%)
U_1	95
U_2	100
U_3	100
U_4	12

DISCUSSION

Glitz & Dekker (1963) first isolated an extracellular ribonuclease of *Ustilago sphaerogena*. From the culture media of the same organism we have succeeded in isolating four ribonucleases designated ribonucleases U_1, U_2, U_3 and U_4. Of the four ribonucleases, ribonuclease U_1 is the main one, so it may be the extracellular ribonuclease described by Glitz & Dekker (1964*a*,*b*). Properties such as pH optimum (pH 7·5) and molecular weight (about 10000) of both enzymes coincide. Moreover, as described in the following paper (Arima *et al.* 1968), ribonuclease U_1 is a guanyloribonuclease like the ribonuclease described by Glitz & Dekker (1963, 1964*a*,*b*).

The production of ribonucleases U_1 and U_4 is greatly enhanced in the RNA medium. So ribonuclease U_4, one of the minor components, was purified from culture in this medium. This, however, makes the purification difficult. Although

most of the degradation products of RNA are insoluble in the concentrated medium of step 2 and a major portion of soluble degradation products is removed by gel filtration, some RNA remains and can be removed only by DEAE-cellulose chromatography. Ribonuclease U_4 even thus prepared is far from homogeneous, as seen from the Sephadex G-75 gel-filtration pattern (Fig. 3*d*).

The specific activities of the most purified preparations of ribonucleases U_1 and U_2 are one-tenth of that of ribonuclease T_1 and almost equal to that of ribonuclease T_2 (Egami, Takahashi & Uchida, 1964). Nishimura (1960) reported that the specific activity of the crystalline ribonuclease of *Bacillus subtilis* is almost equal to that of pancreatic ribonuclease, and Takahashi (1961) found highly purified ribonuclease T_1 to be four times as active as pancreatic ribonuclease. So the specific activities of ribonucleases U_1 and U_2 may be regarded as of the same order as those of other highly purified ribonucleases. The specific activity of ribonuclease U_3 is a little lower, suggesting that it is less pure.

Several enzymic properties of the four ribonucleases differ. Ribonuclease U_4 is inhibited by bivalent metal ions, and ribonucleases U_2 and U_3 are activated by Mn^{2+}. Ribonucleases U_1, U_2 and U_3 are thermostable, as are most endoribonucleases, whereas ribonuclease U_4 is heat-labile. Phosphate apparently enhances the activity of ribonuclease U_1 and U_4 in the acidic pH region. Unlike the ribonuclease described by Glitz & Dekker (1963, 1964*a*,*b*), the stabilizing effect of certain proteins was not observed with our ribonucleases. These enzymic properties remain to be reinvestigated with completely purified preparations.

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