

## Some Properties of Ribonucleases from *Aspergillus saitoi* and Seminal Vesicles immobilized on Sepharose 4B activated by Cyanogen Bromide

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Many trials have been made to produce water insoluble derivatives of enzymes with retaining their activities. For ribonucleases, insoluble form of bovine pancreatic ribonuclease A,<sup>2)</sup> ribonuclease S-protein,<sup>3)</sup> and ribonuclease T<sub>1</sub> from *Aspergillus oryzae*<sup>4)</sup> were reported hitherto.

Two ribonucleases which are thought to be useful for base analysis of RNA were purified in our laboratory. They were a ribonuclease from *Aspergillus saitoi* (RNase M) known to be base-nonspecific RNase<sup>5)</sup> and a ribonuclease from seminal vesicles (RNase Vs<sub>1</sub>)<sup>6)</sup> known to be active for both non-helical and double helical RNAs.<sup>7)</sup> RNase M or the combination of RNase M and RNase Vs<sub>1</sub> could possibly hydrolyze the most of RNA except t-RNA containing 2'-O-methylated ribose to the mixture of mononucleotides completely.

Immobilization of the above enzyme seems to make it easy to separate the enzyme from the hydrolysis product of RNA and to use the enzyme repeatedly. Thus in this paper, we reported the immobilization of the above two enzymes and some properties of the immobilized enzymes were also studied. As tools for structural studies and base analysis of RNA, the enzyme immobilized should be very stable for longer storage and incubation and active even under the extreme experimental conditions in which helical structure of double stranded RNAs and the interaction of RNA and the coexisting protein become less tight. Therefore, the stability of the immobilized RNases at acidic and alkaline pH's and that in the presence of denaturant, 8M urea, were also studied.

### Experimental

**Reagent**—Sepharose 4B activated by cyanogen bromide was purchased from Seikagaku-kogyo Co., Ltd. RNA was obtained from Kojin Co., Ltd.

**Enzyme**—RNase M was prepared from commercial digestive "Molsin" of Seshin Pharm. Co., according to the method of Irie, *et al.*<sup>8)</sup> RNase Vs<sub>1</sub> was prepared according to the method of Hosokawa and Irie<sup>6)</sup> from seminal vesicle gland.

**Enzyme Assay**—Assay method of RNases was essentially as follows; To a 2 ml of 0.25% RNA solution, 20–300  $\mu$ l enzyme was added and incubated at 37° for 5 min. To stop the reaction, one ml of MacFadyen reagent<sup>9)</sup> was added. The reaction mixture was centrifuged for 5 min at 3000 rpm and 0.3 ml of the supernatant was diluted with water to 2.3 ml. The optical density of the diluted solution was measured at 260 nm. The pH of the buffers used for assay of RNase M and RNase Vs<sub>1</sub> were 5.0 and 7.5, respectively, if not otherwise mentioned. The amount of enzyme which increases optical density of 1.0 by the assay method described above was defined as 1 unit.

- 1) Location: a) Ebara, 2-4-41, Shinagawa-ku, Tokyo; b) Yoshidashimoadachi-cho, Sakyo-ku, Kyoto.
- 2) D. Givol, Y. Weinstein, M. Gorecki and M. Wilchek, *Biochem. Biophys. Res. Comm.*, **38**, 825 (1970).
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- 5) M. Irie, *J. Biochem.*, **62**, 509 (1967).
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- 9) D.A. MacFadyen, *J. Biol. Chem.*, **107**, 297 (1934).

**Preparation of Immobilized Ribonucleases**—Sephacryl 4B activated by cyanogen bromide<sup>10</sup> (500 mg) was swollen in 1 mM HCl. The swelling Sepharose was filtered on a glass filter and washed with 200 ml of distilled water, then with 50 ml of 0.1M NaHCO<sub>3</sub> containing 0.5M NaCl, pH 8.0. The washed Sepharose was added to the solution of RNase (5 mg per 5 ml of the above described buffer) and the mixture was shaken gently at 20°. At appropriate time intervals, the Sepharose was filtered through with a glass filter and absorbancy of the filtrate was measured at 280 nm to estimate the amount of RNase not bound on the Sepharose. The filtrate was then mixed with the Sepharose again and the mixture was allowed to react further. When the optical density at 280 nm of the filtrate did not change further, Sepharose-bound RNase was filtered and washed 6 to 7 times with each 5 ml of buffer solution, 0.01M Tris-HCl (pH 7.5) for RNase Vs<sub>1</sub> and 0.01M acetate buffer (pH 5.0) for RNase M. The optical density at 280 nm of each washing was measured. From the absorbancy of the first filtrate and washings, amount of the enzyme not bound was estimated. The Sepharose was suspended in the same buffer used for washing and kept at 5°. At this temperature, the enzyme activity of RNase Vs<sub>1</sub> and RNase M bound to Sepharose did not change for at least 6 months.

**Buffers Used**—Buffers used were, glycine-HCl buffer for pH 1 and 2, acetate buffer for pH 3 to 5, Tris-HCl buffer for pH 7, 8 and 9 and glycine-NaOH buffer for pH 11 and 12.

## Results and Discussion

Time course of the binding of RNase M and RNase Vs<sub>1</sub> with Sepharose 4B activated by cyanogen bromide are shown in Fig. 1. Under the experimental conditions described in the experimental, maximum binding of RNases on Sepharose was about 90–95% and 70% for RNase Vs<sub>1</sub> and RNase M, respectively. The rate of binding of RNase Vs<sub>1</sub> was much faster than that of RNase M and it reached to the maximum binding in less than 5 min. In contrast to RNase Vs<sub>1</sub>, the binding of RNase M became maximum at about 4 to 5 hr.

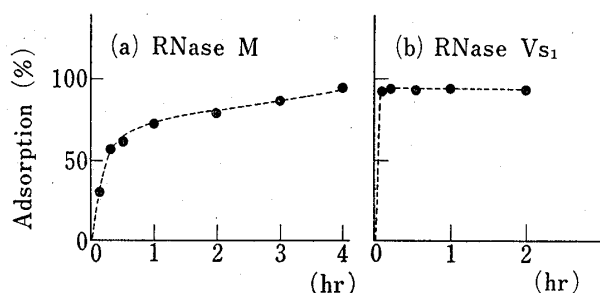


Fig. 1. Time Course of the Binding of RNase M and RNase Vs<sub>1</sub> with Sepharose 4B Activated by Cyanogen Bromide

The experimental conditions were described in the text. The ordinate was expressed as the percentage of the maximum binding.

### Specific Activity of Sepharose-Bound RNase M and RNase Vs<sub>1</sub>

The specific activities of Sepharose-bound RNase M (Sepharose RNase M) and Sepharose-bound RNase Vs<sub>1</sub> (Sepharose RNase Vs<sub>1</sub>) prepared as above were about 7.2% and 10% of the native enzyme, respectively. The trials to prepare Sepharose RNase having higher specific activity, by changing the ratio of enzyme/Sepharose 4B, pH and nature of buffers used for binding experiments were not successful enough so far.

### Michaelis Constants of Sepharose RNases

Michaelis constants of Sepharose RNases were obtained using RNA as substrate from Lineweaver-Burk's plot.<sup>11</sup> Michaelis constant obtained were about twice larger than those

TABLE I. Michaelis Constant of Sepharose RNase M and Sepharose RNase Vs<sub>1</sub>, at pH 6.0 and 8.0, respectively

Enzymes	RNase M	Sepharose RNase M	RNase Vs <sub>1</sub>	Sepharose RNase Vs <sub>1</sub>
K <sub>m</sub> (mg RNA per ml)	0.50	0.81	1.9	3.1

The Michaelis constants were calculated from the Lineweaver-Burk's plot<sup>11</sup> using RNA concentration from 0.5 to 4.0 mg/ml. Enzymes used were 0.13, 0.18, 0.23 and 0.23 units for RNase Vs<sub>1</sub>, Sepharose RNase Vs<sub>1</sub>, RNase M and Sepharose RNase M, respectively.

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11) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

of the native enzymes (Table I). This may indicate some steric interaction between RNase and Sepharose molecule or the modification of some functional groups on RNase with Sepharose make the binding of RNases with RNA more unfavorable. The results were different from those of RNase  $T_1$  bound to Sepharose reported by Lee.<sup>4)</sup>

### pH Optimum of Sepharose RNases

The enzymatic activity of RNase M, RNase  $V_{s_1}$  and Sepharose RNases as a function of pH are shown in Fig. 2. Sepharose RNase  $V_{s_1}$  showed the same pH optimum as RNase  $V_{s_1}$ , but that of Sepharose RNase M is about half pH unit lower than that of the native one. Thus, at

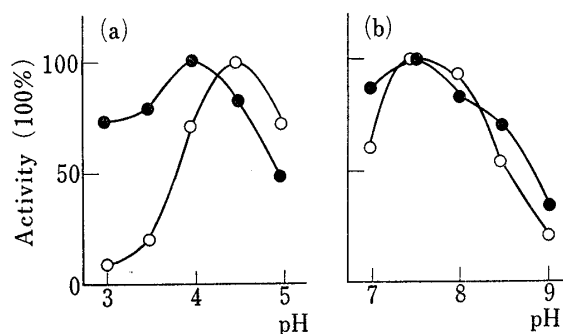


Fig. 2. Effect of pH on the Enzymatic Activity of Sepharose RNases

Enzyme assay was carried out essentially by the standard assay method using the following amount of enzymes for each assay tube.

(a) RNase M (0.2 units); —○—, and Sepharose RNase M (0.08 units); —●—  
 (b) RNase  $V_{s_1}$  (0.13 units); —○—, and Sepharose RNase  $V_{s_1}$  (0.18 units); —●—

The ordinate was expressed as the percentage of maximum activity. Buffer concentration was 0.1M.

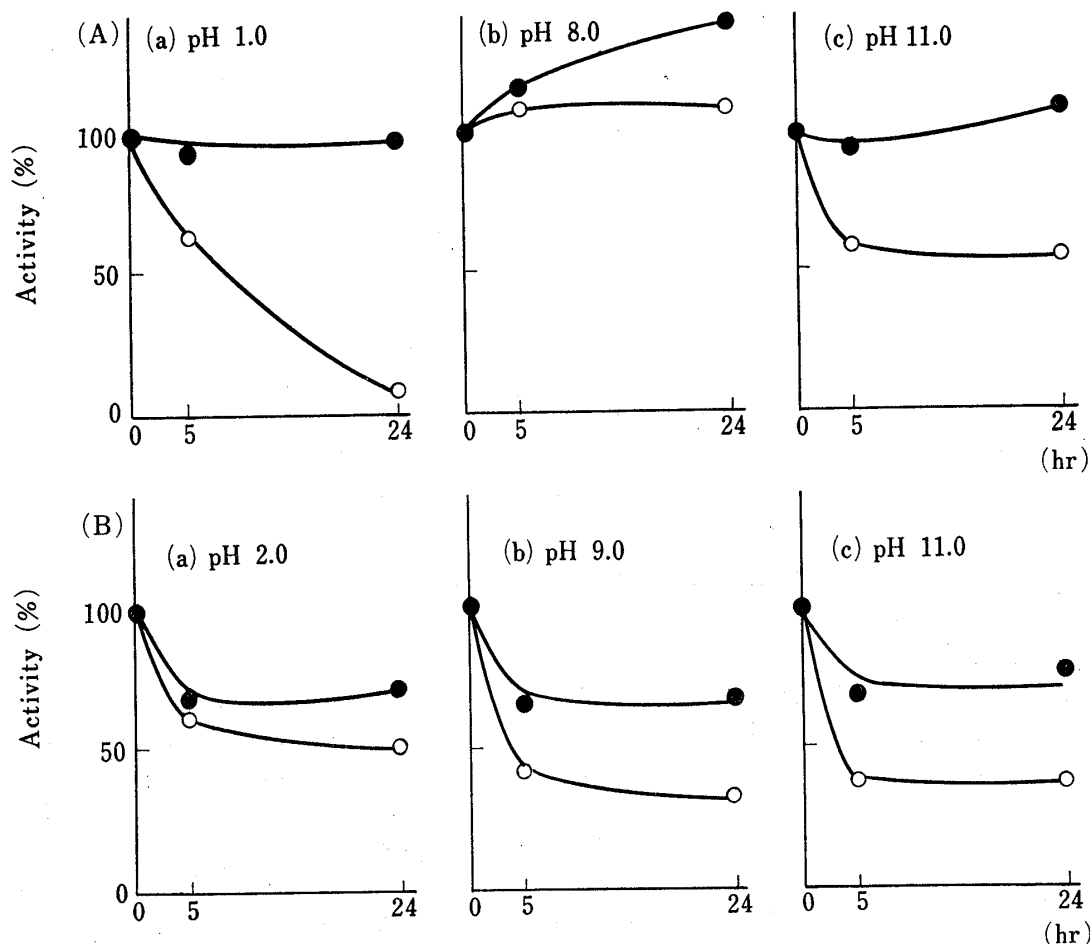


Fig. 3. Stability of Sepharose RNases at Various pH's (I)

(A) To a 0.3 ml of buffer of various pH's (0.01M), added 0.08 units and 0.2 units of RNase M and Sepharose RNase M, respectively. After incubation for appropriate time at 37°, 2 ml of 0.5% RNA solution (0.05M acetate, pH 5.0) was added to the enzyme solution. The hydrolysis of RNA was carried out for 5 min at 37°, then treated as the standard method as described in the text. (a) pH 1.0, (b) pH 8.0 and (c) pH 11.0

RNase M; —○— and Sepharose RNase M; —●—

(B) The same experiments as Fig. 3(A) were carried out except the use of 0.05M Tris-HCl, pH 7.5 for enzyme assay using 0.13 units of RNase  $V_{s_1}$  and 0.18 units of Sepharose RNase  $V_{s_1}$ . (a) pH 2.0, (b) pH 9.0 and (c) pH 11.0. RNase  $V_{s_1}$ ; —○— and Sepharose RNase  $V_{s_1}$ ; —●—

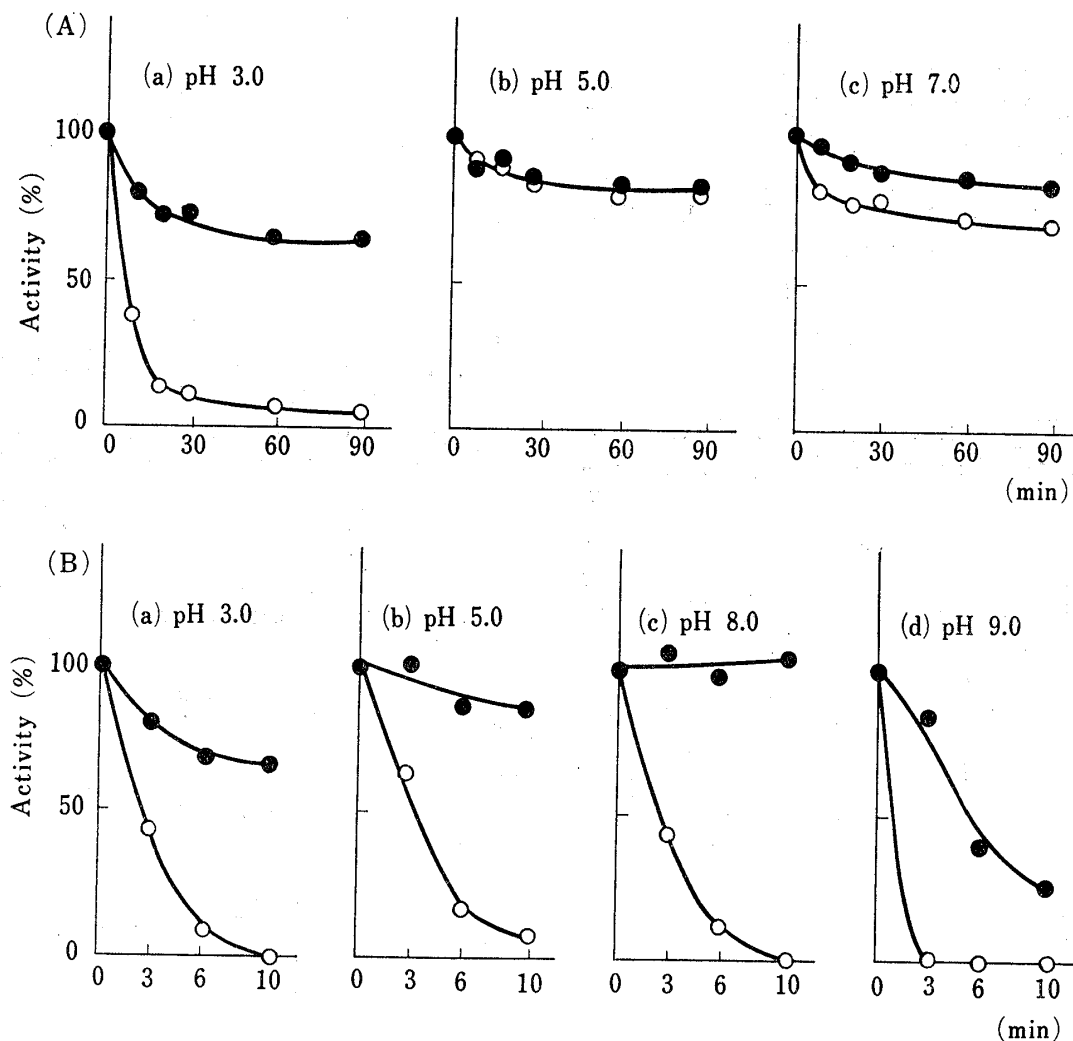


Fig. 4. Stability of Sepharose RNases at Various pH's (II)

(A) Stability of RNase M and Sepharose RNase M. The experimental conditions were the same as those in Fig. 3(A) except the temperature of preincubation, 50° and the buffers used were pH 3.0, 5.0 and 7.0.

(B) Stability of RNase Vs<sub>1</sub> and Sepharose RNase Vs<sub>1</sub>. The experimental conditions were the same as those in Fig. 3(B) except the preincubation temperature of 90° and buffers used were pH 3.0, 5.0, 8.0 and 9.0. RNase M or RNase Vs<sub>1</sub>; —○— and Sepharose RNase M or Sepharose RNase Vs<sub>1</sub>; —●—

optimal pH, the specific activity of Sepharose RNase M could be about 10.7% of the native RNase activity at pH 4.5.

#### Stability of Sepharose RNases at Various pH's (I)

Since RNase M was known to be very stable at 37° and pH from 3.0 to 9.0, the stability of Sepharose RNase M was studied at pH 1.0, 8.0 and 11.0 (Fig. 3). At pH 1.0, the activity of the native RNase M decreased markedly, but Sepharose RNase M was stable at least for 24 hr. In contrast to the decrease of native enzyme activity at pH 11.0 to about 50% in 24 hr, Sepharose RNase M was stable at this condition too.

RNase Vs<sub>1</sub> was very stable between pH 3 and 8 at 37° for 24 hr (not shown here) and its activity decreased gradually at pH 2.0, 9.0 and 11.0 (Fig. 3). The activity of Sepharose RNase Vs<sub>1</sub> decreased at the condition described in Fig. 3, but the rates of inactivation were significantly smaller than those of native enzyme at alkaline pH's (pH 9.0 and 11.0). These results indicated that Sepharose RNase M and Sepharose RNase Vs<sub>1</sub> were more stable than native enzymes.

#### Stability of Sepharose RNases at Various pH's (II)

Since RNase M was rather unstable at temperature higher than 50° even at pH 5.0 and 7.0, the most stable pH's at room temperature, the stability of Sepharose RNase M were

tested at pH more close to neutral than described above and at higher temperature. Although slow decrease in enzymatic activity was observed at these two pH's, but some degree of protection from inactivation by the binding with Sepharose was observed at pH 7.0 and the stability of Sepharose RNase M was more marked at pH 3.0. RNase  $V_{s_1}$  is very stable at neutral and weakly acidic pH at 60°. Therefore, the stability of Sepharose RNase  $V_{s_1}$  was tested at 90°. In contrast to the native RNase  $V_{s_1}$ , Sepharose RNase  $V_{s_1}$  was stable at pH 5.0 and 8.0 at 90°, and the rate of decrease of the activity at pH's 3.0 and 9.0 was smaller than that of the native enzyme. Experiments described above also indicated that Sepharose RNases were more stable than corresponding native RNases at higher temperature and at both acidic and alkaline pH's.

### Stability of Sepharose RNases in 8 M Urea

The possible decrease of interaction of double helical RNA chains or protein and RNA in 8M urea, may support the attack of RNA by RNases, if it is provided that RNases are still reasonably active in the presence of 8M urea. For this anticipation, the stability of Sepharose RNases in 8M urea were studied. RNase M was stable at room temperature in 8M urea as in the absence of urea at pH 2.0 and 7.0, but its activity decreased gradually at 40°. However, Sepharose RNase M was still fully active at pH 2.0 to 7.0 even 2 hr incubation. Similar experiments performed for RNase  $V_{s_1}$ , at pH 4.0 and 9.0 indicated that Sepharose RNase  $V_{s_1}$  is more stable than the native RNase as shown in the experiments carried out at pH 9.0 in the presence of urea.

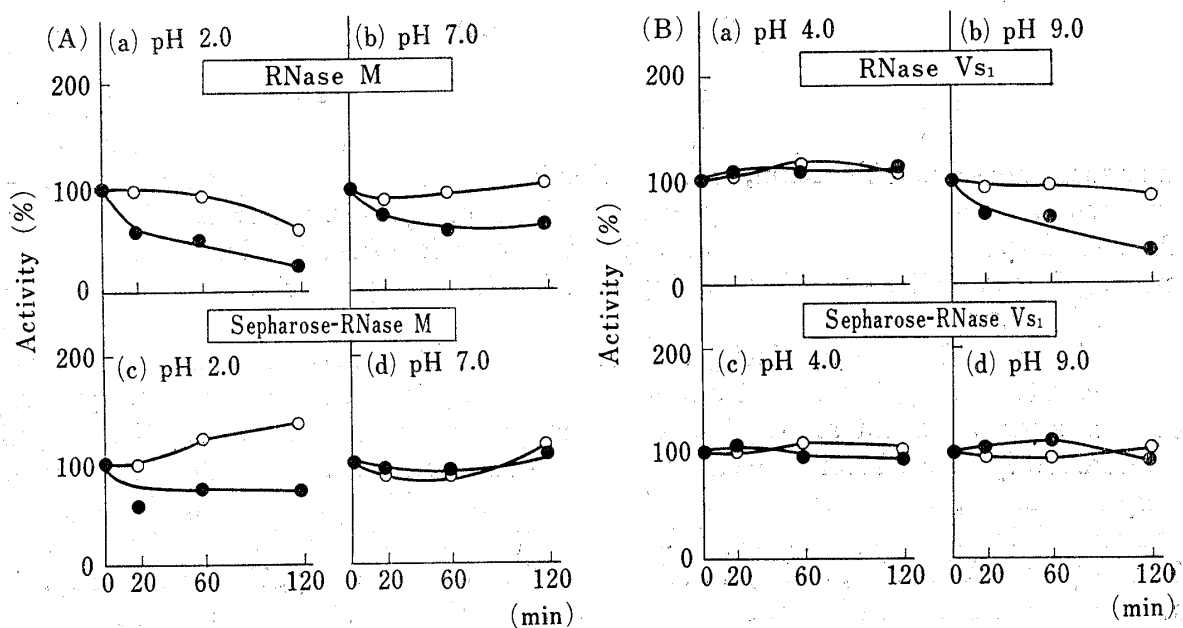


Fig. 5. Stability of Sepharose RNase in the Presence of 8M Urea at 40°

(A) Stability of Sepharose RNase M in the presence of urea  
 (a), (b) RNase M; and RNase M in 8M urea; —●—  
 (c), (d) Sepharose RNase M; —○—, and Sepharose RNase M in 8M urea; —●—  
 (B) Stability of Sepharose RNase  $V_{s_1}$  in the Presence of Urea  
 (a), (b) RNase  $V_{s_1}$ ; —○— and RNase  $V_{s_1}$  in 8M urea; —●—  
 (c), (d) Sepharose RNase  $V_{s_1}$ ; —○— and Sepharose RNase  $V_{s_1}$  in 8M urea; —●—  
 The experimental conditions were the same as Fig. 3(A) and (B) except that buffer for the preincubation contain 8M urea. The amount of enzyme used were RNase M; 0.19 units, Sepharose RNase M; 0.11 units, RNase  $V_{s_1}$ ; 0.13 units and Sepharose RNase  $V_{s_1}$ ; 0.13 units.

These studies described above indicated that Sepharose RNase  $V_{s_1}$  and Sepharose RNase M are more stable than the corresponding native enzymes in respect of the change of environment, pH, temperature and presence of denaturant, though the specific activity was about 10% of the native enzyme.