

[Chem. Pharm. Bull.]  
30(3)1069—1072(1982)

Preparation of  $^{35}\text{S}$ -Labeled Ribonucleic Acid and Deoxyribonucleic Acid and Their Use for the Assay of Nucleolytic Enzymes (Nucleosides and Nucleotides. XXXIX)<sup>1)</sup>

KAZUNOBU MIURA, SHOKO SATO, KUMIKO TAKAGI, JUN-ICHI TOHYAMA, and TOHRU UEDA\*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12,  
Nishi-6, Kita-ku, Sapporo, 060, Japan

(Received August 19, 1981)

Radioactive sulfur was introduced into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) molecules by chemical means. The thio group in 4-thiouracil residues, which were introduced into RNA and DNA by sulfhydrolysis, was exchanged with radioactive  $\text{Na}_2\text{S}$  via a 4-thiocyanatouracil intermediate. Specific activity of the  $^{35}\text{S}$ -labeled RNA and DNA was around  $10^5$  cpm/ODU<sub>260</sub>. The labeled RNA and DNA were successfully applied as substrates for the assay of nucleolytic enzymes or ribonuclease inhibitor. The sensitivity of the assay was 100 times better than that of the current method using non-labeled nucleic acids as the substrate.

**Keywords**—sulfhydrolysis; 4-thiouracil residues;  $^{35}\text{S}$ -labeled 4-thiouracil residues;  $^{35}\text{S}$ -labeled RNA or DNA; ribonucleases; ribonuclease inhibitor; deoxyribonuclease

We have recently reported on the introduction of 4-thiouracil residues into RNA and DNA molecules by means of a chemical transformation of cytosine residues with hydrogen sulfide.<sup>2)</sup> This procedure is useful since the 4-thiouracil residues thus introduced could be a key intermediate for further transformation of the pyrimidine base in nucleic acids. For instance, the thio group of the 4-thiouracil residue in *E. coli* tRNA has been photoactivated to form an intramolecular link between the 8th and 13th bases of tRNA,<sup>3)</sup> and replaced with radioactive sulfur via a 4-thiocyanatouracil residue.<sup>4)</sup>

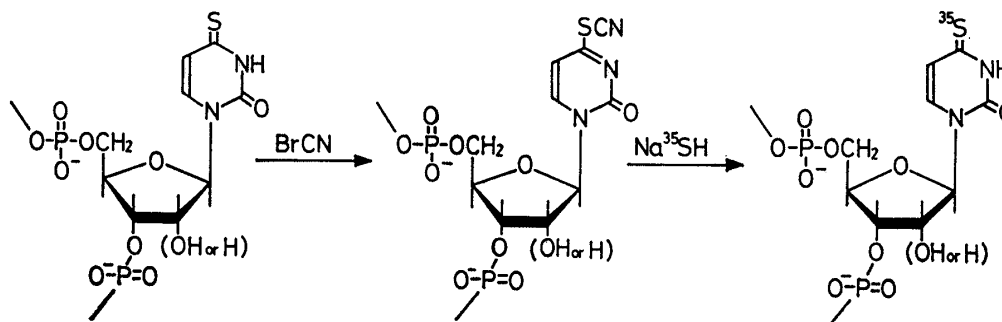


Chart 1

In this paper, we report the preparation of  $^{35}\text{S}$ -labeled RNA and DNA from the RNA and DNA containing 4-thiouracil residues and its application for the assay of nucleolytic enzymes.

#### Materials and Methods

**Materials**—Yeast RNA and calf thymus DNA were purchased from Kohjin Co., Ltd. and Worthington Biochemical Co., respectively. Enzymes were obtained from the following sources: ribonuclease A from Sigma Chemical Co., ribonucleases  $\text{T}_1$  and  $\text{T}_2$  from Sankyo Co., Ltd., nuclease  $\text{P}_1$  from Yamasa shoyu Co., Ltd. and deoxyribonuclease I from Worthington Biochemical Co.  $^{35}\text{S}$ - $\text{Na}_2\text{S}$  (67.5 mCi/mmol) was purchased from New England Nuclear Co.

**Preparation of RNA and DNA containing 4-Thiouracil Residues**—Introduction of 4-thiouracil residues into yeast RNA or heat-denatured calf thymus DNA by reaction with liquid hydrogen sulfide in pyridine-H<sub>2</sub>O was carried out according to the reported method.<sup>2)</sup> For example, yeast RNA (1572 ODU<sub>260</sub> in 10 ml of H<sub>2</sub>O) was treated with liquid H<sub>2</sub>S (20 ml)-pyridine (10 ml) in a sealed tube at 40°C for 60 h. After vaporization of H<sub>2</sub>S and repeated evaporation with pyridine, RNA was recovered by precipitation with EtOH. Yield was 1011 ODU<sub>260</sub>.

**Preparation of RNA and DNA containing <sup>35</sup>S-4-Thiouracil Residues**—A solution of RNA containing 4-thiouracil residues (600 ODU<sub>260</sub>) in 50 mM phosphate (pH 8.0) was treated with cyanogen bromide (freshly distilled, 10 equimolar against 4-thiouracil residues) and the mixture was kept for 15 min at 27°C. The reaction mixture was concentrated to dryness under high vacuum as quickly as possible to avoid degradation of the 4-thiocyanatouracil intermediate. The residue was dissolved in H<sub>2</sub>O (original volume) and <sup>35</sup>S-Na<sub>2</sub>S (1.2 equimolar against 4-thiouracil residues) was added. The substitution reaction was performed by keeping the mixture at 27°C overnight. The RNA was recovered by precipitation with EtOH, and was washed with 5% trichloroacetic acid to remove the contaminating <sup>35</sup>S-Na<sub>2</sub>S and other acid-soluble materials. Finally, the RNA was dissolved in water and reprecipitated with EtOH. The recovery of RNA was 415 ODU<sub>260</sub> and the specific activity was  $1.2 \times 10^5$  cpm/ODU<sub>260</sub>.

DNA containing <sup>35</sup>S-4-thiouracil residues was prepared in the same way; its specific activity was  $3.7 \times 10^5$  cpm/ODU<sub>260</sub>.

TABLE I. Conditions for the Assay of Nucleolytic Enzymes

Enzyme	Buffer	Substrate
Ribonuclease A	30 mM Tris-HCl(pH 7.5)-0.03% BSA <sup>a)</sup>	
Ribonuclease T <sub>1</sub>	30 mM Tris-HCl(pH 7.5)-0.03% BSA	<sup>35</sup> S-RNA
Ribonuclease T <sub>2</sub>	10 mM NH <sub>4</sub> OAc(pH 4.5)-0.03% BSA	3000 cpm
Nuclease P <sub>1</sub>	40 mM NH <sub>4</sub> OAc (pH 5.3)-0.03% BSA	
Deoxyribonuclease I	25 mM Tris-HCl(pH 8.0)-5 mM Mg(OAc) <sub>2</sub>	<sup>35</sup> S-DNA
Nuclease P <sub>1</sub>	40 mM NH <sub>4</sub> OAc(pH 5.3)-0.03% BSA	3000 cpm

a) BSA=Bovine serum albumin.

**Assay of Nucleolytic Enzymes**—A mixture (30 μl) of enzyme and <sup>35</sup>S-labeled RNA or DNA shown in Table I was incubated at 25°C for 30 min, then the reaction was stopped by addition of cold 10% trichloroacetic acid. After being held in an ice-bath for 30 min, the mixture was filtered through GF/C filter paper. The filter paper was thoroughly rinsed with cold 5% trichloroacetic acid and dried. Then, the radioactivity was measured with a liquid scintillator. The enzyme activity was calculated from the remaining counts on the GF/C filter paper.

**Preparation of Ribonuclease Inhibitor**—Ribonuclease inhibitor from rat liver was prepared according to the method reported by Blackburn *et al.*,<sup>5)</sup> which gave a single band on polyacrylamide gel electrophoresis (data not shown).

**Assay of Ribonuclease Inhibitor**—A mixture of ribonuclease A (100 pg) and an appropriate amount of ribonuclease inhibitor in 40 μl of 50 mM Tris-HCl-2 mM DTT-1 mM EDTA (pH 7.5) was incubated for 10 min at 25°C. <sup>35</sup>S-Labeled RNA (4000 cpm) was added to the preincubated mixture and the mixture was incubated at 25°C for 30 min. The reaction was stopped by addition of cold 10% trichloroacetic acid, then the mixture was worked up as described in the assay of nucleolytic enzymes.

## Results and Discussion

### Preparation of RNA and DNA containing <sup>35</sup>S-4-Thiouracil Residues

RNA or DNA containing isotope-labeled 4-thiouracil residues was prepared by the chemical introduction of 4-thiouracil residues followed by replacement of the thio group in 4-thiouracil residues with <sup>35</sup>S-Na<sub>2</sub>S.

The reaction of yeast RNA or heat-denatured calf thymus DNA with liquid hydrogen sulfide in pyridine and H<sub>2</sub>O at 40°C for 60 h afforded RNA or DNA in which 50–60% of the cytosine residues was converted to 4-thiouracil residues.

The introduction of isotope, <sup>35</sup>S, was carried out according to the reported procedure<sup>4)</sup> with some modifications. From several examinations with varying amounts of cyanogen bromide and <sup>35</sup>S-Na<sub>2</sub>S, it was found that the use of large excess amounts of these reagents

was unnecessary to obtain the maximum exchange of the thio group to  $^{35}\text{S}$ -thio group. As described in "Materials and Methods," a 10-fold molar excess of cyanogen bromide was enough to convert the 4-thiouracil residues to the active intermediate, 4-thiocyanatouracil residues, and addition of an equimolar amount of  $^{35}\text{S}\text{-Na}_2\text{S}$  resulted in a satisfactory replacement. Although the regeneration of 4-thiouracil residues from 4-thiocyanatouracil residues was completed by the addition of a minimum amount of  $\text{Na}_2\text{S}$ , the incorporation of radioisotope was about 50% at maximum. This means that bond-scission between S and CN also occurred along with the substitution of SCN with  $\text{SH}^-$  under the conditions used in this study. The specific activity of RNA thus obtained was  $1.2 \times 10^5$  cpm/ODU<sub>260</sub>, which was sufficient for its use as a substrate for the assay of nucleolytic enzymes.

DNA containing  $^{35}\text{S}$ -4-thiouracil residues was prepared in the same way and its specific activity reached  $3.7 \times 10^5$  cpm/ODU<sub>260</sub>.

The presence of  $^{35}\text{S}$ -4-thiouracil residues in RNA or DNA was confirmed by the nuclease  $\text{P}_1$  digestion of RNA or DNA followed by two-dimensional thin layer chromatography, which showed that the spot of radioisotope coincided with that of 4-thiouridylylate but not other nucleotides.

By the method described here, isotope-labeled nucleic acids can be obtained conveniently and on a relatively large scale. It should be noted that the handling of  $^{35}\text{S}$ -labeled nucleic acids is easier than that of  $^{32}\text{P}$ -labeled nucleic acids, since the lifetime of the isotope is much longer.

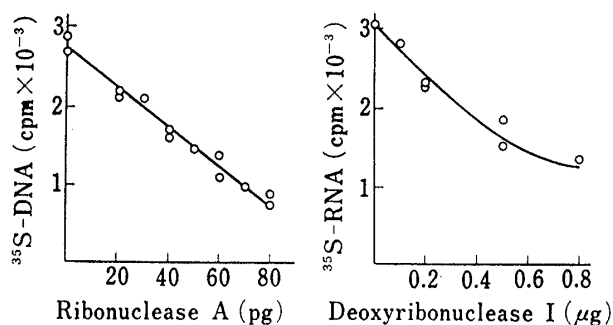


Fig. 1. Decay of  $^{35}\text{S}$ -Labeled RNA or DNA in the Assay of Nucleolytic Enzymes

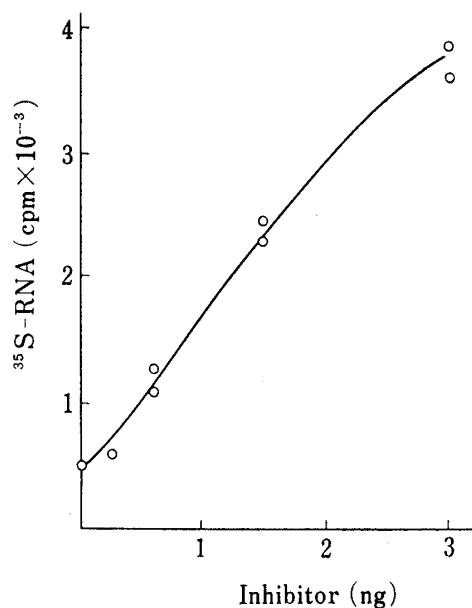


Fig. 2. The Assay of Ribonuclease Inhibitor

TABLE II. The Range of Quantity of Nucleolytic Enzymes Measurables by using  $^{35}\text{S}$ -Labeled RNA or DNA as a Substrate

Substrate	Enzymes	Range of quantity
$^{35}\text{S}\text{-RNA}$	Ribonuclease A	20—80 pg
	Ribonuclease $\text{T}_1$	0.1—0.8 ng
	Ribonuclease $\text{T}_2$	0.05—0.3 unit
$^{35}\text{S}\text{-DNA}$	Nuclease $\text{P}_1$	10—80 ng
	Deoxyribonuclease I	0.1—0.5 $\mu\text{g}$
	Nuclease $\text{P}_1$	10—50 ng

### Assay of Nucleolytic Enzymes using $^{35}\text{S}$ -Labeled Nucleic Acids as Substrates

The assay was performed by the incubation of a mixture of enzyme and  $^{35}\text{S}$ -labeled nucleic acid in an appropriate buffer at  $25^\circ\text{C}$  for 30 min and measurement of the radioisotope activity in the acid-insoluble materials from the digest. Although the enzymes activity was measured generally in terms of the amount of acid-soluble materials after digestion, the present experiment measures the acid-insoluble materials collected on the filter paper, which is less laborious and faster.

In Fig. 1, the results of digestion with ribonuclease A or deoxyribonuclease I are shown as a function of the decay of the substrate against the amount of enzyme. In the case of ribonuclease A, linearity of the decay of the RNA was observed within the range of 20–80 pg of ribonuclease A. The order of the amount of ribonuclease A measurable by this method is 100 times lower than that measurable by the conventional method using non-labeled RNA as a substrate.<sup>6)</sup> The results with other enzymes are summarized in Table II. It is clear that the sensitivity of the assay of nucleolytic enzymes was increased by using  $^{35}\text{S}$ -labeled nucleic acids.

### Application of Ribonuclease A- $^{35}\text{S}$ -Labeled RNA System for the Assay of Ribonuclease Inhibitor

Many studies on ribonuclease inhibitors of alkaline ribonucleases have appeared in order to clarify the function of this protein in the biosynthesis of nucleic acids. In these studies,<sup>5–7)</sup> the assay of ribonuclease inhibitor was performed by measurement of the extent of inhibition of the nucleolytic degradation of yeast RNA by ribonuclease A. Accordingly, the procedure for the assay of nucleolytic enzymes described in this paper can be applied to the assay of ribonuclease inhibitors.

The amount of ribonuclease A used in this assay was set at 100 pg, where the degradation of  $^{35}\text{S}$ -labeled RNA reached the plateau as shown in Fig. 1. The addition of more than 3 ng of the inhibitor caused almost complete inhibition of ribonuclease A. Fig. 2 showed the relation between the inhibition and the amount of the inhibitor. The linear relation is maintained in the range of 0.3–2 ng of ribonuclease inhibitor. As compared with the reported method for the assay of ribonuclease inhibitor, the quantity requirement of ribonuclease inhibitor in the present method is reduced to 1/100.

In conclusion, the procedure for the assay of nucleolytic enzymes and ribonuclease inhibitor was improved by using  $^{35}\text{S}$ -labeled nucleic acid as a substrate, and enzymic activity in the extract from the tissue or cells could be quantitatively analyzed using the curve shown in Fig. 1 as the standard.

**Acknowledgement** The authors are grateful for a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

### References and Notes

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