Counting of ^{32}P -Labeled Ribonucleic Acid Utilizing Cerenkov Radiation

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1. Introduction

Hard beta-emitters which have beta-particles with the energy larger than 0.265 MeV are subjects of direct counting, without using any scintillator systems, *via* Cerenkov radiation^{1,2)} in aqueous solutions using a commercially available liquid scintillation spectrometer. Counting conditions by this method were studied in detail by Haberer³⁾ with ^{137}Cs , ^{204}Tl , ^{40}K , ^{90}Sr , ^{90}Y , ^{144}Ce , ^{144}Pr , and ^{106}Rh , and by Braunsberg and Guyver⁴⁾ with ^{24}Na and ^{32}P . Recently, Clausen⁵⁾ also applied this method to counting ^{32}P -orthophosphate (^{32}Pi), and it was suggested by these studies that this method was useful in determining the radioactivities of various biological compounds labeled with ^{32}P . Bolton, et al.⁶⁾ applied this method for the determination of the elution profile of ^{32}P -labeled bacterial DNA fragments from the hydroxyapatite column. Their application was based on the following useful features of this method: samples can be counted without drying and without being adulterated, and valuable materials can be reprocessed.

In this paper, we will describe the application of "Cerenkov counting"^{7,8)} for measuring ^{32}P -labeled ribonucleic acid (^{32}P -RNA). The present study showed that the ^{32}P -RNA, not only in the high molecular weight form, but also in the hydrolyzed form, could be counted in aqueous solutions without being affected by quenchers, carriers, and high concentrations of salts and sugar, in a wide range of sample volumes. The ^{32}P -RNA adsorbed on a membrane-filter was also counted satisfactorily by this method.

2. Materials and Methods

2.1 Preparation of ^{32}P -labeled RNA

Pseudomonas schuylkilliensis strain *P* was grown at 30°C with aeration for 3 hr and 15 min to the mid-log phase in the nutrient broth-polypeptone medium (8 g nutrient broth (Difco) + 5 g polypeptone per 1 liter of distilled water, pH 6.8) containing 0.33 mCi (per 13.5 ml of the medium) of carrier-free ^{32}Pi (The Radiochemical Centre, Amersham, England). Cells from 27 ml of the culture were harvested by centrifugation (10,000×g, 5 min) in the cold, suspended in 10 ml of $5 \times 10^{-2} M$ Tris-HCl (pH 7.4) containing $10^{-2} M$ MgCl_2 and 0.5 mg/ml bentonite⁷⁾, and subjected to sonic oscillation (10kc, 7 min). To the sonicate, 0.3 ml of 20% sodium lauryl sulfate (SLS) and an equal volume of phenol saturated with 0.1 M acetate buffer (pH 6.0) and containing 0.1% (w/v) of 8-hydroxyquinoline were added. The mixture was stirred at room temperature (29°C) for 60 min, then was centrifuged (8,000×g, 15 min) in the cold, and the water layer was separated. Nucleic acids were precipitated by adding 2% sodium acetate and 2 volumes of 99.5% ethanol. After letting the mixture stand over-night at -20°C, the precipitate was collected by centrifugation (3,000 rpm, 10 min) in the cold. The precipitate was dissolved in 1.0 ml of $10^{-2} M$ Tris-HCl (pH 7.4) containing $5 \times 10^{-3} M$ MgCl_2 and was dialyzed against 500 ml of the same buffer for 1.5 hr, being stirred at room temperature. After the dialysis, 50 μg of

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deoxyribonuclease (1×cryst. beef pancreas, Worthington Biochem. Corp.) was added and the mixture was incubated for 30 min at room temperature. Then 50 μ g of pronase E (Kaken-Kagaku Co., Tokyo, self-digested at 37°C for 2 hr) was added and the mixture was incubated for another 30 min at room temperature.

The mixture was shaken for 5 min with an equal volume of phenol (saturated with acetate buffer, containing 8-hydroxyquinoline) in the presence of 0.8 mg of bentonite, and was centrifuged (3,000 rpm, 20 min) in the cold. From the water-layer, RNA was precipitated by adding sodium acetate and ethanol as stated above, and was dissolved in 1.0 ml of distilled water. This solution was applied on a column (1.2 cm×26 cm) of Sephadex G-50 (coarse), eluted with distilled water, and the first portion of the excluded material monitored both by U.V. absorption and radioactivities was collected and used throughout the experiment as 32 P-RNA. This preparation (total volume = 26 ml, total amount of RNA = 1.46 mg) showed a typical U.V. absorption spectrum of RNA: $A_{250\text{m}\mu}/A_{260\text{m}\mu} = 0.521$, $A_{230\text{m}\mu}/A_{260\text{m}\mu} = 0.405$. Ninety-three per-cent of the radioactivity of this fraction was precipitable in the presence of cold 5% trichloroacetic acid (TCA), and 99.6% of it became cold TCA-soluble after incubating with 100 μ g/ml of ribonuclease (cryst., salt-free, Worthington Biochem. Corp., pre-incubated at 80°C for 10 min) at 37°C for 30 min, and 99.8% of it became cold TCA-soluble after being hydrolyzed with 0.3 N KOH at 37°C for 18 hr.

2.2 RNA and protein carriers

Carrier RNA (3.1 mg/ml) was prepared from the liver of trout, *Oncorhynchus nerka*, by the SLS-bentonite-phenol method, which will be stated in another report. Albumin (egg, 5×cryst., Nutritional Biochemicals Corp.) dissolved in $5 \times 10^{-2} M$ Tris-HCl (pH 7.4) was used as a carrier protein.

2.3 Hydrolysis of RNA

A mixture of 28 μ g of 32 P-RNA and 310 μ g of the carrier RNA in the total volume of 2.0 ml was hydrolyzed under one of the following conditions: 0.3 N KOH, 37°C, 18 hr; 1 N HCl, 100°C, 1 hr; 5% TCA, 100°C, 15 min; and 5%

perchloric acid (PCA), 100°C, 15 min.

2.4 Paper-chromatography of the alkaline hydrolysate of 32 P-RNA

The 32 P-RNA was hydrolyzed by 0.3 N KOH at 37°C for 18 hr, neutralized by PCA and the salt was precipitated by centrifugation. The supernatant was applied on a sheet of chromatographic paper (40 cm×40 cm, Toyo Filter-paper Co., Tokyo, No. 51) and developed two-dimensionally using the following solvent systems: 1) isobutyric acid-1 N NH_4OH -0.1 M $\text{Na}_2\text{-EDTA}$ (10:6:0.16, by volume), descending, 2) methanol-ethanol-conc. HCl-water (25:12.5:3:9.5, by volume), ascending. Four 2', (3')-mononucleotide spots were located under the U.V. lamp. Each spot was cut into 2 to 4 pieces, put into a counting vial, and the radioactivity was determined *via* Cerenkov radiation without adding any solvent.

2.5 Adsorption of RNA on a membrane-filter

The mixture of 32 P-RNA (5.6 μ g) and the carrier RNA (93 μ g) in 3.0 ml of 5% TCA was placed in ice for 10 min, then passed through a membrane-filter (diameter: 3.0 cm, Millipore HAWP, mean pore size: 0.45 μ) which was prewashed with 5 ml of cold 5% TCA- $10^{-2} M$ phosphate buffer (pH 6.7), and washed with 5 ml of the same cold TCA-phosphate three times. Under these conditions, 93% of the radioactivities was adsorbed on the filter. The filter was dried at 80°C for 30 min before counting. When various amounts of 32 P-RNA were adsorbed, 0.02 to 0.3 ml of samples were taken from the mixture of 0.8 ml of 32 P-RNA and 0.24 ml of the carrier RNA. Total volume of each sample was adjusted to 3.0 ml with phosphate buffer (pH 6.7) and TCA giving final concentrations of $8 \times 10^{-3} M$ and 5% respectively. Filtration was performed as mentioned above.

2.6 Liquid scintillation spectrometer and scintillator systems

A Tri-Carb 3375 (Packard Instrument Co. Inc.) was used throughout the experiment. The high voltage of the instrument was adjusted to give 47.7% of the counting efficiency for the ^3H -standard (Packard K0412, 121,600 dpm, 9 May 1966, in toluene with 2,5-dipheny-

loxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)) at a gain of 50% with a window setting of 50~1,000.

The following three scintillator systems were used: 1) Bray's system⁵⁾: POPOP was replaced by dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene). 2) Toluene-triton system: PPO 5 g, dimethyl-POPOP 0.3 g, triton X-100 333 ml, toluene 667 ml. 3) Toluene system: PPO 5 g, dimethyl-POPOP 0.3 g, toluene 1,000 ml.

3. Results

3.1 Counting of ^{32}P -RNA in solution

The ^{32}P -RNA dissolved in 1.0 ml of distilled water was counted *via* Cerenkov radiation. As shown in Fig. 1-A, the maximum counting efficiency was given at gains between 30 and 50% with a window setting of 50~1,000. At a gain of 30% with a window setting of 50~1,000, ^{32}P i in 1.0 ml of $10^{-2}M$ phosphate buffer (pH 6.7) gave a counting efficiency of 22%

(by the Cerenkov counting). A window setting of 50~1,000 was used throughout the experiment, although the energy spectrum of the above ^{32}P -RNA sample showed that most of the count (99.7%) was recovered in the window width of 50~800. The counting of ^{32}P -RNA *via* Cerenkov radiation was not affected (Fig. 1-A) by acids (10% PCA, 5% TCA) and alkalis (1 *N* NH_4OH , 0.3 *N* KOH), while, as compared in Figs. 1-B and 1-C, when the ^{32}P -RNA sample was counted in the presence of a scintillator system (Bray's system or toluene-triton system), both the maximum counting efficiency and the gain position yielding the maximum counting efficiency were affected by these quenchers.

As shown by Clausen⁵⁾ for the counting of ^{32}P i, the Cerenkov counting of ^{32}P -RNA was affected by sample volume. Between 5 and 11 ml of the sample volume the counting efficiency reached the maximum, and 88.3% and 79.7% of the maximum value (at 7 ml) was obtained when the sample volume was 1 ml and 20 ml respectively. When samples of a constant volume (2.0 ml) were prepared, good proportional counting was obtained between 0.152 mg and 3.648 mg of the RNA content (Fig. 2).

Fig. 1 Effect of quenchers on the counting of ^{32}P -RNA. A: Cerenkov counting, B: with 10 ml of Bray's system, C: with 10 ml of the toluene-triton system. Window setting, 50~1,000, in each case. To 0.1 ml of the ^{32}P -RNA, 0.9 ml of quencher, as indicated, was added. In A, effects of two quenchers are indicated, points obtained by three other quenchers were either in-between or overlapped.

Fig. 2 Effect of RNA concentration on the Cerenkov counting. From the mixture of 0.1 ml of the ^{32}P -RNA and 5.0 ml of the carrier RNA, samples containing various amounts of RNA, as indicated, were taken, and the total volume in a vial was adjusted to 2.0 ml with distilled water. Window setting, 50~1,000. Gain, 35%.

Additions of carrier RNA (0.004~10.230 mg), protein (0.5~10.0 mg), KCl or NaCl (0.19~1.90 *M*), and sucrose (2.9~28.6%) showed very little effect (the maximum difference from the no-

addition control value was 6.5% when 10.23 mg RNA was added) on the counting of ^{32}P -RNA *via* Cerenkov radiation (Figs. 3-A~3-C).

Fig. 3 Effects of RNA and protein carriers, salts and sucrose on the counting of ^{32}P -RNA *via* Cerenkov radiation. To 0.1 ml of the ^{32}P -RNA, various materials were added to obtain final concentrations as indicated. Total volume was adjusted to 2.0 ml with 0.05 M Tris-HCl (pH 7.4) for protein, and with distilled water for others. Window setting, 50~1,000. Gain, 40%.

Table 1 Counting of ^{14}C and ^3H with or without scintillator

	Cerenkov counting	+Toluene-triton scintillator system (10 ml)
Discriminator setting	50-1,000	50-1,000
Gain (%)	40	10 40
	(counts/min)	
^{14}C -L-leucine ^a	26 ^c	231, 190 —
^3H -adenosine ^b	14	— 532, 540
background	16	20 22

a. ^{14}C -L-leucine (generally labeled) 0.02 ml + 1 N NH_4OH 1.0 ml

b. ^3H -adenosine (generally labeled) 0.01 ml + 5% PCA 1.0 ml

c. background counts are included.

Approximately 1.2 times better counting efficiency was obtained when a polyethylene vial was used instead of a glass vial. ^3H and ^{14}C gave essentially no count under the condition used for the Cerenkov counting as indicated in Table 1.

3.2 Counting of ^{32}P -RNA in hydrolyzed forms

^{32}P -RNA hydrolyzed either by alkali or acid could also be counted *via* Cerenkov radiation. Those hydrolyzed RNA samples gave the maximum counting efficiency at the same gain range—i.e. 35~50%—as with that of the non-hydrolyzed ^{32}P -RNA with a window setting of 50-1,000. When various amounts of hydrolyzed samples were counted *via* Cerenkov radiation or by adding Bray's scintillator system, the linearity of the counts vs. amounts of sample was better with the Cerenkov counting (Fig. 4).

Fig. 4 Counting of ^{32}P -RNA in hydrolyzed forms. Sample volume was adjusted to 2.0 ml with distilled water. Window setting, 50~1,000. Gain, 40% for the Cerenkov counting, 1.5% for the counting with Bray's system (10 ml). As to Cerenkov counting, each point on the graph consists of overlapped points of nve different conditions.

3.3 Counting of ^{32}P -RNA adsorbed on a membrane-filter

The ^{32}P -RNA adsorbed on a membrane-filter in the presence of 5% TCA demonstrated to be counted without adding any solvent. As shown in Fig. 5, the gain range giving the maximum counting efficiency was 40~50% with a window setting of 50~1,000. When the filter-paper was immersed in toluene, higher counts were obtained with shift of the gain position (20~30%), and again, counts were affected by the volume of toluene: 20 ml was the best in this case.

Fig. 5 Counting of ^{32}P -RNA adsorbed on a membrane-filter. A membrane-filter which adsorbed the ^{32}P -RNA was cut into four pieces, and a piece was put on the bottom of a glass counting-vial. Counting was performed without adding solvent or with toluene as indicated or with 10 ml of the toluene scintillator system. Window setting, 50~1,000.

When the filter-paper was immersed in 99.5% ethanol, 5% TCA and 5% PCA respectively, lesser counts were obtained than in toluene: counts with solvent (10 ml)/counts without solvent = 3.03 (toluene), 2.00 (ethanol), 1.56 (5% TCA) and 1.68 (5% PCA). No elution of ^{32}P -RNA into toluene was observed, but significant amounts of ^{32}P -RNA were eluted into other solvents: per cent elution was 0 (toluene), 3.6 (ethanol), 34.2 (5% TCA) and 59.7 (5% PCA).

As shown in Fig. 5, at each peak condition, counts obtained without adding solvent were 25.0% and those obtained by adding 20 ml of toluene were 75.2% of those obtained by add-

ing toluene-scintillator system.

When various amounts of ^{32}P -RNA (14.4~216 μg) were adsorbed on a membrane-filter, satisfactorily linear relationships between counts and amounts of ^{32}P -RNA were obtained by either of the above counting systems. Furthermore, when several pieces of membrane-filter each of which adsorbed ^{32}P -RNA were counted in a vial, good coincidence with calculated values (the summation of separate countings of each piece) was demonstrated (Fig. 6), suggesting that, as far as these numbers of pieces were concerned, the geometry of a piece of filter-paper in a vial did not affect the counting efficiency very much.

Fig. 6 Counting of several pieces of membrane-filter carrying ^{32}P -RNA. One membrane-filter which adsorbed the ^{32}P -RNA was cut into four pieces. These pieces were counted separately or several pieces were counted together in a glass vial as indicated. Window setting, 50~1,000. Gain: 40%, without solvent; 30%, with 10 ml of the toluene; 0.8%, with 10 ml of the toluene scintillator system.

When the filter-paper carrying ^{32}P -RNA was counted without adding solvent in poly-

ethylene vials, approximately 1.5 times higher counts were obtained than in glass vials (window setting, 50~1,000, gain, 50%).

This method was applied for determination of radioactivities of four 2', (3')-mononucleotide spots on the paper-chromatograph of the alkaline hydrolysate of the ^{32}P -RNA. The data in Table 2 indicated that 96.5% of the radioactivities applied was recovered as mononucleotides, and the base composition of the ^{32}P -RNA calculated by the distribution of radioactivities reasonably resembled those of ribosomal RNA, the bulk of cellular RNA.

Table 2 Cerenkov counting of ^{32}P -2', (3')-mononucleotides adsorbed on a chromatographic paper

Spot on the chromatograph	counts/min ^a	Base compositions	
		^{32}P -RNA ^b	<i>Ps. schuylkillensis</i> ribosomal RNA
		(mole %)	
Adenylic acid	1,382	25.9	25.4
Guanylic acid	1,697	31.8	31.7
Uridylic acid	1,128	21.1	21.5
Cytidylic acid	1,130	21.2	21.4
Total radioactivity recovered as mononucleotides 5,337			
Total radioactivity applied 5,530			
^{32}P as mononucleotides 96.5%			

a. gain 40%, discriminator 50~1,000, background counts (16 cpm) were subtracted.

b. calculated from the distribution of counts, assuming that ^{32}P was incorporated uniformly.

4. Discussion

The present studies revealed that ^{32}P -labeled RNA either in solution or adsorbed on a membrane filter could be counted quantitatively via Cerenkov radiation. Acids, alkalis, salts, sucrose, protein, and RNA had no, or very little, quenching effects, thus samples could be counted at their maximum counting efficiencies with a fixed setting of gain and window width (ex. 40%, 50~1,000). ^{32}P -RNA of high molecular weight forms fractionated by the sucrose gradient sedimentation or

column chromatographies, or ^{32}P -RNA hydrolyzed under various acidic or alkaline conditions would be good subjects of counting by this method. As the counting efficiency was affected by the sample volume, it is necessary to make samples of the same volume in a set of experiments. The counting efficiency of ^{32}P obtained by the Cerenkov counting was approximately 20% and is roughly an equivalent level with that obtained by a thin-window gas flow counter. ^{14}C and ^3H gave essentially no count under the conditions of the Cerenkov counting. It is, therefore, useful in double-labeling experiments (^{32}P and ^{14}C , or ^{32}P and ^3H) to use this method first to determine the radioactivity due to ^{32}P . The counting of ^{32}P -RNA or its hydrolysate adsorbed on a membrane-filter or on a chromatographic paper via Cerenkov radiation will have advantages in the following cases, when samples are required to be eluted for further studies, or when samples are adsorbed with quenchers such as some chromatographic solvents, or in double-labeling experiments.

Counting of ^{32}P -RNA adsorbed on a filter-paper in a glass or polyethylene vial without adding any solvent can not be due to the Cerenkov radiation in air, because $\beta (=v/c)$, where v is the velocity of the β -particle, and c the velocity of light, is calculated as 0.973 for ^{32}P , by the following equations: $\beta = \sqrt{(mc^2)^2 - (m_0c^2)^2} / mc^2$, $mc^2 = T_e + m_0c^2 = 1.71 + 0.51 (\text{MeV})$, where mc^2 is the total energy of electron, T_e the maximum kinetic energy of β -particle of ^{32}P , and m_0 the mass of electron. Thus, n , the index of refraction of the medium, should be greater than 1.027 as $n > 1/\beta$ is required to cause the Cerenkov radiation, but n of air at atmospheric pressure is 1.00029. Thus, it is most likely to assume that the counting is due to the Cerenkov radiation in glass or polyethylene of counting vials caused by escaped β -particles of ^{32}P through filter-paper and air. Yield of photons by the Cerenkov radiation is affected not only by n but also by the path length of electrons in the medium^{1,2)}, thus higher counts would be expected by using polyethylene vials or by immersing a filter-paper in solvents. The volume effect on the Cerenkov counting in

solution could also be explained from the balance between electrons converted to photons by the Cerenkov radiation in a solvent and material of vials and electrons or emitted Cerenkov lights absorbed in those media.

Summary

^{32}P -labeled RNA was quantitatively counted *via* Cerenkov radiation, without adding any scintillator solvent, using a liquid scintillation spectrometer. This method could be applied to the counting of ^{32}P -RNA of high molecular weight form or hydrolyzed form either in solution or adsorbed form on a filter-paper. The counting efficiency, approximately 20% in solution, was practically not affected by acids, alkalis, salts, sucrose, protein, and carrier RNA. The sample volume was shown to be a factor which affected the counting efficiency.

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