FURTHER OBSERVATIONS ON THE CARBON DIOXIDE INCORPORATION INTO RNA IN THE EARLY PHASE OF CONIDIA GERMINATION IN ASPERGILLUS ORYZAE WITH SPECIAL REFERENCE TO SOLUBLE RNA SYNTHESIS

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In the previous paper reported by ONO et al. (1), ribosomal RNA (rRNA) was found to be synthesized starting from the very initial period of germination of Aspergillus oryzae conidia as revealed by the fractionation of nucleic acids extracted from conidia which had been germinated in the presence of $^{14}$CO$_2$. They also pointed out that soluble RNA (sRNA) was actively synthesized after 45 min of germination.

The present paper describes that the sRNA synthesis (or modification) in the early phase of germination commenced earlier than 45 min of germination but later than the onset of rRNA synthesis, and that pyrimidine bases of sRNA were labeled with $^{14}$CO$_2$ more strongly than purine bases.

MATERIALS AND METHODS

Organism and cultivation. The conidia of Aspergillus oryzae purchased from Sanzaemon Kojiya & Co., Kyoto, were washed three times with deionized cold water and cultured at 30° in TERUI's medium (2), the inoculum size being 0.5 mg dry conidia per ml medium.

Labeling of conidia with $^{14}$CO$_2$. The labeling of germinating conidia with $^{14}$CO$_2$ was performed principally by the method described previously (1) using a rubber-stoppered flask in the presence of 1 µc Na$_2$$^{14}$CO$_3$ per ml medium. The washed labeled-conidia were suspended in 5 mM Tris-5 mM MgCl$_2$ (pH 7.4) and stored at -20°.

Extraction and fractionation of nucleic acid by the MAK column chromatography. Nucleic acids were extracted from conidia samples ground with quartz sand by the method of ISHIHAMA et al. (3) and they were fractionated by the methyalted albumin-coated kieselguhl (MAK) column chro-

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matography (4). Details of the procedures were the same as those described previously (1).

Isolation and purification of sRNA. The sRNA of $^{14}$C-labeled conidia was extracted and purified by method II-A described in the previous report (5).

Nucleotide analysis of RNA. $^{14}$C-labeled RNA was hydrolyzed and the hydrolyzate was subjected to paper chromatography. In one experiment RNA was hydrolyzed with perchloric acid at 100° for 1 hr, neutralized with KOH, and the hydrolyzate was paper chromatographed one-dimensionally using a solvent system of isopropanol-HCl (6). Then the paper was cut into pieces of 1 cm long and radioactivity of each piece was determined after the elution with 0.1 N HCl. In the other experiment radioactivities of nucleotide components separated by two-dimensional chromatography (7) were estimated by the elution of nucleotides with 0.1 N HCl from spots cut out from the paper chromatogram.

RESULTS

$^{14}$CO$_2$ incorporation into nucleic acids

Conidia were germinated for 3, 15 and 45 min in the presence of $^{14}$CO$_2$ and the labeled nucleic acids were extracted and chromatographed on the MAK column. Figs. 1A to 1D illustrate the elution patterns of the chromatography. As already reported in the previous report (1), profiles were essentially the same showing three major regions, sRNA, DNA and rRNA regions in the order of elution. The radioactivity profiles, on the other hand, were quite different for each one of these nucleic acid fractions. The nucleic acid sample obtained from the 3-min labeled conidia showed only a detectable radioactivity at the region eluted slightly later than that of rRNA (Fig. 1A). In the case of nucleic acid of the 15 min-labeled conidia, labeling was rather distinct at the region slightly behind the rRNA region as well as at those of sRNA and between sRNA and DNA (Fig. 1B). When the conidia were labeled for 45 min, radioactivities of nucleic acids were distributed among various molecular species of nucleic acids indifferent to optical density readings (Fig. 1C). An almost complete correspondency between radioactivity and optical density was demonstrated in a sample which had been labeled for 15 min after 4.5 hr of cultivation (Fig. 1D). From these observations, it should be noticed that in nucleic acid samples extracted from 15- and 45-min labeled conidia, higher specific radioactivities were found in sRNA than in rRNA.

A: Labeled for 3 min immediately after inoculation (reproduced from Fig. 4A of the paper of ONO et al., (1).
B: Labeled continuously for 15 min after inoculation.
C: Labeled continuously for 45 min after inoculation.
D: Labeled for 15 min following the germination of 4.5 hr in the absence of $^{14}$CO$_2$. 
Fig. 1. The MAK column elution patterns of nucleic acids extracted from germinating A. oryzae conidia labeled with $^{14}$CO$_2$. 

- - - OD$_{260}$ nM, - - - - Radioactivity

For labeling conditions see foot note on p. 330.
Comparison of 14C-incorporation into bases of rRNA and sRNA

Conidia were labeled continuously for 45 min with 14CO₂. Nucleic acid mixture was extracted with phenol from the labeled conidia, fractionated by the MAK column chromatography and respective RNA fractions were subjected to hydrolysis with perchloric acid. Four spots shown in the center area are the chromatogram of authentic bases employed as reference standards in the paper chromatography.

**Comparison of 14C-incorporation into bases of rRNA and sRNA**

The nucleic acid extract from 45-min labeled conidia was fractionated by the MAK column chromatography and the rRNA and sRNA fractions were collected, concentrated and hydrolyzed with perchloric acid. The hydrolyzates of both RNA fractions were subjected to the one-dimensional paper chromatography and radioactivity scanned as described previously. As seen in Fig. 2, four bases of rRNA appeared to be labeled almost equally, whereas those of sRNA were labeled rather unevenly showing higher labelings in pyrimidine bases than in purine bases.

**14C-Incorporation into bases of sRNA**

sRNA was extracted and purified by the method of TANAKA et al. (5) from conidia cultured for 45 min in the presence of 14CO₂. In the final step of purification by the DEAE-cellulose column chromatography eluted with a linear gradient concentration of KCl, the elution pattern of radioactivity
was completely superimposed by that of optical density showing uniform labeling of sRNA molecules (Fig. 3). The alkaline hydrolyzate of the 14C-labeled purified sRNA was subjected to the two-dimensional paper chromatography and an autoradiogram was prepared. As seen in Fig. 4, the autoradiographic pattern was qualitatively almost similar to the UV-absorption pattern shown in the previous report (5). Each spot on the paper chromatogram was cut out and eluted with 0.1 N HCl to determine the 14C-distribution among nucleotides. The results listed in Table 1 clearly show that pyrimidine nucleotides were highly labeled. It is also noteworthy that the highest specific activity was shown in a minor component, pseudo-uridylic acid.

**DISCUSSION**

The previous experiments of ONO et al. (1) showed that, among various molecular species of RNA, rRNA and possibly its precursor were found to be synthesized even in the initial 3 min of germination as revealed by the fractionation of 14C-labeled RNA classes by the MAK column chromatography. The present experiments carried out in the similar way afforded an additional
information on the synthesis of the RNA classes in the early phase of germination. After the commencement of the synthesis of rRNA at the very initial period of germination, when no sign of the sRNA synthesis was de-

Fig. 4. Autoradiogram of alkaline hydrolyzate of sRNA isolated from A. oryzae conidia cultured for 45 min in the presence of $^{14}$CO$_2$.

sRNA was isolated and purified as described in the legend of Fig. 3, and hydrolyzed with 0.3 N KOH at 37° for 18 hr. Solvent system of paper chromatography: 1st dimension, isopropanol-NH$_2$OH–water (70:25:5); 2nd dimension, isobutyric acid–0.5 N NH$_2$OH (50:30).
Table 1. $^{14}$CO$_2$ incorporation into nucleotides of sRNA isolated from conidia labeled for 45 min at the initial phase of germination.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Base ratio$^a$</th>
<th>cpm/spot</th>
<th>Specific activity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGp</td>
<td>0.2</td>
<td>30</td>
<td>58.8</td>
</tr>
<tr>
<td>Gp</td>
<td>27.9</td>
<td>2620</td>
<td>39.8</td>
</tr>
<tr>
<td>Up</td>
<td>19.9</td>
<td>4380</td>
<td>93.2</td>
</tr>
<tr>
<td>W-Up</td>
<td>3.6</td>
<td>1105</td>
<td>131.0</td>
</tr>
<tr>
<td>Cp</td>
<td>26.5</td>
<td>4135</td>
<td>66.0</td>
</tr>
<tr>
<td>Ap</td>
<td>19.6</td>
<td>2837</td>
<td>61.2</td>
</tr>
<tr>
<td>Tp</td>
<td>1.7</td>
<td>187</td>
<td>46.2</td>
</tr>
<tr>
<td>MeGp</td>
<td>0.4</td>
<td>60</td>
<td>58.8</td>
</tr>
</tbody>
</table>

$^a$ Base ratio was expressed as per cent moles of identified nucleotides.

$^b$ cpm per μmole nucleotide.

...synthesis of sRNA has appeared to proceed at the 15th min of germination. From these observations together with those of KIMURA et al. (8), the sequential commencement of the syntheses (or modification) of various RNA classes in the early phase of germination may be depicted as follows: rRNA, at the very initial period of germination; sRNA, sometime before 15 min of germination; and mRNA, from 45 to 60 min of germination.

As already pointed out by ONO et al. (7), a portion of the rRNA synthesized during the initial 45 min of germination was not necessarily characterized by the completed form of rRNA as deduced from its elution pattern of the MAK column chromatography. The present experiment showed that when the conidia were labeled for 45 min in the initial period of germination four bases of rRNA were labeled rather evenly.

Interesting enough is the finding that the labeling pattern of sRNA bases was quite different from that of rRNA. In sRNA, an uneven labeling characterized by higher specific activities in pyrimidine nucleotides than in purine nucleotides was demonstrated. Although it is not yet clear why such an uneven labeling was occurred only in sRNA but not in rRNA, a possibility that some special molecules of sRNA is synthesized during the initial 45 min of germination may be excluded, since the elution patterns of DEAE-cellulose column chromatography of labeled and non-labeled sRNA fractions were superimposed almost entirely over a wide region of the main peak. It may possibly be considered that the formation site of building blocks of these RNA classes may be, in part, different in their localizations in the cell and, in necessity, the degree of labeling with $^{14}$CO$_2$ of these building blocks becomes...
variable from one site to the other, this in turn leads to the variable labelings of bases of different RNA classes.

SUMMARY

Using *Aspergillus oryzae* conidia, timing of the syntheses of various RNA classes in the early phase of germination was investigated. The methylated albumin-coated kieselguhl column chromatography was employed for the fractionation of RNA classes extracted from the conidia which had been labeled with $^{14}$CO₂ for different periods of germination.

Following the synthesis of ribosomal RNA (rRNA) at the very initial step of germination, the synthesis or the modification of soluble RNA (sRNA) was found to commence not later than 15 min of cultivation. After the hydrolysis of the $^{14}$C-labeled rRNA and sRNA, the hydrolyzates were subjected to paper chromatography and radioactivities of bases (or nucleotides) scanned. The results showed that bases of rRNA were almost evenly labeled, while those of sRNA quite unevenly (higher specific activities in pyrimidine nucleotides than in purine ones).

REFERENCES