Fixation and Incorporation of CO_2 into Ribonucleic Acid by Germinating Uredospores of Uromyces phaseoli

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ABSTRACT

The percentage of ^{14}C incorporated into ribonucleic acid (RNA), assayed by radioactivity present in cytidylic acid (CMP), adenylic acid (AMP), guanylic acid (GMP), and uridylic acid (UMP), was greater in the purine nucleotides than in the pyrimidine nucleotides at all times sampled. The concentrations of mononucleotides increased from 15.3 μmole/250 mg spores 5 minutes after the start of germination to 17.5, 8 hr after germination, indicating an increase in RNA during germination. The mole per cent of GMP and UMP in the RNA hydrolysate was always highest, and CMP lowest. During germination, the purine:pyrimidine ratio decreased from 1.34 to 1.02 in 8 hr.

When nucleic acids from germinating bean rust uredospores were separated on methylated albumin columns during the first 30 min of germination, significant radioactivity was present only in the oligonucleotide fraction. Radioactivity was present in both light and heavy ribosomal RNA 1.5 hr after initiation of germination; the highest activity was in the elution profile just past the heavy ribosomal RNA. Six hours after germination, radioactivity was present in the soluble RNA peak, in the ribosomal RNA. Six hours after germination, the highest activity was once again found in the region just beyond the heavy ribosomal RNA; radioactivity was also present in the soluble RNA peak and in the ribosomal RNA peaks. Radioactivity was highest in the oligonucleotide peak for each experiment, with no activity in either transfer RNA or deoxyribonucleic acid peaks. The sequence of synthesis of RNA species, as determined by ^{14}C incorporation, appears to be (i) an adduct of both light and heavy ribosomal RNA (sometimes interpreted as mRNA); (ii) ribosomal RNA; and (iii) soluble RNA. Phytopathology 61:400-405.

Data are available concerning the fixation of CO_2 by germinating spores of *Aspergillus* sp. and the subsequent incorporation of CO_2 into various spore metabolites, particularly the nucleic acid fraction (11, 24, 25, 26). Results of Yanagita (28) indicate that CO_2 fixed by *Aspergillus* sp. was extensively incorporated into RNA by both germinating and nongerminating spores. Yanagita found that the CO_2 fixed was rapidly incorporated into RNA at the initial stages of germination.

Ono et al. (11) further investigated the participation of CO_2 incorporation into RNA by determining the sequential synthesis of the various molecular species of RNA by germinating spores of *Aspergillus oryzae*. They found that ribosomal RNA (rRNA) synthesis occurs at the initial stages of germination, and that the synthesis of rRNA or its precursor may be one of the reactions initiating germination. Incorporation of $^{14}CO_2$ into transfer RNA occurred 15 min after initiation of germination; and messenger RNA (mRNA), 60 min after initiation of germination.

Staples et al. (21) described the physico-chemical properties of ribosomes contained in germinated and nongerminated uredospores of *Uromyces phaseoli*, and also a polyuridylic acid-directed phenylalanine incorporation into polyphenylalanine by these ribosomes in vitro. Later, Ramakrishnan & Staples (12, 13) reported the synthesis of RNA species in germinating bean rust uredospores. Using orotic acid-6-^{14}C, they (12) noted that radioactivity first appeared in rRNA and after 6 hr in tRNA. Using pulse-labeling techniques with ^{3}H-uridine, Ramakrishnan & Staples (13) found a greater incorporation of tritium in differentiated spores (spores which formed appressoria) than in nondifferentiated spores, and the synthesis of a limited amount of mRNA in the differentiated spores. Staples (20) reported that resting bean rust uredospores incorporate exogenous amino acids into protein, and later, Staples et al. (22) reported the presence of polyribosomes in these same uredospores which have the capability of incorporating amino acids in a cell-free system. This suggested the presence of a stable mRNA which was necessary for germ tube development. Ramakrishnan & Staples (14) conclude that synthesis of mRNA does not occur in nondifferentiated uredospores of *U. phaseoli,* but does occur at the time of differentiation; i.e., appresorium formation.

Biosynthetic pathways described by Schulman (18) and Roberts et al. (16) indicate that compounds derived from the tricarboxylic acid cycle are utilized for nucleic acid synthesis. They also describe the direct involvement of CO_2 incorporation into the purine and pyrimidine bases of these acids. Data by Rick & Mirocha (15) show that the malic enzyme is operative in germinating bean rust spores; thus, CO_2 fixed by the spores could be incorporated into nucleic acids. Data by Stallknecht & Mirocha (19) showed that germinating uredospores of the bean rust fungus fix CO_2 in the dark, and that significant amounts of CO_2 are incorporated into RNA. The synthesis of various species of RNA during spore germination is of interest, particularly the synthesis of mRNA, since information is transcribed on the RNA from DNA for the synthesis of enzymes which would be required in the initial stages of spore germination.

In the study of the host-parasite relationship between *U. phaseoli* and its host, *Phaseolus vulgaris*, a large amount of ^{14}C from $^{14}CO_2$ fixed in the dark was found to be incorporated immediately around the young infection site (4 days after infection) on the host (29). In order to determine the extent that the fungus contributed to this ^{14}C pool, it was necessary to study the CO_2 fixing enzymes of the fungus apart from the host. The malic enzyme was found to be constituent in the uredospores, which accounted for $^{14}CO_2$ fixation (15). In order to show the significance of CO_2 fixation by the germinating uredospores, it was necessary to determine what role CO_2 played in the metabolism of the fungus spore. The latter prompted the study of CO_2 incorporation into nucleic acids, and is the subject of this research. The proximate objectives of the present study were to determine the patterns of CO_2 incorporation into the four nucleotide bases of RNA (guanylic, adenylic, cytidylic, and uridylic acids), the patterns of CO_2 incorporation into species of RNA during the germination of bean rust uredospores, and the probable significance of this phenomenon in the infection process on the host.

MATERIALS AND METHODS.—The source and maintenance of the bean rust (*Uromyces phaseoli* [Pers.] Wint. var. *typica* Arth. race 32) spores, methods of spore germination, exposure of spores to $^{14}CO_2$, and extraction of RNA are as previously described (15, 17, 19). The precipitated RNA was washed 3 times with ethanol, and the ethanol assayed for radioactivity from incorporated $^{14}CO_2$. No radioactivity was found in the ethanol. In all experiments, 250 mg dry wt of uredospores were used.

Determination of ^{14}C incorporation into the nucleotide bases of uredospore RNA.—The RNA obtained from the phenol extraction as described by Stallknecht & Mirocha (19) was added to 5.0 ml of 0.3 N KOH and hydrolyzed at 30 C for 24-30 hr. The solution was adjusted to pH 8.0 by adding cold 3 M perchloric acid and letting it stand for 15 min at 0 C. The potassium perchlorate precipitate and residue were removed by centrifugation at 12,100 *g* for 10 min.

Cytidylic acid (CMP), adenylic acid (AMP), guanylic acid (GMP), and uridylic acid (UMP) were separated on a Dowex 1-X8 resin (200-400 mesh) column. The column was prepared by washing a Dowex 1-X8 resin (chloride form) with 4 N ammonium formate until the effluent from the column gave a negative test for silver chloride as assayed by the addition of 0.1% silver nitrate. Four ml of the RNA hydrolysate was then added to a 7 × 150 mm column. The hydrolysate was washed into the column with 10 ml of distilled water. The four mononucleotides were then eluted as follows, following the method of Ewing & Cherry (3): CMP with 100 ml of 0.15 N formic acid; AMP with 100 ml of 1.0 N formic acid; GMP with 140 ml of 3.0 N formic acid; and UMP with 100 ml of a 4.0 N formic acid-0.1 M ammonium formate mixture. The eluates were collected in 10 ml fractions, and the absorbance was read at 280 mμ for CMP and at 260 mμ for AMP, GMP, and UMP.

Determination of specific activity of mononucleotides.—Individual fractions of mononucleotides were collected and reduced in volume by flash evaporation at room temperature (ca. 24 C). The concentration of the respective mononucleotides was calculated from their molar extinction coefficients, CMP-6,800, AMP-14,200, GMP-11,800, and UMP-9,800 at 260 mμ at pH 2 (27).

For assay of radioactivity, a 1-ml aliquot of each mononucleotide was added to 10 ml of dioxane scintillation solvent which consisted of 150 g of naphthalene, 10 g 2,5-diphenyloxazole (PPO), and 0.3 g of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) in 1 liter of *p*-dioxane. The vials were counted on a dual channel Beckman liquid scintillation counter. Specific activity was calculated as counts/min per μmole of nucleotide.

Determination of RNA species by methylated albumin column chromatography.—Spore RNA was extracted by the phenol method as previously described (19). The RNA was then fractionated on a methylated albumin-coated Kieselguhr (MAK) column according to the method of Mandell & Hershey (10). The RNA was eluted with a linear NaCl gradient of 400 ml each of 1.2 M NaCl and 0.3 M NaCl in a 0.05 M phosphate buffer (pH 6.7). Ten-ml fractions were collected in all experiments.

After each fraction was collected and read at 260 mμ, the test tubes were placed at 4 C for 1 hr. Bovine serum albumin, 0.2 mg/tube, was added as a coprecipitant prior to adding the trichloroacetic acid. Five ml of cold 10% trichloroacetic acid were then added to each test tube, and the tubes were allowed to stand for 0.5 hr in the cold. The RNA fractions were then collected on membrane filters (Schleicher and Schuell, type B6), dried, and placed in standard vials containing 10 ml of scintillation fluid. The scintillation liquid was prepared by adding 42 ml of Liquifluor (a commercial preparation of PPO and POPOP by Pilot Chemical) and 1 liter of toluene. The filter apparatus used was a Schleicher and Schuell microfilter Model SS 25.

RESULTS.—*Analysis of RNA mononucleotides from germinating bean rust uredospores.*—The mole per cent of each mononucleotide and the nucleotide ratios of RNA extracted from the bean rust uredospores are given in Table 1. The mole per cent of CMP varied

TABLE 1. Determination of RNA mononucleotide composition of bean rust uredospores, *Uromyces phaseoli*, during various stages of germination

	Germination time		
RNA nucleotides[a]	5 min	4.5 hr	8 hr
Cytidylic acid CMP	14.5	13.5	16.2
Adenylic acid AMP	21.8	21.2	23.2
Guanylic acid GMP	33.7	32.7	27.2
Uridylic acid UMP	30.0	32.6	34.3
Purine:pyrimidine	1.24	1.18	1.02
6 Am:6 Keto[b]	0.57	0.53	0.63

[a] CMP, AMP, GMP, and UMP = cytidylic, adenylic, guanylic, and uridylic acids, respectively. (Concentration in mole per cent)

[b] The mole concentration of adenylic acid + cytidylic acid/guanylic acid + uridylic acid.

from 14.5 to 16.2, for AMP from 21.8 to 23.2, and for UMP from 30.0 to 34.3 as assayed as spore germination times of 5 min, 4.5 hr, and 8 hr. The mole per cent of GMP decreased as germination progressed, the concentration being 33.7% after 5 min of germination and dropping to 27.2% after 8 hr. Nucleotide ratios of AMP:UMP and GMP:CMP tended to decrease as the length of germination increased. The purine:pyrimidine ratio also decreased during germination, the value being 1.24 at 5 min, 1.18 at 4.5, and 1.02 at 8-hr germination time. The purine:pyrimidine ratio changes indicate that the pyrimidine nucleotide concentrations increased slightly more than the purine during germination. In considering the 6 amino:6 keto nucleotide ratio, the value varied from 0.53 to 0.63, these values being much lower than those given by Traub & Elson (25), who indicate that the usual 6 amino:6 keto nucleotide ratios from RNA of most plant species vary from 0.80 to 1.0.

The total concentration of the four mononucleotides extracted from germinating bean rust spores increased with the length of germination (Table 2), indicating an increase in RNA synthesis during spore germination. The actual concentration in moles of CMP, AMP, GMP, and UMP (Table 2) increased during germination, except for GMP. The specific activity (CMP/μmole) of each nucleotide increased as the length of exposure to $^{14}CO_2$ increased (Table 2). GMP was highest, followed by AMP, CMP, and UMP in that order. Results indicate that a greater amount of $^{14}CO_2$ was incorporated into the purines than the pyrimidines of RNA.

Incorporation of $^{14}CO_2$ into various species of RNA extracted from germinating bean rust uredospores.—The identification of the various species of RNA in this study are as described for other fungi and higher plants (1, 3, 6, 8, 24).

Results of bean rust nucleic acid separation on methylated albumin Kieselguhr columns are given in Fig. 1. The identity of the peaks are as follows: I = oligonucleotides plus small amounts of residual phenol; II = soluble RNA; III = DNA; IV = light rRNA; V = heavy rRNA; and VI = adduct of light and heavy rRNA interpreted by some as mRNA. The elution profile and incorporation of $^{14}CO_2$ into nucleic acids indicates that after a 30-min exposure and germination time, a significant incorporation of radiocarbon was present only in the oligonucleotide peak (Fig. 1-A), whereas only small amounts of radiocarbon from $^{14}CO_2$ was incorporated into any of the RNA fractions. That nongerminated spores show an elution profile similar to the 30-min germination profile may indicate that the spores contain an appreciable amount of endogenous RNA which may be activated at the initiation of germination. Results from spores exposed to $^{14}CO_2$ and germinated for 1.5 hr show highest radioactivity in the oligonucleotides, slight radioactivity in soluble RNA found between fraction 40 and 60, and slight radioactivity in both the light and heavy rRNA (Fig. 1-B). The greatest amount of ^{14}C incorporated into RNA after 1.5 hr of germination appeared in peak VI, which is considered to be an adduct of both light and heavy rRNA and considered by some to be mRNA. No activity was observed in the DNA peak.

Results of germinating bean rust spores exposed to $^{14}CO_2$ for 6 hr show distinct incorporation of ^{14}C into transfer RNA (tRNA), light and heavy rRNA, and high radioactivity in peak VI (Fig. 1-C). Once again the oligonucleotides were most radioactive.

TABLE 2. Concentrations and specific activity of nucleotides of RNA extracted from uredospores of *Uromyces phaseoli* after initiation of germination

Factor	Nucleotide	Germination time		
		5 min	4.5 hr	8 hr
		μmole nucleotide/250 mg spores		
Concentration	CMP[a]	2.22	2.16	2.82
	AMP	2.33	3.40	3.90
	GMP	5.16	5.36	4.74
	UMP	4.59	5.25	6.00
	Total	15.30	16.17	17.46
		count/min μmole nucleotide		
Specific activity[b]	CMP	350	555	1,630
	AMP	520	1,360	2,010
	GMP	1,120	4,680	6,000
	UMP	260	265	1,770

[a] CMP, AMP, GMP, and UMP = cytidylic, adenylic, guanylic, and uridylic acids, respectively.
[b] Spores were exposed to 70-82 μc of $^{14}CO_2$.

DISCUSSION.—RNA from bean rust uredospores was extracted at 5 min, 4.5 hr, and 8 hr after initiation of germination. The time of the extractions coincides with the stages of germination represented by (i) initial hydration and swelling; (ii) germ tube protrusion; and (iii) germ tube elongation. During germination, the total combined concentration of the four mononucleotides, CMP, AMP, GMP, and UMP increased from 15.3 to 17.46 μmole/250 mg dry wt of spores, indicating RNA synthesis. If one compares the results of experiments in this study with results of other investigators concerned with RNA nucleotide analyses, several distinct differences and a particular similarity can be observed. The first distinct difference we observed was the specific activity of ^{14}C incorporated into the respective mononucleotides. Our results consistently showed that the purine bases had a higher specific activity (count/min per μmole nucleotide) than did the pyrimidine bases. Investigations by previous researchers (15, 21) working with bacteria and *Aspergillus* species exposed to $^{14}CO_2$ indicated that the highest specific activities were always found in the pyrimidine bases. Secondly, it appears that the nucleotide ratio of CMP, AMP, GMP, and UMP differ markedly from those of *Neurospora crassa* (5), *Aspergillus sp.* (24), *Bacillus subtilis* (2), and from higher plant tissues (3). The mole per cent of CMP was found to be consistently high and UMP was found to be consistently low in the aforementioned cases, whereas in our study, UMP and GMP was found to be highest and CMP lowest in mole % concentrations. Our data do agree, however, with the results obtained by Johnson et al. (7) and Kloker et al. (9) concerning mole % and base ratio percent-

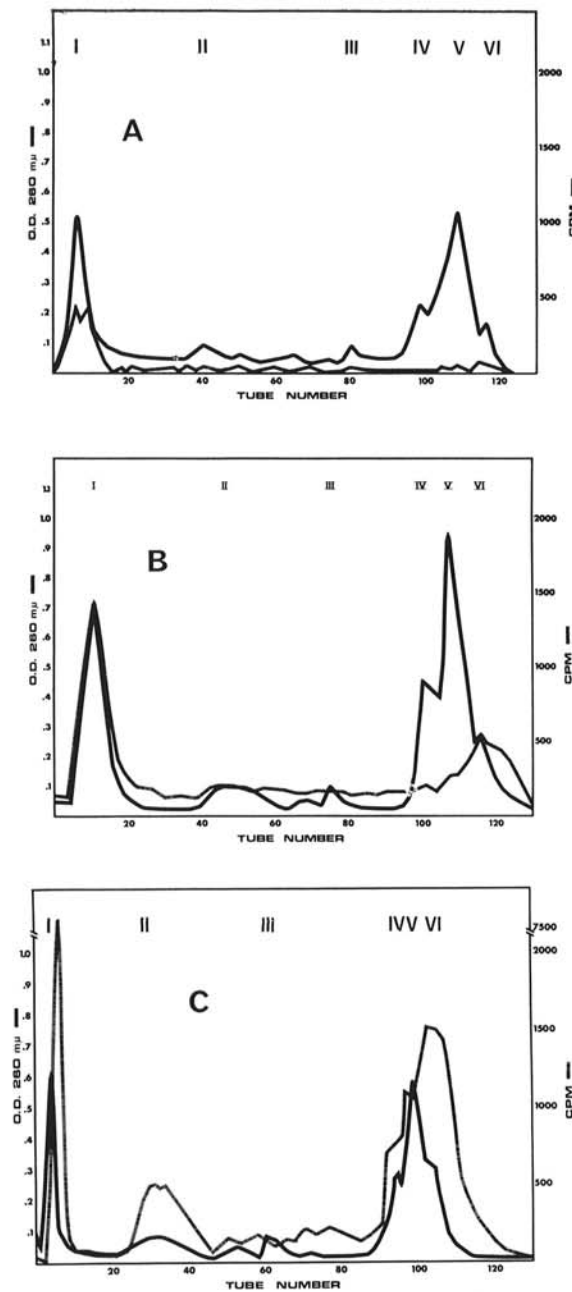

Fig. 1. Separation of molecular species of RNA from uredospores of *Uromyces phaseoli* exposed to $^{14}CO_2$ after various periods of germination: **A)** 30 min after initiation of germination and exposed to 790 μc $^{14}CO_2$; **B)** 1.5 hr and 875 μc; **C)** 6 hr and 875 μc. I = oligonucleotides; II = soluble RNA; III = DNA; IV = light ribosomal RNA; V = heavy ribosomal RNA; VI adduct of IV and V, sometimes interpreted as messenger RNA.

ages of nucleotides of rust uredospores. Johnson et al. (7) noted that the mole per cent nucleotide composition of RNA from uredospores of *Puccinia recondita* was lowest in CMP and highest in GMP. Their purine: pyrimidine ratio and 6 amino:6 keto ratio were comparable to ours. Kloker et al. (9) noted similar results for nucleotides of RNA from uredospores of *Puccinia graminis* f. sp. *tritici,* which showed that CMP was lowest and UMP highest in mole % concentrations. Data by Ewing & Cherry (3), Henny & Storck (5), and Doi & Igarshi (2) show that nucleotide ratios vary among various species of RNA extracted from higher plants, fungi, and bacteria. Transfer RNA is considered to have a greater percentage of GMP and CMP, whereas, rRNA and mRNA have higher percentages of AMP and UMP. Thus, rust uredospores may have an RNA composition which may be distinct and characteristic of obligate parasites.

A similarity between the nucleotides of germinating bean rust uredospores and germinating conidia of *Aspergillus niger* was the drop in the purine:pyrimidine ratio as germination progressed. In spores of *Aspergillus niger* (23), this ratio drops from 1.20 to 1.02 during a 6-hr germination period, as compared to decrease in ratio from 1.24 to 1.02 for an 8-hr germination period in bean rust uredospores. Sussman & Halvorson (23) indicated that a decrease in the purine:pyrimidine ratio with time may indicate mRNA synthesis; however, they do not indicate the basis of their conclusion.

Results from this study indicate that RNA precursors are being synthesized within 30 min after onset of germination, followed later (1.5 hr) by synthesis of rRNA. Synthesis of tRNA takes place during the later stages of germination. Ramakrishnan & Staples (12) indicated that they found incorporation of orotic-6-^{14}C initially in rRNA and that no activity was found in tRNA until after 6 hr of germination which is in agreement with our studies. Ono et al. (11) studied the sequential synthesis of various species of RNA in germinating conidia of *Aspergillus oryzae*. They found that rRNA is synthesized at the initial phase of germination, followed somewhat later by tRNA. They found that mRNA was synthesized only after 60 min of germination.

The appearance of ^{14}C in the various species of RNA during rust uredospore germination occurred at a much later time in our germination experiments when compared to results obtained by Ono et al. (11) for germinating spores of the *Aspergillus* species. They noted that rRNA synthesis occurred immediately after the onset of germination and throughout germination. Transfer RNA synthesis occurred 15 min after onset of germination, and mRNA after 60 min. Their analyses were based on methylated albumin Kieselguhr column chromatography and sucrose density-gradient studies. RNA synthesis was not detected until 1.5 hr after the onset of germination.

Possible significance in the delay of detectable quantities of radioactive substances in the various RNA species during germination of bean rust uredospores could be related to the observation by Staples et al. (22), who showed that polysomes are present in dormant bean rust uredospores; they reported that nongerminating spores have an intact mechanism for protein synthesis which allows the spores to germinate

without significant increases in RNA synthesis during the initial stages of germination. Results by Yanagita et al. (28) and Henney & Storck (4) showed that polysomes are not present in dormant spores of *Aspergillus* sp. or *Neurospora crassa*. Thus, these spores may require more RNA synthesis at an earlier stage in germination than would rust uredospores.

Although no significant amounts of radioactive materials were noted in the various RNA species of bean rust uredospores after 30 min of germination, low radioactivity values were detected in the total RNA fraction within 5 min after the onset of germination. The percentage of ^{14}C in the total RNA fraction was calculated on the basis of total ^{14}C incorporated by spores. Results of our study show that 0.08% was found in the RNA fraction at 5 min, and 0.14% at 30 min, after onset of germination. But when RNA was separated on MAK into distinct RNA species, the radioactivity found in each species was not significant at 30 min after germination. This could be due to the dilution of RNA during the fractionation.

Our results reported here and previously (19) support the contention that at least part of the radioactive metabolites surrounding rust infection sites and derived from $^{14}CO_2$ fixed in the dark are of fungal origin. This does not mean that these products are exclusively of fungal origin, because many products of host photosynthesis also accumulate. This research also supports the fact that uredospores of bean actively fix CO_2 at the time of inoculation and sporulation on the host, and that part of this CO_2 takes part in nucleic acid metabolism. Incorporation into nucleotides takes place within 30 min after initiation of germination, which is within the time of the greatest rate of CO_2 fixation. Between 0.5 and 1.5 hr, a substantial amount of $^{14}CO_2$ is incorporated into soluble RNA (sRNA) and some into fraction VI, which is an adduct of light and heavy rRNA or mRNA. The incorporation into fraction VI is most marked 6 hr after initiation of germination, and also corresponds to the time of differentiation or appresorium formation on the host. It is apparent that CO_2 fixed by uredospores is involved as a substrate in the nutrition of the germinating and differentiating spore on the host.

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