Carbon Dioxide Fixation by Uredospores of Uromyces phaseoli and its Incorporation into Cellular Metabolites

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ABSTRACT

CO_2 fixation by uredospores of *Uromyces phaseoli* was greatest during the initial stages of germination, decreasing rapidly during the first 2 hr of germination. No difference in CO_2 fixation was noted between spores germinating in light (3,000 ft-c) or in total darkness. CO_2 fixed by germinating spores was incorporated into the soluble low mol wt metabolites, RNA, and into other macromolecules of the spores. The greatest amount of ^{14}C was found in the macromolecular fraction of the spores, represented by proteins and polysaccharides. Phytopathology 60:1338-1342.

Fixation of CO_2 by germinating fungus spores has been demonstrated in *Aspergillus* sp., *Botrytis cinerea*, *Puccinia recondita*, and *Uromyces phaseoli* (8, 16, 17, 20). Yanagita (20) demonstrated that CO_2 was essential for germination of *Aspergillus niger* spores, and that it was fixed immediately upon initiation of the germination process. He also noted that macromolecular substances, such as proteins and nucleic acids, rapidly incorporated radioactive carbon from $^{14}CO_2$ during the initial stages of germination.

Staples & Weinstein (16) studied CO_2 fixation in the dark by bean rust uredospores en masse and apart from the host. When they investigated the incorporation of $NaH^{14}CO_3$ by nongerminated spores and separated the cellular components into acidic, basic, and neutral fractions, they found that the components of the acidic fraction had incorporated significant amounts of radioactive carbon.

Rick & Mirocha (12) showed that nongerminating bean rust uredospores had a high specific activity of the malic enzyme, whereas spores germinated for 24 hr showed only slight malic enzyme activity, indicating that the rate of CO_2 fixation during the initial stages of germination was greater. Further, Mirocha & Rick (10) noted that bean leaves inoculated with uredospores fixed the greatest amount of CO_2 within the first 3 hr after inoculation, but CO_2 fixation decreased rapidly afterwards. This initial fixation is attributed to the uredospores during the early stage of their germination rather than fixation by the host.

The results of these investigations (10, 12) may indicate that CO_2 fixed in the dark by bean rust uredospores may be involved as a substrate in the nutrition of the germinating spore. Carbon dioxide fixed via the malic enzyme could contribute to the total metabolism of the spores as an anaplerotic mechanism, as described by Kornberg (7). Thus the fixation of CO_2 to form dicarboxylic acids of the tricarboxylic acid cycle from pyruvate could serve as metabolic intermediates for macromolecule synthesis or to replenish the loss of acids taken out of the cycle for synthesis of spore metabolites.

The object of our study was twofold: (i) to determine the kinetics of the CO_2 fixation process of uredospores apart from the host and after synchronization; and (ii) to determine the metabolic fate of the CO_2 taken up by the spores with particular reference to RNA.

MATERIALS AND METHODS.—*Source and maintenance of the rust.*—The isolate of bean rust used in this study was *Uromyces phaseoli* (Pers.) Wint. var. *typica* Arth. race 32. The rust culture was maintained on *Phaseolus vulgaris* L. 'Top Crop'. Spores were stored in liquid N until used. They were removed from the N storage tank and heated to 40 C, which insured a high percentage of germination according to the method of Loegering & Harmon (9).

Six days after inoculation (method of Rowell & Olien, 13), uredospores appeared on both upper and lower surfaces of the inoculated leaf. Spores were collected from the plants 10 days after inoculation, placed in a desiccator for 4 hr to reduce the moisture content, and then either stored at 4 C or in liquid N.

Spore germination.—The method is essentially that as described by Schipper et al. (14). For ease of handling, 25 mg of uredospores were evenly distributed over the surface of Millipore filters (47 mm diam), type SC (pore size 8 μ). The moist uredospores were treated on the filter pads.

For experiments on dark fixation of $^{14}CO_2$ by bean rust spores, a 5-liter desiccator was painted black to exclude light. For studies on the effect of light on $^{14}CO_2$ fixation by rust spores, three General Electric fluorescent lamps (GE F 15 T8CW, 15) were used to produce a light intensity of 3,000 ft-c. The light transmission of the desiccator cover was checked by use of ISCO spectroradiometer Model SR. The desiccator glass cover transmitted all wave lengths of light emitted from the lamps (380 to 750 nm) as checked by the spectroradiometer.

To expose rust spores to light, the porcelain plate of the desiccator was covered with two layers of Whatman No. 1 filter paper and moistened with glass distilled water. The Millipore filter pads containing the germinating rust spores were then placed in the desiccator.

The temp in the desiccator was maintained at 20 C

by circulating cooled ethylene glycol around it while it was contained in a large plastic vessel.

Carbon dioxide fixation in light and in the dark.— $Ba^{14}CO_3$ of known wt and specific activity was used to generate $^{14}CO_2$ according to a method previously described (21). Bean rust spores were germinated for various periods of time, placed in the desiccator and exposed to $^{14}CO_2$ for 15 min, 30 min, and 1.5 hr. After exposure to $^{14}CO_2$ (50 µc), the uredospores were removed from the desiccator and washed with 2 N HCl containing 100 ppm of Tween-20 (polyoxyethylene sorbitan monolaurate) to remove adsorbed $^{14}CO_2$. The spores were washed by placing the Millipore filter pad containing the spores in the filter apparatus and washing them with acid for 10 sec. The spores and filter pad were then placed in a 30-ml brown glass bottle. Two ml of NCS Solubilizer (a tissue-dissolving reagent, quaternary ammonium hydroxide, Nuclear-Chicago) was added to each bottle. This solution completely digested the Millipore filter and rust spores. After 24 hr, two 0.5 ml aliquots of the digested spore sample were removed from each bottle and pipetted into 1.25-cm planchets previously coated with Tween-20 to insure even distribution of the sample. Radioactivity was measured on a Nuclear Chicago gas flow Geiger tube counter (Model 4342). Each treatment was replicated twice and the experiment repeated once.

Extraction and separation of spore metabolites.— RNA was extracted from bean rust spores using a modification of the phenol technique described by Kirby (6). Ten Millipore filters, with 25 mg of spores on each, were used throughout the following studies to give a total of 250 mg dry wt of spores/experiment. After exposure to gaseous $^{14}CO_2$, the spores were disrupted with a Braun mechanical cell homogenizer, Model MSK (Bronwill Scientific Div., Rochester, N.Y.). Disruption techniques were similar to those used by Rick et al. (11), except that the flow of CO_2 used to cool the disruption flask was controlled to prevent freezing of the buffer. The bean rust uredospores were disrupted for 3 min in 10 ml of 0.005 M Tris[tris(hydroxymethyl)amino methane]-HCl; 0.005 M $MgCl_2$ (pH 7.5); 1 ml of 5% Duponal (sodium lauryl sulfate), and 1.0 ml of bentonite, prepared according to Brownhill et al. (1). After disruption, the spore suspension was mixed with an equal volume of 88% phenol for 5 min at 4 C and centrifuged for 10 min at 18,800 g. The upper aqueous layer containing the RNA, soluble nucleotides, and low mol-wt compounds was carefully removed with a pipette. The lower phenol layer contained denatured protein, DNA, and cellular residue as a precipitate. The aqueous layer containing the RNA was mixed with 1 ml of bentonite and 10 ml of 88% phenol for 3 min and centrifuged for 10 min at 18,800 g. The phenol-residue layer was mixed with 10 ml of Tris-HCl buffer and centrifuged for 10 min at 18,800 g; the aqueous layer removed and extracted with 1 ml of bentonite and 10 ml of 88% phenol and centrifuged for 10 min at 18,800 g. The aqueous fractions containing RNA were then combined and extracted 3 times with diethyl ether in a separatory funnel to remove any residual

phenol. The ether was removed by passing a stream of N_2 over the solution. The aqueous RNA fraction was then mixed with two volumes (equal to that of the RNA) of absolute ethanol and allowed to stand overnight at 4 C. The resulting RNA precipitate was collected by centrifugation at 18,800 g for 20 min. The RNA precipitate was washed with cold absolute ethanol and centrifuged. The RNA thus extracted was hydrolyzed in 5 ml of 0.3 N KOH at 30 C for 24 to 30 hr. The nucleotide solution was then cooled to 0 C in an ice bath, and 0.05 ml of 0.1% phenol-phthalein was added to the solution, as a pH indicator. The solution was then adjusted to an approximate pH of 8.0 by the addition of cold 3 M perchloric acid and allowed to stand for 15 min at 0 C. The potassium perchlorate and nondigested residue were removed by centrifugation at 12,100 g for 10 min.

Separation of compounds by ion-exchange chromatography.—The supernatant fraction obtained from the precipitation of RNA with ethanol was separated into basic, acidic, and neutral fractions by ion exchange column chromatography. This fraction is the aqueous layer obtained during the phenol extraction of RNA, and represents the free amino acids, organic acids, sugars, and nucleotides soluble in the aqueous buffer system. The supernatant fraction was reduced to 25 ml volume in a flash evaporator and applied to an anion exchange column and then to a cation exchange column. The columns consisted of a 10 × 100 mm Dowex-1-formate 200-400 mesh and a 10 × 100 mm Dowex-50-hydrogen form 200-400 mesh. The effluent from the Dowex-50 was designated as the neutral fraction, and contained low mol-wt sugars. The acidic fractions were adjusted to 25 ml, and a 1-ml sample was assayed for per cent activity in dioxane scintillation mixture.

The spore residue and precipitate from the phenol buffer extraction were placed in a 30 ml brown bottle, and 10 ml of NCS was added to digest the material. A 0.5 ml sample of each solution was assayed after 72 hr.

RESULTS.—*Fixation of $^{14}CO_2$ by germinating uredospores in the dark and light.*—To determine if the spores could fix CO_2 during various stages of germination, bean rust spores were germinated for various time periods and then exposed simultaneously to $^{14}CO_2$. Spores exposed to $^{14}CO_2$ in the following experiments were hydrated, and their germination synchronized according to methods described (14). Three 25-mg samples were used for each spore germination treatment. Zero time of germination is defined as the end of the cold treatment (4 C) period of the synchronization process. Attention must be given to the total length of time the spores were allowed to germinate. For example, in the 1.5-hr exposure of spores to $^{14}CO_2$, the spores exposed at zero time would have a total germination time of 1.5 hr; in the 15-min exposure time, a total germination period of 15 min. Thus, the length of exposure time must be added to the length of time the spores were germinated prior to exposure to $^{14}CO_2$.

The fixation of CO_2 in the dark was greatest during

the very early stages of germination (1 hr), and the amount fixed decreased rapidly during the first 2 hr of germination (Fig. 1). The amount of CO_2 fixed by the spores exposed to $^{14}CO_2$ for 15 min was similar to the results of the 1.5-hr exposure in that the curves obtained on the graph differ only in degree. The amount of CO_2 fixed was again greatest during the beginning of germination; however, the decrease in CO_2 fixed as germination time increased was not as striking as in the 1.5 hr exposure study. The effect of light on CO_2 fixation by germinating spores is given in Fig. 2. The results show the same pattern of CO_2 fixation as occurred in the dark; e.g., the greatest amount of CO_2 was fixed during the first 1.0 hr of germination.

Distribution of ^{14}C into various cellular fractions of germinating bean rust uredospores.—During extraction of RNA, four fractions were obtained: the supernatant fraction from which the RNA is precipitated, the RNA fraction, the phenol fraction, and that fraction containing cellular debris. We combined the percentage of radioactivity in the phenol fraction with that obtained from the residue, since the phenol contains protein that was denatured during extraction. This combined fraction is considered to represent the radiocarbon in the macromolecules other than RNA. The greatest amount of radiocarbon was found in the resi-
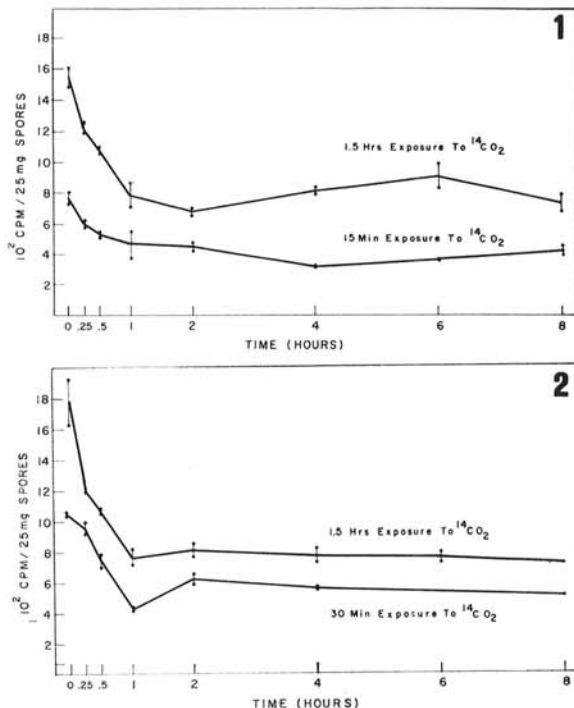

Fig. 1-2. **1)** Fixation of $^{14}CO_2$ in the dark by uredospores of *Uromyces phaseoli* during various stages of germination. The abscissa indicates the actual length of time the spores had been allowed to germinate prior to exposure to 50 μc of $^{14}CO_2$, actual concn about 0.04%. Each point indicates the average of four replicates, and each bar, one standard error of the mean. **2)** Fixation of $^{14}CO_2$ in the light by uredospores of *Uromyces phaseoli* during various stages of germination.

TABLE 1. Percentage of ^{14}C in various cellular fractions of bean rust uredospores, *Uromyces phaseoli*, exposed to $^{14}CO_2$ during germination

Germination time	^{14}C/fraction[a]		
	Supernatant[b]	RNA[c]	Residue[d]
hr	*%*	*%*	*%*
0.083	0.5	0.08	99.42
0.5	1.2	0.14	98.56
1.5	1.0	0.28	98.72
4.5	2.3	0.36	97.34
6	2.1	0.58	97.32
8	4.0	0.84	95.16

[a] Three fractions were obtained during phenol extraction of RNA.[b, c, d]
[b] Aqueous supernatant solution after RNA precipitation which contains soluble organic acids, amino acids, sugars, and nucleotides.
[c] RNA fraction representing total activity found in alkaline hydrolysates.
[d] Residue fraction considered to represent the ^{14}C incorporated into macromolecules of the spore other than RNA.

due (Table 1). Radioactivity in the residue was greater than 95% throughout an 8-hr germination period, as assayed at 5 min, 30 min, 1.5 hr, 4.5 hr, 6 hr, or 8 hr. Radioactivity in the supernatant fraction increased from 0.53% at 5 min to 4.0% at 8 hr after germination. The radioactivity in the RNA fraction, as assayed by nucleotide hydrolysate, was the lowest, increasing from 0.08%, 5 min after onset of germination, to 0.84% after 8 hr of germination.

To determine if any soluble materials were present in the residue, it was washed 3 times with 70% ethanol after the spores had been extracted twice with phenol for the isolation of RNA. No appreciable radioactivity was found in the combined ethanol washings. These results indicate that most of the soluble components of the spores, such as free nucleotides, amino acids, organic acids, and sugars would be in the supernatant fraction from which the RNA was precipitated. We, therefore, separated the supernatant solution into acidic, basic, and neutral fractions by use of cation- and anion-exchange chromatography. Assays of percentage radioactivity in each fraction are given in Table 2. The acidic fraction had the greatest amount of radioactivity (60%); the basic fraction had 34% and the neutral fraction 6% after the first 30 min to 8 hr after germination. Five min after initiation of germination, 72% of the radioac-

TABLE 2. Percentage of ^{14}C in acidic, basic, and neutral portions of the supernatant fraction obtained during the RNA extraction from bean rust uredospores exposed to $^{14}CO_2$ during germination

Germination time	^{14}C/fraction		
	Acidic	Basic	Neutral
hr	*%*	*%*	*%*
0.083	72	14	13
0.5	63	31	6
1.5	61	35	4
4.5	60	34	6
6	60	35	5
8	60	34	6

tivity was present in the acidic fractions, 14% in the basic fraction, and 13% in the neutral fraction.

Each of the three fractions was checked for absorbance at 260 nm and for the presence of nucleotides. The acidic fraction showed a distinct peak at 260 nm, whereas, the basic fraction absorbed ultraviolet radiation very slightly and the neutral fraction not at all. These results indicate that some of the radiocarbon in the acidic fraction could be present in nucleotides which would be eluted with the free acids.

DISCUSSION.—Substantial data is available in the literature concerning the dark fixation of CO_2 by various rust species present in the host tissue (10, 12, 15, 21). But except for the study of Staples & Weinstein (16), little information is available concerning the involvement of CO_2 fixation by rust uredospores apart from the host. The results of the present study demonstrate the ability of germinating uredospores of *Uromyces phaseoli* to fix CO_2 in the dark, and that the CO_2 is incorporated into both the macromolecular components of the spore, as well as the low mol-wt soluble spore components.

Gottlieb (5), in reviewing the data on CO_2 fixation by germinating fungus spores, indicated that although CO_2 fixation is common in germinating spores, the exact role of CO_2 in the metabolism of the spore is not known. The most specific information concerning CO_2 fixation by fungus spores is presented by Yanagita (20). He found that CO_2 was a necessary requirement for spore germination of *Aspergillus* sp. and is incorporated immediately into macromolecules, particularly the RNA fraction, at the onset of germination. The most striking result of our experiments is that the rate of fixation decreased rapidly soon after the initiation of germination.

The amounts of CO_2 fixed by spores which had been germinating for various time lengths are similar to the metabolic activities of germinating spores which were obtained by other investigators. Farkas & Ledingham (4) and Williams & Allen (18) found that the metabolic activity of *Puccinia graminis* f. sp. *tritici* uredospores was highest during the initial stages of germination. These investigators utilized manometric gas exchange techniques involving CO_2 evolution and O_2 uptake. Data from both investigations show that O_2 uptake and CO_2 evolution were highest during the first 3 hr after the onset of germination, and that afterward, gas exchange was minimal and constant. Bush (2) observed similar results of high O_2 consumption during the initial 50 min of germination of wheat stem rust uredospores. Oxygen consumption was determined polarographically with a biological oxygen monitor. Plots of O_2 consumption show that the highest rates of O_2 uptake occurred at the onset of germination, and dropped rapidly as germination progresses. Wynn & Gajdusek (19) show a similar plot of exogenous substrate uptake by germinating spores of bean rust uredospores. Their results show that the incorporation of either mannitol-1-^{14}C or glucose-1-^{14}C into the glucomannan-protein fraction was greatest during the initial stages of germination (2 hr), and decreased rapidly afterwards.

The pattern of carbon dioxide fixation was the same in the light as in the dark. In both treatments, fixation was greatest during the initial stages of germination, and then decreased rapidly after the initial 1.5 hr of exposure to $^{14}CO_2$.

Studies on the distribution of ^{14}C in various spore fractions showed that more than 95% of the radioactivity was found in the residue. Since Wynn & Gajdusek (19) reported a high rate of glucomannan-protein synthesis upon initiation of germination, it is possible that much of the CO_2 taken up may be incorporated into this fraction (i.e., during cell wall synthesis). An appreciable amount of ^{14}C was also present in the RNA fraction of the spore. Although the total percentage of radioactivity present in the RNA fraction was small, the percentage of RNA compared to the total spore constituents in the spore also was small.

Results from the separation of the low mol wt and soluble spore components show that the acidic fraction was highest in radioactivity and the neutral fraction was the lowest. The acidic fraction, which is considered to be made up predominantly of free organic acids, also contained free nucleotides, and at least a portion of the radioactivity present in the acidic fraction may have been in these nucleotides. It appears evident that germinating bean rust uredospores do use CO_2 as an exogenous substrate, but the total significance of the CO_2 fixed in relation to the nutritional requirements of the spores is not clear.

Wynn & Gajdusek (19) thought that perhaps the spore has some regulatory system which is responsible for buildup of a short-term reserve of polysaccharide rapidly synthesized upon initiation of germination and then utilized during the first 2 hr of germination after initiation of germination. Daly et al. (3) indicated that the most conspicuous event during germination is the rapid deposition of germ tube cell walls. Thus, the large amount of $^{14}CO_2$ incorporated into the residue fraction in the study may reflect the importance of the requirement for polysaccharide synthesis upon initiation of germination as described by them (19).

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