Atmospheric Transfer of Carbon-14: A Problem in Fungus Translocation Studies

Abstract. *The carbon-14-labeled carbon dioxide that is released by respiration after glucose labeled with carbon-14 is applied to fungal mycelium can be reabsorbed in highly significant amounts by distant mycelium and agar media in the same petri dishes. Atmospheric transfer of carbon-14 must be considered when using labeled organic compounds to study translocation in fungi.*

It has been well established that various substances are translocated in mycelium (*1*), although the necessary conditions are not always clear. In studying ectotrophic mycorrhizal fungi of *Pinus taeda* L., it was of interest to determine whether substances could be translocated by mycelium in pure culture. In preliminary investigations it became apparent that when glucose-C^{14} was applied to fungus mycelium, $C^{14}O_2$ was readily evolved and was probably reabsorbed by distant portions of mycelium. Studies in which $C^{14}O_2$ was used indicate that CO_2 can enter by fixation into citric acid, pyruvic acid, lactate, oxalic acid, succinic acid, or other cell constituents, depending on the particular fungus (*2*). Lewis and Weinhouse (*3*) demonstrated that mycelial mats of *Aspergillus niger* suspended in aqueous solution with $NaHC^{14}O_3$ could assimilate labeled CO_2 into citrate and oxalate. Various other techniques have been used to demonstrate the translocation of organic and inorganic substances in fungi (*4*), but reports of the use of compounds labeled with C^{14} are few. The use of sucrose-C^{14} and glucose-C^{14} in techniques similar to those of Schutte (*4*) has been reported by Thrower and Thrower (*5*).

Before attempting to demonstrate the translocation of C^{14} in fungus mycelium, it was necessary to determine (i) how much $C^{14}O_2$ is evolved by respiration, (ii) to what extent it is reabsorbed by either fungus or substrate, and (iii) what precautions should be taken to prevent reabsorption of $C^{14}O_2$ when rates and quantities of C^{14} translocation are being studied.

In the experiments reported here, isolates of *Pisolithus tinctorius* (Pers.) Coker and Couch and *Thelephora terrestris* (Ehrh.) Fr. were grown on Hagem's agar medium (*6*), either in standard 100-mm petri plates or 90-mm divided plates. The age of each culture is indicated in the results (Table 1).

Fungus cultures were inoculated with D-glucose uniformly labeled with C^{14} in 14 percent ethanol. The labeled glucose had a specific activity of 150 mc/mmole and was introduced to surfaces of mycelial mats by applying 10 μc (22,200,000 counts per minute) in a droplet of 10 μl. The addition was made midway between the center of the colony and the growing edge. Cultures (without lids) were maintained in individual airtight bell jars. Experiments were conducted under normal room illumination during the day at approximately 25°C.

To determine the content of C^{14}, samples of agar and agar plus mycelium were obtained from tested cultures by removing a cylindrical 4-mm core from the medium by means of individual disposable pipettes with rubber bulbs. Samples of mycelium plus agar were taken approximately 1.5 cm from the distal growing edge; agar samples were taken at the point most distant from the mycelial mat. Partitioning of agar and mycelium was not attempted. Each sample was placed in a 20-ml scintillation vial together with 1 ml of methyl alcohol and was crushed with a glass rod. To this was added 9 ml of a dioxane scintillation solution for liquid scintillation counting (*7*).

The $C^{14}O_2$ released by respiration was trapped in vials containing 0.5 ml of 5*N* NaOH; the NaOH was neutralized with $HClO_4$, and then 9 ml of dioxane solution was added. All samples were counted at −1°C in a Packard liquid scintillation counter. Results are expressed in activity as counts per minute per sample after correction for background and counting efficiency.

To determine the release of $C^{14}O_2$ (experiment 1), a single fungus culture and one vial with 0.5 ml of NaOH were placed in each airtight bell jar, and 10 μc of glucose-C^{14} was added to the mycelium of the culture. After 2, 26, 50, and 74 hours, the vial of NaOH was replaced by a new vial. Counting each vial gave an indication of the $C^{14}O_2$ trapped during the particular time interval. Count data were averaged for each age group and plotted as a function of time (Fig. 1).

To determine the reabsorption of $C^{14}O_2$ (experiment 2), two physically separated fungus cultures were placed in each airtight bell jar. Two cultures, either separated by a partition in a petri dish or grown in separate petri dishes, were used in each trial. One culture was inoculated with 10 μc of glucose-C^{14} and the other was sampled for C^{14}. (The only means of exchange of C^{14} was atmospheric transfer.) Samples of agar plus mycelium and agar alone were removed at various intervals and were counted (Table 1).

In experiment 1, large quantities of $C^{14}O_2$ were released by the cultures within 24 hours (Fig. 1). The activity recovered in individual samples of NaOH during the first 2-hour interval ranged from zero activity to 155,000 count/min. By the end of the third 24-hour sampling interval, the rate of loss of $C^{14}O_2$, for most cultures, had begun either to level off or to decline. The percentage of total activity recovered during the 74 hours ranged from a low of 0.57 percent for a replicate of *P. tinctorius* to a high of 4.46 percent for a replicate of *T. terrestris*.

The values we obtained tend to be conservative, since at least some of the $C^{14}O_2$ was reabsorbed by mycelium and

Table 1. Relative content of C^{14} in mycelium plus agar and agar alone as a result of reabsorption of $C^{14}O_2$ from atmosphere. The 4-mm sample cores were removed at specified time intervals.

Sample No.	Age of culture (weeks)	Type of sample	Activity (count/min) after following number of hours				
			2	24	48	72	96
		Pisolithus tinctorius					
1	13	Mycelium + agar	0	3	19		44
2	2	Mycelium + agar		967	583	504	
3	2	Agar only		228	34	0	
4	2	Mycelium + agar		572	563	342	
5	2	Agar only		88	50	24	
		Thelephora terrestris					
6	13	Mycelium + agar	1	21	28		19
7	5	Mycelium + agar	7	34	64		29

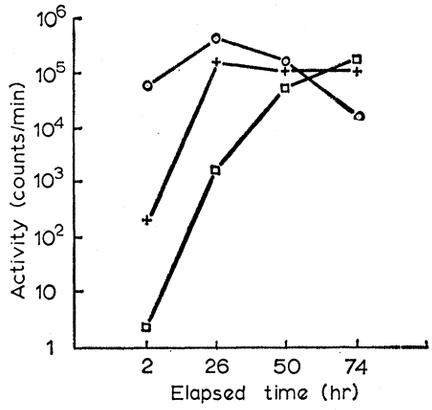

Fig. 1. Release of $C^{14}O_2$ by respiration at various periods after introduction of C^{14}-labeled glucose to mycelium. Carbon-14 activity is expressed as counts per minute per 0.5-ml sample of NaOH. Curves: +—+, average of four replicates of 11-week-old cultures of *P. tinctorius;* □—□, average of two replicates of 5½-week-old cultures of *P. tinctorius*; and ○—○, average of two replicates of 13-week-old cultures of *T. terrestris.*

agar and a small amount was probably lost upon neutralization.

The results of experiment 2 (Table 1) indicate highly significant amounts of activity in samples of all the cultures we tested. In cultures of *P. tinctorius*, samples of agar also showed the presence of C^{14}, although considerably less than in samples of mycelium plus agar. This may have been the result of diffusion of C^{14} in the agar after the C^{14} was absorbed by mycelium, but, more probably, it was a result of direct absorption of $C^{14}O_2$. The peak of activity in the 24- to 48-hour period and the decline with time was unexpected, since samples represent accumulative absorption of $C^{14}O_2$ up to the time of removal. This may have been caused by an initial concentration of C^{14} at the mycelium surface followed by an equilibrating diffusion throughout the substrate, but it would not explain the decrease in activity of the agar samples.

Representative cultures, other than those actually sampled in the experiments, were used to obtain an approximation of the weights of fungi in sample cores. In seven equivalent samples of *P. tinctorius*, the average dry weight was 1 mg. The average dry weight of *T. terrestris* was 2 mg.

In summary, (i) $C^{14}O_2$ was released in large quantities by respiration, and (ii) $C^{14}O_2$ was reabsorbed in some manner by the fungus cultures. Errors in translocation studies due to absorption of $C^{14}O_2$ can be minimized by using both a trap of NaOH and a directional flow of fresh air to remove atmospheric contamination.

Because of the interrelation between the mycelium and the agar medium, it was not possible to conclude definitely that the site of $C^{14}O_2$ reabsorption was fungal hyphae. The $C^{14}O_2$ could also be absorbed first by the agar medium and then diffuse into the mycelium.

Regardless of the exact site of absorption, the ultimate incorporation of C^{14} into the fungus can make the results of translocation studies misleading and should be taken into account. Although the data presented here result from the use of D-glucose-C^{14}, the use of other organic compounds labeled with C^{14} could produce similar problems.

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References and Notes

1. E. P. Hill, in *The Fungi*, G. C. Ainsworth and A. S. Sussman, Eds. (Academic Press, New York, 1965), vol. 1, pp. 457–463.
2. V. W. Cochrane, *Physiology of Fungi* (Wiley, New York, 1958), p. 228.
3. K. F. Lewis and S. Weinhouse, *J. Amer. Chem. Soc.* **73**, 2906 (1951).
4. K. H. Schutte, *New Phytol.* **55**, 164 (1956).
5. S. L. Thrower and L. B. Thrower, *Nature* **190**, 823 (1961).
6. O. Modess, *Symbolae Bot. Upsal.* **5**, 1 (1941).
7. F. N. Hayes, *Tech. Bull. No. 1* (1963), Packard Instrument Co.
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Collagen-Like Fragments: Excretion in Urine of Patients with Paget's Disease of Bone

Abstract. *Patients with Paget's disease of bone excrete, in the urine, polypeptides that have amino acid composition and other properties resembling those of fragments of collagen. The pattern of isotope incorporation in vivo suggests that these fragments are derived from collagen that has been synthesized and rapidly degraded, or that they are rapidly synthesized but not incorporated into tropocollagen molecules.*

The characteristic manifestation of Paget's disease is an increased rate of remodeling of bone accompanied by distortion of its architecture. Patients with this disease excrete in the urine large amounts of peptide-bound hydroxyproline which is thought to reflect the accelerated turnover of bone collagen (*1, 2*). In an attempt to understand the pathogenesis of this disease, we have isolated from the urine relatively large, collagen-related polypeptides and are studying their origin and manner of production.

Eight adult subjects with active Paget's disease of bone were maintained on gelatin-free diets; urine samples were collected and kept in the cold under toluene. The total daily excretion of hydroxyproline (*3*) in these patients ranged from 123 to 980 mg (normal adults excrete less than 40 mg per 24 hours). After exhaustive dialysis of the urine against large volumes of 0.15*M* NaCl followed by water, the nondialyzable material, amounting to approximately 10 percent of the total hydroxyproline, was lyophilized. It was then subjected to gel filtration on columns of Sephadex G-75 (125 by 3 cm), equilibrated with 5*M* LiCl and 0.01*M* tris, *p*H 7.4 (Fig. 1); a single hydroxyproline-containing zone was observed. This material was dialyzed against water, lyophilized, and then passed through smaller columns of Sephadex G-75 (110 by 1 cm) in pyridine–acetic acid buffer, *p*H 4.5. The single protein-containing peak coincided with the peak containing hydroxyproline. This same fraction, subjected to gel filtration on columns of Bio-Gel P-6 (73 by 2.5 cm), emerged with the void volume. This material was not retained by carboxymethyl cellulose at *p*H 4.8 and an ionic strength of 0.06 (*4*), and therefore was subjected to anion-exchange chromatography on columns of diethylaminoethyl- (DEAE) cellulose equilibrated with 0.05*M* tris, *p*H 8.2, with the use of a linear gradient of NaCl (Fig. 2.) The hydroxyproline-containing material was fractionated into two to four major components, each of which was then desalted by filtration on columns of Bio-Gel P-6.

Amino acid analyses (*5*) of material from comparable peaks obtained by DEAE-cellulose chromatography have thus far been performed on samples from four of the eight patients (Table 1). Amino acid composition of the urinary peptides resembled the composition of mammalian collagens in general and are shown in comparison with collagen from human bone (*6*). Ultraviolet absorption spectra revealed low absorbancy at 280 mμ compared with that at 230 mμ, which is consistent with a low content of tyrosine and tryptophan, a characteristic feature of mammalian collagens and gelatins. A 5-mg portion of one of the polypep-