

Changes in Exogenous Palmitic Acid Utilization and Lipid Composition in Aging Cultures of *Penicillium chrysogenum*

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Introduction

Growth characteristics of submerged cultures of *Penicillium chrysogenum* have been related to a predictable change in pH of the culture medium (S. Zoghbi, Master's Thesis, Ball State University, 1975). When grown at an initial pH of 7.4, cultures remained at this pH for approximately 20-40 hours, depending on culture conditions. Then during the next 9 hours, the pH dropped to 3.4-3.8 where it stabilized. The greatest increase in dry weight (growth) of cells occurred during the nine hours of the pH drop after which time 60% of the initial energy source (glucose) still remained in the medium. This indicated that the cultures were not nutritionally depleted at that time, rather it was demonstrated that glucose was not depleted until approximately 20-30 hours later. With these criteria as bases, young and old cultures were defined in this study as those harvested before and after the decline in pH.

This research reports upon the morphological characteristics and the lipid composition of both young and aging cultures. Differences in incorporation and utilization of radioactively labeled palmitic acid by young and aging cultures were studied using biochemical and autoradiographical techniques.

Methods and Materials

Stock cultures of *Penicillium chrysogenum*, Q 176, were maintained on potato-dextrose-agar slants and stored at 4 C. Spores were scraped and washed from slants with 10 ml sterile medium, transferred to cuvettes, adjusted to 30% transmittance at 520 nm, added to 40 ml sterile medium of modified Czapek formula (1) and incubated at 28 C for 16 hr on a reciprocating shaker (180 oscillations/min). This starter culture was added to 450 ml of medium in a 2800 ml Fernbach flask and incubated as above. The pH of the submerged cultures was monitored at 2 to 3 hr intervals until the pH declined and stabilized. Cells were observed by phase microscopy at similar intervals. Young (pH 7.2, 20 hr) and aging (pH 3.4, 40 hr) cultures were labeled with 2 μ Ci of 1- 14 C-palmitic acid which had been made soluble in 1% albumin in 30% ethanol. After 2, 10, 60, and 120 min, 20 ml samples of the cultures were removed, filtered through Miracloth, and washed with distilled water. A small portion of mycelium from each was placed in 3% gluteraldehyde and fixed for electron microscopy. The remainder of the harvested mycelium was placed in 10 ml of methanol and used in lipid analysis.

Lipid Analysis

Mycelial samples were sonified for two minutes by a series of 20 sec pulses

(setting 6, Branson Sonifier, Model J-17A, Heat Systems). Lipids from the ruptured cells were extracted with chloroform/methanol according to a modified Folch procedure (2). Total lipids were separated into lipid classes by thin layer chromatography on plates coated with silica gel G impregnated with 0.02% Rhodamine G using n-hexane/ether/glacial acetic acid/absolute methanol (90:2:2:3, by vol) followed by n-hexane/ether (94:6, by vol) (3). Bands were scraped into scintillation vials which contained 2,5-diphenyloxazole-1,4-bis (2-(5-phenyloxazolyl)-benzene (PPO-POPOP) in toluene and radioactivity was determined in a Beckman ambient temperature liquid scintillation system (LS150, Beckman Instruments, Inc.). To separate phospholipids, TLC plates (Adsorbosil-5 prekotes; Applied Science Div., Milton Roy Co.) were prewashed in n-hexane/ether (94:6 by vol) and dried. Total lipids were then applied and neutral lipids separated by Solvent System I, n-hexane/ether (94:6 by vol) followed by Solvent System II, chloroform/methanol/n-propanol/0.25% KCl/ethyl acetate (25:15:25:9:25 by vol) to separate the phospholipids (9). For visualization, plates were sprayed with Phospray (Supelco, Inc.). Separations were identified by comparison with standards scraped from plates and radioactivity determined.

Electron Microscope Autoradiography

Mycelia were fixed in 3% gluteraldehyde at 4 C for 2 hr, washed in buffer, post-fixed in 1% osmium tetroxide, and dehydrated for 5 min or less in each cold alcohol solution. By omitting a propylene oxide dehydration step the amount of incorporated label extracted by solvents was held to less than 7% (7). Mycelia were embedded in Epon 812 (Polyscience, Inc.) according to the procedure of Luft (5).

Ultra-thin sections (0.05 μm , silver or gold interference color) were cut with glass knives using 1KB Ultratome (type 4801A, Ultramicrotome) and positioned on celloidin-coated slides according to a modified procedure of Kopriwa (4). Sections were stained with 1-2.5% aqueous uranyl acetate and Reynold's lead citrate, and carbon coated (5-10 nm). Slides were coated with Ilford L-4 nuclear tracking emulsion (Ilford Ltd., Ilford, Essex, England) and stored in light-sealed black boxes to await exposure. Following exposure they were developed in Kodak Formula D-170 (Eastman Kodak, Rochester, NY), fixed in sodium thiosulfate fixer and washed in running water. Within 15 min after developing, the celloidin film was floated free on a large tank of dust-free distilled water, 200 mesh EM grids were positioned over the sections on the floating film and the support film picked up on the ball of an acetone-cleaned evaporative flask and air dried. Grids were cut free, examined, and photographed using a RCA EMU-3C electron microscope.

Results and Discussion

Mycelia removed from rapidly growing submerged cultures showed changes in cell morphology with increased age (Figure 1). Young hyphae (harvested at 16 hr, pH 7.2) were slender with dense cytoplasm and infrequent end walls. This appearance contrasted sharply with the stubby thick-walled, highly vacuolar and granular appearance of aging cells (30 hr, pH 3.8). These changes do not appear to be caused by depletion of the energy source since most of the initial glucose remained in the medium at that time. Instead, observations suggest a change in utilization of products synthesized by the cell.

Thin layer chromatograms of extracted lipids indicated negligible amounts of triglycerides in young cultures whereas triglycerides were found in older cultures (Figure 2). There appeared, also, to be more diglycerides in young cultures.



FIGURE 1. Phase micrographs of mycelia of *P. chrysogenum* after (A) 16 hr. (pH 7.0) and (B) 40 hr. (pH 3.8) of growth. Magnification = X560

However, the distribution of lipids does not reflect the metabolic activity of the cells as evidenced by the results of incorporation of labeled palmitic acid into young and older cultures.

Both young and old cultures incorporated large amounts of label (Table 1). Young cultures converted palmitic acid rapidly (within 2 min) into phospholipids.

TABLE 1. Incorporation of Labeled Palmitic Acid Into Lipid Classes^a

Time ^b	Percent label ^c incorporated into:				
	PL	DG	FA	TG	CE
2 min					
Young ^d (2)	59.0 ± 4.8	9.9 ± 2.1	13.1 ± 1.7	11.9 ± 0.2	5.8 ± 0.7
Aging (5)	15.5 ± 2.7	6.1 ± 0.9	71.9 ± 1.3	5.0 ± 1.5	1.3 ± 0.6
10 min					
Young (2)	55.4 ± 0	8.7 ± 0.5	14.8 ± 2.6	14.7 ± 3.4	6.3 ± 0.3
Aging (5)	23.8 ± 1.5	6.6 ± 0.8	63.5 ± 3.0	4.0 ± 0.5	1.9 ± 0.6
60 min					
Young (2)	72.5 ± 1.2	4.0 ± 0.0	10.6 ± 3.3	10.9 ± 3.3	2.0 ± 0
Aging (5)	23.0 ± 4.4	5.7 ± 0.9	68.0 ± 6.0	4.2 ± 0.7	1.3 ± 0.8
120 min					
Young (2)	80.2 ± 0.4	3.1 ± 0.1	14.7 ± 0.3	1.7 ± 0.5	1.0 ± 0.3
Aging (5)	24.8 ± 6.5	5.7 ± 2.3	65.0 ± 7.8	3.3 ± 0.3	1.0 ± 0.3

^aThe value represents the mean of the number of experiments indicated in parentheses + S.E. Results are expressed in percent of label incorporated into lipid classes at the times indicated. Of the 218.4 nmol (2μCi) of label in the form of 1-(¹⁴C)-palmitic acid added to each culture, young cultures incorporated 21.84 nmol after 120 min; aging cultures incorporated approximately 2 times this amount after 120 min incubation. Values for young cultures represent the mean of 2 samples, for aging cultures the mean of 5 samples. ^bTime after addition of label. ^cFrom 900 to 85,000 cpm recovered from individual cultures. ^dYoung harvested at pH 7.2; aging, pH 3.5, PL—phospholipids, DG—diglycerides, FA—free fatty acids, TG—triglycerides, CE—sterol esters.

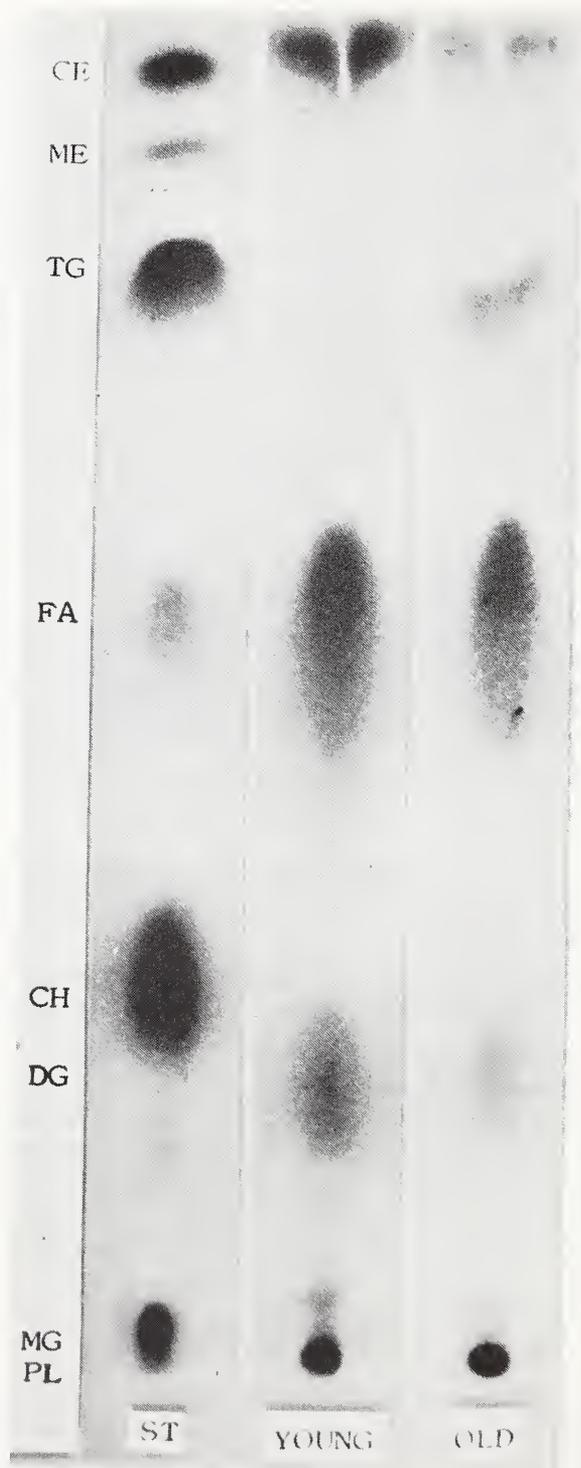


FIGURE 2. *Distribution of Lipids from Young and Old Cultures of P. chrysogenum.* Spots were visualized by spraying with conc. H_2SO_4 and heating for 10 min. in an oven at $100^\circ C$.

Standards top to bottom:

Ce—cholesterol (sterol) esters, ME—methylesters, TG—triglycerides, FA—free fatty acids, CH—cholesterol (sterols), DG—diglycerides, MG—monoglycerides, PL—phospholipids

Largest amount of label was recovered in the phospholipid fraction at all time points; less than 15% of the label remained in the free fatty acid fraction in young cultures. The conversion of the palmitic acid was slow in old cultures and less was converted into phospholipids. In aging cultures 30% of the labeled fatty acid was incorporated into complex lipids. Approximately 65% of the label remained in the free fatty acid fraction.

Incorporation of labeled palmitic acid into diglycerides, triglycerides, and

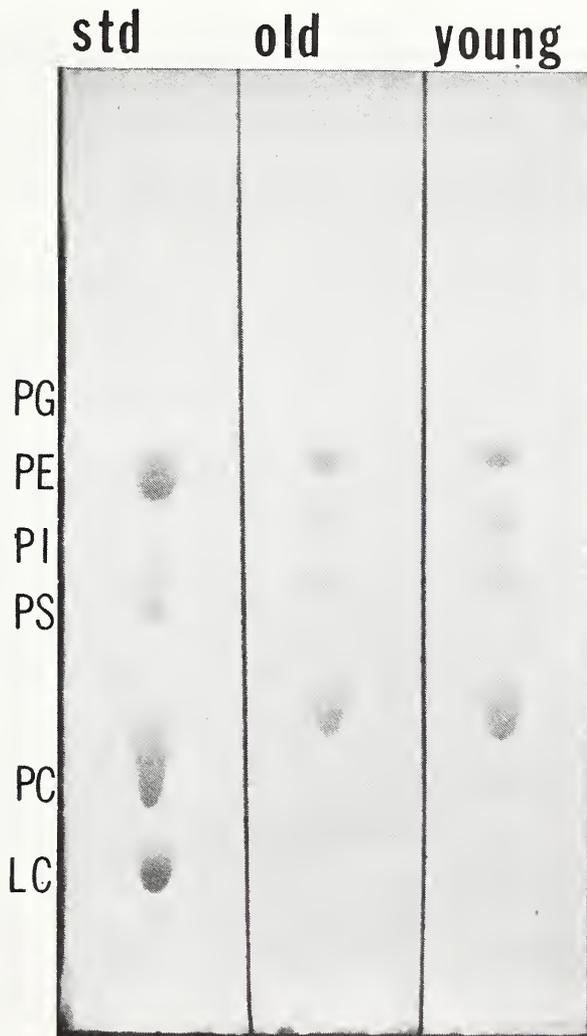


FIGURE 3. Phospholipids of *Penicillium chrysogenum*. TLC plates were prewashed in *n*-hexane/ether (94:6 by vol) and placed in 100° C drying oven for 30 minutes. Total lipids were applied and neutral lipids separated by Solvent System I, *n*-hexane/ether (94:6 by vol) followed by Solvent System II, chloroform/methanol/*n*-propanol/0.25% KCl/ethyl acetate (25:15:25:9:25 by vol) to separate phospholipids. Visualization was by acid molybdate.

Standards: LC—lysophosphatidyl choline
 PC—phosphatidyl choline
 PS—phosphatidyl serine
 PI—phosphatidyl inositol
 PE—phosphatidyl ethanolamine

The spot above PE was identified in other separations to be phosphatidyl glycerol—PG.

sterols was similar in both culture conditions. Diglycerides, triglycerides, and sterols incorporated some label at early time points, but the amount decreased with time suggesting that these substances were not being stored as such in the cells but were being depleted as they were incorporated steadily into synthesizing membranes.

Phospholipid types have not been previously described for *Penicillium chrysogenum*. Five types were identified: phosphatidyl serine, phosphatidyl inositol, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl glycerol (Fig. 3). Small amounts of cardiolipin were also detected. These findings are consistent with analysis of *Cephalosporium* (8) and for a wide variety of fungi (10). Types of phospholipids found in young and aging cultures are similar, however distribution of ^{14}C -label in phospholipids differed (Fig. 4). The phosphatidyl serine component incorporated most label in aging cultures, while young cultures incorporated the most label in phosphatidyl choline. Aging cultures incorporated about

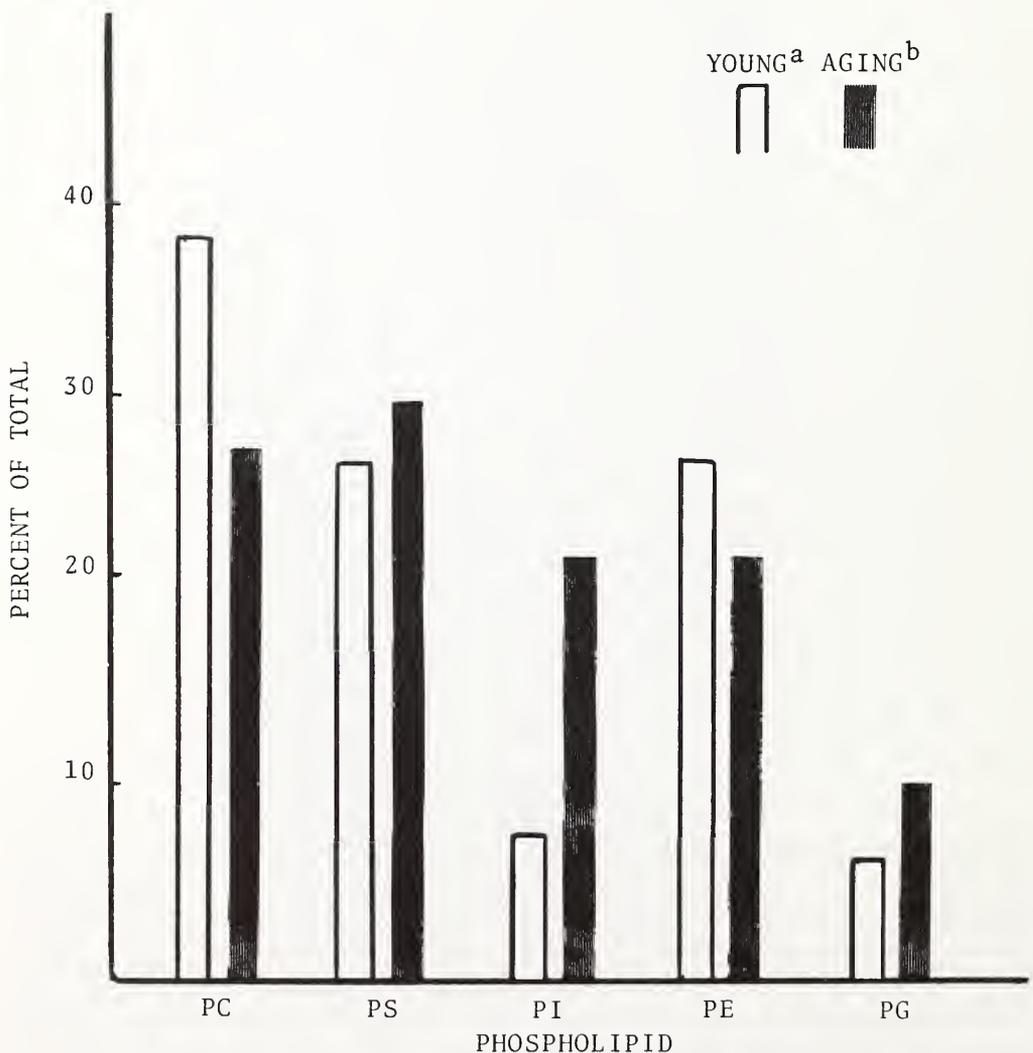


FIGURE 4. Distribution of label in phospholipids of young cultures and aging cultures of *P. chrysogenum* incubated for 30 minutes with 1- ^{14}C -palmitic acid. ^aAverage of two samples, ^baverage of five samples. PC-phosphatidyl choline, PS-phosphatidyl serine, PI-phosphatidyl inositol, PE-phosphatidyl ethanolamine, PG-phosphatidyl glycerol.

TABLE 2. Localization of 1-(¹⁴C)-Palmitic Acid in Electron Microscope Autoradiographs of *P. Chrysogenum*

Location of grains	Present ^a	
	Young cells	Aging cells
Plasma membrane	69	47
Nuclear membrane	3	1
Cytoplasm (general)	26	26
Spherosomes	1	11
Mitochondria	1	15

^aNumber given is the percent of total grains counted; for young cells, from a composite tally of 12 micrographs, for aging cells, a composite tally from 24 micrographs.

20% of label in phosphatidyl inositol, young cultures averaged less than 10% of the label in this fraction. The amount of label in phosphatidyl glycerol varied considerably in samples, with up to 10% seen in many old cultures, but with only 1-2% recovered from young cultures.

High resolution autoradiographs of cells incubated with 1-(¹⁴C)-palmitic acid support the biochemical evidence pertaining to the localization of label. In young cells approximately 70% of the developed grains were found in close association with plasma membranes while in aging cells no more than 47% of the developed grains were over the plasma membranes (Table II, Figure 5). In both young and

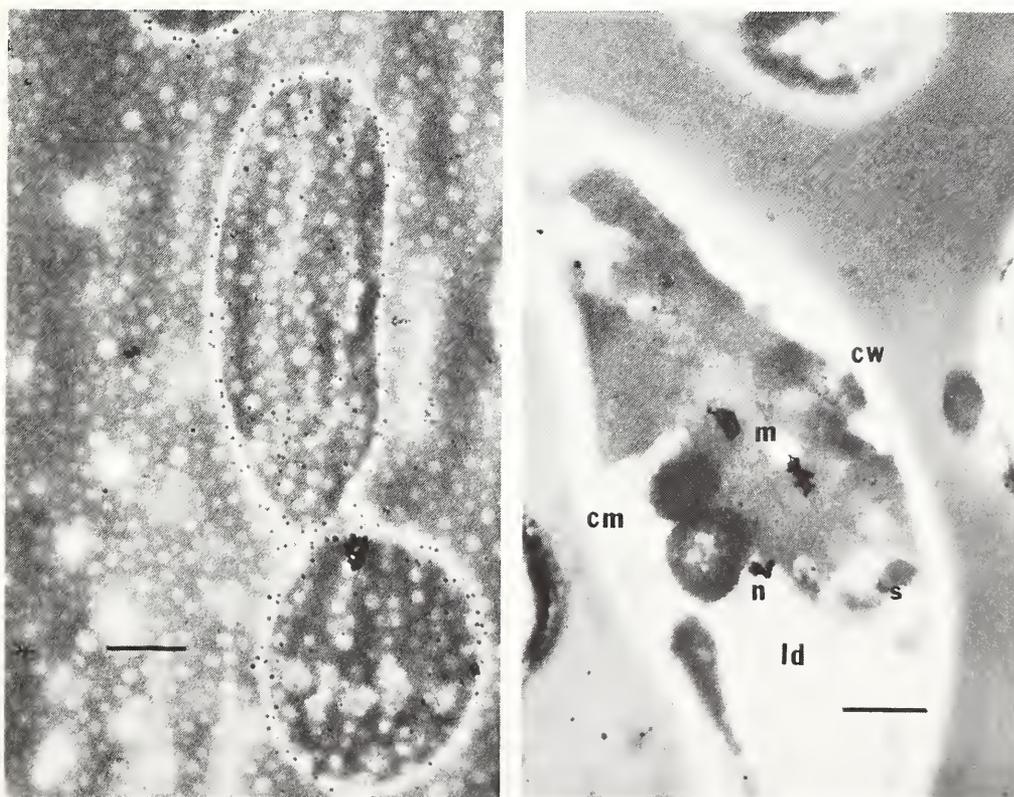


FIGURE 5. Electron microscope autoradiographs of *P. chrysogenum*. Young cells (A) were incubated for two minutes and aging cells (B) were labeled for 60 minutes with 1-(¹⁴C)-palmitic acid. Small punctate grains show generalized distribution of incorporated labels in areas of membranes in young cells. In older cells, grains were highly concentrated in localized lipid deposits.

cm—cell membrane, cw—cell wall, ld—lipid depot, m—mitochondrion, n—nucleus, s—spherosome. Bar = μm

old cells, 26% of the developed grains appeared throughout the cytoplasm. Spherosomes, cell organelles identified as "lipid bodies", but of uncertain function in fungi (11,6) were evident in all cells. However, since only 1% of developed grains was found in spherosomes in young cells, with approximately 10% in aging cells, it appears that spherosomes are not involved in lipid utilization in *Penicillium*.

These results indicate a relationship between age of the culture and utilization of an exogenous source of fatty acid. Old cultures exhibited a decreased ability to incorporate the added palmitic acid into phospholipids. The types of lipids present for synthesis of structural components play a significant role in forming the morphological characteristics of cells. Thus changes in amounts and types of lipids would be reflected in altered cell morphology. Therefore, the age of the culture should be carefully considered in studies involving morphology and lipid metabolism in organisms such as *Penicillium chrysogenum*.

Acknowledgments

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