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Uptake of Glucose-1-^{14}C by *Pullularia pullulans*

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Pullularia pullulans cells were grown in a medium containing yeast extract, malt extract, glucose, and nutrient salts, in addition to glucose-1-^{14}C. The lipids extracted from the cells were fractionated by use of a single column packed with silicic acid. Of the total radioactive carbon added to the culture medium, the neutral lipid fractions contained 24.8%, whereas the phospholipid portions contained only 2.1%. The largest amount (16.5%) of ^{14}C among the neutral lipids was found in the fraction containing the free sterols. Among the phospholipids, the largest amount (1.1%) was found in phosphatidylserine and phosphatidylethanolamine. The second largest amount (9.6%) of the total ^{14}C used was found in trehalose, followed by carbon dioxide (7.3%).

The past decade has witnessed an increasing amount of attention to the study of the yeastlike fungus *Pullularia pullulans* (2, 4, 9, 10). *P. pullulans* is widely distributed in nature (1, 5) and is important industrially because it is a causative agent of the deterioration of paint and of the discoloration of lumber, and is injurious to plants and plant products. It has been isolated from the lymph nodes of patients with Hodgkin's granuloma (16) and from the inflamed joints of patients with rheumatoid arthritis (Arthritis and Rheumatism Council, London, 1966). In previous work from this laboratory, methods were reported for the isolation of trehalose (13), pigment (11), and lipids (14) from cell extracts of *P. pullulans* strain NRRL YB-4515.

As little is known about the metabolism of *P. pullulans*, aside from the above, the present investigation was undertaken to label one or more metabolic products which might be concerned with the biosynthesis of the black pigment, which is of great industrial importance. Since it had been established that the black pigment produced by *P. pullulans* is not melanin (11), the study was directed toward the lipids and trehalose. Glucose-1-^{14}C was introduced into the medium to obtain better quantitation and to facilitate determination of isotope turnover in the pertinent fractions. Special attention was directed toward pigment synthesis and changes in trehalose content.

MATERIALS AND METHODS

The reagents were of high purity or reagent grade, and all solvents were redistilled prior to use. Glucose-1-^{14}C was purchased from Volk Radiochemical Co., Burbank, Calif. Except for a few modifications, the procedures were identical to those described by Merdinger and Kohn (13) and Merdinger et al. (14).

Culture medium. It was ascertained that the components used for the preparation of the culture medium did not contain lipids. Three transfers of *P. pullulans* were made successively into culture medium in amounts of 20, 100, and 1,300 ml. To the last, 10 ml of a solution containing approximately 22.5 μc of glucose-1-^{14}C (5,281,500 counts/min) was added, and the mixture was shaken in a reciprocal shaker for 12 days at 28 C. This period of shaking was necessary to insure maximal formation of black pigment.

A rubber-capped glass tubing which entered each flask allowed the withdrawal by needle and syringe of samples for daily cell counts by hemocytometer, for testing contamination, and for microscopic examination of pigment formation inside the cells of *P. pullulans*. After 12 days, the cells were separated by centrifugation, and the supernatant fluids were examined for extracellular lipids by the methods of Deinema (7) and Ruinen (15). The cells were washed several times with 0.90% sodium chloride and then with distilled water until they were free from chloride; they were then dried in an oven at 50 C and finely ground in a mortar.

Extraction of lipids from cells. The dry cells obtained from three batches, of which only one contained glucose-1-^{14}C, weighed 9.3, 8.7, and 9.1 g, and were extracted by various solvents specified in previous reports (13, 14). Trehalose was not removed from the cells by chloroform-methanol but was exhaustively extracted by prolonged shaking with 95% ethyl alcohol at 28 C.

Extraction of pigment from cells. Following the removal of trehalose, the defatted cells were subjected to successive Soxhlet extractions with mixtures of ethyl alcohol-water in ratios of 3:1, 1:1, and 1:3 (v/v), and finally with distilled water for periods of 48, 72, 96, and 120 hr, respectively. Each extract was centrifuged at 17,000 rev/min, and the supernatant fluid was passed through a fine-porosity fritted-glass filter to remove any residual solids. The amber-colored liquid was then dialyzed in cellophane for 3

to 4 days against running tap water, during which time the pigment usually separated. The pigment was removed by centrifugation at 3,000 rev/min, leaving a light-colored supernatant fluid. Further pigment was extracted until the supernatant portion was essentially colorless. The pigment was dried at 30 C.

Separation of lipids. The separation of the various lipid fractions was accomplished by a combination of the methods of Hanahan et al. (8) and Böttcher et al. (3). A total of 156 fractions were collected. The first 120 fractions contained 3 ml each, and the remainder, 5 ml each. The eluant was changed when three or four successive fluid fractions yielded no lipid. The 156 fractions were further combined on the basis of viscosity and color into 11 pooled fractions.

Determination of radioactivity. Analyses were carried out on samples with the toluene-PPO-POPOP (PPO: 2,5 diphenyl-oxazole; POPOP: 2,2'-dimethyl-*p*-phenylene-bis-5-phenyloxazole) solvent system in a Packard Tri-Carb scintillation spectrometer, model 3324. The scintillation solution, prepared by mixing 1 liter of toluene, 3 g of PPO, and 100 mg of POPOP, was added in the amount of 15 ml to each vial.

RESULTS AND DISCUSSION

The lipids subjected to column fractionation weighed 828 mg, and the overall recovery in the 156 fractions was 818 mg (99%). The lipids fractionated with the silicic acid column consisted of 63% neutral lipids and 37% polar lipids. These values are in good agreement with earlier findings with *P. pullulans* (14) and *Saccharomyces cerevisiae* (8). Repeated preliminary runs established that differences in the amounts of various lipid constituents were due mainly to the length of time allowed for growth and to slight changes in the amounts of certain components of the culture medium (6). An interesting observation was made with regard to the turnover of free and esterified sterols as a function of time. During the first 6 days, a steady increase in sterol esters occurred, reaching a maximum on the sixth day, whereas the free sterols occupied a much lower range. However, after the sixth day, the amount of esterified sterols decreased rapidly, whereas that of free sterols increased. The amount of free sterols reached its peak between the tenth and twelfth days, paralleling a decline in triglyceride content. The cell-free supernatant fluids were free from lipids. The overall distribution of fractions is shown in Fig. 1, and the composition and the radioactive carbon recovery of the combined fractions appear in Table 1.

Table 1 shows that 24.8% of the originally employed ^{14}C occurred in the neutral lipid fractions, and of this about 67% was present in the free sterol fraction. These findings are noteworthy when it is considered that in similar experiments with the allied organism *Debaryomyces hansenii* the largest amount of ^{14}C occurred in the hydrocarbon fraction (12). Only 2.1% of the total ^{14}C activity initially employed was found in the phospholipid fractions of *P. pullulans*, the activity ranging highest in the phosphatidylserine and phosphatidylethanolamine fractions. These findings are in good agreement with the values determined with *D. hansenii* (12). The cell-free supernatant fluid from *P. pullulans* was nearly free from ^{14}C, whereas that of *D. hansenii* contained 66% (12).

The high activity residing in the trehalose is to be emphasized (Table 2). Extraction with hot 95% ethyl alcohol yielded 260 mg of this sugar with a total count of 508,560 counts/min (1,956 counts per min per mg), corresponding to 9.6% of ^{14}C used. This amount of ^{14}C ranks the second highest among the fractions isolated.

Together with the lipids, some proteinaceous material was also extracted from the lyophilized cells. After the removal of the lipid material with organic solvents, there remained 465.8 mg of insoluble matter which contained 4.6% of the radioactive carbon. The carbon dioxide, liberated mainly during the first 3 days of the fermentation and trapped by barium hydroxide, contained 7.3% of the radioactive carbon supplied (Table 2).

The pigment separated by the various mixtures of ethyl alcohol-water showed a decrease in color intensity with each subsequent alcohol-aqueous mixture, from deep black to light brown. All pigment fractions possessed high luster. Only semiquantitative determinations of the ^{14}C content in the pigment were made, because of its insolubility and because only small quantities were extractable; most of the pigment remained in the cells. The ^{14}C content diminished progressively from the first to the fourth extraction. It should be pointed out that the carotenes could not be eluted from the earlier fractions because of the strong adherence of the black pigment to them. The solvent mixture of 5 and 10% methanol in chloroform eluted mixtures of carotenes and pigment. Addition of petroleum ether to the mixture dissolved the carotenes, which were identified by the method of Clausen and McCord (14) and confirmed by thin-layer chromatography with authentic standards.

Thin-layer chromatography of the total phospholipids, as well as of samples from the various fractions separated by column chromatography, yielded single spots. These areas coincided with those of the respective samples run concurrently and with authentic standards.

The radioactive carbon content of the cell residues was 43.1% of the ^{14}C supplied. On subsequent repeated alkaline extractions, the small amounts of pigment liberated contained radioactive carbon. The cells remained black even after 20 extractions. The total recovery of radioactive

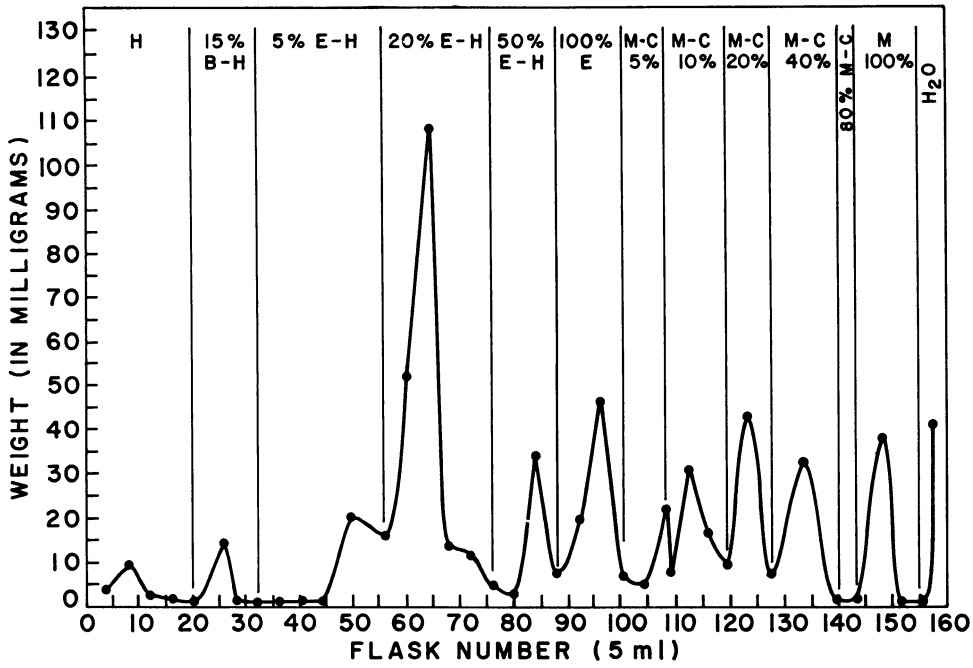

FIG. 1. *Separation of the extractable lipid constituents of Pullularia pullulans by column chromatography on silicic acid in the proportions shown. Solvents were n-hexane (H), benzene (B), diethyl ether (E), chloroform (C), and methanol (M). Removal of the neutral lipids was completed with diethyl ether. Chloroform-methanol was used or elution of the phospholipids.*

TABLE 1. *Quantitative distribution of radioactive carbon in the lipid constituents of Pullularia pullulans grown in medium containing glucose-1-^{14}C*

Eluant[a]	Fractions combined	Lipid recovery[b] (mg)	Lipid constituents	Counts/min	Per cent ^{14}C[c]
n-Hexane	1–20	17	Hydrocarbons and traces of sterol esters	20	None
Benzene-hexane (15:85)	21–29	19	Sterol esters	69	None
Ether-hexane (5:95)	30–57	38	Triglycerides and free fatty acids	579	0.01
Ether-hexane (20:80)	58–77	191	Free sterols	872,602	16.5
Ether-hexane (50:50)	78–88	129	Diglycerides	149,399	2.7
Ether	89–101	79	Monoglycerides and free fatty acids	296,000	5.6
Methanol-chloroform (5:95)	102–108	53	Pigments[d] and traces of carotenes	7,904	0.2
Methanol-chloroform (10:90)	109–119	68	Pigments and carotenes[d]	19,700	0.4
Methanol-chloroform (20:80)	120–128	64	Phosphatidylethanolamine, and traces of phosphatidylserine	21,188	0.4
Methanol-chloroform (40:60)	129–140	56	Phosphatidylserine	23,390	0.4
Methanol-chloroform (80:20)	141–144	Trace	—	—	—
Methanol	145–155	58	Polar lipid material[e]	35,503	0.7
Distilled water	156–158	46	Water-soluble residue	1,896	0.04
			Total		26.95

[a] All ratios are on a volume basis.
[b] Amount of eluted material recovered.
[c] Percentage of total ^{14}C supplied as glucose-*l*-^{14}C.
[d] Glycerol, fatty acids, and phosphates were also present in small amounts.
[e] As yet unidentified material.

TABLE 2. *Distribution of ^{14}C from glucose-1-^{14}C in various cell constituents of Pullularia pullulans*

Name of substance	Amt (mg)	Counts per min per mg	Total counts/min	Per cent ^{14}C[a]
Total lipid[b]			1,428,250	26.95
Residual insoluble matter[c]	465.8	516	240,353	4.6
Trehalose	260	1,956	508,560	9.6
Barium carbonate for CO_2	1,910	201	383,910	7.3
Residual cells[d]	6,750	337	2,274,750	43.1
Cell-free supernatant fluid			479	0.01
Total				91.56

[a] Percentage of total ^{14}C supplied as glucose-*1*-^{14}C.
[b] See Table 1.
[c] Insoluble matter on reextraction of initial lipid extract.
[d] Cellular material remaining after lipid extraction with chloroform plus methanol.

carbon, including the four pigment fractions, amounted to 91.6% of the ^{14}C in the culture medium (Table 2). Microscopically, the initial pigment is light brown and arises at the inner cell wall, spreading uniformly toward the center. It fills the entire cell space within 10 to 12 days, at which time it is deep black.

The pigment is not visible on the first 2 days of incubation but becomes so on the third day. Similarly, trehalose is undetectable during the first 2 days in the cell but appears on the third day. Trehalose reaches a maximum by the sixth day, at which time glucose is no longer detectable.

As cell multiplication continues through the twelfth day and beyond at a rapidly declining rate, pigment intensity increases while the amount of trehalose diminishes (Table 3). These observations suggest that trehalose may serve as the reserve carbohydrate source for *P. pullulans*. Similarly, it appears from Table 3 that the largest amount of triglycerides is 46.1% on the sixth day, decreasing to 4.5% on the twelfth day. This observation indicates that, during the resting and death phases, the cells use up triglycerides for metabolic processes.

Cell reproduction declined rapidly after the sixth day, but phosphatidylethanolamine, phosphatidylserine, and free sterols increased up to the twelfth day, to approximately 4.5 times their 6-day level. Since in most organisms the phospholipids are located in the cell membranes, it is suggested that any excess of phospholipids will be deposited in the membranous area, and the ratio of phosphatidylethanolamine, phosphatidylserine, and free sterols according to Table 3 becomes 1:1:3. At no time could the presence of choline or inositol be detected. It would be interesting to determine whether the cell membrane of *P. pullulans* is electronegative owing to the presence of phosphatidylethanolamine, which has a negative charge that is not counterbalanced by the positive charge of choline in the lecithins.

TABLE 3. *Changes in the concentrations of certain cell constituents of Pullularia pullulans during incubation*

Compound	At 6 days	At 12 days
	%	%
Sterol esters	16.4	2.3
Free sterols	5.5	23.0
Triglycerides	46.1	4.1
Phosphatidylethanolamine	1.7	7.7
Phosphatidylserine	1.4	6.7
Trehalose	1.8	0.4

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