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Production of Luteoskyrin, a Hepatotoxic Pigment, by *Penicillium islandicum* Sopp

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Various factors affecting the yields of luteoskyrin, a hepatotoxic mycotoxin, and related pigments in the liquid medium were studied. Maximal yields of luteoskyrin (0.13% by isolation) and of other pigments were attained in the late phase of the cultivation. The yield of the pigment was increased by supplying malt extract, malonic acid, glutamic acid, or asparagine. A useful material for preparation of ^{14}C-labeled luteoskyrin was 2-^{14}C-malonate.

Luteoskyrin is a hepatotoxic anthraquinone produced by *Penicillium islandicum* Sopp (14). Long-term feedings of mice with rice infected with *P. islandicum* Sopp and with the isolated luteoskyrin cause pathological changes of the liver, including adenoma and hepatoma (13, 14). Shibata and Kitagawa (3) and S. Shibata et al. (Ann. Meet. Natural Products Chem. Abstr., p. 9, 1968) studied the chemistry of this toxin and proposed the structure shown in Fig. 1.

Biochemical investigations revealed that luteoskyrin impairs mitochondrial function and structures of rat liver (4, 10, 11), binds with deoxyribonucleic acid (2, 5, 8) or deoxyribonucleohistone (9), and inhibits the synthesis of nuclear ribonucleic acid in Ehrlich ascites tumor (7). Luteoskyrin is also known to show toxicity to Chang's liver cells and HeLa cells (12) and to protozoa such as *Paramecium caudatum* (A. Kawamota et al., 19th Ann. Meet. Jap. Pharm. Soc. Abstr., p. 157, 1964) and *Tetrahymena pyriformis* (6).

We have examined effects of different growth conditions on the production of luteoskyrin and related pigments in a liquid medium, and the results are reported in this paper.

MATERIALS AND METHODS

P. islandicum Sopp strains E and Jc, kindly supplied by H. Tsunoda of the Food Research Institute, Tokyo, were used. The cultures were inoculated on Czapek agar (30 g of sucrose, 2 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.5 g of KCl, 0.01 g of $FeSO_4$, and 15 g of agar in 1 liter of deionized water) and incubated at 27 C until good sporulation was observed. The spores were washed from agar plates with sterile water and were vigorously shaken with glass beads (0.5 mm in diameter) to give a uniform suspension. The suspension was filtered through glass wool to remove mycelial hyphae and was used as an inoculum.

Culture. A 200-ml Erlenmeyer flask containing 50 ml of the Czapek liquid medium was sterilized for 20 min, and 10^4 to 10^5 conidia were inoculated in duplicate and incubated at 27 C for desired periods. After cultivation, the mycelium was washed with water and dried at 50 to 60 C overnight to measure the dry weight. To obtain preparative quantities of luteoskyrin, the spores were inoculated and incubated at 27 C for two weeks in 100 petri dishes (21 cm in diameter), each containing 20 ml of the above liquid medium. In an experiment designed to test incorporation of ^{14}C-labeled precursors into the pigments, the fungus was cultivated for 3 days at 27 C in a small tube containing 2 ml of the liquid medium supplemented with 10 μc of the ^{14}C-labeled compounds under investigation; after an additional 3 days of incubation, the mycelium was harvested. For the preparation of ^{14}C-labeled luteoskyrin, the fungus was cultured in a Petri dish (9 cm in diameter) containing 20 ml of the liquid medium supplemented with 100 μc of 2-^{14}C-malonate on the 3rd day of the inoculation; the cultivation was continued for additional 11 days.

Extraction and separation of luteoskyrin and other pigments. To obtain the pigments, the dried mycelium was first extracted with *n*-hexane to remove the lipids and then extracted with acetone. The acetone-extracted pigments were separated on plates of silica gel G containing oxalic acid and were developed with acetone–*n*-hexane–water (6:3:1.5). The R_F values were as follows: rubroskyrin, 0.10; skyrin, 0.20; luteoskyrin, 0.40; iridoskyrin, 0.90; and islandicin, 0.95. Each pigment was eluted from the plate with a small amount of acetone, and the optical density of the eluate was measured. The following extinction coefficients were employed: luteoskyrin, 5.54 (448 nm); rubroskyrin, 4.01 (418 nm); iridoskyrin, 4.39 (492 nm); islandicin, 4.09 (492 nm); and skyrin, 4.37 (458 nm). Luteoskyrin was isolated from 20 g of solids of the acetone-extractable material from the mycelium. The solids were dissolved in about 200 ml of acetone,

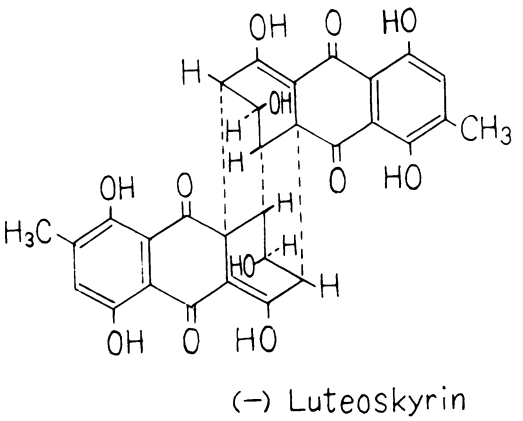

FIG. 1. *Structure of luteoskyrin.*

passed through a charcoal column (0.5 mesh, 6 by 60 cm), and eluted with 3 to 4 liters of acetone. After evaporation of the solvent, luteoskyrin was crystallized from methanol, giving fine, yellow needles.

Preparation of luteoskyrin. *P. islandicum* Sopp strain Jc was cultured at 27 C for 2 weeks in 100 petri dishes (21 cm in diameter), each containing 20 ml of Czapek medium, in the presence or in the absence of malt extract (5 g/liter). In the case of Czapek medium, 200 g of the dried mycelium gave 18 g of acetone-soluble pigments from which 1.5 g of luteoskyrin was obtained. In the case of the malt extract-supplemented medium, 350 g of the dried mycelium gave 40 g of acetone-soluble pigments, from which 4 g of luteoskyrin was obtained.

Materials. Yeast extract, malt extract, proteose peptone, and Casamino Acid were the products of Difco. Organic acids in the culture medium were separated by thin-layer chromatography of silica gel G in 1-propanol–NH_4OH (3:2); they were detected by spraying with a methanolic solution of bromocresol green. Sugar determination was made by the anthrone method. The ^{14}C-labeled compounds were purchased from Daiichi Chemicals, Tokyo. Radioactivity of the pigments was determined by a liquid scintillation counter.

RESULTS

Effects of carbon source on the yield of the mycelia and the pigments. The effect of carbohydrates and alcohols on growth and pigmentation is shown in Table 1. Acetate, succinate, fumarate, citrate, and malonate caused a decrease in mycelial weight and in percentage of pigments to mycelial weight.

Effects of nitrogen. The effects of nitrogen in various forms were investigated with sucrose as a carbon source. As shown in Table 2, $NaNO_3$, asparagine, glutamine, and malt extract were the preferred sources of nitrogen for the mycelial growth and the pigmentation, and they decreased the pigment formation. The addition of peptone, yeast extract, or Casamino Acid caused an increase in weight of mycelium and a decrease in yield of pigments.

Malonic acid and pigmentation. Since it was

TABLE 1. *Effect of carbon sources on the mycelial growth and pigment formation of Penicillium islandicum strain Jc*[a]

Substrate (3% in Czapek medium)	Mycelium[b] (mg/50 ml)	Pigments[c]
Starch	414.8	28.9
Maltose	369.6	23.6
D-Galactose	365.9	24.6
D-Xylose	365.4	28.2
D-Glucose	305.7	27.2
D-Mannitol	290.4	14.6
D-Fructose	270.2	38.2
Sucrose	254.9	24.4
Glycerol	201.0	27.1
D-Sorbitol	177.4	17.6
Lactose	144.5	27.2
Glutamate	80.0	3.0
Aspartate	78.0	4.1
Citrate	49.4	2.0
Malonate	48.1	2.3
Succinate	36.0	1.9
Fumarate	32.0	4.7
Acetate	20.4	—

[a] The medium (50 ml in a 200-ml Erlenmeyer flask) was incubated for 2 weeks at 27 C.
[b] Values are averages from two flasks.
[c] Expressed as per cent of total mycelial weight.

TABLE 2. *Effects of nitrogen sources on the mycelial growth and pigment formation of Penicillium islandicum strain E*[a]

Expt[b]	Nitrogen source (0.2 %)	Mycelium (mg/50 ml)	Pigments[c]
A	Control (Czapek medium)	286	15.8
	NH_4NO_3	137	5.1
	NH_4Cl	200	4.0
	Urea	105	7.6
B	Asparagine	463	27.2
	Glutamine	390	27.9
	Peptone	769	2.8
	Yeast extract	847	2.4
	Malt extract	687	18.4
	Casamino acid	749	5.2

[a] Cultivation conditions were as described in Table 1.
[b] In experiment A, $NaNO_3$ in Czapek medium was replaced as cited in the table; in experiment B, Czapek medium was supplemented with the compounds listed.
[c] Expressed as per cent of total mycelial weight.

noted earlier that malonic acid promoted the synthesis of pigments in *P. islandicum* Sopp, we have reexamined its effect on the growth and pigmentation of the fungus at various stages of cultivation. The addition of 0.02 or 0.04 M malonic acid to the culture medium increased the yield of total pigments (Table 3).

Because malonic acid has been known to be an inhibitor of succinic dehydrogenase, we investigated whether this acid would act as a metabolic inhibitor or as a substrate for the fungus. *P. islandicum* Sopp strain E was cultured for 15 days on Czapek liquid medium in the presence or in the absence of supplemented malonate. The filtrate of the culture medium were acidified, and ether-soluble organic acids were separated by thin-layer chromatography of silica gel G in 1-propanol–NH_4OH (3:2). No difference in relative amounts of oxalic, citric, fumaric, malic, and succinic acids was found between the control medium and malonate-supplemented medium.

TABLE 3. *Effects of malonic acid on the pigment formation by Penicillium islandicum strain E*[a]

Days of cultivation	Malonic acid M	Mycelium[b] (mg/50 ml)	Pigments[c]
5		115	0.9
	0.02	299	1.6
	0.04	226	2.3
10		308	11.2
	0.02	444	24.9
	0.04	497	17.9
15		345	12.5
	0.02	427	22.4
	0.04	484	20.1

[a] Cultivation conditions were as described in Table 1.
[b] Values are averages of three flasks.
[c] Expressed as per cent of total mycelial weight.

TABLE 4. *Incorporation of ^{14}C-labeled compounds into the mycelial lipids and pigments of Penicillium islandicum strain E*[a]

^{14}C-Compounds	Radioactivity in pigments/ radioactivity in lipids
U-^{14}C-Glucose	6.1
2-^{14}C-Acetate	3.8
2-^{14}C-Malonate	8.3
2-^{14}C-Mevalonate	0.7
U-^{14}C-Aspartic acid	7.6
U-^{14}C-Glutamic acid	8.3

[a] Czapek medium (2 ml) was supplemented with 10 μc of ^{14}C-labeled compound on the 3rd day of the incubation; the cultivation was continued for an additional 3 days and the mycelium was analyzed.

TABLE 5. *Incorporation of ^{14}C-malonate and ^{14}C-glutamate into the quiniod pigments of Penicillium islandicum strain E*[a]

^{14}C-Compound	Pigment	Specific radio-activity (μc/ μmole)	Yield	
			mg	*μc*
2-^{14}C-Malonate	Islandicin	0.27	0.72	0.65
	Iridoskyrin	0.53	0.24	0.24
	Luteoskyrin	0.53	4.78	5.24
	Skyrin	0.40	7.20	5.08
	Rubroskyrin	0.57	1.40	1.40
5-^{14}C-Glutamate	Islandicin	0.16	1.02	0.60
	Iridoskyrin	0.45	0.30	0.25
	Luteoskyrin	0.09	5.01	0.90
	Skyrin	2.75	6.00	2.56
	Rubroskyrin	0.88	0.80	1.92

[a] Czapek medium (20 ml) was supplemented with 100 μc of ^{14}C-labeled compounds on the 3rd day of the incubation; the cultivation was continued for an additional 11 days and the pigments in the mycelium were analyzed.

Incorporation of ^{14}C-labeled compounds into luteoskyrin and related pigments. Uptakes of ^{14}C-labeled compounds into mycelial lipids and pigments were examined. All of the compounds tested, except 2-^{14}C-mevalonate, were incorporate into pigments more extensively than into lipids, and the incorporation into pigments was highest in cases of 2-^{14}C-malonate, *U*-^{14}C-aspartic acid and *U*-^{14}C-glutamic acid (Table 4). Examination with larger amounts of the radioactive compounds indicated that 2-^{14}C-malonate was incorporated into the isolated pigments and gave nearly the same specific radioactivity as skyrin, luteoskyrin, iridoskyrin, and rubroskyrin (Table 5). The total incorporation of 2-^{14}C-malonate into luteoskyrin was about 5% of the radioactivity of added ^{14}C-malonate. In the case of 5-^{14}C-glutamate, all five pigments were labeled, but the specific radioactivity of each pigment was much lower than those of ^{14}C-malonate.

DISCUSSION

Relationship among the time of incubation, the growth of fungus, the consumption of sugar, and the yield of pigments was investigated. The content of sugar in the medium was rapidly decreased during the 1st week of the cultivation, and the maximal rate of the pigment formation in the mycelium took place at the late-logarithmic phase of the fungal growth.

Chromatographic analysis of the fungal pigments indicated that luteoskyrin and other quinoid pigments were synthesized in the mycelium

of *P. islandicum* without any particular order. This finding suggested that the pigments were biosynthesized from a common precursor, in agreement with the finding of Gatenbeck (1).

According to the experiments shown in Tables 1 and 2, the best carbon source for growth of *P. islandicum* Sopp were carbohydrates such as fructose, starch, xylose, and glucose, and nitrogen materials such as malt extract, glutamine, or asparagine. The best inorganic nitrogen was sodium nitrate. Very meager growth of the fungus was observed with the use of mannitol, sorbitol, or organic acids as a sole carbon source.

Peptone, yeast extract, and Casamino Acid promoted the mycelial growth and decreased the pigment formation (Table 2). These materials presumably modify the characters of the fungus in the early stage of growth, because the decreased production of the mycelial pigment could not be restored even when the mycelium was returned to Czapek medium after 1 week of the cultivation.

The accelerating effects of glutamine and malonic acid on the pigment formation suggest them to be precursors of pigments. The incorporation of radioactive glutamic acid and malonic acid into pigments and the exhaustion of malonate added to the medium support the abovementioned assumption (Tables 4 and 5). A noteworthy finding is that luteoskyrin contained about 5% of the radioactivity of ^{14}C-malonate added. High efficiency of the uptake indicates that the 2-^{14}C-malonate is a useful material for preparation of ^{14}C-labeled luteoskyrin.

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