Melanin Biosynthesis by *Streptomyces lavendulae*

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SUMMARY

Streptomyces lavendulae was studied in a defined medium. When tyrosine was added to the medium a brown-black pigment was synthesized; without tyrosine, only a trace of this pigment was detected. Growth was the same with or without tyrosine. The addition of (2-¹⁴C) DL-tyrosine to the medium resulted in synthesis of a radioactive pigment, and tyrosinase activity was demonstrated in extracts of disrupted organisms. Of 15 amino acids added to the medium only tyrosine and tryptophan were used for pigment formation. Pigment production was inhibited by *p*-benzyloxy-phenol, an inhibitor of mammalian melanogenesis. In yeast + glucose medium a pH range of 6·8–8·2 was optimal for pigment synthesis; more pigment was formed at 20° than at 28° in spite of the fact that there was twice as much growth at the latter temperature. The pigment was precipitated from culture media by a method described for precipitating melanin from urine and in every way tested was identical with a synthetic dihydroxyphenylalanine-melanin. The data indicate that the brown-black pigment synthesized by *S. lavendulae* is melanin.

INTRODUCTION

Pigment synthesis by streptomycetes has aroused considerable interest for many years. The brown-black pigment has been referred to as 'melanin', and as 'melanin-like' because of demonstrations of an involvement of tyrosine in its synthesis. In this regard Skinner (1938) observed that pigmentation resulted from the addition of tyrosine to a defined medium upon which he was growing some actinomycetes and Douglas & San Clemente (1956) observed a dark brown pigment in flasks in which mycelial homogenates of *Streptomyces scabies* were mixed with tyrosine, dihydroxyphenylalanine, or both. In attempting to correlate pigment formation and antibiotic synthesis by *S. antibioticus* Sevcik (1957) manometrically determined the presence of phenol oxidase activity, with tyrosine and several other substrates. While these studies suggest that the pigment is melanin, apparently no attempts have been made to isolate and characterize the pigment. *Bergey's Manual* (1957) refers to the pigment as a 'soluble brown pigment'. The present paper reports an attempt to determine whether the brown-black pigment of *S. lavendulae* is actually melanin.

METHODS

Streptomyces lavendulae (no. 3515, Institute of Microbiology, Rutgers University, U.S.A.) was used throughout. Stock cultures of the organism were maintained on yeast + glucose agar, and yeast + glucose liquid medium was used as the seed

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medium as well as for some preliminary observations. A defined medium (GGG) was used for most experiments. It contained (g.): glucose, 5.0; glycine, 10.0; monosodium glutamate, 10.0; K_2HPO_4 , 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; (mg.): $CaCl_2 \cdot 2H_2O$, 11.0; $FeSO_4 \cdot 7H_2O$, 15.0; $ZnSO_4 \cdot 7H_2O$, 13.0; $CuSO_4$, 7.5; distilled water to 1 litre. The medium without glucose (glucose autoclaved separately) was adjusted to about pH 7.5 and then autoclaved at 120° for 15 min. The medium after autoclaving and the addition of glucose was at pH 7.2–7.5. Pigment formation was readily observed because the medium itself was colourless.

Each 100 ml. of medium in a 250 ml. Erlenmeyer flask was seeded with 2.0 ml. washed suspension of organism grown for 18–24 hr. in yeast + glucose liquid medium. Organisms used for inoculation of defined medium were washed by centrifugation and resuspension until two successive washings were colourless; usually four to five washings were adequate. Except for temperature studies, cultures were incubated at 28° on a rotary shaker at 220 rev./min. and were routinely harvested by filtration after one week of incubation. Growth was determined as dry weight.

Relative values of pigment synthesis were obtained by determining % transmission of culture fluid at 540 m μ . using a Bausch and Lomb Spectronic 20 Colorimeter. The pigment was precipitated from culture fluids by a modification of a method described by Blackberg & Wanger (1933) for precipitating melanin from urine. For this culture fluid was first adjusted to pH 7.0 and 1 g. potassium persulphate was added for each 100 ml. of fluid and allowed to stand for 2 hr. with occasional shaking. Then 100 ml. of methanol was added for each 100 ml. of culture fluid and the mixture allowed to stand for 3 days; during this time pigment was precipitated, and was collected and washed by centrifugation. Pigment synthesized under various conditions was compared colorimetrically after dissolving the precipitated pigment in 10.0 ml. M-NaOH.

Synthetic DL-dihydroxyphenylalanine (dopa)-melanin was prepared by the method of Arnow (1938); 20 mg. of DL-dopa were dissolved in 50.0 ml. 0.01 M-NaOH and aerated by bubbling air saturated with water through the solution for 1 hr. The resulting brown-black precipitate was collected and washed by centrifugation.

To prepare cell extracts, washed organism (equiv. about 1.0 g. dry wt.) from an 18 hr. shake culture was suspended in 50.0 ml. 0.1 M-phosphate buffer (pH 6.9) and disrupted at maximum power in a 10 Kc Raytheon sonic oscillator for 3 min., whole organisms were separated from the suspension by centrifugation for 10 min. Tyrosinase activity was determined by a method of Cooper & Brown (1956) in which the cell extracts were added to holes cut in a phosphate-buffered agar plate containing tyrosine, dopa, or both together as substrates. Control plates were run without substrate. A positive reaction resulted in the formation around the hole of a pink ring which changed to brown and then black.

An inhibitor of melanogenesis, *p*-benzyloxy-phenol, was dissolved in methanol. Solutions were prepared on the day of use.

Radioactive pigment was assayed in a liquid scintillation spectrometer (Packard Tri-carb).

RESULTS

The effect of tyrosine on pigment biosynthesis in a defined medium

To determine the effect of tyrosine on pigment synthesis by *Streptomyces lavendulae* various concentrations of tyrosine were tested in the defined medium described above. An increase in tyrosine concentration did not result in an increase or decrease in yield of organism nor did it have an effect on the pH value of the culture (Table 1). The principle effect of the tyrosine was on the amount of pigment synthesized: as the tyrosine concentration increased, the amount of pigment increased. To substantiate further the role of tyrosine in the formation of pigment, (2-¹⁴C) DL-tyrosine was added to the defined medium, resulting in the synthesis of a radioactive pigment.

Table 1. *Effect of tyrosine on pigment synthesis by Streptomyces lavendulae in a defined medium*

Tyrosine concentration (g./l.)	Resuspended pigment (% transmission at 540 mμ)*	Yield of organism (mg. dry wt.)*	pH value of culture†
Control	80	201	8.4
(no tyrosine)	83	165	8.3
0.1	76	152	8.4
	68	162	8.5
0.2	52	151	8.5
	52	171	8.5
0.3	39	147	8.5
	36	149	8.5
0.4	12	168	8.5
	16	166	8.5

* Values are those of duplicate flasks run at the same time.

† Duplicate flasks; after incubation for 1 week at 28°.

Demonstration of tyrosinase activity

The presence of a tyrosinase in *Streptomyces lavendulae* was shown by the tyrosinase assay described. At 20°–24° the extract of sonically disrupted organisms began to oxidize dopa within 3 min. as evidenced by the appearance of a pink ring around the extract-containing hole; this ring became dark brown to black within 1 hr. Autoxidation of dopa was not the cause of the colour change; if it were, the entire plate would have changed to the same colour. Tyrosine alone was oxidized by the extract but at a slower rate than dopa since the oxidation became apparent in 30 min. Catalytic concentrations of dopa added to the tyrosine did not shorten this period. All controls were negative. At 37° only dopa was oxidized by the extract. Removal of copper by adding sodium diethyldithiocarbamate to a system containing dopa + cell extract prevented a positive tyrosinase reaction.

Inhibition of pigment biosynthesis

p-Benzyloxy-phenol is an inhibitor of melanogenesis (Lerner & Fitzpatrick, 1950; Peck & Sobotka, 1941). To investigate the effect of this inhibitor on *Streptomyces lavendulae* different concentrations of it were added to a complete medium

containing 0.3 g. tyrosine/l. With increasing concentration of inhibitor there was a decrease in the amount of pigment synthesized (Table 2). However, cell synthesis appeared to be slightly stimulated by these concentrations of inhibitor. Concentrations of 1.0×10^{-4} M-*p*-benzyloxy-phenol inhibited cell synthesis as well as pigment synthesis. The pH values were only slightly lower in the media containing *p*-benzyloxy-phenol. The effect of this compound was an inhibition of pigment synthesis without inhibition of cell synthesis.

Table 2. *Inhibition of Streptomyces lavendulae pigment synthesis by p-benzyloxy-phenol in defined medium containing 0.3 g. tyrosine/l.*

<i>p</i> -benzyloxy-phenol $\times 10^{-5}$ M	Resuspended pigment (% transmission at 540 m μ)*	Yield of organisms (mg. dry wt.)*	pH value of culture†
3.0	94	190	8.2
	89	180	8.3
2.5	89	237	8.3
	81	204	8.5
2.0	91	205	8.4
	90	218	8.4
1.5	79	160	8.4
	85	175	8.6
1.0	72	160	8.5
	80	174	8.7
0.5	57	178	8.4
	56	178	8.5
Control A‡	60	107	8.8
	54	149	8.7
Control B	97	102	8.9
	94	95	8.9

* Values are those of duplicate flasks run at the same time.

† Duplicate flasks; after incubation for 1 week at 28°.

‡ Control A: no *p*-benzyloxy-phenol.

Control B: no *p*-benzyloxy-phenol, no tyrosine.

Comparison of the pigment from Streptomyces lavendulae with a synthetic dopa-melanin

A comparison of the brown-black pigment synthesized by *Streptomyces lavendulae* and a sample of synthetic dopa-melanin showed them to be identical in several chemical and physical properties (Table 3). Their solubilities were the same, they reacted to oxidation and reduction in the same way, were precipitated by the same techniques, and neither exhibited any characteristic absorption peaks.

Effect of amino acids other than tyrosine on pigment biosynthesis

To examine the possibility that amino acids other than tyrosine have an effect on pigment biosynthesis by *Streptomyces lavendulae*, the following amino acids were added singly to the defined medium at 0.4 g./l.: DL-alanine; L-arginine; DL-serine; DL-lysine monohydrochloride; DL-phenylalanine; sodium aspartate; L-histidine monohydrochloride; DL-leucine; L-proline; DL-threonine; DL-valine; L-tryptophan.

Glycine and sodium glutamate were present as components of the synthetic medium. Of the fifteen amino acids tested only tyrosine and tryptophan were used for pigment synthesis. The pigment which resulted from tryptophan utilization was identical with the tyrosine pigment in all of the properties listed in Table 3. Phenylalanine, reported to be an inhibitor of melanogenesis (Lerner & Case, 1959), actually stimulated pigment synthesis in the presence of tyrosine.

Table 3. *Comparison of pigment of Streptomyces lavendulae with a synthetic dopa-melanin*

Property	Pigment of <i>S. lavendulae</i>	Dopa-melanin
Solubility in water:	Insoluble	Insoluble
Solubility in m-NaOH:	Soluble	Soluble
Colour:	Brown-black	Brown-black
Appearance in water:	Evenly dispersed floc	Evenly dispersed floc
Precipitation by Blackberg-Wanger technique:	Precipitated	Precipitated
Precipitation from NaOH by HCl	Precipitated	Precipitated
Formation of black precipitate with FeCl ₃ which dissolves with excess FeCl ₃	+	+
Reduction by sodium dithionite and re-oxidation by potassium ferricyanide	Black → tan Tan → black	Black → tan Tan → black
Absorption spectrum (350–650 mμ)	No characteristic peaks	No characteristic peaks

The effect of pH value and temperature on pigment biosynthesis

Yeast + glucose liquid medium adjusted to eleven different values from pH 5.2 to 8.5 was inoculated with *Streptomyces lavendulae*. The range pH 6.8–8.2 was most favourable for pigment synthesis. Yeast + glucose liquid medium seeded with *S. lavendulae* and incubated at 37°, 28° and 20° yielded more pigment at 20° than at 28°, in spite of the fact that there was only one half as much yield of organism. At 37° the yield of organism was equal to that obtained at 20°, but only a trace of pigment could be detected.

DISCUSSION

The influence of tyrosine on pigment production by *Streptomyces lavendulae* is like melanin formation in other cells (Raper, 1927; Mason, 1948; Lerner, 1953). Although the disappearance of tyrosine from the medium was not followed it is probable that some tyrosine also was used for growth in spite of the fact that no net increase was observed in amount of organism dry weight. However, the tyrosine appeared to be preferentially utilized for pigment synthesis. A qualitative observation showed its incorporation into the pigment when (2-¹⁴C) DL-tyrosine was supplied in the medium. The utilization of tryptophan for pigment synthesis is not surprising because tryptophan is a precursor of kynurenine and anthranilic acid which can be metabolized to catechol. It is possible that melanin was synthesized by this route with catechol as substrate for tyrosinase. The tryptophan pigment had physical and chemical characteristics identical with the tyrosine pigment and with synthetic dopa-melanin. Conditions of synthesis, inhibition, and the physical and chemical

characteristics of the precipitated pigment correspond to those known for dopa-melanin. Because of these data it is submitted that the brown-black pigment of *S. lavendulae* is in fact melanin.

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