MELANOID PIGMENT PRODUCTION IN A STRAIN OF *TRICHOPHYTON RUBRUM*

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Trichophyton rubrum is a dermatophyte that causes chronic and often serious superficial mycoses in man. Aside from descriptive, clinical, and biochemical studies, relatively little investigation has been done of the intermediary metabolism of the dermatophytes. Only recently have such studies been published (Jensen, Altschuller, and Bard, 1957; Burack and Knight, 1958).

This study is concerned with the mechanism by which *T. rubrum* may elaborate pigment. Earlier experiments with this organism (Zussman, 1959) demonstrated that pigment formation was dependent upon (a) tyrosine or phenylalanine and (b) atmospheric oxygen. Furthermore, ascorbic acid was able to decrease or prevent pigment formation when added to the medium in concentrations that did not seriously inhibit growth. These findings suggested that the pigment may be melanoid in character and formed by a mechanism similar to those described by other investigators (Bloch, 1917; Evans and Raper, 1937; Ito, 1952; Keilin and Mann, 1938; and Lerner and Fitzpatrick, 1950). The results presented here provide further evidence that the pigments of *T. rubrum* are of the melanoid type.

MATERIALS AND METHODS

Pigment production. The pigment was obtained from *T. rubrum* grown in shake cultures as described by Silva, Kesten, and Benham (1955), and Jensen, Altschuller, and Bard (1957). The growth medium (Nickerson and Chadwick, 1946) was dispensed into 250-ml Erlenmeyer flasks in volumes of 135 ml and each flask was then inoculated with 1 ml of fungal homogenate (Vicher, Lyon, and White, 1959). The latter was prepared from one half of a 7-day-old culture grown on Sabouraud's agar, suspended in 35 ml of sterile saline, and homogenized in a Servall Omni-Mixer for 1 min at approximately 6,000 rpm. The cultures were continuously agitated

from 3 to 4 weeks on a rotary shaker set at about 150 rpm at 27 C. After incubation, spherical clones, heavily laden with pigment, were obtained as previously described (Zussman, 1959).

Pigment extraction. Pigment was extracted by a modification of the method of Wirth, O'Brien, Schmidt, and Sohler (1957). The pigment-bearing fungus was separated from the medium with a Büchner funnel, yielding a filtrate which contained water-soluble pigments. The fungal tissue was ground with white sand in a mortar and the resultant watery, pastelike mass was transferred to a Soxhlet extractor. Acidified acetone was used in the extractor and the pigment was removed from the paste in 2 hr. The acetone was removed by distillation in vacuo. The remaining suspension was acidified to pH 3 with HCl and the pigment was extracted with ethylene dichloride. After centrifugation the ethylene dichloride extract was separated from the aqueous phase by decantation and washed several times with acidified water until the wash water became negative to ninhydrin.

The amorphous residue of crude pigment remaining after evaporation of the ethylene dichloride was treated with cyclohexane and carbon tetrachloride to remove substances soluble in lipid solvents. Losses were minimal since the pigment was only slightly soluble in cyclohexane and completely insoluble in carbon tetrachloride.

Pigment purification. The crude pigment was dissolved in ethylene dichloride and adsorbed onto a column of terra alba no. 1. The column (8 mm diameter) was developed with ethylene dichloride containing 0.07 per cent glacial acetic acid flowing at a rate of 1.0 ml of eluent per 4 hr. Total development time was 24 hr.

After development, the contents of the column were extruded onto glass and the individual pigment bands were cut out with a spatula. The pigments were extracted from these sections with ethylene dichloride containing 1.0 per cent acetic acid. Each pigment was tested for the presence

TABLE 1
*Decomposition ranges of Trichophyton
rubrum pigments*

Pigment	Decomposition Range
	C
Yellow.....	380-390
Red.....	385-390
Orange.....	380-390
Purple.....	185-190

of impurities with Molisch's, Millon's, and ninhydrin reagents and absorption at 280 m μ was also noted. By this procedure purified yellow, orange, and red pigments were obtained in sufficient quantity for further study.

A water-soluble pigment, purple at pH 8, was precipitated by acidification of the culture medium filtrate to pH 3 with HCl. The precipitate was an amorphous reddish-orange mass which dissolved in alkalized water. Alternate acidification and alkalization (6 cycles) coupled with centrifugation purified the pigment, but the preparation was not homogeneous.

Nitrogen and carbohydrate analyses. Since tyrosine and glucose are associated with pigment production (Zussman, 1959), the yellow and purple pigments were assayed for nitrogen by Nesslerization after prolonged acid hydrolysis, and for carbohydrate with Molisch's reagent.

Thermal decomposition data. The thermal decomposition ranges of the yellow, orange, red, and purple pigments were determined with a micromelting point apparatus (table 1). Charring or a change in color was the criterion of decomposition for the first three pigments; loss of ability to change color caused by a change in pH was the criterion of decomposition for the purple pigment.

Solubility data. The relative solubilities of the pigments were determined in acidified and alkalized water, acetone, ethanol, methanol, carbon tetrachloride, cyclohexane, ethylene dichloride, petroleum ether, and xylene. Approximately 1 mg of pigment was shaken at room temperature with 3 ml of solvent for 1 min. If all the pigment dissolved, it was called "soluble"; if all the pigment did not dissolve but the solvent became colored, it was called "slightly soluble"; and if the pigment did not impart a color to the solvent it was called "insoluble". The results of this differentiation are reported in table 2.

TABLE 2
Solubilities of Trichophyton rubrum pigments*

Solvent	Pigment			
	Yellow	Red	Orange	Purple
Water				
Acidified.....	I	I	SS	SS
Alkalized.....	I	I	I	S
Solvents				
Miscible with water				
Acetone.....	S	I	S	SS
Ethanol.....	SS	SS	SS	SS
Methanol.....	SS	SS	SS	I
Immiscible with water				
Carbon tetrachloride...	I	I	I	I
Cyclohexane.....	SS	SS	SS	I
Ethylene dichloride...	S	SS	S	SS
Petroleum ether.....	I	I	I	I
Xylene.....	I	I	I	I

* Soluble, S; slightly soluble, SS; insoluble, I.

Spectrophotometric analyses. Tracings of the yellow and purple pigments and of freshly oxidized 3,4-dihydroxyphenylalanine (dopa) were obtained with a Beckman recording spectrophotometer in the range between 340 and 600 m μ .

Dopa oxidase activity. Homogenates of *T. rubrum* were assayed for dopa oxidase activity as follows: a 0.2-ml sample of the supernatant from a fungal homogenate (prepared by grinding an 8-day-old colony in a cold mortar with 10 ml of cold water for 3 min and centrifuging at 1000 rpm for 2 min) was added to 10 ml of Sorensen buffer (pH 7.1) containing 5 mg of dopa. Three milliliters of dopa solution alone (control) and of dopa solution plus supernatant were pipetted into cuvettes. Optical density readings were recorded every 10 min for 1 hr at 390 m μ . Each cuvette was briefly aerated 2 min prior to each reading.

Radiotyrosine studies. C¹⁴-Tyrosine, labeled randomly or in the alpha position, was added separately to a chemically defined medium (Zussman, 1959) in which *T. rubrum* was incubated for 3 weeks. At the end of the incubation period, yellow pigment was extracted and purified as described above and the amount of C¹⁴ incorporated into the pigment was determined in the usual manner by end-window counting with a gas flow Geiger-Müller detector.

RESULTS AND DISCUSSION

Chromatographic analyses of pigments. Two typical chromatograms were observed, depending upon whether the predominant pigment in a given culture was orange or yellow. When orange, the descending pattern was (a) a brown sludge of unknown composition; (b) a faint purple band; (c) a nonhomogeneous orange band which, as reported by Wirth et al. (1957), could be crystallized into orange plates and red needles; and (d) an apple green band. Under ultraviolet light, three fluorescent bands were observed: (a) a light blue band which was eluted from the column prior to any significant separation of the bands visible in ordinary light; (b) a diffuse green band in the region between the purple and orange bands; and (c) a yellow band accompanying the upper half of the orange band.

When the yellow pigment predominated, a different chromatographic pattern was observed: (a) a brown sludge similar in appearance and consistency to that described above; (b) a very faint, diffuse red band; and (c) a canary yellow band. Under ultraviolet light, the previously described light blue and green bands were observed but the fluorescent yellow band was absent.

Although the conditions that prevail during the growth period of *T. rubrum* probably determine which group of pigments will form, the specific determinants are as yet unknown and uncontrollable. Cultures which yield orange pigment grow in the form of highly compact, spherical clones and the culture medium is never pigmented. Cultures from which the yellow pigment can be extracted grow in the form of noncompact stellar clones and the culture medium always contains a considerable amount of a water-soluble, yellow-orange pigment.

The water-soluble pigment, purple at pH 8, was obtained from cultures which yielded either of the predominant pigment forms. The purple pigment undergoes a sharp transition in color between pH 7.2 (orange-red) and pH 7.8 (deep indigo). A water-soluble contaminating pigment, yellow in color, was observed on a circular filter paper chromatogram spotted with alkalized purple pigment. This contaminant, occupying approximately 10 per cent of the area covered by the purified purple pigment, was eluted with acetone or alkalized water, whereas the purple

pigment could not be eluted with any of the common solvents listed previously.

Nitrogen and carbohydrate analyses. Qualitative analysis of the purple pigment revealed the presence of nitrogen. However, because of its nonhomogeneity, it was not clear whether the nitrogen was contributed by the purple pigment, by the contaminating substance(s) present, or both. Samples of relatively pure yellow pigment, (not the yellow contaminant referred to above), contained approximately 4 per cent nitrogen. Although Wirth et al. (1957) found no nitrogen in several pigments of *T. rubrum*, it should be pointed out that (a) this fungus may form both nitrogen-containing and nitrogen-free pigments and (b) the extraction procedure described herein is different from that employed by Wirth et al. Neither the purple nor yellow pigment gave a positive test for carbohydrate with Molisch's reagent.

Thermal decomposition data. None of the pigments exhibited a melting point; the thermal decomposition ranges are presented in table 1. The similarity in decomposition temperatures of the yellow, orange, and red pigments may indicate a common structural relationship.

Solubility data. The relative solubilities of the four pigments are summarized in table 2.

The solubility data suggest that the purple pigment may have ionizable groups while the other pigments probably do not. It is interesting to note that the yellow and orange pigments were soluble in acetone and in ethylene dichloride while the purple was relatively insoluble in these solvents.

The nitrogen-free orange pigment isolated by Wirth et al. (1957) was resolved into orange plates and red needles, a finding confirmed in this study. Furthermore, as described above, a yellow component was observed under ultraviolet light. Therefore, on the basis of the data of Wirth et al. and the chromatographic behavior and thermal decomposition and solubility data presented herein, it appears reasonable to assume that *T. rubrum* may synthesize one group of closely related substances including the orange, yellow, and red pigments, and the purple pigment may be representative of another group. The differentiation may be related to the degree of polymerization of these pigments (*vide infra*).

Spectrophotometric analyses. Tracings of oxidized dopa, purified yellow pigment, and partially

Figure 1. Spectrophotometric tracings of oxidized dihydroxyphenylalanine, purified yellow pigment, and partially purified purple pigment.

purified purple pigment are shown in figure 1. Identical transmittance minima at 390 $m\mu$ suggest that these pigments of *T. rubrum* are related to each other through certain functional groups and to the oxidation products of dopa.

Dopa oxidase activity. The influence of the supernatant from a *T. rubrum* homogenate on the rate of oxidation of dopa is indicated in figure 2. This activity was not observed at pH 4, or with autoclaved supernatants. The catalytic substance in the supernatant has several of the properties of dopa oxidase: (a) it accelerates the rate of oxidation of dopa to various colored products, (b) its optimal pH lies close to neutrality, and (c) it is thermolabile. If the above evidence is sufficient to establish the identity of this catalyst with dopa oxidase, it would imply a capacity of *T. rubrum* to form melanoid pigments.

Burack and Knight (1958) have reported a high rate of deamination of various amino acids by this fungus with the liberation of ammonia and a consequent rise in pH. This activity may explain why an initial period of growth must precede the formation of pigment in cultures with an initially low pH; pigmentation in *T. rubrum* does not occur until the pH of the medium rises to 7.

Figure 2. Dihydroxyphenylalanine oxidase activity of a *Trichophyton rubrum* homogenate. Readings taken at 390 $m\mu$.

Radiotyrosine studies. The process by which melanoid pigments are formed in plants and animals may be summarized as follows (Bloch, 1917; Evans and Raper, 1937; Lerner and Fitzpatrick, 1950; and White et al, 1954): (a) Tyrosine is oxidized to dopa by tyrosinase, which may be identical to dopa oxidase (Ito, 1952; Keilin and Mann, 1938; and Lerner and Fitzpatrick, 1950). (b) Dopa is oxidized to dopa quinone by dopa oxidase (tyrosinase). (c) A leuko-compound with an indole nucleus forms when the nitrogen of the quinone becomes bonded to carbon. At this time the carboxyl group is cleaved and presumably removed as CO_2 . (d) The leuko-compound is rapidly oxidized to hallochrome, a red-colored compound. (e) This sequence of reactions terminates with the polymerization of hallochrome to form the melanoid pigments. Although the polymerization mechanism has not been clarified, the longer polymers are known to be darker in color.

These studies were carried out to see whether the mechanism described above might function in pigment formation by *T. rubrum*. The extent of incorporation of C^{14} into yellow pigment is indicated in table 3. The specific activity of pigment recovered from cultures grown in the presence of tyrosine-2- C^{14} was 99 per cent of the specific activity of the added labeled amino acid. This randomly labeled tyrosine yielded pigment with a specific activity approximately 80 per cent that of the exogenously labeled amino acid.

The specific activity data suggest that (a) the intact carbon skeleton of the tyrosine mole-

TABLE 3
Incorporation of C^{14} -tyrosine into a yellow
pigment of *Trichophyton rubrum*

Compound	Specific Activity	Specific Activity Ratio
	counts:min:μg	pigment: C^{14} -tyrosine
Tyrosine-2- C^{14}	1.475×10^3	0.99
Pigment.....	1.460×10^3	
Tyrosine- C^{14}	1.870×10^4	0.79
Pigment.....	1.480×10^4	

cule may be incorporated into the pigment polymer, (b) the pigment formed from labeled tyrosine is not significantly diluted with endogenous tyrosine, and (c) tyrosine probably does not undergo fragmentation to any significant extent prior to its incorporation into pigment. The specific activity ratio of 0.79 suggests that some radioactive carbon atoms may be replaced by nonradioactive carbon atoms in the intermediate stages of melanin formation.

Requirements for pigmentation in T. rubrum. Previous observations demonstrated that tyrosine or phenylalanine is necessary for pigment formation in *T. rubrum*. Tyrosine and phenylalanine are structurally related, and various mammalian systems are known in which phenylalanine may be readily converted to tyrosine (White et al., 1954). In certain microorganisms shikimic acid, synthesized from carbohydrate, may be a precursor common to both tyrosine and phenylalanine, as well as to tryptophan, *p*-aminobenzoic acid and *p*-hydroxybenzoic acid (Barratt et al., 1956; Davis, 1951; Kalan et al., 1956; and Srinivasan et al., 1956). If shikimic acid were synthesized, the pigmentation observed following the addition of phenylalanine to the medium might increase the availability of energy and raw materials for the formation of pigments by the fungus. Earlier studies (Zussman, 1959) have shown that arginine, aspartic acid, histidine, glutamic acid, lysine, and serine may also initiate pigment formation but to a much lower degree than phenylalanine or tyrosine.

Pigmentation in *T. rubrum*, like melanogenesis, is dependent upon oxygen. Furthermore, both pigmentation processes are inhibited by ascorbic acid. Ito (1952) has demonstrated that melanin formation may be delayed or completely prevented in vivo by ascorbic acid, which presum-

ably acts as a reducing agent. The evidence also suggests that the pool of pigment precursors may increase during anaerobic growth since subsequent aerobic growth for a few days results in the formation of relatively large amounts of pigment.

Data obtained in this study have confirmed the findings of Lewis and Hopper (1941) and of Silva et al. (1955) that glucose or other suitable carbohydrates, such as sorbose, in low concentrations (Zussman, 1959), must be added to a culture medium for the formation of pigment by *T. rubrum*. However, the specific role of carbohydrate metabolism in pigment formation is unknown.

SUMMARY

Pigment formation was studied in submerged and aerial cultures of the dermatophyte *Trichophyton rubrum*.

The requirements for pigmentation include oxygen, a suitable carbohydrate, and certain amino acids, principally tyrosine or phenylalanine.

Water-insoluble pigments were extracted with acidified acetone, dissolved in ethylene dichloride, and separated and purified chromatographically on terra alba no. 1.

The purified water-insoluble pigments and a water-soluble purple pigment were characterized according to solubility and thermal decomposition characteristics, and by chemical and spectrophotometric analysis.

A thermolabile substance, with properties and activity similar to dihydroxyphenylalanine oxidase, was demonstrated in homogenates of *T. rubrum*.

Studies with tyrosine-2- C^{14} and tyrosine randomly labeled with C^{14} indicated that probably all or most of the carbon atoms of the tyrosine molecule are incorporated into the pigment molecule.

The nutritional and metabolic evidence presented suggests that *T. rubrum* forms melanoid pigments.

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