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Pigment Production from Tryptophan by an *Achromobacter* Species

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ABSTRACT

DUERRE, JOHN A. (University of North Dakota, Grand Forks), AND PATRICK J. BUCKLEY. Pigment production from tryptophan by an *Achromobacter* species. J. Bacteriol. **90**:1686–1691. 1965.—A microorganism was isolated from the soil near the University of North Dakota. Biochemical and morphological characteristics indicated that this organism would best be classified as a member of the family Achromobacteraceae, genus *Achromobacter*, species unknown. The organism produced a red pigment when grown in a medium containing yeast extract and tryptophan. The *p*H optimum for pigment production was about 8.0 and the optimal temperature was 25 C. During a study of the nutritional requirements for growth and pigment production, it was found that the organism would grow and produce pigment in a medium containing tryptophan and nucleosides, but the rate of both growth and pigment formation in this medium was slower than that observed with tryptophan and yeast extract. The organism grew well in the presence of acid-hydrolyzed casein and nucleosides without producing pigment, indicating that the pigment is not necessary for growth. Resting-cell experiments definitely established tryptophan as the sole exogenous requirement for pigment production. The pigment was extracted from yeast extract-tryptophan medium with chloroform. Thin layer chromatographic analysis of the crude pigment extracted from this medium revealed the presence of two other pigments in addition to the major red pigment. One of these was a highly fluorescent orange pigment and the other a pink pigment. Only the red pigment was produced by resting cells in the presence of tryptophan alone. This pigment served as an electron acceptor when coupled with formic dehydrogenase, indicating its possible function as an oxidation-reduction pigment. The oxidized pigment had absorption peaks at 506 and 304 mμ. The peak at 506 mμ disappeared upon reduction with sodium sulfite. Shaking the reduced pigment in air proved to be an unsatisfactory method for returning the reduced pigment to the oxidized, colored state.

Tryptophan has been associated with pigment production in several different organisms. Kuhn (1943) reported that tryptophan was the initial substrate for the synthesis of the ommochrome pigments found in a large number of insects. Using radiolabeled tryptophan, Fuzeau-Braesch (1957, 1958) showed that this compound was the precursor to the pigments produced in the integument of crickets. Bulenandt, Weidel, and Becker (1940) found that α-hydroxytryptophan affected eye-pigment production in Drosophila and Ephestia. Tatum and Beadle (1940) further demonstrated that this phenomenon was due to the production of a hormonal substance by certain bacteria from tryptophan.

Tryptophan in connection with pigment production by microorganisms has been demonstrated only in isolated cases, generally only as a minor aspect of some other research. A red pigment produced by *Clostridium corallinum* was described by Prévot and Raynaud (1944). They found that the addition of tryptophan caused a reduction in the period of time normally necessary for this organism to produce the pigment. Swack and Miles (1960), while studying indigotin production by *Schizophyllum commune*, noted that the addition of tryptophan to the growth medium increased the production of a red pigment. This pigment had been previously found by Miles, Lund, and Raper (1956) to be associated in very small amounts with indigotin. Recently, Polster and Svobodova (1964) showed that enterobacteria of the *Proteus-Providencia* group form a reddish-brown pigment from tryptophan. An organism isolated from the soil near this laboratory demonstrates a similar property: the production of a red pigment from tryptophan. The classification of this organism proved to be

difficult. The key to the families of Eubacteriineae in *Bergey's Manual* suggested the family Achromobacteraceae. Within this family, Achromobacter genus is the only possibility, although the organism could not be identified with any species.

The present study deals with the nutritional and physical requirements of this organism for growth and pigment production, the isolation of the pigment, and a description of some of the properties of this pigment.

MATERIALS AND METHODS

Culture and preparation of cells. The organism was isolated from soil on the campus of the University of North Dakota on a medium containing DL-tryptophan as a source of carbon. The organism was maintained on stock culture agar (Difco). Larger quantities of cells for resting-cell experiments were grown in a medium containing the following: nutrient broth (Difco), 8 g; Casamino Acids (Difco), 10 g; demineralized water, 1,000 ml. The *p*H of the medium was adjusted to 8.0 with NaOH, and the medium was distributed in 200-ml amounts in 500-ml Erlenmeyer flasks. The flasks were inoculated from a fresh stock culture of the organism, placed on a rotary shaker, and incubated for 18 to 24 hr at 23 C. The cells were separated from the medium by centrifugation at 16,000 × *g* with a GSA rotor for 15 min in a Sorvall refrigerated centrifuge. The cells were washed twice in sterile demineralized water, and were suspended in sterile water for immediate use.

Measurement of growth and pigment production. Growth and pigment production were measured in the following manner. A 5.0-ml amount of the contents of a flask under study was removed with a sterile pipette and placed in a small Pyrex test tube. The contents were centrifuged for 10 min at 8,000 × *g* with the SS-34 rotor in the Sorvall centrifuge to separate the cells from the medium. The optical density of the supernatant fluid was determined on a Coleman spectrophotometer at 500 mμ against a sterile blank taken from the flask under consideration before inoculation. The cells were suspended in 5.0 ml of water, and the turbidity was read at 450 mμ against water.

Extraction and purification of the pigment. Medium consisting of 30.0 mg of boric acid, 15.0 mg of $ZnSO_4$, 15.0 mg of $MnSO_4$, 7.5 mg of $FeCl_3$, 1.5 mg of $CuSO_4$, 1.5 mg of KI, 3.0 mg of $MgSO_4$, 112.5 g of NH_4Cl, 0.02 M phosphate buffer (*p*H 8.0), 30 g of DL-tryptophan, and 15 g of yeast extract in 15 liters of demineralized water was added to a 20-liter carboy. The contents were autoclaved for 1 hr at 121 C. The inoculum was 3.0% by volume of an 18-hr culture grown in the same medium. The carboy was aerated by passing sterile air through the culture at the rate of 9 to 10 liters per min. Silicone antifoam compound (Dow Chemical Co., Midland, Mich.) was added to prevent foaming. The carboy was incubated at 23 C for 48 hr. The cells were separated from the medium by a Szent-Gyorgi-Blum continuous-flow apparatus at 37,000 × *g*. The supernatant fluid was acidified with HCl to a *p*H of 3.0 and the red-brown pigment was extracted with chloroform. Separate extractions were carried out with equal volumes of chloroform. The resulting chloroform-pigment solution was placed in a flash evaporator to isolate the pigment and recover the chloroform for future extractions. The dry pigment was redissolved in 95% ethyl alcohol and was filtered through a Millipore filter. A small amount of pigment was further purified by paper chromatography. Whatman 3mm paper was used and an ethyl alcohol-water-acetic acid solvent system (65:34:1) was employed. The bright-red pigment was eluted from the paper with ethyl alcohol and water (1:1). The absorption spectrum of the pigment from 210 to 800 mμ was determined in both aqueous and ethanolic solutions by use of a Beckman DB recording spectrophotometer.

Thin-layer chromatography of the pigment. Thin-layer chromatography was employed to determine the purity of the pigment. The Serva thin-layer chromatography system (Gulliard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.) was employed with the use of a Serva-TLC cellulose. A slurry, made up of seven parts of water to one of cellulose, was prepared. This was homogenized in a Waring Blendor to break up larger particles. The slurry was poured onto glass plates (10 by 17.8 cm) which had been thoroughly cleaned. A layer of 500 μ thickness was left on the plate by use of the Serva applicator. The plates were dried overnight at room temperature before use. Various amounts of the crude pigment extracted from the yeast extract-tryptophan medium, pigment extracted from the resting-cell experiments, and the paper-purified pigment, ranging from 2 to 5 μliters, were placed in spots about 3.0 cm from the bottom of the glass plate. Ethyl alcohol-water-acetic acid (65:34:1), ethyl alcohol-water-1.0 N NaOH (65:34:1), and benzene-ethyl alcohol (50:50) solvent systems were employed. The plates were placed on end in a cylindrical, covered, glass container with the solvent system at a depth of 1.5 cm.

Chemicals. All vitamins, purines, pyrimidines, nucleosides, and nucleotides were obtained from Calbiochem. DL-Tryptophan and vitamin-free, acid-hydrolyzed casein were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. All other chemicals were supplied by Fisher Scientific Co., Pittsburgh, Pa., or Mallinckrodt Chemical Works, St. Louis, Mo.

RESULTS

Properties of the microorganism. The organism is a gram-negative, nonmotile rod, 0.8 by 1.0 to 2.0 μ, with rounded ends. In broth culture, the organisms occur singly and in pairs. On gelatin or nutrient agar, the organism forms small, round, smooth, milky-white colonies. Table 1 gives the reactions with various carbohydrates

TABLE 1. *Action of the organism on carbohydrates**

Carbohydrate	Day			
	1	2	3	5
Glucose	Acid	Acid, slight gas	Acid, gas	Acid, gas
Mannose	Acid	Acid, slight gas	Acid, gas	Acid, gas
Lactose	Alkaline top, slight acid bottom	Alkaline top, slight acid bottom	Alkaline top, slight acid bottom	Alkaline
Maltose	Alkaline top, slight acid bottom	Alkaline top, slight acid bottom	Alkaline top, slight acid bottom	Alkaline top, slight acid bottom
Mannitol	Acid	Acid	Acid	Acid, slight gas
Salicin	Acid	Acid	Acid	Acid, slight gas
Saccharose	Acid	Slight alkaline top, Acid bottom	Slight alkaline top	Acid, slight gas
Adonitol	Acid	Acid, slight gas	Acid, slight gas	Acid, gas
Sorbitol	Acid	Slight alkaline top	Acid	Acid, slight gas
Inositol	Acid	Acid	Acid	Acid

* Fermentation tests were performed with phenol red broth base (Difco) with 0.5% carbohydrate added. Tubes were inoculated with 24-hr nutrient broth culture and were incubated under stationary conditions at 25 C.

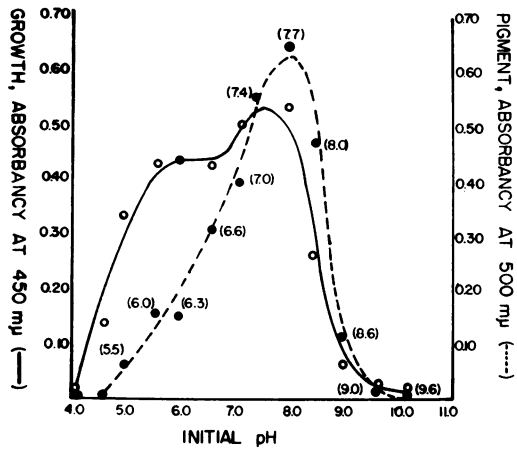

FIG. 1. *Effect of pH on growth and pigment production. Medium employed contained salts, 2.0 g of* DL-*tryptophan and 1.0 g of yeast extract per liter; pH values from 4.0 to 8.0 were obtained with 0.02 M phosphate buffer, and pH values from 7.5 to 10.0 were obtained with 0.02 M Tris buffer. Vigorous aeration was obtained by placing 100 ml of medium in 500-ml flasks on a rotary shaker. Each flask was inoculated with 0.5 ml of a solution of washed cells giving an optical density reading of 0.64 at 450 mμ. Incubation time was 24 hr. Numbers in parentheses indicate final pH of medium.*

after 5 days of incubation at 23 C. The organism did not hydrolyze starch or gelatin, hydrolyzed urea, was catalase-positive, produced nitrites from nitrates, was methyl red-positive, Voges-Proskauer-negative, and indole-negative. Litmus milk remained unchanged, and citrate was utilized as a sole source of carbon. The optimal temperature for growth was found to be 25 C, with little growth occurring below 18 C or above 38 C.

Effect of pH on growth and pigment production. The effect of *p*H on growth and pigment production is shown in Fig. 1. Growth was maximal from *p*H 5.5 to 8.0. The cells became chromogenic, assuming a dark red-brown color at *p*H values above 7.5, which may account for some of the increase in turbidity over this region. Pigment production was optimal at 8.0, falling off rapidly above 8.0 and below 7.0.

Nutritional requirements for growth and pigment production. The organism grew well and produced abundant pigment when grown in a medium containing yeast extract and tryptophan (Table 2). Experiments were undertaken to define the nutrients present in yeast extract since tryptophan alone would not support growth or pigment production (Table 2). The organism would grow and produce pigment when a mixture of nucleosides was substituted for yeast extract, but growth and pigment production occurred at a slower rate. Nucleotides substituted for yeast extract did not support growth or the production of pigment as well as nucleosides did. The further addition of a mixture of the following vitamins was without effect: 70 μg of choline, 75 μg of ascorbic acid, 1 μg of biotin, 200 μg of thiamine, 200 μg of riboflavine, 10 μg of niacin, 2 μg of pyridoxal, 200 μg of pantothenate, 300 μg of inositol, 200 μg of *p*-aminobenzoic acid, and 100 μg of folic acid per 100 ml of medium.

When vitamin-free acid-hydrolyzed casein was

TABLE 2. *Effect of nutritional conditions on growth and pigment production*[a]

Supplement[b]	12 hr		24 hr	
	Growth	Pigment	Growth	Pigment
Tryptophan (0.2 g/100 ml)	0.05	0.04	0.14	0.1
Tryptophan + yeast extract (0.1 g/100 ml)	0.48	0.52	0.86	0.63
Tryptophan + nucleosides[c]	0.09	0.07	0.26	0.22
Tryptophan + nucleotides[d]	0.06	0.03	0.13	0.10
Tryptophan + nucleosides + vitamins	0.1	0.05	0.24	0.15
Hydrolyzed casein[e] (1.0 g/100 ml)	0.77	0.05	1.0	0.1
Hydrolyzed casein + tryptophan	0.95	0.77	1.26	0.7
Hydrolyzed casein + nucleosides	0.90	0.05	1.0	0.1
Hydrolyzed casein + nucleosides + tryptophan	1.0	0.68	1.3	0.6

[a] Growth and pigment are expressed as the optical density at 450 and 500 mμ, respectively.
[b] Medium employed contained salts and 0.02 M phosphate buffer (*p*H 8.0) in addition to components listed on the table. Other conditions similar to Fig. 1.
[c] Nucleosides were adenosine, guanosine, uridine, cytidine, and thymidine (1.0 mg each per 100 ml).
[d] Mixture of 2′,3′-monophosphate of corresponding nucleosides.
[e] Vitamin-free, acid-hydrolyzed casein.

added to a medium containing tryptophan and nucleosides, excellent growth and pigment production were achieved (Table 2). Since tryptophan is destroyed during acid hydrolysis of casein, its effect on pigment production could be measured in such a medium. If tryptophan was deleted, the microorganisms grew well, although not quite to the extent obtained with tryptophan. The effect of tryptophan on pigment production was quite pronounced, with the organisms producing only traces of pigment in its absence. When the nucleosides were omitted, growth and pigment production were not reduced significantly. Experiments were undertaken to determine whether nucleosides or amino acids other than tryptophan were essential for pigment production. Figure 2 shows that only tryptophan was required for the production of pigment with resting cells. The further addition of nucleosides enhanced pigment production slightly, whereas hydrolyzed casein supressed pigment production by resting cells for the first 2 to 3 hr. The deletion of salts from the medium did not affect the ability of the organism to produce pigment from tryptophan in 0.02 M phosphate buffer (*p*H 8.0). In resting-cell experiments, pigment production was quite rapid, appearing in less than 1 hr at 23 C. It was also found that pigment production did not occur when indole was substituted for tryptophan.

Pigment properties. Difficulty was encountered at first with the chloroform extraction of the pigment. It was discovered, however, that lowering the *p*H to about 3.0 with HCl decreased the solubility of the pigment in water, facilitating the extraction with chloroform. The addition of

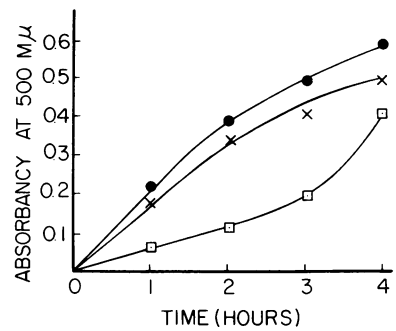

FIG. 2. *Pigment formation by resting cells. Cells were harvested as described in Materials and Methods. Washed cells were added to the incubation mixture to give a final optical density reading of 0.75 at 450 mμ. Flasks were incubated at 23 C on a rotary shaker. In addition to components listed below, the incubation mixture contained salts and 0.02 M phosphate buffer (pH 8.0). Symbols: × = DL-tryptophan (0.2 g/100 ml); ● = tryptophan and nucleosides; ⊡ = tryptophan and acid-hydrolyzed casein (1.0 g/100 ml).*

acid turned the cell-free pigment-medium solution a much darker red-brown color. During the extraction, a thick, dark-brown substance formed between the water and chloroform phases. The nature of this material was not determined.

Thin-layer chromatography of the crude pigment extracted from the yeast extract-tryptophan medium revealed the presence of two other pigments in addition to the major red pigment. Employing the ethyl alcohol-water-1.0 N NaOH (65:34:1) solvent system, the following was noted: a small amount of a highly

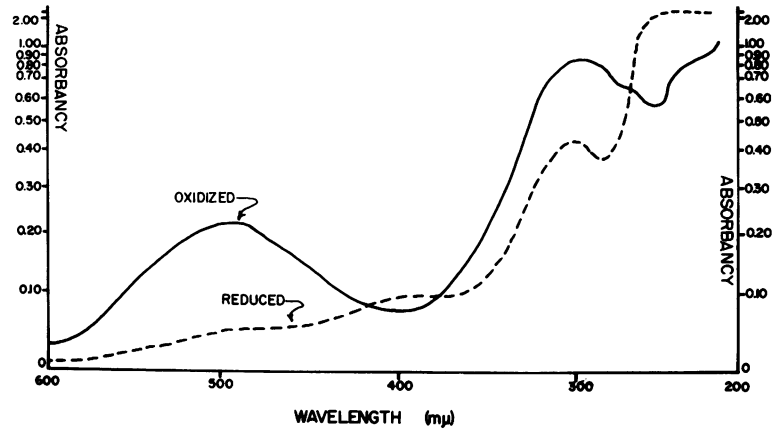

FIG. 3. *Spectrum of the oxidized and reduced pigment. The pigment was dissolved in ethyl alcohol and reduced with 20 mg/ml of* Na_2SO_3.

fluorescent bright-orange pigment, R_F 0.65; a large amount of the red pigment, R_F 0.71; and a small amount of a faintly-pink pigment, R_F 0.92. The ethyl alcohol-water-acetic acid (65:34:1) solvent system resolved only two pigments, the red pigment with an R_F of 0.74 and a red-orange pigment having an R_F of 0.96. The presence of the orange pigment was also noted in the paper chromatographic purification of the crude pigment extract. It was concentrated in an area just ahead of the red pigment.

The appearance of the pink and orange pigments raised some question as to whether these were also produced from tryptophan or from some other substance in the yeast extract. To determine this, pigment produced by resting cells in a salts-phosphate buffer-tryptophan medium was extracted and subjected to thin-layer chromatography by use of the ethyl alcohol-water-1.0 N NaOH solvent system. The only pigment detected was the red pigment.

It was found that the red pigment which had been purified by paper chromatography could be reduced by adding Na_2SO_3 (20 mg/ml) to the pigment in either ethyl alcohol or water. Upon reduction, the pigment became colorless. Shaking the pigment in air proved to be an unsatisfactory method for returning it to the oxidized (colored) state. The absorption spectrum of the oxidized pigment (Fig. 3) shows peaks at 506 and 304 mμ. The reduced pigment lost the absorption peak at 506 mμ. A decrease in absorbancy at 304 mμ was also observed. If pigment was purified further by thin-layer chromatography, it did not have a significantly different absorption spectrum. The possibility that the pigment may function as an electron acceptor in oxidation-reduction reactions was tested by the Thunberg technique for the estimation of dehydrogenase activity (Umbreit, Burris, and Stauffer, 1964). The pigment was diluted 1:10,000 with water and substituted for methylene blue as the electron acceptor, with formate as substrate and washed-cell suspensions of *Escherichia coli* as the source of formic dehydrogenase (Billen and Lichstein, 1950). It required about 20 min to reduce the red pigment to the colorless state as compared with 4 min to reduce methylene blue under similar conditions.

DISCUSSION

Polster and Svobodova (1964) reported the production of a red-brown pigment from tryptophan. They suggested that this property might be used as a characteristic of the *Proteus-Providencia* group of enterobacteria. A careful study of this group and the other enterobacteria led us to conclude that the organism dealt with in this paper cannot belong to this group. The fact that the organism hydrolyzed urea dismisses the *Providencia* group and the lack of motility, lack of indole production, and lower temperature optimum demonstrated by this organism make membership in the *Proteus* group very doubtful. With the present information, the genus *Achromobacter* in the family Achromobacteraceae seems to be the best choice for the organism.

The organism was first isolated from the soil and grown in a yeast extract medium in which tryptophan was employed as a source of carbon. The fact that the organism did not produce pigment on ordinary nutrient media as it did in the presence of tryptophan indicated that this amino acid was necessary for pigment production. Pigment production also seemed to be dependent upon the concentration of cells; pigment was pro-

duced slowly and only in small amounts by cultures not exhibiting good growth. These findings led us to study the nutritional elements present in the yeast extract which contributed to growth and pigment production.

Having noted that the B vitamins or purines and pyrimidines would not support growth of the organism, we tested nucleosides and found them to support growth to a certain extent. The reason nucleotides did not function as well in this capacity may be one of permeability. The stimulatory effect of acid-hydrolyzed casein on growth as well as pigment production was demonstrated. Resting-cell experiments were performed to establish whether nucleosides or acid-hydrolyzed casein had any direct effect on pigment production or whether their ability to stimulate growth was the cause of the increase in pigment. Data from this as well as other experiments point to the fact that tryptophan is the sole exogenous requirement for the production of pigment by the organism. The possibility that metal ions are required has not been ruled out. However, their further addition to the resting cells was unnecessary for pigment production. The fact that metal ions may have been present in large enough concentrations within the cells is a matter for further investigation.

The separation of both pink and orange pigments from the crude red pigment by thin-layer chromatography raised some question as to the role of tryptophan in their production. Pigment extracted from resting cells did not contain these additional substances. This indicates that they are not produced by the organism from tryptophan alone. These substances are probably produced from tryptophan and some other compound(s) in the yeast extract or perhaps from these compounds alone.

The data have established that the pigment is not necessary for growth. This raises the question of what function the pigment has, if any. The fact that the pigment is reduced to a colorless state outside the cell by Na_2SO_3 suggests its possible function as an oxidation-reduction pigment. The role of the pigment as an electron acceptor when coupled with formic dehydrogenase lends further credence to this hypothesis. Further experimentation to elucidate the structure and additional properties of this compound, as well as possible metal ion requirements, promises to throw more light on this subject.

ACKNOWLEDGMENTS

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