PHYSIOLOGICAL ASPECTS OF VIOLACEIN BIOSYNTHESIS IN NONPROLIFERATING CELLS[1]

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Received for publication April 20, 1959

The chemical synthesis of violacein, a pigment formed by *Chromobacterium violaceum*, has been reported by Ballantine *et al.* (1958). The biologically formed pigment had been previously suggested to consist of three moieties, 5-hydroxyindole, oxindole, and a β-hydroxypyrrole (Ballantine *et al.*, 1957*a*, *b*). The nutritional requirements and certain physiological factors involved in growth of the organism have been described (DeMoss and Happel, 1959). It was of interest to investigate in detail the biosynthetic pathway, since the results of such studies would be expected to permit elucidation of the factors which control pigment synthesis in relation to those metabolic sequences which are necessary to the growth process. The apparent separability of the pigment-generating and growth processes was observed in previous experiments, in conjunction with nutritional studies (DeMoss and Happel, 1959). The present report describes experiments leading to the development of conditions for violacein biosynthesis in nonproliferating cell suspensions.

METHODS AND MATERIALS

Bacteriological. C. violaceum strain ATCC 553 was grown on CV[2] medium and maintained as described previously (DeMoss and Happel, 1959). For the production of fresh cells, 1.0 ml of a 24-hr culture (30 C with shaking) was introduced into 90 ml of medium in a 500-ml Erlenmeyer flask and incubated 16 hr at 30 C with shaking. The cells, harvested by centrifugation, were usually of a buff or light purple color, and were washed twice in one third the original growth volume of salts F, pH 6.5 (per 100 ml: $FeSO_4 \cdot 7H_2O$, 0.5 mg; $MgCl_2$, 100 mg; K_2HPO_4, 600 mg; K_2SO_4, 50 mg) and resuspended in a suitable volume of the same salts solution.

Pigment determination. The reaction mixtures were incubated in 125-ml Erlenmeyer flasks fitted with rubber stoppers. The flasks were agitated on a rotary shaker at 30 C and samples were removed at suitable intervals.

Pigment was quantitated as optical density units at 565 mμ, in the Evelyn colorimeter, using 18-mm tubes or in the Beckman spectrophotometer, model DU. Under this application, optical density in the Beckman at 565 mμ may be multiplied by the factor 1.52 to convert to Evelyn units. Unless otherwise expressed, the amount of pigment (mμmoles) was calculated from Evelyn readings, using the value 17.0×10^6 cm^2 per mole as the extinction coefficient.

To a reaction mixture containing pigment were added 2 volumes of acetone. The mixture was chilled in an ice bath for 15 min, centrifuged, and the optical density of the supernatant determined at 565 mμ. Where necessary, the rate of pigment synthesis is expressed as the change in optical density per unit time per mg of cells (dry weight). Turbidity readings of cell suspensions were converted to dry weight values by means of a previously constructed curve relating the two parameters.

Source of chemicals. The amino acids were purchased from Nutritional Biochemicals Corporation. Chloromycetin was provided by Dr. A. Nason. Aureomycin, tetracycline, erythromycin, tyrothricin, filipin, and endomycin were obtained from Dr. A. T. Jagendorf. A generous sample of 3-hydroxyanthranilic acid was supplied by Dr. M. Speeter. Dr. L. E. Rhuland kindly provided samples of isatin hydrazone, 2-isonitroso-3-phenylpropionic acid, 2-carboxy-4-hydroxyquinoline, *N*-(*o*-carboxyphenyl)-glycine, and *o*-formamidotoluene. The indole derivatives listed in table 4 were generously supplied by Smith, Kline, and French Laboratories, or Lederle Laboratories, as indicated.

RESULTS AND DISCUSSION

A study of growth and violacein production as a function of time had demonstrated that pig-

[1] This research was supported in part by grants from the U. S. Public Health Service (E-1467, E-1626) and the National Science Foundation (G-4023).

[2] Refers to medium used for production of cells of *Chromobacterium violaceum*.

ment synthesis was not evident until the culture had incubated for 16 to 20 hr. Under the conditions employed (CV medium, 30 C incubation) the rate of growth ceases to be logarithmic at about 16 hr. Thus, it would appear that concomitant growth is not a requirement for pigment synthesis.

Following the observation that cells harvested from nutrient broth cultures were capable of synthesizing pigment when supplemented with an amino acid mixture or spent medium, i. e., the supernatant from harvested cultures, the requirements for pigment synthesis were determined. The standard assay procedure consisted of supplementing the test cells with tryptophan and alanine, or tryptophan alone, in potassium phosphate buffer, and incubating the reaction mixture with shaking at 30 C (table 1). At intervals, aliquots were removed for the determination of pigment.

The data of table 1*A* suggest that 16 to 18-hr cultures, grown in CV medium, provided cells with maximal activity for pigment synthesis under the conditions used. For technical reasons, the synthetic activity of younger cultures was not tested. The resuspension of harvested cells from a 10 to 12 hr-culture almost invariably

TABLE 1

Pigment synthesis: age of cells

A		B	
Age of culture	Activity	Storage time	Activity
hr	*mμmoles/hr/mg cells*	*hr*	%
14	10.4	0	100
16	14.4	24	51
18	13.9	48	10
20	13.2		

A: The activity of freshly harvested cells was determined under standard conditions. Protocol: fresh cell suspension; 24 μmoles L-tryptophan; 400 μmoles phosphate buffer, pH 7.0; total volume, 15.0 ml. The reaction mixture was incubated in a rubber stoppered 125-ml Erlenmeyer flask at 30 C on a New Brunswick model VS rotary shaker operating at 200 rpm. Two-ml samples were taken at 1-hr intervals and pigment measured as described in the text.

B: The activity of washed cell suspensions was tested under standard conditions after storage intervals at 4 C. Activity is expressed as per cent of original activity remaining.

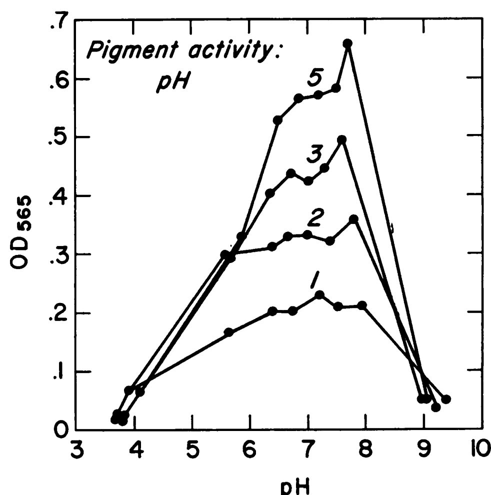

Figure 1. Effect of pH on pigment synthesis. The amount of pigment synthesized, expressed as optical density (Evelyn) at 565 mμ, is plotted as a function of pH at 1-, 2-, 3-, and 5-hr reaction times. At the time intervals indicated, samples were removed for estimation of both pigment and pH. A Beckman pH meter, model G was employed. The reaction mixture, containing 40.8 mg cells, dry weight, complied with standard conditions, with the exception that pH was varied by adding appropriate amounts of phosphoric acid or its potassium salts, while maintaining a constant phosphate concentration.

resulted in rapid and extensive lysis, as determined by optical density readings. This phenomenon was particularly evident in distilled water. In fact, the low activity of 14-hr cells is due to partial lysis. The loss of activity from cells grown in CV medium and stored at 4 C is shown in table 1*B*, a result which dictated the use of freshly harvested cells for each experiment.

The data presented in figure 1 indicate that the maximal rate of pigment synthesis occurs between pH 7 and 8 under the conditions employed. It may be noted that during the growth, the final pH of cultures tends to approach pH 7.0. The substitution of Veronal or tris-(hydroxymethyl)aminomethane for phosphate at pH 8.0 resulted in only slight differences in the rate of violacein synthesis, whereas borate effected an 80 per cent inhibition.

To determine the effect of tryptophan concentration on pigment synthesis, a series of reaction mixtures was prepared, containing varying amounts of DL- or L-tryptophan. It may

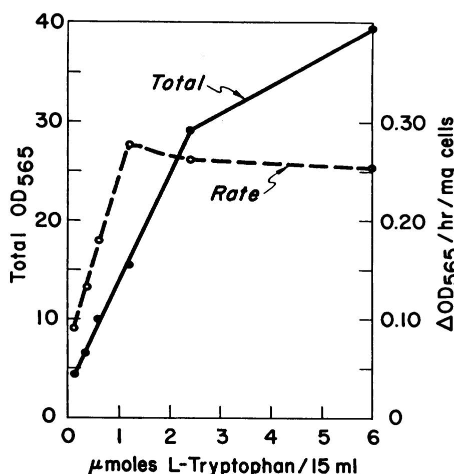

Figure 2. Effect of tryptophan concentration on the rate and extent of pigment synthesis. Standard conditions were used with 47.6 mg cells, dry weight. Total pigment was taken from the value estimated after 4 hr incubation. No further increase in pigment occurred except at the highest tryptophan concentration. Optical density was estimated in the Evelyn colorimeter and is expressed as the calculated value equivalent to the Beckman reading.

be noted from the data of figure 2 that, although the total pigment formed is a linear function of tryptophan concentration, the cells synthesized additional pigment from endogenous material. To correct for pigment produced from endogenous sources and for pigment which may have been present at zero time, the blank value, with no tryptophan added, was calculated by extrapolation of the data of figure 2. This method of correction was followed, in place of a separate blank reaction mixture, since the effect of added tryptophan on endogenous pigment synthesis is unknown. From the data of figure 2 and of 19 additional experiments, 1 μmole of L-tryptophan was calculated to yield a total amount of pigment which is equivalent to 9.11 OD_{565} units (Beckman). This would correspond to an extinction coefficient of 18.22×10^6 cm^2 per mole at 565 mμ, assuming that two moles of tryptophan yield one mole of pigment. This assumption seems reasonable in view of the possible structures for violacein suggested by Ballantine *et al.* (1957*b*). Their analyses were consistent with a 2,3-substituted pyrrole, a 1,3-substituted pyrrole or a 2,4-substituted pyridine. A more recent report (Ballantine *et al.*, 1958) indicates that the correct structure for violacein is 5-[3-(5-hydroxyindolyl)]-3-(3-isatinyl)-2-pyrrolone. It should be pointed out that in the estimations of pigment yield the optical density of crude pigment samples was measured.

Using chromatographically purified, but noncrystalline, pigment (blue component), three separate determinations of the extinction coefficient at 565 mμ yielded 14.92, 16.92, and 15.28×10^6 cm^2 per mole. The calculations assumed a molecular weight of 357 for the blue component of the pigment. The corrected extinction coefficient is probably near 17×10^6 cm^2 per mole, since the purified pigment sample was known to contain at least 1.09 per cent metals by weight, including aluminum. The latter was derived from the alumina chromatographic column by means of which the pigment had been purified.

An additional complication is introduced by the fact that the crude pigment samples always contain both the blue and purple components. In numerous experiments, the blue and purple components have been separated either by paper sheet or alumina column chromatography. The mean ratios of the total optical densities of the blue to purple components was 5.9 and 15.9, respectively, by the two methods used. Since the alumina column separation requires less time, the ratio 15.9 is probably more representative of the situation in the reaction mixture. Thus, the purple component probably contributes significantly in the pigment yield determinations, but the extent of error introduced is unknown, since it has not yet been possible to determine the extinction coefficient for the purple component.

Pigment formation is not affected by the addition of D-tryptophan to reaction mixtures containing L-tryptophan. Pigment was not synthesized from D-tryptophan alone. Thus, with respect to pigment formation, D-tryptophan is inert.

As noted in table 2, the presence of additional substrates in the standard reaction mixture inhibited the rate of pigment synthesis. It is unlikely that the inhibition can be attributed to utilization of tryptophan for growth of the cells since methionine, which is required for growth, was not added to the reaction mixture. The ability of the organism to oxidize the added substrates was verified in separate experiments.

TABLE 2

Pigment synthesis: effect of added substrate

Addition	Activity	
	mμmoles/hr/mg cells	*% of control*
None	34.6	100
Citrate	30.0	86
Succinate	23.3	67
Glucose	22.1	64
Pyruvate	19.3	56
Glycerol	18.6	54
Acetate	15.8	45

Standard conditions of assay were used with 25.6 mg cells, dry weight. Each added substrate was present at 0.02 M final concentration. The acids indicated were added as the sodium salts.

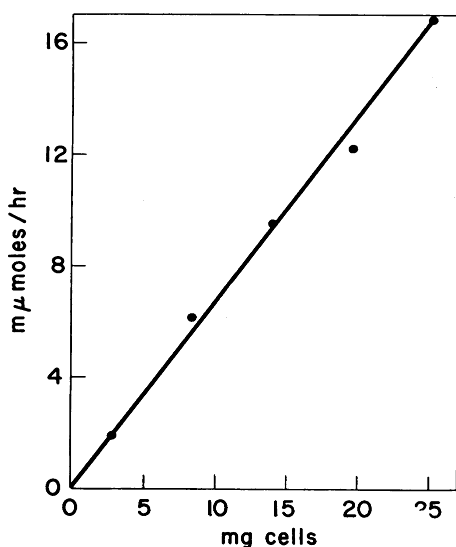

Figure 3. Rate of pigment synthesis as a linear function of cell concentration. Activity was estimated under standard conditions except that cell concentration was varied.

Under the conditions employed, the rate of violacein synthesis was found to be directly proportional to cell concentration. At each cell concentration tested in figure 3, the rate of pigment formation was linear to at least 5 hr. Although the rate of pigment synthesis varied slightly from one cell suspension to another, a linear relation between activity and cell concentration was always observed.

Under an atmosphere of air, 134 mμmoles of pigment were formed, whereas under nitrogen, either with or without added nitrate, no pigment was synthesized. It is concluded that molecular oxygen is required for the conversion of tryptophan to violacein, although possible electron acceptors other than nitrate were not tested. The results obtained were not unexpected since the pigment contains a 5-hydroxyindole residue, which, for formation from tryptophan, would be expected to require a hydroxylation step. Most biological hydroxylation reactions thus far reported exhibit a requirement for molecular oxygen (Mason, 1957).

The effect of growth temperature on the pigment synthesizing activity of the cells is presented in table 3. It is apparent that cells grown at 30 C are more active than cells grown at 37 C. Although the initial rate of pigment synthesis is greater when tested at 37 C, the total amount of pigment formed is larger at 30 C. These observations are consistent with the results which indicate that the pigment forming system is relatively unstable (table 1).

The effect of possible inhibitors on the pigment-forming system was tested with results as presented in tables 4, 5, and 6. Indole analogues were used in attempts to either inhibit or alter pigment synthesis. No change in the absorption peak was noted, nor was the blue-purple component ratio altered. Assuming that pigment formation is endergonic, dinitrophenol and azide were included in an attempt to distinguish between substrate level and oxidative phosphorylation as possible energy sources. Arsenate was tested as a means of indicating possible

TABLE 3

Pigment synthesis: effect of temperature

Temperature		Pigment Synthesis			
Growth	Incubation	Rate		Total	
C	*C*	*mμmoles/hr/mg cells*	*%*	*mμmoles*	*%*
30	30	6.7	100	1873	100
30	37	9.1	131	1386	74
37	30	4.7	70	1146	61
37	37	6.0	89	1215	65

Activity estimated under standard conditions with 44 mg cells, dry weight. Cells were harvested from 16.5-hr cultures at both growth temperatures. The yields of cells were nearly identical in the two cultures. Total pigment was the amount estimated after no further increase was observable.

TABLE 4

Pigment synthesis: effect of indole analogues

Source*	Compound Added		OD_{565}
		μg/ml	% of control
	None		100
LL	5-Methyltryptophan	6.25	87
U	3-Hydroxyanthranilic acid	6.25	87
LL	5-Ethylcarbazole	12.5	94
LL	1,2,3,4-Tetrahydrocarbazole	12.5	82
LL	2,3-Cycloheptenoindole	12.5	110
LL	3-(*o*-Chlorobenzyl)-1-hydroxyoxindole	3.1	113
LL	2,3-Dioxo-6-nitroindoline	12.5	35
LL	6-Ethylisatin	12.5	94
LL	6-Propylisatin	12.5	102
LL	4-Chloroisatin	12.5	83
LL	4-Chloro-7-methoxyisatin	12.5	75
LL	4,5-Tetramethyleneisatin	12.5	75
SKF	2-Methylindole	12.5	89
SKF	2,3-Dimethylindole	12.5	68
SKF	3-Ethyl-2-methyl-5-nitroindole	12.5	71
SKF	2-Phenylindole	12.5	62
SKF	2,3-Diphenylindole	12.5	87
SKF	β-Diethylaminoethylindole-3-acetate	12.5	93
LER	Isatin hydrazone	12.5	93
	Kynurenine	12.5	77
LER	2-Isonitroso-3-phenylpropionic acid	12.5	83
LER	2-Carboxy-4-hydroxyquinoline	12.5	94
LER	*N*-(*o*-Carboxyphenyl)-glycine	12.5	88
LER	*O*-Formamidotoluene	12.5	101

Activity estimated under standard conditions with 18 mg cells, dry weight. Each compound was added as a solution, usually in 95 per cent ethanol. A control was included with an equivalent amount of ethanol; no effect was observed in comparison to a control without ethanol. Activity is expressed as the relative change in optical density during the 1 to 4 hr interval.

* Sources of compounds were: LL, Lederle Laboratories; SKF, Smith, Kline, and French Laboratories; LER, Dr. L. E. Rhuland; U, Dr. M. Speeter, The Upjohn Company.

TABLE 5

Pigment synthesis: effect of inhibitors

Addition		Activity	
	M	mμmoles/hr/mg cells	% of control
None		11.0	100
KCN	10^{-3}	5.3	48
NaN_3	10^{-3}	10.7	97
Na_2HAsO_4	2×10^{-3}	10.7	97
Na_2HAsO_3	2×10^{-3}	0.5	4
DNP	10^{-4}	6.1	55
N_2H_4	10^{-2}	1.3	12
$NaHSO_3$	10^{-2}	10.9	99

Activity estimated under standard condition with 44 mg cells, dry weight. The final concentration of added inhibitors is given.

TABLE 6

Pigment synthesis: effect of metal ions

Addition	Activity	
	mμmoles/hr/mg cells	% of control
None	31.6	100
Mg^{++}	32.3	102
Na^{+}	31.6	100
Ca^{++}	31.6	100
Fe^{+++}	28.9	91
Mn^{++}	26.6	84
Co^{++}	23.8	75
Sn^{++}	18.5	58
Cu^{++}	0.0	0
Hg^{++}	0.0	0

Activity estimated under standard conditions with 36 mg cells dry weight. All metal ions were present as the chloride salts at a final concentration of 0.005 M.

TABLE 7

Pigment synthesis: effect of antibiotics

Addition		Activity	
	μg/ml	mμmoles/hr/mg cells	% of control
None		17.5	100
Tyrothricin	67	4.1	23
Tyrothricin	47	6.6	38
Tyrothricin	20	10.3	59
Tyrothricin	7	14.7	84
Gramicidin	10	6.9	39

Activity estimated under standard conditions with 36.6 mg cells, dry weight. The final concentration of antibiotics is given.

substrate level phosphorylation or acyl transfer. The possibility of an intermediate oxidation of α-keto acid (e. g., indole pyruvate) was supported by the inhibition due to arsenite. Cyanide, hydrazine, and bisulfite were added as carbonyl trapping agents. In addition to the compounds listed, the following antibiotics were without inhibitory activity when tested at a final concentration of 67 μg per ml: penicillin, chloromycetin, streptomycin, aureomycin, erythromycin, filipin, endomycin. Tetracycline and tyrothrycin, at the same level, 67 μg per ml, inhibited the rate of violacein formation by 40 and 90 per cent, respectively. The results of further experiments with tyrothricin are recorded in table 7, along with a test of gramicidin activity. It would appear that the tyrothricin activity is primarily the result of the gramicidin component since tyrothricin contains 10 to 20 per cent gramicidin. The mechanism of this inhibition is not clear on the basis of the data presently available. Penicillin was tested at somewhat higher concentrations in the growth medium. At levels of penicillin from 0 to 200 μg per ml, growth and pigment synthesis after 24 hr were essentially unaffected. These experiments were initiated to determine the feasibility of using the penicillin technique for selection of mutants. However, these results, together with the knowledge that the organism is morphologically and physiologically similar to the pseudomonads provide evidence that *C. violaceum* is not an optimal candidate for the application of the penicillin technique.

From the fact that *C. violaceum* requires added methionine for growth (DeMoss and Happel, 1959), it follows that little growth should occur in reaction mixtures devoid of methionine. The contention that pigment synthesis, under the conditions used here, has proceeded in the absence of growth is further supported by observations in several experiments. After 4 hr incubation, the final optical density of the cell suspension, measured at 720 mμ in the Evelyn colorimeter, usually decreased, and did not, in any experiment, increase by more than 13 per cent of the original value. Further support for the belief that growth of the cells did not occur may be derived from the data of table 3 in which total pigment production is shown to be a function of limiting tryptophan concentrations. In the experiments of table 3 it is apparent that the quantity of tryptophan added is insufficient to satisfy the energy, carbon, and nitrogen requirements for growth.

SUMMARY

The purple pigment violacein is synthesized by nonproliferating cells of *Chromobacterium violaceum*. L-Tryptophan was the sole required carbon source for pigment synthesis. D-Tryptophan was not converted to pigment and had no effect on pigment synthesis. The amount of pigment formed is proportional to the amount of L-tryptophan present when the latter is in limiting concentration. The extinction coefficient, determined by two independent methods, was calculated to be approximately 17×10^6 cm^2 per mole at 565 mμ.

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