INCORPORATION OF C^{14}-LABELED SUBSTRATES INTO VIOLACEIN[1]

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Previous studies on the physiological aspects of violacein biosynthesis (DeMoss and Evans, 1959) suggested that only L-tryptophan would serve as the primary source of carbon for pigment formation by *Chromobacterium violaceum*. In view of the pigment structure suggested by Ballantine *et al.* (1958), 5-[3-(5-hydroxyindolyl)]-3-(3-isatinyl)-2-pyrrolone, it seemed advisable to determine the actual source of the pigment carbon, and to determine if the pyrrolone residue is formed via an independent metabolic pathway.

The data presented suggest that tryptophan is probably the sole precursor of violacein carbon; furthermore, the formation of the pyrrolone moiety is a process involving tryptophan metabolism and does not involve the mediation of the glycine-succinate pathway for pyrrole synthesis described by Shemin (1955).

MATERIALS AND METHODS

C. violaceum strain 553 was grown and harvested as previously described (DeMoss and Evans, 1959). Violacein synthesis by nonproliferating cells in the presence of C^{14}-labeled substrates formed the basis for incorporation studies. The reaction mixtures always contained tryptophan in addition to the labeled substrates. Reaction mixtures were shaken at 30 C in rubber-stoppered 125-ml Erlenmeyer flasks for 5 to 20 hr as indicated.

Two methods, involving paper sheet or alumina column chromatography were employed to purify pigment samples for assay of radioactivity.

Paper sheet chromatography. At the end of the incubation period, the entire reaction mixture was extracted exhaustively with ethyl acetate to remove pigment. The pigment solution was evaporated to dryness *in vacuo*, and the residue extracted with 2 to 3 ml of absolute methanol. The methanolic solution was streaked on Whatman no. 1 filter paper and irrigated overnight at room temperature in a solvent system consisting of isopropanol-NH_4OH (concentrated reagent)-water; 8:1:1 (v/v). The portions of the chromatogram containing the blue (R_f approximately 0.53) and purple (R_f approximately 0.79) components were cut into long narrow strips and extracted with 10 to 15 ml absolute methanol in an apparatus suggested by Dr. Harry Beevers. The strips were hung from a cold finger inside a slightly larger tube and extracted under reflux conditions. The blue component was determined quantitatively by suitable dilution and measurement at 565 mμ in the Beckman model DU spectrophotometer. The extinction coefficient was previously determined to be approximately 17.0×10^6 cm^2 per mole (DeMoss and Evans, 1959). After the quantity of blue pigment was determined, the ethanolic solution was evaporated to a small volume or to dryness and assayed for C^{14} content.

Alumina column chromatography. The residue from the ethyl acetate solution of crude pigment was dissolved in 5 to 8 ml acetone and adsorbed on an alumina column. Aluminum oxide, Merck reagent grade, was washed with 2 N HCl, followed by repeated washing with distilled water until the pH of the wash water had risen to at at least 5.0. After drying overnight at 95 C, the alumina was activated by heating for 2 hr at 300 C. The adsorption column was prepared after suspending the activated alumina in reagent grade benzene. The column, 12 cm by 18 mm diameter, was capped with a filter paper disc to minimize deformation of the top of the column by addition of solvent. After addition of the acetone solution of pigment, the column was washed briefly with 10 to 15 ml of acetone. The purple component was eluted with acetone-methanol (95:5, v/v) after which the blue component was eluted with acetone-methanol (50:50, v/v). Good separation of the two components was usually obtained. If the separation was not clean, the fractions which contained both

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components were discarded. The fractions containing blue pigment were combined, evaporated, and assayed for C^{14} content. The purple component was not studied in detail because of the apparent low yield from tryptophan and because of its instability as judged by disappearance of color.

As a control in each experiment, a zero time reaction mixture was prepared with added unlabeled pigment. The pigment was carried through the same isolation procedure which was employed in the experimental flask to eliminate the possibility that labeled substrate might contaminate the isolated pigment. Such contamination was not found in any control flask.

DL-Alanine-2-C^{14}, DL-alanine-1-C^{14}, Na-acetate-2-C^{14}, ribose-1-C^{14}, K-succinate-2,3-C^{14}, glycine-1-C^{14}, glycine-2-C^{14}, glucose-U-C^{14}, and DL-lactate-3-C^{14} were supplied by and used in the laboratory of Dr. Martin Gibbs. L-Lysine-U-C^{14} was a gift from Dr. R. Dawson, and DL-tryptophan-3a,7,7a-C^{14} was kindly supplied by Dr. L. M. Henderson. $NaHC^{14}O_3$, DL-tryptophan-2′-C^{14}, DL-5-hydroxytryptophan-3′-C^{14}, DL-tryptophan-3′-C^{14}, DL-serine-1-C^{14}, and DL-serine-3-C^{14} were obtained from commercial sources. L-Tryptophan-1′-C^{14} and L-tryptophan-3′-C^{14} were prepared from indole and appropriately labeled DL-serine, using tryptophan synthetase from *Escherichia coli* (Crawford and Yanofsky, 1958) as the condensing agent. The product L-tryptophan was isolated by an ion exchange column procedure. A lyophilized preparation of *E. coli* strain T3, the source of tryptophan synthetase, was kindly furnished by Dr. C. Yanofsky.

Assay of C^{14} content. The early C^{14} incorporation experiments were performed using the paper chromatographic method for pigment separation. The pigment samples were combusted using the reagents of Van Slyke *et al.* (1951) in an apparatus similar to that described by Stutz and Burris (1951). The $BaCO_3$ precipitate obtained was plated on glass fritted planchets and dried. The C^{14} content was estimated in a Packard gas flow counter.

The solvents used in the separation method were invariably found to contain a small amount of carbonaceous residue after evaporation *in vacuo* which could not be removed by careful fractional distillation. In addition, although the Whatman no. 1 filter paper used was thoroughly washed with oxalic acid followed by extensive water rinses, blank paper controls for pigment elution always yielded a small amount of contaminating material which behaved as carbon. Similar carbon-like residues were always observed in the alumina column eluates. The errors contributed by these sources of extraneous carbon were not precisely determined but appeared to be relatively small. To eliminate the error due to carbon contamination, all C^{14} assays subsequent to those reported in table 1 were performed by direct plating of the pigment solutions on stainless steel cupped planchets. Specific activity calculations were then based upon pigment content rather than carbon content. Self absorption was not significant with the level of material which was plated. In view of the results actually obtained with the earlier method, it was possible to form at least qualitative conclusions, since the combustion procedure assured a relatively constant error.

TABLE 1

Incorporation of C^{14}-labeled substrates into violacein

Substrate	Specific Activity		
	Substrate	Blue pigment	Dilution ratio, substrate to blue pigment
	mμc/mg C	*mμc/mg C*	
$NaHC^{14}O_3$	112.8	0.07	1,610
Acetate-2-C^{14}	115.9	0.017	6,800
DL-Lactate-3-C^{14}	154.1	0.00	
Succinate-2,3-C^{14}	15.0	0.00	
Ribose-1-C^{14}	11.1	0.025	444
Glucose-U-C^{14}	1900.0	0.033	57,000
Glycine-1-C^{14}	5.7	0.025	228
Glycine-2-C^{14}	189.8	0.066	2,875
DL-Alanine-1-C^{14}	61.5	0.11	559
DL-Alanine-2-C^{14}	10,000.0	0.12	83,000
L-Lysine-U-C^{14}	8.4	0.00	
Anthranilic-1′-C^{14}	862.0	0.51	1,690
DL-Tryptophan-2′-C^{14}	37.88	26.8	1.41
DL-Tryptophan-3a,7,7a-C^{14}	3.57	0.78	4.6

Specific activities were estimated from $BaCO_3$ samples after combustion. The standard reaction mixture contained 24 μmoles L-tryptophan; 50 to 100 μmoles of substrate; 400 μmoles potassium phosphate, pH 7.0; fresh cell suspension, 40 mg dry weight; total volume, 15.0 ml.

RESULTS AND DISCUSSION

It is apparent from the data of table 1 that of the several substrates tested, tryptophan was the only significant added source of pigment carbon. The low C^{14} dilution observed with tryptophan-2′-C^{14} suggests that at least a portion of the tryptophan side chain enters pigment directly and without dilution from other carbon sources. Since alanine, lactate, and acetate do not contribute either directly or indirectly to pigment synthesis, it is probable that the tryptophan molecule, with the possible exception of the carboxyl carbon, is incorporated intact into pigment. Further support for this conclusion is afforded by the data of table 2, derived from experiments with various tryptophan-C^{14} species. These and all subsequent data were calculated from the direct plating procedure rather than the combustion procedure. It is clear that the carboxyl carbon of tryptophan is eliminated during pigment synthesis, and it is quite probable that all other carbon atoms of the tryptophan molecule are incorporated as a unit. These results may be expected from a consideration of the pigment structure, although no conclusions can be formed concerning the synthetic pathway.

Theoretically, the specific activity ratios of pigment to tryptophan in table 2 should be expected to be 2.0. Since the theoretical value was not observed, it was concluded that cellular carbon may add to the carbon of exogenous tryptophan in contributing to pigment synthesis. The endogenous carbon may be expected to arise from tryptophan precursors if endogenous tryptophan is synthesized during the course of pigment formation, or from possible pools of metabolic intermediates which are situated on the pathway between tryptophan and pigment.

TABLE 2

Incorporation of tryptophan-C^{14} into violacein

Configuration; Position of C^{14}	Specific Activity		Ratio, Blue Pigment to Tryptophan
	Substrate	Blue pigment	
	cpm/μmole	*cpm/μmole*	
L-1′	903	0	0
L-1′	3742	0	0
L-3′	439	453	1.03
L-3′	439	540	1.23
DL-3′	487	614	1.26
DL-3′	487	696	1.41
DL-3′	487	643	1.32
DL-3′	487	629	1.29
DL-2′	1038	1427	1.37
DL-3a,7,7a	399	533	1.34

Specific activities were estimated after direct plating of samples. Sample concentrations were estimated spectrophotometrically at 565 mμ for pigment, $\epsilon = 17.0 \times 10^6$ cm² per mole, and at 280 mμ for tryptophan, $\epsilon = 5.37 \times 10^6$ cm² per mole.

The origin of unlabeled carbon in tryptophan precursors is unlikely if exogenous tryptophan is acting in a negative feedback capacity. It is known from the work of Monod and Cohen-Bazire (1953) that endogenous tryptophan synthesis is inhibited by exogenous tryptophan. The addition of serine-C^{14} to a reaction mixture containing tryptophan does not result in significant C^{14} incorporation as shown in table 3. It was concluded that no significant amounts of tryptophan were formed endogenously during the reaction time period.

If pigment intermediates represent the source of unlabeled carbon, then it could be expected that cells grown in the presence of labeled tryptophan would accumulate the endogenous intermediates and incorporate them into pigment in an appropriate reaction mixture containing unlabeled tryptophan. This hypothesis was tested by growing cells in CV medium (DeMoss and Evans, 1959) containing DL-tryptophan-3′-C^{14} or DL-serine-3-C^{14}. The labeled cells were harvested and exposed to unlabeled tryptophan under conditions which promoted pigment synthesis. No significant amount of C^{14} was found in the isolated pigment. It was concluded that the diluent carbon found in pigment must arise from sources in the cell other than tryptophan precursors and intermediates between tryptophan

TABLE 3

Incorporation of DL-serine-3-C^{14} into violacein

Serine Added	Specific Activity		
	Serine	Blue pigment	
		4 hr	22 hr
μmoles	*cpm/μmole*	*cpm/μmole*	*cpm/μmole*
10	2284	0	1
50	2284	0	26

Specific activities were estimated after direct plating of samples. Reaction mixtures were incubated for 4 and 22 hr as indicated.

and pigment. Although the hypothesis has not been further tested, consistent evidence was obtained in physiological experiments which demonstrated a rapid decrease in the ability of cells to form pigment after short periods of incubation in the absence of exogenous tryptophan (table 4). The rapid loss of pigment forming activity is to be contrasted with essentially no loss of activity when cells either are held in an ice bath or are incubated with tryptophan for the same time period.

Attempts to elucidate the pathway of pigment synthesis involved the determinations both of direct incorporation and of isotope competition using suspected intermediates. Mitoma *et al.* (1956) have observed 5-hydroxytryptophan as a product of tryptophan metabolism in *C. violaceum*. This compound is a suspected intermediate from the fact that the pigment contains a 5-hydroxyindole residue. The data of table 5 suggest that exogenous 5-hydroxytryptophan does not contribute significantly to the quantity or rate of pigment synthesis. The hypothesis that 5-hydroxytryptophan is an intermediate was further tested by using C^{14}-

TABLE 4

Effect of preincubation time on rate of violacein synthesis

Preincubation Time	Rate of Pigment Synthesis
hr	*mμmoles/hr/mg cells*
0.0	11.8
0.5	7.3
1.0	3.8
1.5	1.6
2.0	0.7

Replicate standard reaction mixtures were incubated without added tryptophan for the indicated time periods. At the time indicated, 24 μmoles of L-tryptophan were added and the rate of pigment synthesis determined during the 1 to 3 hr interval after tryptophan addition. Both pre- and post-incubations were at 30 C with shaking.

TABLE 5

Contribution of 5-hydroxytryptophan to violacein synthesis

L-Tryptophan Added	Total Pigment		Increment Due to 5HT
	Without 5HT*	With 5HT	
mμmoles	*mμmoles*	*mμmoles*	*mμmoles*
600	371	470	99
1200	739	771	32
2400	1465	1536	71

The indicated amounts of L-tryptophan were added to otherwise standard reaction mixtures.

* 5-Hydroxytryptophan (5HT), 9100 mμmoles, was added where indicated.

TABLE 6

Incorporation of DL-5-hydroxytryptophan-3′-C^{14} into violacein

Cells	Specific Activity	
	5HT	Blue pigment
	cpm/μmole	*cpm/μmole*
1	3562	66
2	3562	73
2a	3562	46
3	3562	207

Type 1 cells were grown under normal conditions. Type 2 cells were grown under normal conditions, but with 50 mg of 5-hydroxytryptophan (5HT) added per 100 ml of CV medium. Type 2 and 2a cells represent different batches of identically grown cells. Type 3 cells were grown under normal conditions, but 5 mg of sterile 5-hydroxytryptophan were added per 100 ml of the growth medium (above) 2 hr prior to harvesting the cells. The reaction mixtures contained 13.6 μmoles of 5-hydroxytryptophan-3′-C^{14} in addition to the standard components.

TABLE 7

Effect of suspected intermediates on incorporation of L-tryptophan-3′-C^{14} into violacein

Addition		Ratio, Pigment (cpm/μmole) to Tryptophan (cpm/μmole)
	μmoles	
None		1.03
5-Hydroxyindole-3-acetate	15	1.07
5-Hydroxytryptamine	17	1.19
None		1.23
5-Hydroxytryptophan	13.6	1.09
Indole-3-acetate	17	1.76
Tryptamine	19	1.06

Reaction mixtures contained the indicated compounds in addition to the standard components.

labeled material (table 6). The lack of observed incorporation or increased pigment synthesis due to 5-hydroxytryptophan could not be attributed to a simple permeability barrier. Added 5-hydroxytryptophan is easily converted to 5-hydroxyindoleacetate by fresh whole cells. Further, the experiments of table 6 include controls designed to obviate the possible impermeability of the cell toward 5-hydroxytryptophan. However, because of the results obtained, it could be argued that the effective permeability barrier is a more complex one, possibly due to an inability to activate 5-hydroxytryptophan.

To perform isotope competition experiments, all of the available suspected intermediates were tested for their ability to displace labeled tryptophan during pigment synthesis. None of the added substrates was observed to dilute C^{14} incorporated into pigment (table 7), although indole-3-acetate repeatedly suppressed the normally observed dilution mentioned above. All efforts to ascertain the precise effect of indole-3-acetate have been unsuccessful.

Previous studies on the metabolism of tryptophan by this organism (DeMoss and Evans, 1957) demonstrated the presence of tryptophan-α-ketoglutarate transaminase. It is possible that the product of transamination, indole-3-pyruvate, may serve as a ready source of indole-3-acetate. In view of the suppressive effect of indole-3-acetate, its intracellular formation via transamination could represent a mechanism for control of pigment synthesis. However, a metabolic pathway initiated by transamination of tryptophan and proceeding via indole-3-acetate does not seem to be a likely mechanism for pigment synthesis, in spite of the opportunity for exclusion of the carboxyl carbon.

Because of the instability of the pigment forming system (DeMoss and Evans, 1959) and the possible permeability problems with suspected intermediates, further studies on the pathway of pigment synthesis must await the successful preparation of subcellular systems. To date, pigment synthesis has never been observed in dried or broken cell preparations, in mixtures of mutant cells which are alone unable to form pigment, or in mixtures of mutant cells and extracts from cells which, when unbroken, would form pigment.

SUMMARY

Isotope incorporation studies suggest that only L-tryptophan can serve as the required carbon source for violacein synthesis by *Chromobacterium violaceum*. From experiments with specifically labeled tryptophan molecules, it was concluded that the carboxyl carbon is eliminated, whereas the remainder of the tryptophan molecule is incorporated intact into the pigment structure. Studies on direct incorporation or isotope competition with suspected metabolic intermediates, including derivatives of indole and 5-hydroxyindole, were not fruitful in defining the pathway of pigment synthesis.

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