NUTRITIONAL REQUIREMENTS OF *CHROMOBACTERIUM VIOLACEUM*[1]

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The topic of bacterial pigment synthesis has been a source of intriguing but relatively straightforward studies since the early days of bacteriology. The primary considerations have usually encompassed nutritional requirements for pigment production and chemical identification of the chromophores involved. Occasionally, pigmentation has been utilized in problems of taxonomy as a secondary or even as a primary factor in identification, e. g., species of the genera *Pseudomonas*, *Xanthomonas*, *Chromobacterium*, *Micrococcus*, *Flavobacterium*, and *Serratia*. Nutritional investigations into pigment formation by various bacterial species have been instituted, in general, with the objective either of simplifying the medium required or of increasing the yield of pigment (Georgia and Poe, 1931; Kharasch *et al.*, 1936; Dewey and Poe, 1943; Bunting *et al.*, 1949).

Pigment formation in many cells may be considered as an activity not essential to growth, on the basis of present knowledge. That is, the pigment does not seem to function in a role which is essential to growth, and further, the growth process seems to be entirely independent of the pigment synthesizing pathway. There is no implication, however, that common metabolic precursors do not exist. This concept is substantiated by the observations that with many species, (a) controlled conditions allow growth in the absence of pigment formation (Turfitt, 1936; Poe and Hawkins, 1949), and (b) nonpigmented mutants may be derived from the original pigmented species (Ark, 1951; Corpe, 1953; Kelner, 1947). To the extent that the data permit comparison, it is probable that the cells obtained by either method are, from a physiological point of view, entirely unimpaired by the lack of pigment. Thus, in some respects, pigment formation may be considered to be a "luxury" operation by the bacterial cell. Within this concept of nonessential processes could be included such other activities as bioluminescence and excess synthesis of vitamins whereas exceptions would include the synthesis of carotenoids by photosynthetic organisms in which pigment may serve in a protective capacity.

The nutritional requirements for violacein synthesis are essentially unknown; a few pertinent observations appear in reports by Gilman (1953) and Corpe (1953). As a basis for further investigation into the biochemistry of pigment synthesis, the nutritional requirements for growth of the organism have been studied. Data on pigment synthesis have not been included since the two processes, growth and pigment formation, are independent and can be dissociated.

MATERIALS AND METHODS

Bacteriological. Chromobacterium violaceum strain ATCC 553 was maintained on nutrient agar slants, grown at 30 C, stored at 5 C, and transferred at 30-day intervals. Prior to storage at 5 C, the slants were incubated at 30 C for 1 to 2 days only; longer incubation resulted in decreased viability. The working culture was transferred daily (0.05 ml inoculum) in 15 ml of CV[3] medium in a 125-ml Erlenmeyer flask; composition per 90 ml: glycerol, 0.9 ml; Tween 80 (10 per cent solution v/v), 0.6 ml; salts F (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), 9.0 ml; DL-alanine, 144 mg; DL-tryptophan, 21.6 mg; 0.6 mg each of DL-threonine, L-lysine, DL-methionine, DL-histidine, DL-phenylalanine, L-leucine, DL-isoleucine, DL-valine, L-arginine; adjusted to pH 7.2 before sterilization at 120 C for 15 min. The flask cultures were incubated at 30 C with shaking. For growth experiments 15 ml cultures in the appropriate medium were

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

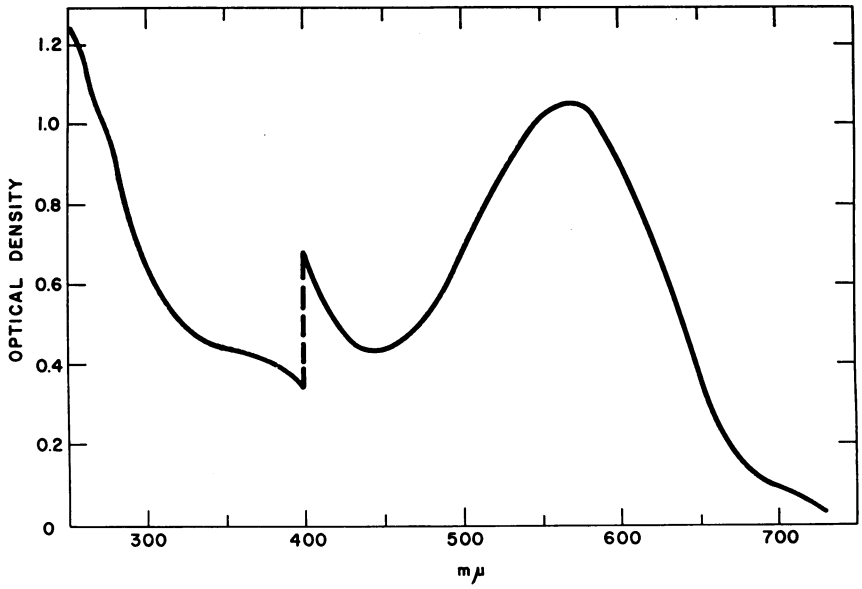

Figure 1. Spectrum of crude violacein in methanol. A culture of *Chromobacterium violaceum* was extracted exhaustively with ethyl acetate. The ethyl acetate layer was evaporated to dryness and the residue partially redissolved in absolute methanol. Below 400 mμ, the curve represents a twofold dilution of the sample used in the visible range.

TABLE 1

Effect of complex media on growth

Components Added	Growth	Relative Growth
g/100 ml	*optical density*	%
Nutrient broth	0.042	100
Salts, yeast extract (0.2)	0.019	45
Salts, tryptone (0.2)	0.033	79
Salts, tryptone, $(NH_4)_2SO_4$ (0.1), KH_2PO_4 (0.1)	0.036	86
Yeast extract, tryptone	0.061	145
Yeast extract, peptone (0.2)	0.026	62
Yeast extract, glutamic acid (0.2)	0.062	147

Cultures incubated at 30 C for 72 hr with shaking.

Final concentration of salts: per 100 ml; $MgSO_4 \cdot 7H_2O$, 8 mg; $FeSO_4 \cdot 7H_2O$, 0.4 mg; NaCl, 0.4 mg; $MnCl_2 \cdot 4H_2O$, 19 mg.

incubated with shaking either at 23 C or at 30 C as indicated. It has been established, by studies on growth kinetics, that the turbidity does not decrease after maximum growth is attained.

Determination of growth. A consideration of the spectrum of crude violacein (figure 1) suggested that growth could be measured as optical density at about 445 mμ or 700 mμ without excessive interference due to the presence of pigment. The pigment is only slightly soluble in aqueous media and during formation rapidly precipitates either as discrete particles or on cells or cell clumps. It was therefore necessary to dissolve the pigment particles to avoid error in optical density measurements. The pigment is soluble in several organic solvents, and for the present purposes, ethanol has been used as the reagent of choice.

To a sample of culture was added one volume of 95 per cent ethanol and the well mixed suspension was read immediately in 18-mm calibrated tubes in the Evelyn colorimeter using the 720 mμ filter. The alcohol treatment resulted in complete solution of the pigment in the culture and, further, did not effect immediate precipitation of the cells.

It was established that the presence of as much as 1 μmole of pigment per ml of cell suspension did not affect the optical density determinations in the Evelyn colorimeter. (However, if measurements were made in the Beckman spectrophotometer, a slight error due to pigment was observed.)

Chemicals. L-Cystine sulfoxide, S-allyl-L-cysteine, and (+)-S-allyl-L-cysteine sulfoxide

(alliin) were obtained through the generosity of Dr. H. M. Sell. All other amino acids were purchased from Nutritional Biochemicals Corporation. Methionine sulfoxide was free of methionine as determined chromatographically in an isopropanol:ammonia:water (8:1:1; v/v) solvent. The vitamin B_{12} preparation used was Cobione, a Merck product.

RESULTS AND DISCUSSION

Nutritional requirements. In the preliminary stages of this investigation, nutrient broth was used for daily transfer of the culture. Accordingly, nutrient broth was utilized as a standard in the early nutritional experiments. Table 1 shows the results obtained for growth in various complex media. In the late experimental samples, pigment had been deposited, as particles, possibly associated with cells or cell clumps, and in general cells of older cultures tended to aggregate. To obviate possible error due to clumping, Tween 80 was introduced into the medium.

The results presented in table 1 suggested that free amino acids (rather than peptides) and vitamins comprised a suitable growth medium. Consequently, the effect of vitamins (table 2) and amino acids (table 3) was investigated. The vitamins tested were apparently unnecessary for growth although a complete complement (yeast extract) in the medium permitted maxi-

TABLE 2

Effect of vitamins on growth

Addition to Basic Medium	Growth	Relative Growth
μg/ml	*optical density*	%
None	0.045	100
Yeast extract (200)	0.119	264
Thiamin (2)	0.037	82
Riboflavin (1)	0.058	129
Pyridoxine (1)	0.052	116
Pantothenic acid (4)	0.061	135
p-Aminobenzoic acid (1)	0.056	124
Nicotinamide (4)	0.056	124

The basic medium contained 0.2 per cent tryptone and salts (see table 1). Cultures were incubated 72 hr at 23 C with shaking.

TABLE 3

Effect of amino acids on growth

Component Omitted	Growth	Relative Growth
mg/15 ml	*optical density*	%
None	0.319	100
DL-Alanine (24.0)	0.132	41
DL-Tryptophan (3.9)	0.238	75
DL-Threonine (1.0)	0.286	90
L-Lysine (1.0)	0.284	89
DL-Methionine (1.0)	0.032	10
DL-Histidine (1.0)	0.286	90
DL-Phenylalanine (1.0)	0.277	87
L-Leucine (1.0)	0.271	85
DL-Isoleucine (1.0)	0.273	86
DL-Valine (1.0)	0.294	92
L-Arginine (1.0)	0.230	72
L-Tyrosine (1.0)	0.333	104

The complete medium was CV medium (see text) plus L-tyrosine.

TABLE 4

Specificity of methionine requirement for growth

Expt	Addition	Growth	Relative Growth
		optical density	%
1	None	0.022	4
	DL-Methionine	0.611	100
	D-Methionine	0.527	86
	L-Methionine	0.638	104
	DL-Lanthionine	0.013	2
	(+)-S-allyl-L-cysteine sulfoxide	0.019	3
2	None	0.000	0
	S-allyl-L-cysteine	0.076	14
	L-Cystine-disulfoxide	0.014	3
	DL-Homocysteine	0.013	2
	Methionine sulfone	0.000	0
	Methionine sulfoxide	0.527	100
	N,N-dimethyl glycine	0.019	4
	DL-Sarcosine	0.012	2
	Betaine	0.004	1
	DL-Homoserine	0.012	2
3	None	0.022	9
	Vitamin B_{12} (Cobione)	0.246	100

The basal medium was CV medium (see text) less methionine. Each component to be tested was sterilized by filtration and added aseptically just prior to inoculation. The final concentration in each case was 30 μg per ml, except for the flask of experiment 3 which contained 0.006 mμg of B_{12} per ml. The cultures were incubated at 30 C with shaking for 41 hr.

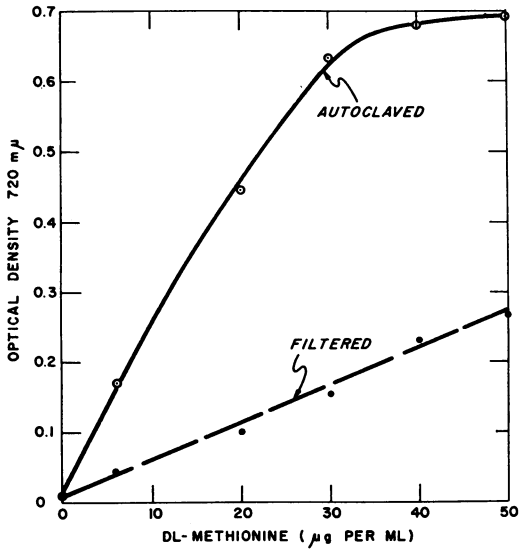

Figure 2. Effect of autoclaved or filtered DL-methionine on growth. DL-Methionine, 300 μg per ml, was sterilized by filtration through sintered glass and added either to CV medium (without methionine) before autoclaving or aseptically to previously autoclaved CV medium (without methionine), as indicated. Each flask was brought to 15 ml final volume, with distilled water. After incubation at 30 C for 41 hr with shaking, growth was determined.

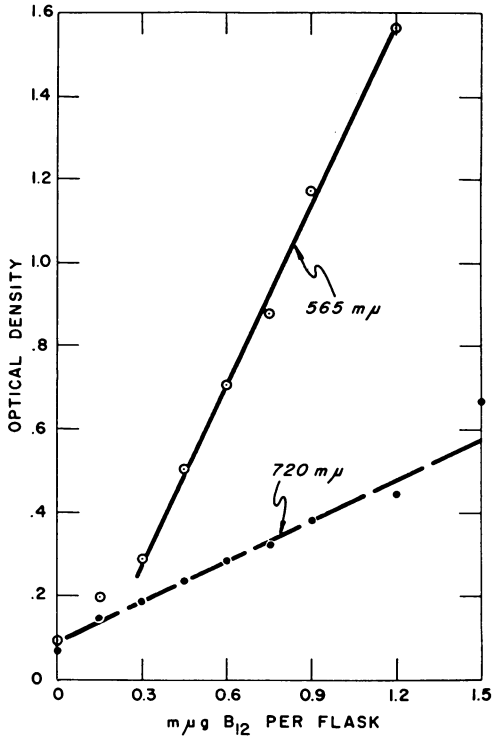

Figure 3. Effect of B_{12} concentration on growth (720 mμ) and violacein (565 mμ) biosynthesis. Vitamin B_{12} (Cobione), 0.0015 μg per ml, was added to CV medium (without methionine) before autoclaving (15 min). Each flask was inoculated with 0.05 ml of a washed 24 hr culture (CV medium), and incubated at 30 C for 48 hr with shaking. Pigment was measured on the same ethanolic suspension of cells and was taken as the 565 mμ reading corrected for turbidity due to cells, i. e., pigment = $OD_{565} - 1.3(OD_{720})$.

mum response, as is usually the case with other bacterial species.

Of the amino acids tested in table 3, only methionine was absolutely required for growth. In the presence of all of the other amino acids listed, alanine, arginine, and tryptophan stimulated growth. The growth stimulation observed in table 3 by arginine was not expressed in a medium which contained alanine, methionine, and tryptophan as the only other amino acids.

Table 4 shows the results of attempts to substitute other compounds for methionine. Of the compounds tested, only methionine, methionine sulfoxide, and vitamin B_{12} stimulate growth and no methionine stereospecificity is exhibited by the organism. The fact that either D- or L-methionine fulfills the growth requirement suggests that an active methionine racemase is present in the organism. Growth as a function of either filtered or autoclaved DL-methionine concentration is illustrated in figure 2 and shows that a considerable stimulation of methionine activity is obtained upon autoclaving. It is not evident from the data presented here whether or not this phenomenon is due to the possible formation of a more active methionine derivative, such as methyl methionine sulfonium (McRorie *et al.*, 1954). The utilization of methionine sulfoxide in place of methionine and the inactivity of methionine sulfone parallels the results obtained with *Streptococcus faecalis* by Shockman and Toennies (1954).

The ability of vitamin B_{12} to at least partially replace the methionine requirement is significant. In many instances, a methionine growth requirement is replaceable only by combinations such as vitamin B_{12} plus homocysteine. *C. violaceum* therefore may represent a more direct

means for the elucidation of a vitamin B_{12} function.

The fact that added vitamin B_{12} is not required for rapid growth in the presence of methionine strongly supports a suggested role of B_{12} in methionine biosynthesis. Further, the same observation implies that B_{12} is actually synthesized by the organism but at a very low rate, since the vitamin is probably required for other metabolic reactions. The organism in fact will grow in CV medium in the absence of either methionine or B_{12}, but growth is initiated only after 7 to 10 days. The same lag was observed upon sequential transfer to the same medium, indicating that neither selection nor adaptation had occurred.

The data of figure 3 show that under the conditions used both growth and pigment formation are linear functions of the amount of vitamin B_{12} added to the medium. *C. violaceum* therefore represents a potential microbiological agent for the assay of vitamin B_{12}. The medium employed is somewhat simpler than in nearly all other assay procedures, while the range of B_{12} concentration detectable compares favorably. If the assay method with *C. violaceum* were more fully developed, the measurement of pigment formation during growth as a function of B_{12} concentration might offer a procedure which is considerably more sensitive than other bacterial assay systems (Jukes and Williams, 1954).

SUMMARY

A study of the nutritional requirements of *Chromobacterium violaceum* demonstrated that either D- or L-methionine or vitamin B_{12} was required for growth. Under certain conditions, both pigment formation and growth were linear functions of the vitamin B_{12} concentration. The organism may be useful for the microbiological assay of vitamin B_{12}.

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