EFFECT OF AMINO ACIDS ON GROWTH AND CAROTENOGENESIS

IN CORYNEBACTERIUM SPECIES STRAIN 7E1C

THESIS

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Studies were evaluated on the effects of known growth
factors on the growth and carotenogenesis of <u>Corynebacterium</u>
species strain 7E1C.

The complex medium, Tryptic Soy Broth, was found to
stimulate growth and production of more pigment in the light
and in the dark than did a mineral salts-glucose medium. A
complete amino acid mixture added to LSG enhanced caroteno-
genesis in the dark in <u>Corynebacterium</u> 7E1C, while B-vitamins
retarded carotenogenesis. No absolute requirement for one or
more amino acids was found, indicating a multiple amino acid
requirement. The fewest amino acids found to stimulate caro-
tenogenesis in the dark were a combination of those in the
Serine and Histidine families which include serine, glycine,
cysteine, and histidine.

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CHAPTER I

INTRODUCTION

Carotenoids form a class of pigments, hydrocarbons and their oxygenated derivatives, red to orange to yellow in color, which are widely distributed in nature. As defined by the International Union of Pure and Applied Chemistry (15), carotenoids are "a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) consisting of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule."

Depending on the organism, available nutrients and other environmental factors affect the production of carotenoid pigments and the type of pigment produced. A number of studies have shown that light, carbon/nitrogen ratio, carbon sources, amino acids, and B-vitamins affect pigmentation.

The effect of light on the quantity of carotenoids was investigated with Mycobacterium lacticolum, strain 35, by Nikitina (21). He found that illumination with a flux of 1000 lux stimulated carotenogenesis. In a later study using Mycobacterium lacticolum and Mycobacterium flavum, Nikitina (23) found that carotenoid pigments were several times greater in cells recovered from cultures exposed to

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light than from cells grown in the dark (dark-grown cells).
The blue-violet portion of the visible light spectrum was
found to be most effective in stimulating carotenoid syn-
thesis in these organisms. The pigment content of Myco-
bacterium flavum, Mycobacterium lacticolum, Mycobacterium
smegatis, and Mycobacterium rubrum was 5 to 7 times higher
when cultures were grown in the dark compared to cultures
grown in the light (22). There are conflicting data con-
cerning the effect of light on pigment production in
Staphylococcus aureus. Hammond and White (11) found that
light has no effect on carotenoid synthesis. On the other
hand, Wilson and Nunez (31) found that visible light has a
variable, temperature-related effect on pigment production.
In Mycobacterium marinum, light was required for carotenoid
synthesis (19). Cultures of Micrococcus reseus and Flavo-
bacterium dehydrogenans also require light for maximum
growth and induction of pigment synthesis (28, 30). With
Flavobacterium dehydrogenans, the total amount of carotenoid
found was between 0.2 and 1.8 ug/l. These quantities were
equivalent to 0.003 and 0.03 ug/mg cell nitrogen when grown
in the light as opposed to being almost entirely free of
carotenoids when grown in the dark (30).

Carotenoid pigments are also produced in some fungi.
When cultures of Blakeslea trispora were grown in the dark,
the dry weight of these cells was indistinguishable from

that of cultures grown in the light. However, β-carotene
production was lower in cells which were grown in the dark
(27). Goodwin et al (7) also showed that light exerted a
stimulatory effect on carotenogenesis in Phycomyces blakes-
leeanus. Seviour and Codner (24) found that carotenoids
were produced in the mycelial form of Cephalosporium
diospyri only after exposure to light. Codner said
"Carotenoid synthesis induced by light was found to consist
of two steps: (a) A temperature-independent photochemical
reaction that needs light and oxygen, (b) A temperature-
dependent dark stage consisting of a lag phase and actual
synthesis of carotenoids." However, Cribanovski-Sassu and
Foppen (10) observed that surface cultures of Eipcoceum
nigrum produced more pigment in darkness than cultures grown
in bright daylight.

The effects of various nutrients on carotenoid synthesis
have been investigated in non-photysynthetic bacteria and
fungi. The effects of different alcohols and polyols on
growth and carotenoid synthesis in Mycobacterium phlei were
tested by adding isopropyl alcohol, ethyleneglycol, tetra-
methyleneglycol, and glycerol to a minimal salts medium.
When glycerol was substituted for glucose, the growth rate
decreased and pigment content increased (13). Batra, et al
(1) produced the same effect in Mycobacterium marinum by
replacing glucose with glycerol. Studies by Greehushkina

et al (9) showed that both growth and synthesis of caro-
tenoids increased with increasing levels of KNO_3 (0.1-1.0%)
with Mycobacterium lacticolum in a minimal medium containing
n-hexadecane as the carbon source.

Codner and Polatt (2) found pigmentation to be more
intense when the fungus Cephalosporium was grown on media
with a high carbon/nitrogen ratio. A 5% glucose solution
produced the largest amount of pigment in cultures of
Rhodotorula glutinis when compared with different concentra-
tions of glucose, maltose, and glycerol (20). In Phycomyces
blakesleeanus burgeff, maltose and glucose stimulated more
pigment production than fructose and xylose (6).

Vitamins also may affect carotenogenesis. Qualitative
and quantitative changes in carotenoid pigments of Coryne-
bacterium poinsettiae were observed when the amount of
thiamine was altered. Red colored cells grew under condi-
tions of low thiamine concentration (0.1 ug/100 ml basal
medium), with the two major pigments being spirilloxanthin
and lycoxanthin. Orange-yellow cells were formed with high
concentrations of thiamine (100 ug/100 ml). Under these
conditions, three major pigments were produced: lycoxanthin,
eryptexanthin, and a small amount of spirilloxanthin (26).
Mycobacterium luteum and Nocardia corallina have also been
found to require thiamine for growth and carotenogenesis (14).

Zalokar (32) showed that biotin is the only vitamin
required for pigmentation in wild type Neurospora. In young

(4 day) growing cultures of <u>Phycomyces</u> <u>blakesleeanus</u>, ribo-
flavin at a concentration of 1 part per 20,000 parts of
media (W/V) stimulated growth and carotenogenesis; greater
concentrations showed a marked inhibition (7). Carotene
synthesis was inhibited in <u>Phycomyces</u> <u>blakesleeanus</u> grown
in media containing less than 0.2 ug thiamine/100 ml, while
growth was inhibited at concentrations lower than 2.0 ug
thiamine/100 ml of medium (5). When growing <u>Flavobacterium</u>
<u>dehydrogenans</u>, the concentration of thiamine was shown to
be critical both for growth and carotenoid biosynthesis
(30).

The role of amino acids in carotenoid synthesis has
also been investigated in several organisms. Thirkell (29)
showed that omission of aspartate from the growth medium
reduced pigmentation in <u>Micrococcus</u> <u>radiodurans</u>. Growth and
pigmentation were retarded in <u>Mycobacterium</u> <u>phlei</u> when the
concentration of asparagine in a mineral-salts glucose
medium was lowered from 0.5 to 0.1% or 0.2%. When the con-
centration of asparagine was increased to 1.0%, 10 day
growth was similar to that obtained under standard conditions
(0.5% asparagine) but the pigment content reached a maximum
concentration at 4 days and then rapidly decreased as auto-
lysis proceeded (13). In experiments by Cooney and Thierry
(3) using <u>Micrococcus</u> <u>roseus</u>, omission of certain amino
acids from the media indicated that the pyruvate group,
except alanine, supported growth and pigment synthesis.

Methionine and isoleucine, from the aspartic acid family, contributed to growth and pigmentation. When members of the glutamic acid or serine families were omitted, there was a decrease in growth and pigment synthesis.

Zalekar (3) showed that most individual amino acids have no effect on pigmentation in Neurospora, although glycine increased the level of all carotenoids and restored pigmentation in peptone-grown cultures. He also found that valine and leucine, which stimulated carotenogenesis in Phycomyces, had no effect in Neurospora and, in fact, depressed pigmentation when used as the sole nitrogen source. Garton et al (6) also showed that glycine stimulates carotenogenesis in Phycomyces blakesleeanus burgeff.

The non-photosynthetic bacterium, Corynebacterium species strain 7E1C, was used in this investigation. This organism was isolated from soil with propane as the sole source of carbon and energy (16). Gordon (8) has referred to this bacterium and similar organisms as occupying an uncertain taxonomic position and proposed that they be classified as Mycobacterium rhodochrous. However, since the "rhodochrous" group of bacteria are still poorly defined, the genus designation used in the original publication (Gordon) will be used in this report.

Previous research in this laboratory has dealt with the effects of pH, light, available nutrients, and available

oxygen on pigment production and growth in <u>Corynebacterium</u> strain 7ElC (12). In addition, it has been shown that white light stimulated carotenoid production in both defined and complex media such as Tryptic Soy Broth, carotenoid production was significantly higher than when the organism was grown on a defined medium.

The fact that carotenoid synthesis was stimulated in the dark when the organism was grown on a complex medium raises the question of the identity of the factor (or factors) responsible for stimulation of pigment production. The purpose of this investigation was to deterine the identity of nutrient(s) responsible for carotenogenesis in <u>Corynebacterium</u> spp. strain 7ElC.

CHAPTER II

MATERIALS AND METHODS

Organism and Inoculum

Corynebacterium species strain 7E1C was maintained on agar slants of LSG (Leadbetter and Foster's mineral-salts medium (17) supplemented with 1% glucose) at pH 6.8-7.2. A standardized inoculum was prepared from late log phase cells grown in the light in 50 ml LSG broth on a rotary shaker (Eberbach) at 140 rpm, at 25-28°C. Continuous illumination for the inoculum was provided by two Westinghouse 30 watt cool white fluorescent lights, 2 x 10^3 ergs/ cm^2 per second. The energy from these lights was measured with a YSI Kettering Model 65 radiometer (Yellow Springs Instruments Co., Yellow Springs, Ohio), placed 29 cm above the surface of the shaker. The cell density of the inoculum was adjusted to optical density of 0.30 using sterile LSG for zero setting at 620 nm on a Bausch and Lomb Spectronic 20 spectrophotometer (Analytical Systems Division, Rochester, New York). This cell suspension was then used to inoculate the test flasks with a 2% (v/v) inoculum. Inoculum purity was checked by microscopic examination and streak plates on TSA (Tryptic Soy Agar, Difco).

The test flasks were incubated for seven days on a
rotary shaker as described above. Cultures were grown in
250 ml Erlenmeyer flasks containing 50 ml of media.
Cultures grown in the dark were grown in red glass flasks
(Pyrex "low actinic" glass) and wrapped with two layers of
aluminum foil. To prevent possible light diffusion
through the cotton plug, the top and neck of each flask
was covered with foil. Purity checks were made on each
flask at the end of the incubation period.

Vitamin Requirements

Stock solutions of B-vitamins were prepared by the
addition of the following B-vitamins (mg/100 ml) in stock
flasks with distilled water: thiamine-HCl, 50; pyridoxine-
HCl, 1.12; pyridoxal, 30; pyridoxamine-HCl, 30; folic acid,
3.2; biotin, 0.4; B_{12}, 1.0; calcium pantathenate, 50;
niacin, 100; para-amino benzoic acid, 1.0; and riboflavin,
50 (19). Then 0.1 ml of each stock was added to 49 ml LSG
medium prior to sterilization by autoclaving.

Amino Acid Requirements

Stock solutions of amino acid were prepared in dis-
tilled water at concentrations of 0.08% (W/V). Cystine
and tyrosine were dissolved in 100 ml distilled water to
which one drop of 1N NaOH was added to increase solubility.
The following L-amino acids were used in the experiment:

alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phelyalanine, proline, serine, threonine, tryptophan, and valine.

Five different media ranging from complex to minimal were used: TSB, LSG and amino acids and B-vitamins, LSG and B-vitamins, LSG and amino acids, and LSG.

Single amino acid elimination experiments using 20 L-amino acids were performed to determine essential amino acid requirements for pigmentation in dark grown cultures. Each flask for the experiment contained 30 ml of LSG and 1 ml each of the amino acid stock solutions, exept the one being eliminated. The amino acids were grouped into families on the basis of their biosynthetic pathways. The following families were used: Glutamate family (glutamine, arginine, and proline); Aspartate family (asparagine, methionine, threonine, isoleucine, and lysine); Aromatic family (phenylalanine, tyrosine, and tryptophan); Serine family (serine, glycine, and cysteine), Pyruvate family (alanine, valine, and leucine); and Histidine family (histidine) (25). The amino acid "family" elimination experiments were done to determine whether a "family" of amino acids or a group of families were responsible for pigment synthesis in the dark. When a single amino acid family was eliminated, a final volume of 50 ml was made by the addition of the remaining families and LSG. For example,

if the Pyruvate family was eliminated, 16 amino acids re-
mained. Therefore, 1.3 ml supplement of each of the 16
remaining amino acids were used, making a volume of 24 ml.
Twenty six ml with 2X LSG concentration were added to the
24 ml of amino acids, giving a final volume of 50 ml at a
concentration of 0.04% (w/v) of amino acids. Experiments
were also done excluding two and three families at a time.

Cell Harvest and Assay of Total Pigment

Cells were harvested at the end of seven days at which
time they were in early stationary phase. Cell turbidity
was measured by diluting 1 ml of culture with 14 ml sterile
LSG and reading O. D. at 620 nm on the spectrorophotometer.
To determine pigment levels, 30 ml of culture were centri-
fuged in a Sorval centrifuge, Model SS-3 (Norwalk, Connecti-
cut) at 10,000 rpm for 10 minutes. Carotenoid pigments
were extracted from the pellet with a 1:5 (v/v) solution of
carbon disulfide: methanol, followed by further extractions
with 1:2 (v/v) solution of carbon disulfide: methanol until
the pigments were completely extracted (12). The separation
of cells and extract was done at 2,500 rpm for 10 min in an
International centrifuge Model HN (Needham Heights, Massachu-
setts). Total amounts of pigments were determined (ug
pgiment/mg dry weight) by a reading of the O. D. at 465 nm
of the extract and by the use of an extinction co-efficient
of ($E_{1\ cm}^{1\%}$) of 2500 for all determinations (4).

Optical Density vs Dry Weight

Flasks were prepared to contain 50 ml TSB and 50 ml

LSG. These flasks were inoculated with the standard

inoculum and grown on the shaker for seven days. The cells

(seven days old) were prepared in dilutions of 1:2,

1:4, 1:6, 1:8, 1:10 by adding 5 ml TSB or LSG to 5 ml

sterile water and measuring the optical density at 620 nm

for the 1:2 dilution. The 1:4 dilution was made by adding

5 ml of cells to 15 ml sterile water; 1:6 dilution was made

by adding 5 ml of cells to 25 ml sterile water; etc. From

each dilution, 10 ml samples were also taken and centrifuged

at 10^4 rpm in a Sorvall SS-3 (Norwalk, Connecticut) centri-

fuge for 10 minutes. The pellet was washed in 10 ml

distilled water, mixed thoroughly, centrifuged at 10^4 rpm,

and washed again to remove residual media. The cells were

resuspended in 1 ml distilled water, and poured into

weighing boats which had been heated in the oven and cooled

in the dessicator until a constant weight was reached. The

cells from the various dilutions were dried to a constant

weight in a vacuum oven at $90^{\circ}C$. Dry weight was plotted

versus optical density.

CHAPTER III

RESULTS

In order to relate pigment production to cellular weight, standard curves of optical density versus dry weight were obtained. These were obtained on both LSG and TSB media and are shown in Figures 1 and 2. All calculations of pigment yield were made by using these curves.

The effect of medium composition on pigment production was initially studied. The results, shown in Table I, indicate that in both TSB and LSG media there was approximately 3 times more pigment produced in the light than in the dark. However, cell growth was similar in both the dark and the light grown cells in these media. About twice as much pigment was produced in TSB in the light compared to cells grown on LSG in the light and about 2.5 times as much pigment was produced in cells grown in TSB in the dark when compared to cells grown on LSG in the dark. Growth was only slightly greater in TSB when grown in the light than when grown in the dark.

These data indicate that there is some factor(s) in complex media which stimulates carotenogenesis in this organism. When amino acids and B-vitamins were added to LSG medium, it was found that the B-vitamins retarded
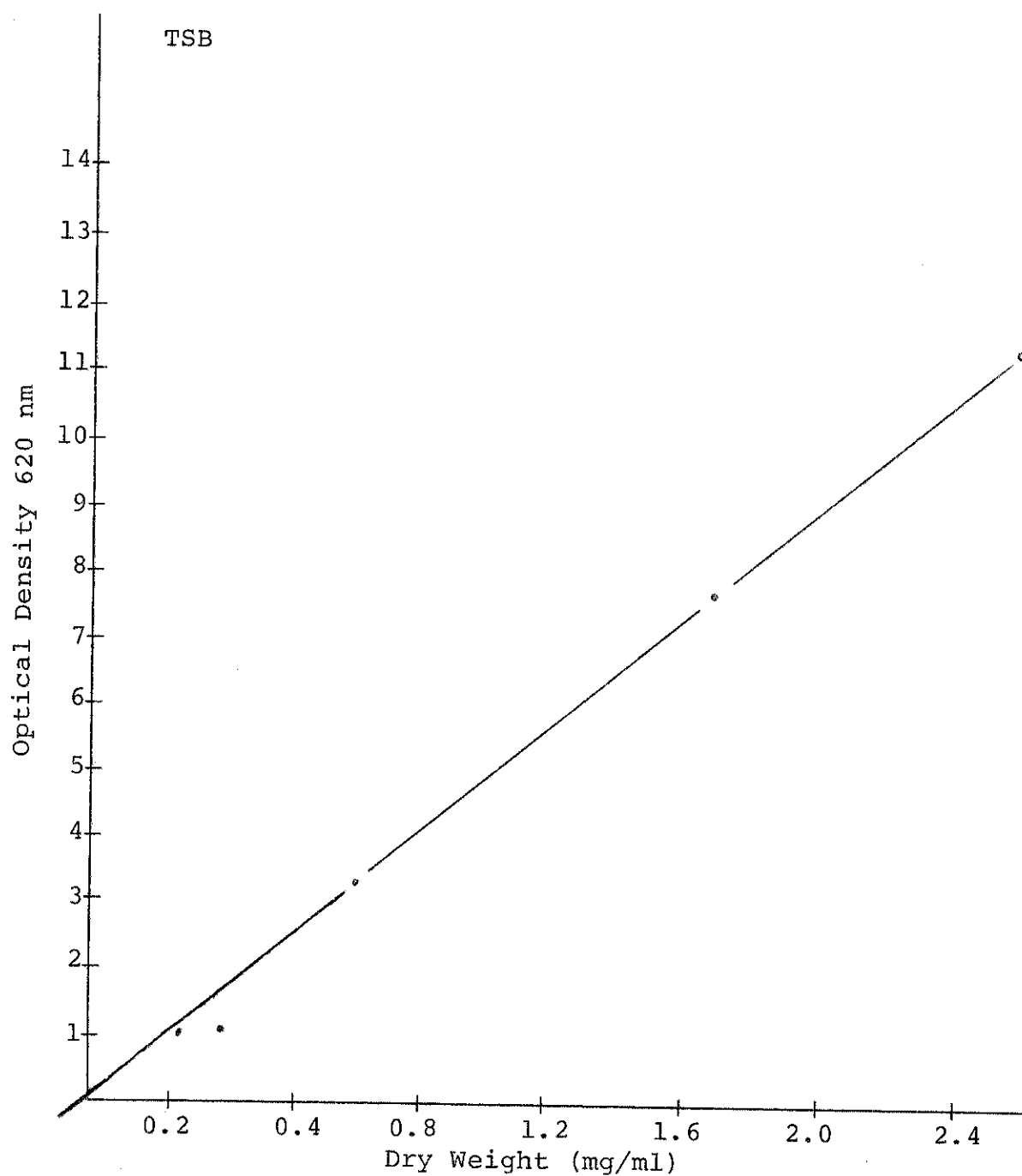
Fig. 1--Dry weight of Corynebacterium 7E1C cells (mg/ml) grown on TSB vs. the carotene pigments extract from Corynebacterium 7E1C as a function of Optical Density at 620 nm.
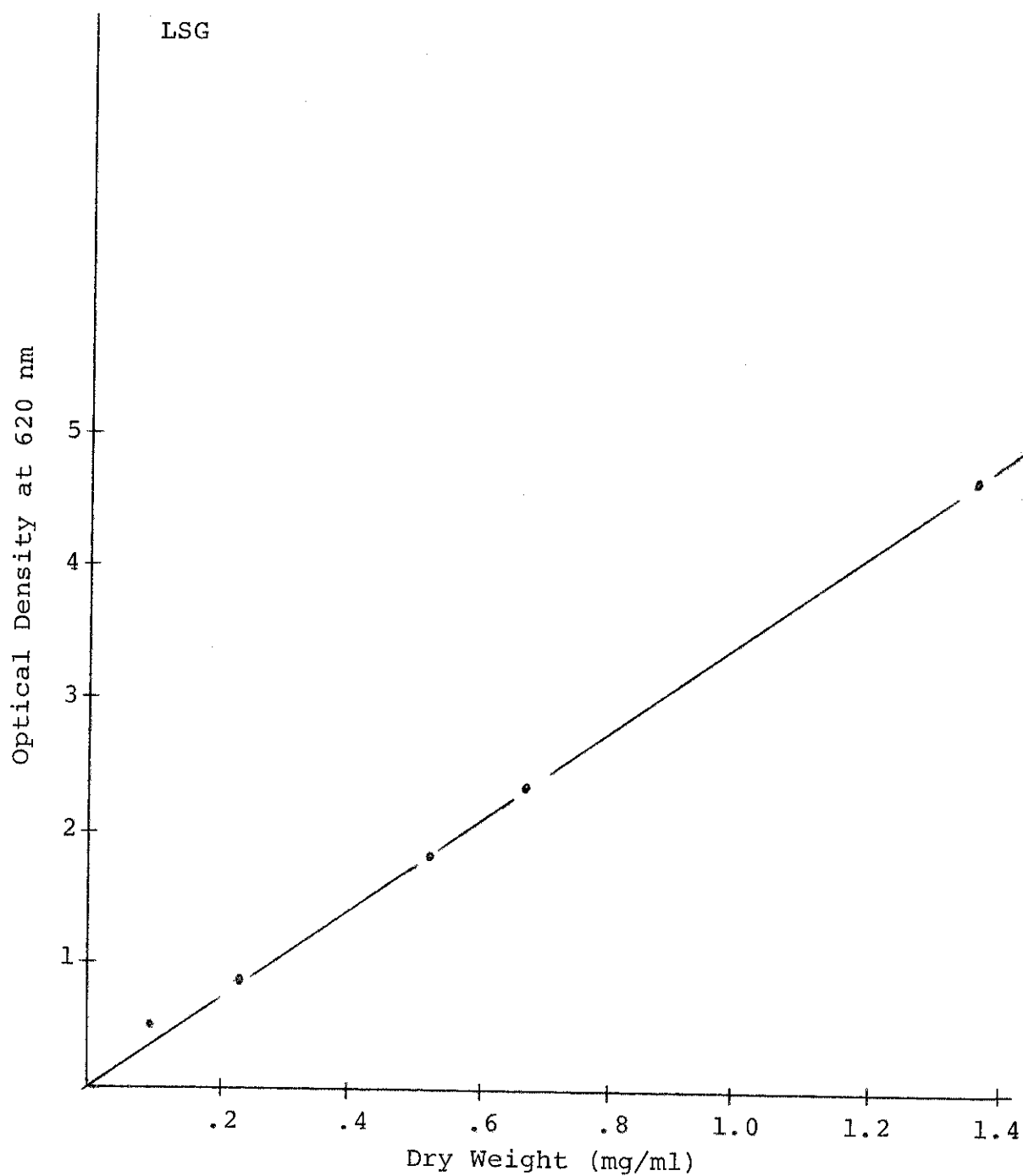
Fig. 2--Dry weight of Corynebacterium 7E1C cells
(mg/ml) grown on LSG vs. the carotene pigments extract from
Corynebacterium 7E1C as a function of Optical Density at
620 nm.

TABLE I

EFFECT OF MEDIUM COMPOSITION ON GROWTH AND PIGMENTATION
OF CORYNEBACTERIA 7E1C

Medium	Light Grown		Dark Grown	
	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.
TSB	1.27	4.29	1.24	1.19
LSG	1.10	1.94	1.06	0.45
LSG + Amino Acids	0.72	2.83	0.66	0.69
LSG + B-Vitamins	1.38	1.43	1.31	0.29
LSG + Amino Acids & B-Vitamins	0.67	1.12	0.64	0.50

pigment synthesis. Amino acids, although effective in LSG, were not as effective as TSB. The amino acids give a 36% increase in LSG when cells were grown in the light and a 63% increase when grown in the dark. Cell growth was inhibited somewhat by the amino acid mixture but not by the vitamins.

Effects of single amino acid exclusion on the pigmentation of Corynebacterium 7E1C are shown in Table II. The light-grown cells produced more pigment than the dark grown cells. These data show that when the organism was grown in the light the elimination of any one amino acid, except isoleucine, did not markedly affect pigmentation any more than the control containing the complete amino acid mixture. Elimination of isoleucine, however, led to enhanced pigment production. In the dark-grown cells when alanine, arginine, and cystine were eliminated pigment levels increased. Elimination of glutamine and isoleucine also resulted in a modest increase in pigment. In no case did the removal of a single amino acid from the mixture result in pigment levels equivalent to that found in the LSG control indicating that the effect is due to a multiple amino acid requirement.

In order to determine what combination of amino acids were required, further studies were conducted with amino acids grouped on the basis of their biosynthetic derivation are shown on Table III. Since some of the amino acids could

TABLE II

EFFECTS OF SINGLE AMINO ACID ELIMINATION OF
CORYNEBACTERIUM 7E1C

Medium	Light Grown ug pigment/ mg dry wt.	Dark Grown ug pigment/ mg dry wt.
I. Amino Acid Eliminated		
Alanine	2.22	1.76
Arginine	2.56	1.04
Asparagine	2.20	0.86
Aspartic Acid	2.24	0.96
Cysteine	2.20	0.78
Cystine	1.70	1.08
Glutamine	2.62	0.91
Glutamic Acid	2.54	0.72
Glycine	2.79	0.64
Histidine	2.86	0.61
Isoleucine	3.34	0.92
Leucine	2.73	0.49
Lysine	2.04	0.65
Methionine	1.14	0.65
Phenylalanine	1.90	0.58
Proline	1.63	0.88
Serine	1.82	0.62
Threonine	1.79	0.79
Tryptophan	1.94	0.75
Tyrosine	2.25	0170
Valine	1.76	0.59
II. Controls		
TSB	2.28	0.89
LSG	1.45	0.36
LSG + Amino Acids	1.84	0.81

TABLE III

AMINO ACID BIOSYNTHETIC DERIVATIONS

Family	Precursors	Products
1. Glutamate	a-ketoglutarate ⟶ glutamate	arginine glutamine proline
2. Aspartate	oxalacetate ⟶ aspartate	Asparagine methionine threonine isoleucine (in part) lysine (in part)
3. Aromatic	PEP + Erythrose-4 PO_4	phenylalanine (in part) tyrosine (in part) tryptophan
4. Serine	3 PO_4 - glyerate ⟶ serine	glycine cysteine
5. Pyruvate	Pyruvate	alanine valine leucine (in part)
6. Histidine	phosphoribosyl PP + ATP ⟶	histidine (in part)

belong to more than one family (25), they were arbitrarily grouped in families as indicated in the table. In future reference to amino acid families, all family names are capitalized and individual amino acids are not.

Effects of single amino acid family elimination on the growth and pigment of Corynebacterium 7E1C are depicted in Table IV. In dark-grown cultures, the elimination of the Aspartate family resulted in more pigmentation. Removal of the Aromatic family and the Pyruvate family resulted in diminished growth in the light and the dark, but had little effect on pigmentation in the dark. However, with cells grown in the light the elimination of the Pyruvate family led to increased pigment production. The overall response of Corynebacterium 7E1C was not clear and no one family appeared to be necessary for enhancement of pigment production.

The effects of the elimination of two amino acid families are shown in Table V. These data indicate the importance of the Serine family for pigmentation in the dark. In every case, when the Serine family was omitted, except when omitted with the Pyruvate family, pigmentation was reduced to levels less than the LSG control. Cell growth was stimulated in these cases. When the Serine and Pyruvate families were eliminated, normal growth and stimulation of pigment synthesis occurred. There was no comparable effect of Serine in the light.

TABLE IV

EFFECTS OF SINGLE AMINO ACID FAMILY ELIMINATION ON
GROWTH AND PIGMENTATION OF CORYNEBACTERIUM 7E1C

Medium	Light Grown		Dark Grown	
	Dry Wt. mg/ml	ug pigment/ mg dry wt.	Dry Wt. mg/ml	ug pigment/ mg dry wt.
I. Family Eliminated				
Glutamate family	0.54	1.59	0.60	0.80
Aspartate family	0.40	1.99	0.55	1.20
Aromatic family	0.68	1.39	0.53	0.89
Serine family	0.73	1.73	0.77	0.62
Pyruvate family	0.40	2.09	0.55	0.76
Histidine family	0.67	1.83	0.67	0.70
II. Controls				
TSB	1.84	2.35	1.88	1.30
LSG	0.66	0.89	0.66	0.39
LSG + Amino Acids	0.71	1.75	0.69	0.75

TABLE V

EFFECTS OF DOUBLE AMINO ACID FAMILY ELIMINATION ON
GROWTH AND PIGMENTATION OF CORYNEBACTERIUM 7E1C

Medium	Light Grown		Dark Grown	
	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.
I. Families eliminated				
Glutamate & Aspartate	0.59	1.88	0.77	0.47
Glutamate & Aromatics	0.72	1.51	0.84	0.67
Glutamate & Serine	0.85	1.71	1.67	0.34
Glutamate & Pyruvate	0.69	1.78	0.67	0.50
Glutamate & Histidine	0.82	1.99	0.71	0.52
Aspartate & Aromatics	0.55	2.58	0.91	0.57
Aspartate & Serine	0.83	1.03	1.30	0.40
Aspartate & Pyruvate	0.76	1.34	0.96	0.54
Aspartate & Histidine	0.81	1.61	0.93	0.50
Aromatics & Serine	0.79	1.56	1.75	0.19
Aromatics & Pyruvate	0.71	1.84	0.82	0.66
Aromatics & Histidine	0.82	1.20	0.90	0.79
Serine & Pyruvate	0.77	2.20	0.79	1.03
Serine & Histidine	0.91	1.49	1.46	0.34
Histidine & Pyruvate	0.76	1.73	0.73	0.63

TABLE V--<u>Continued</u>

Medium	Light Grown		Dark Grown	
	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.
II. <u>Controls</u>				
TSB	1.68	2.82	1.65	1.00
LSG	0.39	0.68	0.58	0.55
LSG + Amino Acids	0.87	1.62	0.81	0.73

There is some indication that the Glutamate and Aspartate familes are also important for pigmentation in the dark. Pigment production was lower when these families were omitted than in the LSG control. The elimination of Aspartate and Serine families caused some reduction in pigmentation in the light as did elimination of the Aromatic and Histidine families.

The effects of growth and pigmentation when three amino acid families were eliminated (or three amino acid families were added) are shown on Table VI. All combinations of three amino acid families allowed production of pigment in the dark to reach a level at least equivalent to that of the LSG control except the Aspartate-Pyruvate-Histidine, Aspartate-Serine-Histidine, Glutamate-Aromatic-Histidine, and Glutamate-Aspartate-Pyruvate families. Combinations which promoted increased pigment levels in the dark over the levels of the control were the Serine-pyruvate-Histidine, Aromatic-Pyruvate-Histidine, and the Glutamate-Aspartate-Histidine families. Aromatic-Serine-Histidine, Aspartate-Pyruvate-Serine, and Glutamate-Serine-Pyruvate families also stimulated pigmentation in the light.

A number of combinations, especially the Aromatic-Serine-Pyruvate; Aspartate-Pyruvate-Histidine; Aspartate-Aromatic-Histidine; Aspartate-Aromatic-Pyruvate; and Glutamate-Aromatic-Histidine families stimulated growth in the dark.

TABLE VI

EFFECTS OF GROWTH AND PIGMENTATION WHEN THREE AMINO
ACID FAMILIES WERE ELIMINATED

Medium	Light Grown		Dark Grown	
	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.
I. Amino Acid Families Added				
Ser., Pyr., & His.	0.61	1.05	0.67	0.82
Aro., Pyr., & His.	0.82	1.34	0.16	0.57
Aro., Ser., & Pyr.	0.83	1.37	3.0	0.39
Aro., Ser., & His.	0.60	2.59	0.72	0.37
Asp., Pyr., & His.	0.95	1.56	2.1	0.20
Asp., Ser., & His.	1.00	1.74	0.77	0.19
Asp., Aro., & His.	1.05	1.18	3.00	0.26
Asp., Aro., & Pyr.	1.20	1.26	2.1	0.35
Asp., Aro., & Ser.	0.67	1.79	0.71	0.42
Asp., Pyr., & Ser.	0.66	2.20	0.88	0.37
Glut., Pyr., & His.	0.12	0.06	0.94	0.28
Glut., Ser., & His.	0.00	0.00	0.91	0.60
Glut., Ser., & Pyr.	0.70	2.15	1.06	0.39
Glut., Aro., & Pyr.	0.96	1.35	0.70	0.29
Glut., Aro., & Ser.	0.90	1.66	0.84	0.43
Glut., Aro., & His.	0.84	0.93	2.00	0.22
Glut., Asp., & His.	0.90	1.88	1.20	0.58
Glut., Asp., & Pyr.	0.85	1.36	0.92	0.21
Glut., Asp., & Ser.	0.88	1.54	0.77	0.40
Glut., Asp., & Aro.	0.87	1.90	0.77	0.56
II. Controls				
TSB	1.66	3.18	1.63	1.34
LSG	0.63	1.02	0.71	0.33
LSG + Amino Acids	0.92	1.68	0.88	0.52

Table VII indicates the growth and pigmentation of Coryne-bacterium 7E1C in an LSG medium which contains two amino acid families. The data indicate that both the Histidine-Serine, and Aromatic-Serine family-combinations allow for pigment production equivalent to that of the complete amino acid mixture, but do not allow for pigment production equal to that of the cells grown in TSB. This table also shows that glucose is not necessary for pigmentation. When the complete amino acid mixture served as the sole carbon source, pigment yields equivalent to those of the amino acids and glucose were obtained.

TABLE VII

GROWTH AND PIGMENTATION OF CORYNEBACTERIUM 7E1C IN
A MEDIUM CONTAINING TWO AMINO ACID FAMILIES

Medium	Light Grown		Dark Grown	
	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.	Dry Wt. (mg/ml)	ug pigment/ mg dry wt.
I. Amino Acid Families Added				
His. & Ser.	0.44	0.90	0.42	0.54
Aro. & Ser.	0.19	1.98	0.35	0.58
II. Controls				
TSB	1.67	2.88	1.50	1.12
LSG	0.49	0.96	0.75	0.32
LSG + Amino Acids	0.93	1.50	0.85	0.55
LS + Amino Acids Without Glucose	0.26	1.36	0.26	0.58

CHAPTER IV

DISCUSSION

When the effect of medium composition on pigment
production in Corynebacterium 7E1C was tested, the results
indicated that more pigment was produced in a complex
medium, in both the light grown cells and the dark grown
cells than in a minimal medium containing only one carbon
source (LSG). When known growth factors, such as amino
acids and B-vitamins, were tested, it was found that the
amino acids enhanced and the B-vitamins retarded pigment
synthesis. However, the response to amino acids was not as
great as that obtained with TSB. One explanation of this
response may be that the concentration of amino acids was
not optimal. The results might also reflect the necessity
of another factor or factors in maximum pigment yield.
A third reason for this response might be that some of the
amino acids stimulate pigment synthesis and others retard
it. The levels of the individual amino acids in TSB are
not known although they were all added in essentially the
same concentration in the LSG medium. Some evidence for
inhibition of pigment production by certain amino acids is
shown in Table II. These data indicate that the omission

of either alanine, arginine, or cystine from the amino acid mixture results in increased pigment production.

The amino acids and the complex medium TSB stimulated pigmentation in the light as well as in the dark. However, in no case did the amount of pigment produced in the dark equal the amount produced in the light. Light-grown cells normally had four times the amount of pigment as dark-grown cells. The implication of this is that two mechanisms are operative; one light dependent, the other substrate dependent.

That the amino acid effect is not due to an absolute requirement for one or more amino acids is shown in Table II. If the requirement was an absolute one, then the pigment produced when it was omitted should drop to the level found in the LSG control. Since the drop in level of pigment production did not occur, the results indicate a multiple requirement.

When combinations of amino acid families were eliminated from the media, the Serine family was found important in stimulating carotenogenesis in the dark since its elimination with nearly all other families resulted in a reduction of pigment synthesis. However, the requirement for the Serine family was not absolute. As indicated in Table VI, the combination of the Glutamate-Aspartate-Histidine families and the aromatic-Pyruvate-histidine families also stimulated pigment production. Apparently

the organism has the ability to interconvert certain amino acids so that no absolute requirement exists.

The amino acid families tested also effected growth, although growth stimulation did not necessarily correlate with stimulation of pigment synthesis. When the Pyruvate family and the Serine family were eliminated together, there was an increase in pigment and a decrease in growth in dark grown cells. When the Aromatic and Serine families were eliminated together there was an increase in growth and a decrease in pigment production. The explanation for this is as yet unknown.

The minimum number of amino acids found so far which will stimulate pigment production in the dark are a combination of those in the Serine and Histidine families which include four amino acids: serine, glycine, cysteine, and histidine. Which of the three amino acids in the Serine family is important has yet to be determined.

The results reported here show that amino acids exert a stimulatory effect on carotenogenesis in the dark in Corynebacterium 7ElC. Furthermore, this response is not due to an absolute requirement for one or more amino acids. A combination of as few as four amino acids has been found to stimulate carotenogenesis. In addition, some amino acid combinations have been found to stimulate growth but not pigment synthesis. The optimum concentration of amino acid

combinations and the mechanisms by which they exert their effects await further research.

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