ISOLATION AND PARTIAL CHARACTERIZATION OF PIGMENT MUTANTS OF CORYNEBACTERIUM POINSETTIAE ATCC 9682

THESIS

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By

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tion of Pigment Mutants of Corynebacterium poinsettiae ATCC
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Carotenoid pigments were extracted from Corynebacterium
poinsettiae (wild type) ATCC 9682, and from 108 mutants ob-
tained by exposure of a streptomycin resistant strain of C.
poinsettiae to ultra-violet light irradiation and N-methyl-
N'-nitro-N-nitrosoguanidine. The pigments were characterized
by their absorption maxima, Rf-values, and partition ratios
in petroleum ether and methanol. Thin layer chromatography
was used to compare pigments of the wild type with those of
the mutants. Possible biosynthetic pathways in carotenoid
synthesis of the wild type were postulated on the basis of
the observed genetic blocks. Mutants were found which sug-
gested the existence of a linear pathway in carotenoid syn-
thesis from the aliphatic C40 molecule to the bi-cyclic
C50-diol. Other mutants suggested possible alternative
pathways in the biosynthesis of these pigments or the
presence of intermediates not detectable by thin layer
chromatography.
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INTRODUCTION

Carotenoids are known to perform certain vital functions. In photosynthetic organisms, carotenoids absorb light for photosynthesis, thereby protecting chlorophylls from light destruction (5, 26, 33, 37). In Corynebacterium poinsettiae, a non-photosynthetic bacterium, carotenoids appear to serve as protection against photodynamic killing (11).

The application of sophisticated physical and chemical methods such as mass spectrometry, thin layer chromatography, X-ray crystallography, optical rotatory dispersion, and circular dichroism, have facilitated the identification of the large number of carotenoids known today. Although some carotenoids are specific to higher plants and animals, many carotenoids are encountered in microorganisms.

Most of the studies on the biosynthesis of carotenoids have been in plant systems. The available evidence indicates a general pathway for the formation of the primary C40 unit from low molecular weight precursors in all carotenogenic systems. The desaturation of the C40 unit appears to be of a general nature. The final steps, including the insertion of oxygen functions, have been found to be specific for the different groups of organisms. The following is the pathway for carotenoid biosynthesis up to the C40 level, as outlined by Lynen et al. (25):
Mevalonic acid (MVA)
  ↓
Mevalonic-5-phosphate (MV5-P)
  ↓
Mevalonic-5-pyrophosphate (MV5-PP)
  ↓
Dimethyl-allyl pyrophosphate (DMAPP)
  →
Isopentenyl-pyrophosphate (IPP)
  ↓
Geranyl-pyrophosphate (GPP)
  ↓
Farnesyl-pyrophosphate (FPP)
  ↓
Geranyl geranyl-pyrophosphate (GGpp)
  ↓
Phytoene

From studies with mutant strains of tomatoes, Porter and Lincoln (31) proposed a stepwise dehydrogenation of phytoene to form Lycopene:

Phytoene
  ↓-2H
Phytofluene
  ↓-2H
Zeta-carotene
  ↓-2H
Neurosporene
  ↓-2H
Lykopene

In this system, dehydrogenation is followed by cyclization of the acyclic precursor to form alpha and beta-ionone ring systems. The stereochemical course of the reactions from the MVA precursor has been defined (10). Despite the initial difficulties in studies on the enzymatic synthesis of carotenenes, it is now possible to discuss carotenoid biosynthesis.
in terms of demonstrable reactions, cofactor requirements and, to some extent, properties of the responsible enzymes. Most of the information on the early stages in enzymatic biosynthesis of carotenoids has been obtained from studies of animal enzyme systems which are identical to those of steroid biosynthesis. Knauss, Porter, and Wasson (20) have demonstrated the synthesis of mevalonic acid from acetate in a study using a combination of microsomes and soluble enzymes of rat liver. Porter and Anderson (32) demonstrated that the conversion of one mole of mevalonic acid to isopentenyl pyrophosphate required three moles of adenosine triphosphate (ATP) and the presence of magnesium ion. Tchen (36) and Levy and Popjak (23) have demonstrated that the initial reaction in the transformation of MVA to IPP is the conversion of MVA to MV5-P. The latter is catalyzed by MVA kinase which has been demonstrated in a number of plant tissues including Hevea latex (5, 12) and pea seeds (29), and in the bacterium Staphylococcus aureus (28). The enzyme for the second reaction in the conversion of MVA to IPP, the phosphorylation of MV5-P to yield MV5-PP, has been isolated from yeast (3, 14), pig liver (13) and H. latex (34). The last enzyme involved in the sequential formation of IPP also has been isolated from pig liver (13). The route to the higher terpenoid precursors from IPP is well understood. The initial step is the isomerization of IPP to DMAPP (2, 31) which initiates chain elongation. The enzyme,
prenyl-transferase, responsible for the transfer of one IPP to DMAPP to form geranyl pyrophosphate (GPP, C\(_{10}\)) (1, 2, 7, 17) is also responsible for the transfer of one IPP to GPP to give farnesyl pyrophosphate (FPP, C\(_{15}\)) (8, 19, 30) as well as the transfer of IPP to FPP to yield geranyl-geranyl pyrophosphate (GGPP, C\(_{20}\)) (18, 19, 26). It is now generally agreed that phytoene is the first C\(_{40}\) compound. The cyclization of acyclic precursors has been investigated and the basic problems have been enumerated. These include (a) the stage at which cyclization takes place and (b) the mechanism of formation the alpha- and beta-ionone ring systems. There is no evidence that cyclization occurs before the neurosporene stage. Evidence for the participation of lycopene in cyclization has been reported in chloroplasts (15, 22).

The interesting aspect in the biosynthesis of carotenoids in non-photosynthetic bacteria involves the formation of carotenoids with 45 and 50 carbon atoms and the photo-induction of carotenoid synthesis. The first C\(_{50}\) carotenoid, decaprenoxanthin, was discovered in *Flavobacterium dehydrogenans* (4, 9). Other C\(_{50}\) pigments have been discovered and characterized. They all carry an additional isopentenyl residue at the C-2 and C-2' positions and can be acyclic, e. g., bacterioruberin, monocyclic, as with C.p 473 from *C. poinsettiae*, or bicyclic with either two alpha rings as in decaprenoxanthin or two beta rings as in C.p 450 from *C. poinsettiae* (16). So far the C\(_{45}\) and C\(_{50}\) groups of
Carotenoids have been found only in Gram positive aerobic bacteria with the exceptions of *Halobacterium* species and *F. dehydrogenans*. The biosynthetic pathways of the C$_{45}$ and C$_{50}$ carotenoids have been studied and follow similar but not identical routes in all organisms thus far examined. The outline for the synthesis of a bicyclic C$_{50}$-diol from lycopene in *C. poinsettiae* outlined by Norgard et al. (27) is depicted below:

There are no reports on the genetics of carotenoid synthesis in *C. poinsettiae*. This organism was chosen for this study because: (a) the pigments have been identified; and (b) this bacterium has been the subject of previous investigations on photodynamic killing (11). The purpose of this study was to isolate and partially characterize
pigment mutants of \textit{C. poinsettiae} ATCC 9682. Through such a study, we hoped to better resolve the biosynthetic pathways of carotenogenesis in this organism.
MATERIALS AND METHODS

Culture.--Corynebacterium poinsettiae ATCC 9682 was obtained from the American Type Culture Collection. The organism is Gram-positive, motile and phytopathogenic. The organism was maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Michigan) slants and was subcultured every three weeks.

Media.--Tryptic soy broth (TSB) (Difco) was the growth medium used for growing the cells in the various experiments. In addition a modified medium of Starr and Saperstein (35) was employed for confirming the identity of two pigments. The basal medium of Starr and Saperstein has the following composition (per 100 ml):

\begin{align*}
\text{NH}_4\text{Cl} & \quad 0.1\text{g} \\
\text{Acid-hydrolyzed Casein (General Biochemical, Inc.)} & \quad 0.1\text{g} \\
\text{K}_2\text{HPO}_4 & \quad 0.5\text{g} \\
\text{KH}_2\text{PO}_4 & \quad 0.5\text{g} \\
\text{Sodium citrate} & \quad 0.01\text{g} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 0.02\text{g} \\
\text{Calcium pantothenate} & \quad 0.1\text{g} \\
\text{Biotin} & \quad 0.1\text{g} \\
"\text{Trace}" \text{ metal solution} & \quad 1.0\text{ml}
\end{align*}
The pH was adjusted to 6.6. Glucose was sterilized separately by auto claving and added to yield a final concentration of 0.5%. The thiamine solution (10.0 mg/100 ml, pH 4.5) was also sterilized separately by auto claving and 1.0 ml of it was added to 100 ml of medium. The "trace" metal solution was of the following composition per 1000 ml and is the one used by Leadbetter and Foster (22).

\[
\begin{align*}
\text{CuSO}_4 \cdot 5\text{H}_2\text{O} & \quad 5.0\mu\text{g} \\
\text{H}_3\text{BO}_3 & \quad 10.0\mu\text{g} \\
\text{MnSO}_4 \cdot 5\text{H}_2\text{O} & \quad 10.0\mu\text{g} \\
\text{ZnSO}_4 \cdot 7\text{H}_2\text{O} & \quad 70.0\mu\text{g} \\
\text{MoO}_3 & \quad 10.0\mu\text{g} \\
\text{KCl} & \quad 0.04\text{g} \\
\text{CaCl}_2 & \quad 0.015\text{g} \\
\text{Deionized water} & \quad 1\text{ liter}
\end{align*}
\]

Chemicals.—Mutants, termed chemical mutants (cm), were induced by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Sigma Chemical Co.). \(\text{NH}_4\text{Cl}\) reagent grade was used occasionally to facilitate transfer of the pigments to the organic phase during saponification with five per cent aqueous KOH.

Solvents.—Reagent grade methanol was used for extraction of pigments. A petroleum ether:acetone mixture (8:2 v/v) was used in the development of chromatograms. Pigments were partitioned during extraction with acetone and diethyl ether. Other solvents employed were chloroform, benzene,
ethanol, hexane, and ethyl acetate. All solvents were reagent grade. The boiling range of the petroleum ether used was 35-60 C.

**Culture conditions.**—Seed flasks of TSB were inoculated from stock slants. The flasks were incubated under light on a rotary shaker at 30 C for 15 hours. After purity checks, the absorbancy of the cultures was read at 580 nm with a spectrophotometer (Bausch and Lomb digital double beam spectronic 210UV) and adjusted to 0.3 with sterile distilled water when necessary. A one percent inoculum (v/v) was used. The flasks were incubated as already described and harvested by centrifugation at 7710 x g for 10 minutes. The cells were washed with sterile water three times before extraction.

**Isolation of a streptomycin resistant (Str\(^R\)) strain of C. poinsettiae ATCC 9682.**—Eighteen test tubes, each containing 5 ml sterile double strength TSB, were set in two rows of nine. Appropriate volumes of filter sterilized streptomycin solution (5,000 \(\mu\)g/ml) and sterile water were added to give graded concentrations of streptomycin of 5, 10, 25, 50, 75, 100, and 200 \(\mu\)g/ml. One drop of a 15 hour culture of *C. poinsettiae* was added to each tube except tube number nine. Tube eight served as a positive control while tube nine served as a negative control (Table I). The tubes were incubated for 48 hours at room temperature under light with continuous shaking. Str\(^R\) mutants were isolated by
TABLE I

EXPERIMENTAL DESIGN FOR DETERMINATION OF THE MINIMAL LETHAL DOSE OF STREPTOMYCIN FOR CORYNEBACTERIUM POINSETTIAE

<table>
<thead>
<tr>
<th>Tube Numbers</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml. of streptomycin solution</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>4.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ml. of sterile water</td>
<td>4.9</td>
<td>4.8</td>
<td>4.5</td>
<td>4.0</td>
<td>3.5</td>
<td>3.0</td>
<td>1.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>ml. of double strength TSB</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Streptomycin concentration</td>
<td>5.0</td>
<td>10.0</td>
<td>25.0</td>
<td>50.0</td>
<td>75.0</td>
<td>100.0</td>
<td>200.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C. poinsettiae inoculum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Appropriate volumes of filter sterilized streptomycin solution (5,000 μg/ml) and sterile water were added to the two sets of nine tubes to give final concentration of streptomycin of 5, 10, 25, 50, 75, 100 and 200 μg/ml. All tubes were inoculated with a drop of a 15 hour culture except tube number nine and incubated for 48 hours. Tube number eight served as positive control while tube number nine served as the negative control.
streaking TSA plates containing 50 µg streptomycin/ml from those tubes in which growth appeared in 48 hours but not in 24 hours. Purity checks at each step were made by microscopic examination and streak plates.

Growth curve of the Strr strain of C. poinsettiae.--The absorbancy readings of cultures were taken at two- or three-hour intervals from hour zero to hour 21 (Figure 1).

Ultra-violet (UV) exposure time for 99% kill of Strr C. poinsettiae.--Twenty milliliters of culture were placed in a glass petri (15 x 60 mm) dish containing a magnetic stirring rod and the plate placed on a magnetic stirrer. A 1.0 ml sample was transferred to a sterile tube for determination of the initial population density. A UV-light model 420-Ul (George W. Gates & Co. Franklin Square, L. I., N. Y.), intensity (186 x 10³ µJ/cm²), positioned 12 inches over the culture was turned on and 1.0 ml samples were taken at 45, 90, 135, 180, 225, 270, 315, and 360 second intervals and placed in aluminum foil wrapped sterile tubes. Ten fold serial dilution of each sample were made (up to 10⁻⁷). Two-tenths ml of the 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions were plated in quadruplicate from the sample exposed for 45, 90 and 135 seconds. Two-tenths ml of the 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions were also plated in quadruplicate from the samples exposed from 180 to 360 seconds. The spread plate technique was used. In order to prevent photoreactivation, all operations
were carried out under a red light and plates were incubated in the dark at 30°C. Colonies were counted after 24 hours. Per cent survival was calculated using the following relationship:

\[
\frac{\text{Cells/ml at exposure time} \times 100}{\text{Cells/ml at zero time}}
\]

**Mutation of Str^r C. poinsettiae using NTG.** Twenty milligrams of NTG were dissolved in 5 ml sterile TSB. Two-fold serial dilutions from $2^{-1}$ to $2^{-10}$, were made in 5 ml TSB at pH 6.5. One drop of an overnight culture of the Str^r strain of *C. poinsettiae* was pipetted into each tube aseptically. The tubes were incubated under light at 30°C for 24 hours. Ten-fold serial dilutions of the $2^{-4}$, $2^{-5}$, and $2^{-6}$ dilutions which showed some growth after 24 hours of incubation were made in sterile water. One-tenth milliliter of the $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilution was plated out in quintuplicate using the spread plate technique. The plates were incubated in the light at 30°C for 48 hours. Mutants were picked aseptically and purified by repeated streaking until only one colony type was observed.

**Extraction of pigment.** One liter of a 24 hour shake culture was centrifuged at 7710 X g for 10 minutes. The cells were washed three times with sterile water and then extracted with methanol (5 ml portions) until the cells
became white. The extracted pigment was evaporated to dryness using a flash evaporator and then dissolved in 3.0 ml of a petroleum ether:acetone mixture (8:2, v/v). Sometimes it became necessary to saponify the pigment extract before concentration in order to eliminate oily substances which clouded the extracts.

Saponification.—A volume of 10% aqueous KOH was added to an equal volume of pigment extract in methyl alcohol to make a final concentration of 5% KOH. The mixture was left to stand for 1 hour. The pigment was then extracted from the mixture with chloroform. Occasionally it was necessary to add a small amount of NH₄Cl to assure that all of the pigments were transferred to the organic phase. The chloroform extract was washed with water, dried over ammonium sulfate, evaporated to dryness and then dissolved in 3.0 ml petroleum ether:acetone mixture (8:2, v/v).

Preparation of thin layer chromatography (TLC) plates.—A slurry of silica gel G (type 60) was freed of air bubbles by gently mixing 60 g of powdered silica gel/150 ml of distilled water. The thick silica gel slurry was then layered on glass plates (20 cm x 20 cm) to a thickness of 0.275 mm as measured by the Kessington Scientific Corporation gauge. Plates were air-dried. Prior to use the gel on the plates was activated by heat (100 C for 15 minutes) and promptly cooled to room temperature before applying the pigments.
Characterization of pigments

Chromatography.--The concentrated pigment in the petroleum ether:acetone solvent mixture was loaded on the thin layer chromatograph plate in the form of a band 2.0 cm from the lower edge of the plate. The plate was placed in a solvent tank containing and equilibrated with the petroleum ether:acetone solvent mixture. After development (about 30 minutes), the different pigments could be visualized and their Rf values calculated. The five bands of pigment obtained were separately scraped off and placed in two sets of five clean test tubes. The pigments in one set of tubes were re-extracted with petroleum ether. Those in the second set of tubes were re-extracted with acetone. The pigments were decanted off the silica gel and re-extraction continued until the silica gel was white. The pigment extracts were pooled and then concentrated as described previously. Fifteen μl of pigment extracts from the colored mutants were spotted side by side on thin layer chromatograph plate with pigment extracts from C. poinsettiae (wild type) using a 5 μl pipette. The chromatogram was developed as described. Genetic block points were determined by observing which pigment(s) in the wild type was/were lacking in the mutant type.

Spectrophotometry.--The different pigments obtained by chromatography and re-extracted in either petroleum ether or
acetone were scanned spectrophotometrically in the visible region (740-410 nm). The absorption maximum of each pigment was recorded and the results compared with those of Norgard et al (27).

Partition ratio.--The absorbancy of the various pigments in petroleum ether was determined. The petroleum ether was then evaporated to dryness and each of the pigments redisolved in equal volumes of a mixture of petroleum ether saturated with methanol and methanol saturated with petroleum ether. The petroleum ether phase was pipetted off and the absorbancy read. The partition percentage in petroleum ether was then calculated using the relation:

\[
\text{Optical density in petroleum ether partitioned with methanol} \\
\times 100
\]

\[
\text{Optical density in petroleum ether alone}
\]

The % partitioning in methanol was then determined by the difference.

Pigment per cell dry weight determination.--From two-hundred ml broth cultures of nine representative mutant types and of the wild type, 20 ml of each culture was centrifuged, washed three times with sterile water and placed in tared aluminum weighing boats. The weighing boats and contents were dried at 100 C overnight and weighed to determine the dry weights of cells. The 180 ml of each culture that remained was also centrifuged and the pigment
extracted as previously described. The absorbancies of the pigments were read at their absorption maxima. The amount of pigment was assayed using an extinction coefficient of $E_{\lambda m} = 2500$. 
RESULTS

Isolation and characterization of the Str^r mutant.--The test organism C. poinsettiae ATCC 9682 was first made streptomycin resistant in order to establish a genetically distinct strain and to reduce the chances of isolating possible pigmented contaminants. Using the procedure given in Materials and Methods, 25 µg of streptomycin/ml of medium was found to be the minimal lethal dose (Table II). Streptomycin resistant mutants were isolated by streaking TSA plates containing 50 µg streptomycin/ml from those tubes in which growth appeared in 48 hours but not 24 hours.

The growth characteristics of the isolated Str^r strain are represented in Figure 1. This curve indicates that the exponential growth phase at which cells were harvested occurred during the period of from 9 to 15 hours after incubation.

The number of cells/ml of culture of the Str^r strain was also determined and the results are shown in Figure 2. This was necessary to determine when 99% kill of this strain was obtained with UV-light irradiation. By exposing the Str^r strain to a dose of UV-light at various time intervals, the exposure time for a 99% kill was determined. Figure 3 is a plot of exposure time versus per cent survival.
<table>
<thead>
<tr>
<th>Tube Numbers</th>
<th>Streptomycin concentration (µg/ml)</th>
<th>Growth (+) or no growth (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>25.0</td>
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<td>4</td>
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<td>8</td>
<td>0.0</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>0.0</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE II
MINIMAL INHIBITORY CONCENTRATION OF STREPTOMYCIN FOR CORYNEBACTERIUM POINSETTIAE
Fig. 1--Growth curve of streptomycin resistant strain of *C. poinsettiae*. Test flasks were inoculated at a level of 1.0% (v/v) from an overnight culture with an absorbancy adjusted to 0.3 at 580 nm. The flasks were incubated at 30°C on rotary shaker. Absorbancy was read at 2 hour intervals.
Fig. 2--Relationship between optical density (OD) and cell numbers of C. poinsettiae. Test flasks were inoculated at a level of 1% (v/v) from a 24 hour culture of C. poinsettiae 0.3 OD. Samples were taken every 2 hours until 21 hours. Absorbancy of samples were determined as described previously. Spread plates were made in quadruplicate using 0.2 ml at dilutions of $10^{-4}$ - $10^{-7}$ and OD between 0.3 and 1.2.
Fig. 3--Ultra-violet light survival curve of streptomycin resistant strain of *C. poinsettiae*. A UV-light model 420-Ul (George W. Gates & Co. Franklin Square, L. I., N. Y.) positioned 12 inches above the culture was used.
From these data it was determined that exposure of the Str\(^r\) 
*C. poinsettiae* cultures to UV-light for 360 seconds resulted 
in a 99% kill.

**Isolation of pigment mutants:**

Mutants obtained by UV-light irradiation designated as 
photomutants (pm) and those obtained by NTG treatment desig- 
nated as chemical mutants (cm) were determined by visual 
observation of color differences of colonies on agar plates 
after incubation for 48 hours under continuous light. Sus- 
pected mutants were purified by a streaking on TSA containing 
50 μg/ml streptomycin.

**Characterization of the pigments of wild type C. poinsettiae 
ATCC 9682:**

Norgard et al. (27) in their investigation of carotenoids 
produced by *C. poinsettiae* have characterized and assigned 
the following names to the pigments:

- **C.p 482** = 2-isopentenyl-3,4-dehydro-rhodopin.
- **C.p 470** = 3,4,3',4'-tetrahydro-bisanhydrobacterioruberin.
- **C.p 496** = Bisanhydrobacterioruberin.
- **C.p 473** = 1'-hydroxy-1',2'-dihydro-2-isopentenyl-2'-(hydroxy- 
isopentenyl) torulene.
- **C.p 450** = 2-(dihydroxy-isopentenyl)-2-isopentenyl-β- 
carotene.

In this study the pigment bands numbered 1, 2, 3, 4, and 5 
on the chromatographic plate (from lower edge upwards) rep- 
resent, respectively the pigments found by Norgard. Table III
<table>
<thead>
<tr>
<th>Band Number</th>
<th>Rf-value</th>
<th>Partition Ratio</th>
<th>Absorption Maxima</th>
<th>Assignment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>Reported</td>
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<td>303,455, 356,374,</td>
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<td>483,412, 454,482,</td>
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<td>1</td>
<td>0.41</td>
<td>72:28</td>
<td>329,456, 377,458,</td>
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<td>485,516, 486,518.</td>
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<td>2</td>
<td>0.44</td>
<td>42:58</td>
<td>329,458, 345,362,</td>
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<td>476,506, 448,473,</td>
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<td>3</td>
<td>0.50</td>
<td>35:65</td>
<td>366,374, 471,498,</td>
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<td></td>
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<td></td>
<td>470,533, (390.</td>
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<td>498.</td>
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<tr>
<td>4</td>
<td>0.53</td>
<td>34:66</td>
<td>335,347, (365),451,</td>
<td>C.p 473</td>
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<td>368,441, 478,509.</td>
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<td>480,401.</td>
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<td>5</td>
<td>0.61</td>
<td>30:70</td>
<td>330,429, (427),454,</td>
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<td>450,480. 481.</td>
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Bausch and Lomb spectronic 210 UV was used in the determination of absorption maxima. Petroleum ether saturated with reagent grade methanol and methanol saturated with petroleum ether was used for partitioning. Solvent system petroleum ether: acetone (8:2 v/v) was used to develop the chromatogram. Pigments were extracted methyl alcohol from exponential phase cells. Before spotting, the methyl alcohol was evaporated and the pigments re-dissolved in petroleum ether:acetone mixture (8:2 v/v). For Rf determination, the pigments were spotted on TLC plate. For determination of partition ratio and absorption maxima, the pigments were banded on TLC plate.
shows: (a) the Rf-values of these pigments in petroleum ether:acetone mixture (8:2, v/v), (b) the partition ratios of the pigments in petroleum ether saturated with methanol and methanol saturated with petroleum ether, and (c) the absorption maxima of the pigments in petroleum ether and acetone respectively. Also included in the table are parallel data reported by Norgard et al. (27). From these data the pigments at each location have been identified as indicated. Figures 4a-j represent the absorption maxima peaks of these pigments. Both the partition ratios and absorption maxima agree well with those obtained by Norgard.

Starr and Saperstein (35), working with *C. poinsettiae* grown in a thiamine-deficient basal medium (1 μg/100 ml), found two pigments which have been characterized as C.p 470 and C.p 496: C.p 496 comprised 85% of the pigment mixture. By growing the test organism in this study in such a medium, and by use of TLC, it was possible to further confirm the assignment of pigments 2 and 3 as C.p 470, and C.p 496, respectively.

Characterization of pigments from mutants of *C. poinsettiae* ATCC 9682.—Figure 5 is a diagramatic representation of the pigment bands obtained from the mutants by TLC. They represent different combinations of the five major pigments obtained from *C. poinsettiae*. Ten distinct genetic groupings marked by ten different possible blocked locations were assigned. Each genetic variation was characterized by the
Fig. 4--Absorption maxima of the five major pigments in *Corynebacterium poinsettiae* (wild type). The Bausch & Lomb double beam digital spectronic 210UV with its recorder attachment was used in the determination.

(a) Abs. max. of C.p 482 in petroleum ether.

(b) Abs. max. of C.p 482 in acetone.

(c) Abs. max. of C.p 470 in petroleum ether.

(d) Abs. max. of C.p 470 in acetone.
(e) Abs. max. of C.p 496 in petroleum ether.
(f) Abs. max. of C.p 496 in acetone.
(g) Abs. max. of C.p 473 in petroleum ether.

(h) Abs. max. of C.p 473 in acetone.
(i) Abs. max. of C.p 450 in petroleum ether.

(j) Abs. max. of C.p 450 in acetone.
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<tr>
<th></th>
<th>Cp450</th>
<th>Cp473</th>
<th>Cp496</th>
<th>Cp470</th>
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<td>Group ii</td>
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<td>pm 2, 3, 4 cm 20, 24, 25</td>
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<td>Group x</td>
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Fig. 5--Diagrammatic reproduction of pigment separation of wild type Corynebacterium poinssetiae, (wt), Photomutants (pm), and Chemical mutants (cm) by thin layer chromatograph using silica gel G. The chromatogram was developed in a mixture of petroleum ether and acetone in the ratio of 8:2 (v/v). Groups ii-x based on pigments observed are represented. Group (i) comprised of colorless mutants which did not show any pigment bands on TLC is not included.
absence of different groups of pigment, viz:

Group (i) was made up of colorless mutants. There was total and complete block of pigment synthesis as no pigment band was evident.


Group (iii) lacked C.p 496, C.p 473, and C.p 450 with the block after C.p 470.

Group (iv) lacked C.p 473 and C.p 450, the genetic block occurring after C.p 496.

Group (v) lacked C.p 482 and C.p 470.

Group (vi) lacked C.p 482, C.p 473, and C.p 450. Two genetic blocks were observed. The first block was before C.p 482 and the second after C.p 496.

Group (vii) lacked C.p 470 and C.p 450, the blocks occurring after C.p 482 and C.p 473.

Group (viii) lacked C.p 473. The genetic block occurred after C.p 496.


Pigments from a total of 108 mutants were chromatographed and the frequency of mutation at each locus determined. The gene(s) and therefore the enzyme(s) coded for between C.p 470 and C.p 496 appeared to be the most
vulnerable having the highest mutation frequency of 20.37%. The frequency for complete block in pigment synthesis was 14.81%. The frequency of mutation between C.p 482 and C.p 470 was 11.11%. The frequency of mutation between C.p 496 and C.p 473 was 3.70%. Mutation frequency in mutants with blocks at two different loci was lowest at 1.85%.

Levels of pigment production in mutants.—Total pigment production per cell dry weight of representative mutant types and of the wild type C. poinsettiae are shown in Table IV.
<table>
<thead>
<tr>
<th>Absorption maximum in nanometers</th>
<th>Mutant Type</th>
<th>Pigment (mg/100 mg of cell dry weight)</th>
<th>% increase or decrease relative to wild type</th>
</tr>
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<tr>
<td>471</td>
<td>PM 35</td>
<td>8.82</td>
<td>74.87</td>
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<tr>
<td>452</td>
<td>PM 33</td>
<td>13.33</td>
<td>113.16</td>
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<tr>
<td>470</td>
<td>PM 2</td>
<td>16.04</td>
<td>136.16</td>
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<td>474</td>
<td>PM 28</td>
<td>8.96</td>
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<td>474</td>
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<td>PM 36</td>
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<td>PM 16</td>
<td>7.31</td>
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<td>453</td>
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<td>452</td>
<td>PM 9</td>
<td>15.6</td>
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<tr>
<td>473</td>
<td>wild type</td>
<td>11.78</td>
<td>100.00</td>
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</table>
DISCUSSION

Our results are in agreement with those of Norgard et al. (27) who suggested the following sequential biosynthetic pathway:

C.p 482 → C.p 470 → C.p 496 → C.p 473 → C.p 450

However, there are indications of alternative pathways. This possibility was suggested by the chromatograph of pigments from mutants lacking more than one pigment. The appearance of such mutants at a frequency of about 1.85%, and the fact that such mutants were obtained both with UV-irradiation and NTG treatment, further strengthens this suggestion. Those mutants we have placed in groups (i-iv) would seem to follow the biosynthetic pathway proposed by Norgard because of their patterns of sequential genetic blocks. In the first group there was total and complete block in pigment biosynthesis. In the second group the block was after C.p 482. In the third group the genetic block was observed after C.p 470. The block was observed after C.p 496 in group (iv). Groups v, vi, vii, viii, ix, and x present patterns which are not consistent with the linear biosynthetic sequence. In group v, C.p 496, C.p 473, and C.p 450 were present but the presumed precursors, C.p 482 and C.p 470, were missing. A possible explanation of this could be that the three pigments seen in this case may
have precursors other than C.p 482 and C.p 470 not detected with TLC either because they are colorless or because the pigments are present at very low levels. Alternatively, C.p 482 and C.p 470 may be present but at levels too low to detect. The same explanation might also apply to groups vi, vii, viii, ix, and x where end products are observed when presumed precursors are absent. Group vii marked by the absence of C.p 470 and C.p 450, might have an alternative explanation since in the sequence C.p 482 → C.p 470 → C.p 496, the C50-diols C.p 470 and C.p 496 differ only in the degree of saturation. It is possible, by a simultaneous dehydrogenation, to convert C.p 482 to C.p 496 without the intermediate step of C.p 470. Group viii lacked C.p 473. In lieu of the explanation given above, the only alternative would be the unlikely one of a dehydrogenation and cyclization at both ends of the molecule occurring simultaneously, resulting in the formation of C.p 450 without the intermediate stage C.p 473. A similar explanation might be offered for the pattern found in group ix. In group x where only C.p 482 and C.p 496 were found, it is possible that C.p 496, which is an aliphatic C50-diol, could be formed directly from C.p 482 which is an aliphatic C45-mono-ol by the addition of a C5-unit followed by dehydrogenation and hydroxylation without cyclization ever occurring.
REFERENCES


