BIOSYNTHESIS OF THE PHENAZINE

PIGMENT PYOCYANINE.

by

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April, 1964.
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Date April 1964
Abstract

Metabolic changes which occur in cultures of *Pseudomonas aeruginosa* prior to, and during pyocyanine production, as well as the biochemical precursors of the pigment, were investigated. Production of the pigment was found to occur at the end of the logarithmic growth phase and was paralleled by cell death and autolysis.

The degradation products of L-tryptophan, namely 3-hydroxyanthranilate and anthranilate were shown to be precursors of the pyocyanine molecule. It is proposed that anthranilate is methylated, at the expense of L-methionine, and that 3-hydroxyanthranilate and N-methyl anthranilate condense to form the phenazine nucleus. The suggestion of this pathway was based on enhancement studies, use of methyl analogues and isomeric forms of L-tryptophan in inhibition studies, and the use of auxotrophic strains.

A correlation between the levels of pyocyanine and oxidized nicotinamide nucleotides was also reported.
Acknowledgement.

I wish to thank Dr. J.J.R. Campbell for his help and guidance throughout this study. I would, also, like to thank my fellow graduate students who have freely offered their thoughts and constructive criticisms. I would, especially, like to thank Robin McKelvie, who isolated the auxotrophic strains of *Pseudomonas aeruginosa* and so generously donated them to my research.
ERRATA

page 23  line 15. "approach" should read "approach".

page 24  line 22. "four show only" should read "two show only".

page 28  "Bill-length  6  8  3" should read "Bill-length  8  6  3".

page 29  Under the heading "Bill-length", "P. bidentata" and "R. cardinalis" should read "X" instead of "-".

page 34  "Pipilio erythrophthalmus" should read "Pipilio erythrophthalmus".

page 46  Under the heading "Puerto Vallarta", "Thryothorus felix" should read "-4 weeks" instead of "-7 weeks".

page 73  line 5. "five" should read "four".

line 8. "four" should read "three".

line 25. "analysis" should read "analysis".

page 74  Under the heading "Tropical Deciduous", "P. pitiayumi" should read "X" instead of a blank space.

page 77  line 15. "Svardson" should read "Svårdson".

page 97  line 10. "Neslon" should read "Nelson".

page 166  line 13. "laurencii" should read "lawrencii".

page 170  line 17, 23-24. "von Madarasz" should read "Madarázs".

page 184  Under the heading "Mainland" insert "I" above "5" and "II" above "4"; under the heading "Island" insert "I" above "II" and "II" above "I".

page 189  Under the heading "Nest building", "T. felix" M should read "May 22-28" instead of a blank space; delete "May 29-June 24" from the same line.

page 215  line 1. "von Madarasz" should read "Madarázs".

line 2. "Termeszetrajzi" should read "Természetrájzi".

page 35  adjacent to the heading "Nos.", "Mainland mean larger" should read "Island mean larger", and "Island mean larger" below should read "Mainland mean larger"; under the heading "Tail", "54" should read "52".

page 177  the headings of the six vertical columns of figures should read, from left to right, "Adult σ", "Adult ω", "Immature σ", "Adult ω", "Adult ω" and "Immature ω".
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Introduction

The genus *Pseudomonas* is classified partially on the ability or inability of various species to excrete highly coloured pigments into the growth medium. The pigments, mainly red, green or blue in colour, have been the subject of investigation since the beginning of taxonomic studies. They have been found to belong to a variety of chemical families including pyrroles (pyoluteorine and fluorescein), quinolines (heptyl-hydroxy quinolines), and, by far the most renowned group, the phenazines. It is with this last group that the majority of investigations have been carried out and which will be the subject of this study.

Although there are several phenazine pigments such as pyocyanine, oxychlororaphin (a carboxyamide derivative), phenazine carboxylic acid, and iodinium (dihydroxy-Di-N-oxide derivative), the vast majority of work has been conducted on the α-hydroxy, N'methyl phenazine pyocyanine, which was first crystallized and defined by Wrede and Strack in 1924 and correctly characterized by Michaelis in 1935. Before that time and since, there has been a concerted effort to isolate bacterial strains and to develop media which will produce the pigment in high yield.

Pyocyanine is produced by the species *aeruginosa* (previously *pyocyanae*) of the genus *Pseudomonas*, an organism found widespread in nature, particularly in dilute aqueous suspensions and in sewage. The organism is often pathogenic and is a common wound contaminant in hospitals. Although it may
give rise to general infections and pathogenic lesions in man and animals, it is usually saprophytic - isolable from outer and middle ears, nasal passages, meninges, bronchii, udders, and other organs. Infections are characterized by a blue-green pigmentation - the green taint arising from several yellow-orange compounds also excreted by this organism which modifies the blue of pyocyanine. The pigments are mildly antibiotic, a property associated mainly with the less renowned and less obvious pigments of *P. aeruginosa*, although pyocyanine, itself, has little antibiotic activity. *P. aeruginosa* has been shown to act as an undesirable tainting agent in sheep wool, and some dairy products.

The role of pyocyanine in metabolism is not specific. It appears to have diverse effects on carbohydrate oxidation (Kurachi (1960); Campbell *et al.* (1957); and Ramakrishnan *et al.* (1955)), amino acid oxidase reactions (Marcus and Feeby (1962)), oxidative phosphorylation (McQuillan (1957)) and also to facilitate the growth of the organism in a hydrocarbon medium (Senez *et al.* (1961)) such as may exist in nature in petroleum plants, oil fields, and in sheep wool. Of chief significance metabolically is its oxidation-reduction ability and its role in hydrogen transport systems.

Voluminous data has been made available on the amino acid (Burton *et al.* (1947); Goodall (1961)), carbohydrate (Kay (1963)), and mineral requirements (Burton *et al.* (1948)) during pigment production under growth conditions and also nutritive requirements prior to production of pyocyanine in resting cells (Grossowicz *et al.* (1957)). However, little work has been done on the immediate precursors of the pigment or
upon metabolic or biosynthetic mechanisms involved in the
formation of pyocyanine or any other of the phenazine pigments.
The present study was undertaken to determine the precise nature
of pigment formation and to study metabolic changes which occur
prior to formation of pyocyanine.
Pyocyanine, the blue pigment produced by *Pseudomonas aeruginosa* was first reported and named in 1865 by a French worker, M. Fordos, who observed the blue-green pigmentation of pus in some surgical dressings and deduced that it was of bacterial origin. The causative agent was isolated by O. Gessard, who grew the organism and demonstrated pigment production on glycerol-peptone agar in 1882.

Since 1882, *P. aeruginosa* has been shown to produce not only pyocyanine but also several other pigments. A selection of the excreted compounds are described below:

1) **Pyocyanine** (Wrede and Strack (1929)). A prominent blue pigment with slight antibiotic activity and distinct redox and pH variability.

2) **Chlororaphin** (Kögl and Postowsky (1930)). A yellow-green pigment with related properties to pyocyanine.

3) **Quinoline derivatives** (Wells et al. (1952); Takeda (1959); Cornforth et al. (1956)). Compounds possess antibiotic activity. 
   \[ R_1 = \text{heptyl, nonenyl } \Delta, \text{ or undecyl.} \]
   \[ R_2 = -OH \text{ or } -H \]

4) **Pyoluteorine** - with chlorinated pyrrole ring (Takeda (1959)). A strongly antibiotic pigment which is inhibitory to *Staphylococcus, Microbacterium, Trichomonas*, and *Tetrahymena* but not to molds or yeasts. Hydroxyl groups may be methylated.

5) **Fluorescein** - A compound which gives a yellow-green fluorescent appearance to cultures. Structure has not been elucidated but its empirical formula has been given as C$_4$H$_7$O$_2$N and described as a pyrrole compound functioning in the respiration of the organism (Lenhoff (1965)).
Pseudomonads, other than *P. aeruginosa*, have been shown, under certain conditions, to produce pyocyanine as well as other phenazine derivatives, e.g. *P. chlororaphis* produces oxidized and reduced chlororaphin (6); *P. iodinum* yields iodinin (7), while *P. aureofaciens* yields phenazine-1-carboxylic acid (8).

\[
\text{Chemical structures}
\]

1) Kogl and Postowsky (1950).
2) Clemo and Daglish. (1950).
3) Dufraisse et al. (1952).
4) KLuyver (1956).
5) Haynes et al. (1956).

Robinson (1941) concluded that pyocyanine formation was induced only by substances necessary for the growth of *P. aeruginosa*. He reported that the two most important factors in pigment production were the concentration of substrates and the degree of oxidation of the media. He concluded that maximum growth and pigment production by *P. aeruginosa* required phosphate, magnesium ions, nitrogen and carbon, other ions being unnecessary (presumably with exception of a few in trace amounts). He also stated that high phosphate levels inhibited pyocyanine production. Hoffs (1934) showed that in his complex medium minus phosphate and magnesium ions pyocyanine formed but required an extended incubation period.

Laurie (1945), who studied the production of both pyocyanine and fluorescein, concluded that glycerol stimulated the production of both pigments, although fluorescein was enhanced at
high phosphate levels while increasing levels of phosphate had a
reciprocal effect on pyocyanine levels. Laurie also reported that
in aged pyocyanine cultures a red pigment appeared and apparently
increased at a corresponding rate to a loss in pyocyanine. This
red pigment, originally reported by Meader et al. (1925), forms
more rapidly at high temperatures and on solid medium and is init-
ially noted in areas of the culture most accessible to air, indicat-
ing an oxidative process. This apparent pyocyanine derivative is
water soluble - not extractable by chloroform and not pH variable.
However, it is chemically reducible to a colourless compound and
may be reoxidised to the original state. Holliman (1960) isolated
and reported the production of two red pigments with similar prop-
erties to Laurie's apparent pyocyanine derivative. His proposed
structures were 2-amino-6-carboxyl-10-methyl phenazinium hydroxide
and a related structure with an addition of a methyl- and sulfo-
group.

Burton et al. (1947 and 1948) carried out the most
complete survey to date on the nutritive requirements for maximal
production of pyocyanine by using a glycerol basal medium and
various combinations of amino acids, to determine amino acid
requirements. Mineral requirements were determined using acid
washed glassware and predetermined optional concentrations of
amino acids and glycerol. The optimal medium was concluded to
be:- glycerol 1.0%, alanine 0.4%, leucine 0.8%, K$_2$HPO$_4$ 0.04%,
MgSO$_4$•7H$_2$O 2.0%, FeSO$_4$•7H$_2$O 0.001%.

McDonald (1948) and Hellinger (1957) agreed that Burton's
medium was superior although the latter stated that potassium and
iron inclusion was not necessary for pyocyanine production.
Goodall (1961) improved on Burton's medium by removing leucine, decreasing Fe$^{++}$ concentration to 0.0005% and raising alanine concentrations to 1.0%. Medium changes were concluded to be due to chemical impurities in Burton's 1947 experiments. Chmura (1957) enhanced pyocyanine production by growing mixed cultures of *P. aeruginosa* with *Serratia marcescens* but as he did not distinguish between cell growth and pyocyanine production little could be concluded from his data.

In 1889 Bauchard worked on the antagonistic properties of *P. aeruginosa* towards other bacterial species. He found that the typical blue-green cultures were due to the blue pigment, pyocyanine, superimposed upon a yellow pigment. Early workers found that the pigment could readily be extracted from basic solutions with chloroform. However, difficulties were encountered in purification and it was not until 1925 that Wrede and Strack proposed a novel phenazine system as the structure of pyocyanine. They suggested a double structure due to empirical formula calculations ($C_{20}H_{20}N_4O_2$) (1).

![Chemical structure of pyocyanine](image1)

1)  

2)  

3)  

![Chemical structures](image2)
Michaelis (1935) simplified the structure to a monomolecular moiety (2). In 1929 Wrede and Strack proposed the structure which is widely accepted at present (3).

Further studies on pyocyanine production were carried out by Grossowicz et al. (1957) who demonstrated pigment production in non-poliferating suspensions of two soil strains of *P. aeruginosa* and showed that glutamic acid and γ-amino butyric acid were the best stimulators of the pigment. They also showed that pigment production did not commence until after the L-glutamate (≈1.0%) had been used up as shown by amino acid analysis. It is significant to note that the respiratory poisons sodium azide, potassium cyanide, etc., first inhibited pyocyanine and then, at higher concentrations, growth, indicating the need for energy sources in pyocyanine production. Halpern et al. (1962) doing further work on their system showed that pyocyanine production was induced after a six hour lag. The time lag and subsequent pigment formation were not accompanied by an increase in cellular mass (Figure 1).

Frank and DeMoss (1959) were unable to duplicate the results of Grossowitz et al. using strain A.T.C.C. 9027 of *P. aeruginosa* and therefore studied pyocyanine production in growing cultures. They reasoned that growth and pigment production were so closely related that specific precursors of the pigment under these conditions were difficult to determine. They felt that exogenous amino acids used in pigment production were modified extensively to facilitate growth. Using C^{14} alanine in the growth medium they showed that the pigment could be derived solely from this amino acid. They did not, however, investigate the label
Fig. 1. Time-course of pyocyanine formation (◼ ◼ ◼), protein concentration (▵ ▵ ▼), dried weight (○ ○ ○), and viable cell count (⊙ ⊙ ⊙). Reproduced from Halpern et al. (1962).
distribution in the pigment as did Ingram and Blackwood (1962) with $^{14}C_{1-3}$ glycerol. In 1957 Blackwood and Neish established an order of preference of labelled carbohydrates and amino acids which were added to a modified Burton's medium, containing 0.4% DL-alanine, 0.8% L-leucine, 0.2% MgSO$_4$·7H$_2$O, 0.05% K$_2$HPO$_4$, 0.001% FeSO$_4$·7H$_2$O, 1.0% Glycerol and 0.1% CaCO$_3$. Labelled substrates were added at concentrations of 0.05 millimoles per 100 ml. of medium. Glycerol and dihydroxyacetone, shown by R. von Tigerstrom (personal communication) to be metabolized by similar routes in P. aeruginosa, were the only substrates which formed pyocyanine with a higher specific activity higher than the average level in cell carbon or respiratory carbon dioxide. L-alanine had the next most preferred relative incorporation rate at approximately one-fifth the glycerol label incorporation. In a subsequent paper Blackwood with Ingram (1962) showed, using a method of pyocyanine degradation, that glycerol $^{14}C_{1-3}$ was incorporated into the phenazine as shown:

They suggested condensations to occur as follows:

A) 3, 2 carbon units/
Benzene ring

B) 1, 4 carbon unit +
1, 2 carbon unit/
Benzene ring
Their experiments indicated no equilibration of the pathway compounds due to the specificity of the labelling. The condensation of a six carbon compound forms the ring and is in turn derived from non-phosphorylated trioses.

Sellers and Wynne (1961) in a review of pigment production, suggested that aldehydic sugars were inhibitory to synthesis and polyhydric alcohols and keto hexoses induced production; however W.W. Kay (1963) demonstrated this to be false, suggesting low pH values to be the inhibiting factor as buffered media containing aldehydic sugars often gave high pigment levels.

In 1958-59 M. Karachi published a series of papers on the biosynthetic pathway of pyocyanine production, and after doing general studies to establish conditions of production concluded that 2.5% glycerol, 0.2% urea, 0.05% MgSO₄·7H₂O, 0.025% K₂HPO₄, 0.0005% FeSO₄·7H₂O was a good basal medium. He demonstrated that in many P. aeruginosa strains methionine enhanced production but not growth of the organism and also that an aromatic derivative found in cows' milk and yeast extract stimulated the pigment. He showed that 6-hydroxy phenazine was found in the medium and that it must be a degradation product as even with methionine and carbon sources it could not be converted to pyocyanine. However, P. aeruginosa cultures could reconvert a degradation product of Aspergillus niger or A. oryzae - incubated with pyocyanine - into pyocyanine. As precursors of the pigment Kurachi tried both aromatic amines and poly phenol compounds in an attempt to establish the biosynthetic pathway. Most compounds tried, notably aniline, acetanilide, o-phenylene diamine, resorcinol and pyrogallol inhibited production. The inhibitory effect of the
aromatic amines was neutralized by addition of anthranilic acid 
\((5 \times 10^{-4} - 2 \times 10^{-3} \text{M})\) which when added by itself stimulated production. The inhibition by polyphenols, with the exception of resorcinol, was not affected by anthranilate additions. Tryptophan (DL or L) had no effect. Acetone powder preparations with anthranilate, methionine, sodium succinate and NaCl gave no pyocyanine but did yield a product with a \(\lambda_{\text{max}}\) at 260 nm. He also showed that in peptone medium containing aniline, acetanilide, o-phenylene diamine or p-amino benzoic acid there was an accumulation of anthranilate in the medium demonstrating a point of inhibition.

MacDonald (1963) using an isotope competition technique concluded that quinic acid and shikimic acid of the aromatic amino acid biosynthetic pathway provided the best specific activity relative to cell carbon of a wide variety of carbohydrate, amino acid and other metabolic compounds tested. He claimed that in the presence of anthranilic acid pyocyanine production was inhibited until all of the anthranilate was metabolized and that his competitive isotope technique indicated that anthranilate did not affect the specificity of the pyocyanine formed. However, at the concentrations used (0.8 - 1.0%) it is difficult not to consider that the overall effects of compounds added might seriously interfere with production. Millican (1962, 1 & 2) followed the incorporation of anthranilic (C\(^{14}\)) and shikimic acids (C\(^{14}\)) into pyocyanine by addition to glycerol-alanine medium reasoning that shikimic acid is a known precursor of anthranilate which Kurachi had shown to stimulate production. He found that he could not duplicate the anthranilate stimulation and only 0.04% of the total reactivity of the labelled compounds, was found in the pyocyanine - much
more was found in bacterial protein. In the case of shikimic acid he obtained only 23% disappearance from the medium 16% of which was recovered in pyocyanine (4% incorporation). The low rates of incorporation suggested to him that these were not precursors of the pigment.

Studies conducted on other phenazine pigments give some indications of the biosynthetic mechanisms of phenazine formation.

Levitch (1961) investigated phenazine-α-carboxylic acid biosynthesis and showed that by addition of various labelled compounds he could establish an order of preference of incorporation. He reported the following decreasing order of $^{14}$C incorporation into pigment: DL-tryptophan-$3-O^{14}$, glycerol-$1,3-O^{14}$, DL-alanine-$2-O^{14}$, Na acetate-$1-O^{14}$, DL-tyrosine-$U-O^{14}$, DL-serine-$3-O^{14}$, Glucose-$6-O^{14}$, Na DL-lactate-$1-O^{14}$, L-threonine-$U-O^{14}$, Na pyruvate-$1-O^{14}$, Na formate-$O^{14}$, and glucose-$1-O^{14}$ (unlabelled pigment).

Carter and Richards (1961) claimed that at least one ring of oxychlororaphin (phenazine-1-carboxamide) was derived in their experiments from anthranilic acid. They concluded that the ring of the phenazine bearing the carboxyl group was not synthesized via 3-hydroxyanthranilic acid as this pathway would result in the loss of the carboxyl label of anthranilate during reaction with ribose-$5$-phosphate to form indole prior to tryptophan synthesis.

In the biosynthesis of related chemical compounds such as the phenoazones, studies indicate that tryptophan degradation products may be direct precursors. Sivak, Nobili and Katz (1960) reported that the phenoazinone chromophore (actinocin) of the actinomycins was isotopically labelled when *Streptomyces antibioticus* was grown in the presence of DL-tryptophan-$7-C^{14}$. 
They also reported that the methyl groups attached to the chromophore were derived from \( \text{L}-\text{methionine} \) as demonstrated by \( {^{14}\text{C}}\text{H}_2 \text{L}-\text{methionine} \) incorporation into the moiety. In a subsequent paper Sivak and Katz (1962) demonstrated that the inhibition of actinocin biosynthesis by methyl analogs of tryptophan (\( \alpha-, 4-, 5- \) and \( 6- \) methyl derivatives) could readily be circumvented by additions of \( \text{L}-\text{tryptophan}, \text{L}-\text{kynurenine}, 3\text{-hydroxy DL-kynurenine} \) or \( 3\text{-hydroxyanthranilic acid} \). They postulated the following condensation:

\[
\begin{align*}
\text{COR} & \quad \text{NH}_2 \quad \text{OH}_2 \quad \text{O} \\
\text{COR} & \quad \text{NH}_2 \quad \text{OH}_2 \quad \text{O} \\
\text{H}_2 & \quad \text{O} \\
\text{H}_2 & \quad \text{O} \\
\text{H}_2 & \quad \text{O}
\end{align*}
\]

\[
2, 4 \text{ Methyl-3-hydroxyanthranilate} \quad \text{Actinomycin - R} \\
R^* = \text{-OH or peptide} \quad \text{Actinomycin - R} = \text{peptide} \\
\text{Actinocin - R} = \text{-OH} 
\]

In classic papers A. Butenandt et al. (1955 and 1956) showed that a \( \text{Drosophila} \) eye pigment, Xanthommatin was produced by condensation of 2 \( 3\text{-hydroxykynurenine} \) molecules. Xanthommatin is the phenoxozone derivative shown below.

Pyocyanine has been shown to function in the oxidation of a number of compounds by \( \text{P. aeruginosa} \). Senex and Azouley (1960 & 1961) showed that dried cell preparations would emulsify and oxidize \( n\)-heptane to \( 1\)-heptene in the presence of pyocyanine but not in controls without pyocyanine. By studying redox potentials (\( -0.03 \text{V at pH 7.0} \)) and other requirements of the
system they established the following equations:

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_5\text{CH}_3 & \rightarrow \text{CH}_3(\text{CH}_2)_4\text{CH}^=\text{CH} \rightarrow \text{Alcohol} \rightarrow \text{Aldehyde} \\
\text{NAD} & \rightarrow \text{NADH} + \text{H}^+ \\
\text{cytochrome c} & \rightarrow \text{pyocyanine} \rightarrow \text{leuco-pyocyanine (+2H)} \\
& \text{(Intense green fluorescence)}
\end{align*}
\]

The same workers (1960) showed that \textit{P. aeruginosa} under anaerobic conditions could utilize nitrate as a terminal hydrogen acceptor to obtain growth. Under these conditions it was found that pyocyanine could be substituted for the nitrate with an efficiency of 40% while methylene blue allowed no growth when used as the sole hydrogen acceptor.

In \textit{P. aeruginosa} pyocyanine has been shown to enhance the rate of oxidation of gluconic acid to 2-ketogluconic acid, by cell free extracts, with increasing concentrations up to a level of approximately 200 \(\mu\)m while cytochrome c, flavin mononucleotide, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD) or the 3' phosphorylated derivative (NADP) did not affect the enzymatic rate, the latter two compounds not being reduced in the reaction although cytochrome c did undergo reduction (Ramakrishnan and Campbell; 1955). Strasdine et al. (1961) suggested that the inhibition of glucose oxidation at 2-ketogluconic acid, initially reported by McQuillan (1956) was due to magnesium chelation by the pyocyanine chromophore.

In a study of the physiological role of pyocyanine in \textit{P. aeruginosa}, Kurachi (1960) obtained results similar to those of Senez and Azouley. He showed that pyocyanine produced some
recovery of auto-respiration of resting cells inhibited by high
concentrations of KCN, however, it had no effect on normal respi-
ration. Pyocyanine did, however, stimulate respiration in cell
extracts and suggested that it replaced NAD or flavoproteins
destroyed in extract preparation.
Materials and Methods

i) Bacteriological:

Various strains of Pseudomonas aeruginosa were used in this study. Those used were as follows: ATCC 9027, G-8 (Greek cheese isolate), 5-64 (Gossowicz et al.), Jap. M3 and Jap. Bl (Kurachi), W409 (U.B.C. Bacteriology dept.) B100, B101, B97, and 120NA (all from U.B.C. Dairying dept. stocks). Two auxotrophic strains of ATCC 9027, isolated by R.M. McKelvie following the procedure given below, were also used. These mutant strains required tryptophan (Try-) and an organic source of sulfur (Org. S-) for growth respectively. In comparison studies P. chlororaphis B977 (Haynes et al.), which produced some pyocyanine, was also used.

All organisms were maintained in glycerol-peptone broth and transferred twice before use. Incubation temperature in all experiments was 35.5°C. Organisms were inoculated into media at approx. 2x10⁷ cells/ml. following centrifugation from transfer medium and resuspension in sterile, pH 7.8, 0.05 M, tris (tris (hydroxy methyl) amino methane) buffer.

Experimental media were adapted from Goodall (1961) and contained 0.05 M, pH 7.8 tris buffer, 0.0005% FeSO₄·7H₂O, 0.04% K₂HPO₄, 2.0% MgSO₄·7H₂O, 0.5% alanine and 0.4% of either glycerol or Ca 2-ketoglucconate. The latter three compounds were occasionally added at different concentrations as indicated in the results. Media components were sterilized together in an autoclave, at 15 p.s.i. for 15 minutes, except the MgSO₄ which was sterilized in the autoclave separately and added after
cooling and Ca 2-ketogluconate which was sterilized by filtration and added prior to inoculation. Glassware, Erlenmeyer flasks and test tubes, were washed, rinsed with hot, 10% nitric acid and re-rinsed repeatedly with distilled water. Erlenmeyer flasks were initially stoppered with cotton wool, however, as this led to variable yields, due to the tightness of the plug, flasks were covered with an inverted beaker while test tubes were covered with metal caps. Erlenmeyer flasks used were 125 ml. and contained 10 ml. of media. Test tubes were 18x150 mm. and contained 2 ml. of media.

Amino acids and other compounds added to media were used in isomeric forms and at concentrations, in the final media volume, as indicated. Standard solutions were made at 0.1% and 1.0% as necessary, sterilized by millipore filtration and stored at 4°C until used.

The procedure for auxotroph isolation was as given by Matney et al. (1962) with the modification of ultraviolet treatment of the cells in solution prior to millipore filtration, and increasing penicillin levels to $2.5 \times 10^4$ I.U./ml.

Resting or non-proliferating cell cultures were made following the procedure of Gossowicz et al. (1957) with the modification that cells were grown in broth cultures containing the basal medium with glycerol (0.4%) instead of on nutrient agar. Cells were harvested by centrifugation at 20 hours, washed twice with tris buffer (pH 7.8, 0.05 M) and resuspended in the same buffer at approx. 14 mg/ml. dried weight. Final volume at incubation was 2.0 ml. by addition of 1 ml. of cell suspension to 1 ml. of buffered nutrient solution.
ii) Chemical.

Anthranilic acid and N-methyl anthranilic acid were obtained from Eastman Kodak Co. (Rochester, N.Y.), and 3-hydroxyanthranilic acid from Light and Co. (London). N-formyl anthranilic acid was synthesized by addition of formaldehyde to a 10% ethereal solution of anthranilic acid at 0°C., stirring for 1 hour and collecting the precipitate formed by filtration.

o-Amino benzyl alcohol was made by a modification of the procedure of Canover and Tarbell (1950) as follows:— To 506 ml. of 2% methyl anthranilate in ethyl ether was added 4% LiAlH₄ and the mixture stirred at room temperature for 15 minutes. The reaction mixture was then neutralized carefully with 10% sodium hydroxide (200 ml.) and the aqueous layer extracted with chloroform and, subsequently, concentrated by flash evaporation at 35°C. to one tenth volume. The crude alcohol was precipitated by addition of warm petroleum ether (b.p. 55~5°C.) and recrystallized from petroleum ether chloroform.

Tryptophan derivatives (6-methyl-L-, 4-methyl-DL-, a-methyl-DL- and D isomers) were obtained as a gift of the U.B.C. dept. of Neurophysiology.

iii) Procedures used.

Pyocyanine was assayed by extraction of a known volume of culture with an excess of chloroform (usual dilution factor ¼) and optical density (O.D.) readings taken at 690 µ on the Beckman model "B" spectrophotometer. These results were compared with those obtained by re-extracting the chloroform solutions
with aqueous acid, neutralizing the pyocyanine in aqueous solution, and then reading at 690 \( \mu \text{m} \), and shown to be comparable although slightly higher. Optical density readings were converted to concentrations (\( \mu \text{g/ml.} \)) by comparison to a standard curve made using crystalline pyocyanine·HCL.

Protein was determined using the method of Lowry et al. (1951). Desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) by method of Schneider (1957). Amino acids were measured by the method of Yemm and Cocking (1950) modified by boiling samples with 0.5N sodium hydroxide to drive off dissolved ammonia (efficiency 99% as demonstrated by control 1 mg/ml ammonia solution) and then the sample neutralized before the assay was carried out. Ammonia was determined by the method of Conway (1950). Nicotinamide nucleotides were determined by the potassium cyanide method of Kaplan (1957).
Experimental results and discussion

As a wide variety of experimental results have been published on the production of pyocyanine under an equally wide variety of conditions it was felt that the strains of *P. aeruginosa*, both pigmented and non-pigmented, should be studied to determine pyocyanine production under standard conditions. A total of twelve strains were examined and grown on a basal medium of L-alanine (1.0%), MgSO₄·7H₂O (2.0%), K₂HPO₄ (0.04%), FeSO₄·7H₂O (0.0005%) in tris buffer (0.05 M, pH 7.8) with a carbohydrate source of either glycerol (1.0%) or 2-ketogluconate (Ca⁺⁺)(1.0%). Results may be seen in figure 2.

The organisms exhibit a wide range of pyocyanine levels under the standard conditions. Glycerol can readily be seen to stimulate the greatest yield of pyocyanine in ten out of twelve strains, the exceptions being strain B97, classified as a non-pigmented strain, and B977, a strain of *P. chlororaphis*. However, a rate study of these organisms showed that growth on 2-ketogluconate was more rapid than on glycerol and pigment production also commenced much sooner (figure 3).

In the highly pigmented, rapidly growing strains pigment production began at approximately 13.5 hours in 2-ketogluconate while in glycerol media growth is slow and pigment production lags until 18.0 to 19.0 hours.

It is of interest to note that strains B977, B100 and B101 produced an orange-yellow pigment, typical of *P. chlororaphis*, in the glycerol medium while none was detectible in 2-ketogluconate media.
Fig. 2. Pyocyanine production by strains of P. aeruginosa\textsuperscript{+} at 68 hours in media containing either glycerol or Ca\textsuperscript{++}-2-ketogluconate

* - strains show chlorophaphin production in glycerol media only.
+ - except B977 which was classified as P. chlororaphis.
Fig. 3. Pyocyanine and growth levels at 20 hours in several strains of *P. aeruginosa*.
A = Glycerol medium.
B = Ca²⁺ 2-ketogluconate medium.
As both pigment production and growth occurred rapidly and predictably in 2-ketogluconate medium it was felt that the actual nature of the culture's growth should be examined in an effort to determine the changes, if any, which occur at the time of initiation of pigment production and to attempt to interpret them in terms of the metabolism of the cell.

Strain G-8 was inoculated into basal-salts-buffered medium containing 0.5% 2-ketogluconate (Ca++) and several levels of L-alanine (1.0%, 0.5%, 0.25%, 0.1%, and 0.05%) with the knowledge that at the two lower levels pigment production would not occur to any appreciable extent. In figure 4 (a)(b) and (c) it may be seen that the point of commencement of pyocyanine production was coincident with the liberation of ammonia into the cell supernatant (confirms observations of Kay (1965)), termination of the logarithmic growth phase and a rapid disappearance of alanine from the medium. The pH of the medium tends to climb slightly, increasing from 7.7 to 8.0, however, this is presumably due to the liberation of ammonia. The cells grown on 0.05% and 0.10% L-alanine had exhausted the supply of amino acid prior to attaining the level of growth achieved in higher alanine concentrations and prior to the time of normal pyocyanine production.

In the case of 0.10% alanine pyocyanine production appeared to lag several hours as did the liberation of ammonia (figure 4(a)). In these cultures, grown on low levels of alanine, there was a slight but significant rise in amino acids in the medium in aging cultures. This rise was interpreted to be due to cell autolysis with subsequent liberation of pool amino acids. Due to the rapid decrease in alanine levels in the medium it was
Fig. 4(a). Strain G-8 in basal medium containing 0.1% L-alanine.

Fig. 4(b). Strain G-8 in basal medium containing 0.25% L-alanine.
Fig. 4(c). Strain G-8 in basal medium containing 0.50% L-alanine.

Fig. 4(a), (b) and (c). Rate of growth (△—△), pyocyanine production (-----), ammonia release (△—△) and alanine uptake (○—○) by *P. aeruginosa* (G-8) in Ca**++**-2-ketogluconate basal medium.
thought that possibly the ammonia may arise via deamination reactions. However determinations of α-keto acids in the cell supernatant showed no detectible pyruvate and only trace amounts of a slow reacting α-keto acid (as α-keto glutarate) in only the 12 hour sample of the 1.0% alanine medium, immediately prior to pyocyanine formation. This agrees with observations of Kay (1963). Although no assay method was available 2-ketogluconate could be readily demonstrated to be present in some quantity by the silver nitrate-sodium hydroxide reaction on a paper chromatogram.

As pyocyanine production commences at the end of the logarithmic growth phase (also reported by Harris (1951), Kurachi (1958), and McDonald (1963)) it was decided to follow cell viability, cell dried weight and pyocyanine production as well as to determine levels of protein, ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) in the cells. It may be seen in figure 5 that immediately upon reaching maximal cell concentrations the viable cell count dropped rapidly as pyocyanine was produced. Dried weight of the cells paralleled the viable count as did protein, RNA and DNA levels (Table 1). It appears that upon dying, as they entered the stationary phase, the cells autolyzed.

The above data appears to show that pyocyanine production is dependent upon the cells entering the stationary phase in the presence of an organic nitrogen source. Pyocyanine production would seem to be proportional to the decrease in viable cells.

The data of Halpern et al. (1962) (figure 1) showed similar loss in viability during pyocyanine production in resting cells. These workers (1957) reported, however, that in the non-
Fig. 5. Rate study of viable cell count (---), dried weight (○—○), and pyocyanine production (△—△) in Ca++ 2-ketogluconate medium.
proliferating cultures exogenously supplied glutamate was used up prior to pigment production.

Table 1. Changes in levels of protein, RNA, DNA and dried weight in a growing culture of *P. aeruginosa* strain G-8.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Protein (μg/ml)</th>
<th>RNA (μg/ml)</th>
<th>DNA (μg/ml)</th>
<th>Dried weight (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>440</td>
<td>110</td>
<td>50</td>
<td>0.75</td>
</tr>
<tr>
<td>15.0</td>
<td>1120</td>
<td>240</td>
<td>130</td>
<td>2.40</td>
</tr>
<tr>
<td>19.0</td>
<td>1640</td>
<td>280</td>
<td>180</td>
<td>2.60</td>
</tr>
<tr>
<td>22.0</td>
<td>1300</td>
<td>240</td>
<td>130</td>
<td>1.70</td>
</tr>
<tr>
<td>34.0</td>
<td>1500</td>
<td>180</td>
<td>130</td>
<td>1.50</td>
</tr>
<tr>
<td>40.0</td>
<td>880</td>
<td>160</td>
<td>100</td>
<td>1.10</td>
</tr>
</tbody>
</table>

In glycerol cultures the situation appears analogous to 2-ketogluconate grown cultures although as stated previously, growth and pyocyanine production lagged somewhat (table 2).

Table 2. Increase in cell density and pyocyanine during growth of G-8 in glycerol medium.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Pyocyanine (μg/ml)</th>
<th>Optical density</th>
<th>Viable cell count (x10^9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>-</td>
<td>0.41</td>
<td>3.7</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>1.55</td>
<td>6.5</td>
</tr>
<tr>
<td>21</td>
<td>30</td>
<td>1.95</td>
<td>8.2</td>
</tr>
<tr>
<td>24</td>
<td>200</td>
<td>2.00</td>
<td>9.5</td>
</tr>
<tr>
<td>30</td>
<td>225</td>
<td>2.00</td>
<td>5.4</td>
</tr>
</tbody>
</table>

As 2-ketogluconate and glycerol have been reported to give high yields of pyocyanine when included in an amino acid medium it was felt that there may be some common pathway of oxidation of these two carbohydrates. According to the Gilson metabolic charts (1961) glycerol may be oxidized to hydroxypyruvate, then transaminated to give L-serine with the amino group being donated by L-alanine, the preferred amino acid in pyocyanine production. It has been tentatively proposed by Gronlund (1962)
that 2-ketogluconate may undergo a 3-3 split during oxidation, a step which could yield hydroxypyruvate. It was then decided that possibly L-serine might be able to be partially substituted for either the carbohydrate and/or the amino acids of the basal medium. However, preliminary experiments showed that this was not the case and that any but low levels of L-serine in the medium inhibited growth and pigment production. Low levels of L-serine added to a limiting medium of L-alanine and carbohydrate showed no stimulatory effects while similar levels of either of the normal ingredients of the basal medium enhanced pyocyanine production.

Pyocyanine biosynthesis

It has been suggested that the origin of the phenazine nucleus is via the condensation of one or more anthranilic acid moieties (Kurachi (1958); Carter and Richards (1961)). This postulate was not confirmed by the radioactive experiments of Millican (1962). Levitch (1961) showed that DL-tryptophan-5-C\(^{14}\) was incorporated into a phenazine nucleus with a higher specific activity than any other compound studied. McDonald (1963), using an isotope competition technique, suggested that compounds of the aromatic amino acid biosynthetic pathway were precursors of the pyocyanine molecule.

i) Stimulation experiments

Although the actual cultural conditions during pyocyanine production appear to involve rapid cellular degradation it is unlikely that the benzenoid moieties of the phenazine nucleus arise directly from any one component of cellular protein, as on a quantatative basis it would require that about one-fifth of the
amino acids would have to be precursors of pyocyanine in that 300 μg/ml of pyocyanine are formed from approximately 2.5 mg/ml dried weight of cells. Therefore it is suggested that a specific pathway may be stimulated at the end of the logarithmic growth phase or during cell autolysis, which would provide precursors for pigment production.

As considerable debate has been published on the role of anthranilic acid and its derivatives in pyocyanine biosynthesis it was decided to attempt to stimulate the production of the pigment using these and related compounds added to the growth medium.

Initially compounds were added at the time of inoculation, however, this appeared to promote a variable effect on the growth rate of the culture and hence conclusions could not be drawn. It was then decided that additions made after pyocyanine production had commenced would be preferable. Additions were made at 20 hours in glycerol media and at 15 hours in 2-ketogluconate media.

Difficulties in comparing one experiment to a second arose due to the fact that, particularly in 2-ketogluconate media, duplicate experiments yielded distinctly different levels of pigment if carried out using different inoculum. Within one experiment results were relatively constant. Glycerol (0.4%) was used where possible in stimulation experiments.

Compounds used in attempts to stimulate pyocyanine production were as follows: - D- and L-tryptophan, L-methionine, o-amino benzyl alcohol, 3-hydroxy-, N-methyl-, and N-formyl-anthranilic acids and anthranilic acid itself. Other aromatic amino acids were also used. o-Amino benzyl alcohol was made and used on the suggestion by Dr. M.F. Mallette that the aerobic
organism *P. aeruginosa* may condense the anthranilate derivative via an aldehydic functional group and may be able to carry out the formation of this group in a much more efficient manner oxidatively rather than reductively. The tested precursors were added to the medium at concentrations usually between $10 \mu g/ml$ and $200 \mu g/ml$. In normal cultures pyocyanine production varied between $200 \mu g/ml$ and $400 \mu g/ml$ maximum.

It was found that the following compounds stimulated production at concentrations given:-- L-tryptophan (10-50 $\mu g/ml$), 3-hydroxyanthranilate (10-30 $\mu g/ml$), N-methyl anthranilate (5-30 $\mu g/ml$), anthranilate (20-100 $\mu g/ml$) and L-methionine (10-50 $\mu g/ml$). Combinations of these compounds yielded stimulation but not significantly in excess of any alone, except in the case of the latter two compounds which gave only slight stimulation when added separately but significantly more when added together. Results of the additions are given in figures 6, 7, 8 and 9. N-formylanthranilate and o-amino benzyl alcohol had either no or inhibitory effects. D-tryptophan inhibited pigment biosynthesis (figure 10).

These results show that although the enhancement of production was not stoichiometric certain conclusions are readily apparent. In figure 7 the addition of 20 $\mu g/ml$ 3-hydroxyanthranilate the production was further enhanced. In figure 6 it can be seen that L-tryptophan significantly stimulated production. N-methyl anthranilate stimulated a level somewhat more than twice the added concentration and was paralleled by the stimulation of anthranilate combined with L-methionine at slightly higher concentrations (figure 8). In general stimulation was considerably in
Fig. 6. Addition of L-tryptophan at 20 μg/ml. (Δ--Δ), and L-tryptophan+L-methionine at 15 μg/ml. (○--○) to growing cultures of G-8.

Fig. 7. Effect of addition of 3-hydroxyanthranilate at 20 μg/ml. (○--○) and 3-hydroxyanthranilate + L-methionine at 20 μg/ml. each (Δ--Δ) on pyocyanine yield in growing cultures of strain G-8.
Fig. 8. Effect of additions of N-methyl anthranilate at 10 μg/ml. (Δ—Δ), and anthranilate + L-methionine at 15 μg/ml. each (O—O) to growing cultures of G-8.

Fig. 9. Effect of additions of 3-hydroxyanthranilate plus N-methylanthranilate at 15 μg/ml. each (Δ—Δ) and 3-hydroxyanthranilate + anthranilate + L-methionine at 10 μg/ml. each (O—O) to growing cultures of G-8.
excess of that expected from contributions to the phenazine nucleus.

The enhancement in strain G-8 is significant but to strengthen our knowledge of the role of these compounds in the production of pyocyanine it was decided to test the effect of the compounds on a variety of strains. Four strains were tested and two showed similar enhancement patterns to strain G-8. These were 5-64 and B977 (P. chlororaphis). Strain Jap. Bl showed no enhancement in any case and generally derivatives added caused inhibition of pyocyanine formation. B97, a non-pigmented strain, showed some pyocyanine formation in cases where L-methionine was added together with an aromatic derivative; N-methyl anthranilate also stimulated low level production (table 3).

Table 3. Effects on pyocyanine production of additions made to various strains of P. aeruginosa.

<table>
<thead>
<tr>
<th>Additions</th>
<th>(µg/ml) of each component</th>
<th>Pyocyanine yield (µg/ml) in strains used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5-64</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>3-OH Anth.</strong></td>
<td>10</td>
<td>296</td>
</tr>
<tr>
<td><strong>3-OH Anth. + L-met.</strong></td>
<td>10</td>
<td>368</td>
</tr>
<tr>
<td><strong>NMA</strong></td>
<td>10</td>
<td>384</td>
</tr>
<tr>
<td><strong>Anth.</strong></td>
<td>20</td>
<td>374</td>
</tr>
<tr>
<td><strong>Anth. + L-met.</strong></td>
<td>20</td>
<td>344</td>
</tr>
<tr>
<td><strong>3-OH Anth. + NMA</strong></td>
<td>20</td>
<td>348</td>
</tr>
<tr>
<td><strong>3-OH Anth. + Anth. + L-met.</strong></td>
<td>20</td>
<td>380</td>
</tr>
<tr>
<td><strong>L-tryptophan</strong></td>
<td>20</td>
<td>388</td>
</tr>
</tbody>
</table>

* Additions made at 20 hours and readings at 92 hours (except B977 due to slow cell growth rate additions at 44 hours and final reading at 140 hours).

** Abbreviations: 3-OH Anth. = 3-hydroxyanthranilate; NMA = N-methylanthranilate; Anth. = anthranilate; L-met. = L-methionine.
In an attempt to simplify the complicated growth situation and pyocyanine production the procedure of Grossowicz et al. (1957) was followed, with some modifications, to produce and attempt to enhance pigment production in non-proliferating (resting) cell suspensions of several strains of \textit{P. aeruginosa}. Strains G-8, 5-64, and ATCC 9027 produced pyocyanine in resting cultures with \textit{L}-glutamate as substrate. The method of Grossowicz was modified by growing the cells in a basal glycerol-alanine broth as opposed to solid nutrient agar cultures. Using the agar grown cells ATCC 9027 failed to produce pyocyanine in resting cell suspensions. Results are given in table 4. Strains B977, B97, and Jap. Bl showed no pigment production in resting cells.

**Table 4.** The effect of 3-hydroxyanthranilate, N-methyl anthranilate and \textit{L}-tryptophan on pyocyanine production in non-proliferating cultures of three \textit{P. aeruginosa} strains.

<table>
<thead>
<tr>
<th>Additions</th>
<th>(\text{ug/ml. of each component added})</th>
<th>\text{Pyocyanine ((\text{ug/ml.}) in strains at times given.}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>* 3-OH Anth.</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>3-OH Anth.</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NMA</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>NMA</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>\textit{L}-tryptophan</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>\textit{L}-tryptophan</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>\textit{L}-tryptophan</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>\textit{L}-tryptophan</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>3-OH Anth. + NMA</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>3-OH Anth. + NMA</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

* Abbreviations - see Table 3.
In ATCC 9027 pyocyanine was detectible in the cultures with high L-tryptophan concentrations several hours before the control or other samples.

ii) Precursor extraction.

In control growing cultures older than about three days the rate limiting process is apparently the condensation of precursors and not the production of them. This was readily demonstrated by addition of gradient levels of Seitz filtered four day old supernatant to a 20-24 hour pigment producing culture and observing the stimulation pattern. Stimulation was not observed by basal medium additions. It is probable that the supernatant of a pyocyanine producing culture contains the precursors of pyocyanine as well as the wide variety of metabolites produced by this organism.

A culture of *P. aeruginosa* producing pyocyanine (pH 8.0) was extracted with chloroform repeatedly then acidified (<pH 4.0) and extracted with ethyl ether. The two extracts were concentrated by flash evaporation (40°C.) and components separated by thin layer chromatography (TLC). Samples taken before pyocyanine production commenced (12.5 hours in 2-ketogluconate medium) showed no fluorescent derivatives present while after that (14-24 hours) as many as seven could be separated from each extract using as solvent chloroform (4): butanol (4): pyridine (1). It is important to note that under ultraviolet light all anthranilic acid derivatives are fluorescent. In the above solvent anthranilic acid and its monomeric derivatives appear as diffuse streaks. In chloroform (4): ethanol (4): ammonia (1) the anthranilic derivatives are distinct spots and are separable. In the ether extract (H⁺) N-
methyl anthranilic acid was tentatively identified, having similar fluorescence and identical Rf value of 0.46 in the latter solvent on Silica gel G.

The isolated compounds from the extracts when added to growing or resting cultures appeared to inhibit production. Since a thorough investigation was not made, however, low concentrations of some of them might enhance production.

iii) Influence of \( \alpha \)-, and 4-methyl-\( \text{DL} \)-, and 6-methyl-\( \text{L} \)-, and \( \text{D} \)-tryptophan.

Katz et al. (1962) have reported that the biosynthesis of the actinomycin chromophore, actinocin, is dependent upon the condensation of two 3-hydroxyanthranilic acid moieties. They found that actinocin synthesis could be inhibited, in vivo, by several methyl analogues of tryptophan. These analogues were reported to inhibit the synthesis and/or degradation of tryptophan by the Streptomycete and that \( \text{L} \)-tryptophan, \( \text{L} \)-kynurenine, 3-hydroxy-\( \text{L} \)-kynurenine or 3-hydroxyanthranilate overcame this inhibition.

Several analogues of tryptophan were shown to be inhibitory to pyocyanine biosynthesis, in vivo. The analogues included \( \text{D} \)-tryptophan, \( \alpha \)-, and 4-methyl-\( \text{DL} \)-, and 6-methyl-\( \text{L} \)-tryptophan. Inhibitory effects of these compounds are given in figures 10, 11, 12 and 13. In agreement with the order given by Katz et al., the inhibitory abilities of the various tryptophan analogues appears to be \( \alpha \rightarrow 4 \rightarrow \text{D} \rightarrow 6 \).

Reversal of these inhibitory effects could be obtained by the addition of \( \text{L} \)-tryptophan and some of the anthranilic acid derivatives previously tested. Unfortunately \( \text{L} \)-kynurenine was not available and could not be used in attempts to overcome the
Fig. 10. Inhibition of pyocyanine production in growing cultures of strain G-8 by D-tryptophan additions.

Fig. 11. Inhibition of pyocyanine production by α-methyl-DL-tryptophan additions to growing cultures of G-8.
Fig. 12. Inhibition of pyocyanine production by 4-methyl-DL-tryptophan in strain G-8.

Fig. 13. Inhibition of pyocyanine production by 6-methyl-L-tryptophan in strain G-8.
inhibition. Figure 14 gives the effect of L-tryptophan on the inhibition by D-tryptophan and figure 15 shows the recovery effect of 3-hydroxyanthranilate with and without L-methionine additions. Neither N-methyl anthranilic acid nor anthranilic acid plus L-methionine reversed D-tryptophan inhibition of pyocyanine production.

In the case of α-methyl-DL-tryptophan (α-MT) inhibition the situation appears more complex in that not only did L-tryptophan and 3-hydroxyanthranilate overcome the inhibition but so did N-methylanthranilate. Figures 16 and 17 show the effects of these compounds. L-methionine and anthranilate when added separately did not compete with the inhibitory processes. When added together N-methyl anthranilate and 3-hydroxyanthranilate overcame the inhibitory effect of α-MT to a level equal to the control with no additions as seen in figure 18. The effect is obviously non-competitive as the levels of the two stimulatory compounds added were not sufficient to stoichiometrically produce this enhancement.

The effect of 6-MT and 4-MT appear very similar to that of the α-methyl analogue in that reversal of inhibition experiments yielded similar results (tables 5 and 6).

Table 5. Reversal of 6-methyl-L-tryptophan inhibition of pyocyanine synthesis in growing cultures of G-8.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Percent of control yield at the various levels of 6-methyl-L-tryptophan given (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>100*</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>97</td>
</tr>
<tr>
<td>3-OH-Anth.</td>
<td>112</td>
</tr>
<tr>
<td>3-OH-Anth. + L-methionine</td>
<td>121</td>
</tr>
</tbody>
</table>

* Control yield = 336 μg/ml at 84 hours.
Fig. 14. Effect of L-tryptophan (50 μg/ml., O—O) on pyocyanine production inhibited by D-tryptophan (100 μg/ml., △—△).

Fig. 15. Effect of 20 μg/ml. 3-hydroxyanthranilate (□—□) and 3-hydroxyanthranilate plus L-methionine (20 μg/ml. each) (O—O) on pyocyanine production inhibited by D-tryptophan (100 μg/ml., △—△).
Fig. 16. Reversal of \( \alpha \)-methyl-\( \text{DL} \)-tryptophan, at 50 \( \mu g/ml \). (O-O) inhibited pyocyanine production by \( \text{L} \)-tryptophan additions (\( \triangle -\triangle \)) and (O-O), 50 and 100 \( \mu g/ml \). respectively.

Fig. 17. Reversal of \( \alpha \)-methyl-\( \text{DL} \)-tryptophan (50 \( \mu g/ml \)). (O-O) inhibited pyocyanine production by 3-hydroxyanthranilate (20 \( \mu g/ml \)). (\( \triangle -\triangle \)) and N-methyl anthranilate (20 \( \mu g/ml \)). (O-O).
Fig. 18. Effect of a combined addition of 3-hydroxyanthranilate and N-methyl anthranilate at 20 μg/ml, each (O--O) on pyocyanine production inhibited by α-methyl-DL-tryptophan at 50 μg/ml. (Δ--Δ).

Time (hours)

Pyocyanine (μg/ml)
Table 6. Reversal of 4-methyl-DL-tryptophan inhibition of pyocyanine synthesis in growing cultures of \textit{P. aeruginosa} strain G-8.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Percent of control yield at the levels of 4-methyl-DL-tryptophan given below ((\mu\text{g/ml}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>100*</td>
</tr>
<tr>
<td>L-tryptophan 20 (\mu\text{g/ml})</td>
<td>100</td>
</tr>
<tr>
<td>L-tryptophan 50 (\mu\text{g/ml})</td>
<td>97</td>
</tr>
<tr>
<td>L-tryptophan 100 (\mu\text{g/ml})</td>
<td>90</td>
</tr>
<tr>
<td>3-OH Anth. 20 (\mu\text{g/ml})</td>
<td>112</td>
</tr>
<tr>
<td>3-OH Anth. 50 (\mu\text{g/ml})</td>
<td>107</td>
</tr>
<tr>
<td>NMA 20 (\mu\text{g/ml})</td>
<td>118</td>
</tr>
<tr>
<td>3-OH Anth. + L-methionine 20 (\mu\text{g/ml}) each</td>
<td>121</td>
</tr>
<tr>
<td>3-OH Anth. + L-methionine 50 (\mu\text{g/ml}) each</td>
<td>118</td>
</tr>
</tbody>
</table>

\* Control yield = 336 \(\mu\text{g/ml}\) at 84 hours.

Table 7. Effect on cell density of additions to 20 hour growing cultures of compounds shown to affect pyocyanine synthesis in \textit{P. aeruginosa}.

<table>
<thead>
<tr>
<th>Additions at 20 hours.</th>
<th>Concentration ((\mu\text{g/ml}))</th>
<th>Percent growth - as optical density at 660 (\text{nm}), 42 hours</th>
<th>68 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100*</td>
<td>100*</td>
</tr>
<tr>
<td>4-ethyl-DL-tryptophan</td>
<td>50</td>
<td>94</td>
<td>95</td>
</tr>
<tr>
<td>4-methyl-DL-tryptophan</td>
<td>50</td>
<td>85</td>
<td>87</td>
</tr>
<tr>
<td>6-methyl-L-tryptophan</td>
<td>100</td>
<td>87</td>
<td>95</td>
</tr>
<tr>
<td>L-methionine</td>
<td>20</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>3-hydroxyanthranilate</td>
<td>20</td>
<td>110</td>
<td>105</td>
</tr>
<tr>
<td>N-methyl anthranilate</td>
<td>20</td>
<td>118</td>
<td>108</td>
</tr>
<tr>
<td>Anthranilate</td>
<td>20</td>
<td>99</td>
<td>97</td>
</tr>
</tbody>
</table>

\* optical densities of 1.62 and 1.90 at 42 and 68 hours respectively.

As inhibitory and stimulatory compounds were added at 20 hours little effect upon growth of organisms was expected. This was confirmed by recording the optical densities of suspensions at 660 \(\text{nm}\) in acid. The tryptophan analogues gave O.D. values 10 to
15% lower than controls while the samples containing 3-hydroxy- and N-methyl anthranilate tended to have slightly elevated readings (Table 7).

The pathway of tryptophan degradation in *Pseudomonas* was established by Suda *et al.* (1950); Stanier *et al.* (1951), Hayaishi and Stanier (1951) and Behrman (1962) who clearly established the oxidation of this compound to empirical carbon units. The oxidation proceeds by the well known pathway found in mammals to the point of formation of kynurenine. In mammals this compound is hydroxylated at the 3-position to give 3-hydroxy-L-kynurenine which is, in turn, cleaved to give 3-hydroxyanthranilate and alanine. In the oxidation of L-kynurenine in *Pseudomonas*, as given by Behrman, the molecule is cleaved directly to give anthranilate and alanine. The anthranilate being degraded by a series of oxygenases to yield acetate, pyruvate and succinate. The isomeric forms of tryptophan are both reported to be oxidized to kynurenine via specific enzymes so that one isomer of tryptophan will yield the same isomeric form of kynurenine (Behrman (1962)). When oxidized in this manner D-tryptophan will specifically enter the quinoline pathway by an oxidative deamination reaction to undergo further oxidation while the L-isomer may enter either pathways described, the quinoline or the anthranilic pathways. A D- to L-racemase for tryptophan was reported to occur in *Pseudomonas* by Behrman and Cullen (1961).

The occurrence of 3-hydroxyanthranilate in the normal degradative pathway has not been reported in *Pseudomonas*, however, its presence has been suggested in *Xanthomonas* as a precursor of nicotinic acid and the nicotinamide nucleotides (Davis *et al.* (1951); and Harris and Binns (1957)).
Hayaishi and Stanier (1951) reported that the 4-, 5-, 6- and 7- methyl analogues of tryptophan were unoxidizable. These workers also reported that the pH optimum for the tryptophan oxygenase reaction to be 7.8 which is identical to that reported for pyocyanine production by Grossowicz et al. (1957).

Hayaishi (1955) reported that the addition of 5-methyl tryptophan to pseudomonas extracts gave 12% inhibition of the tryptophan oxygenase reaction and that 4-, 6- and 7- methyl analogues were less inhibitory. Umbrecht (1955) stated that α-methyl tryptophan inhibits the same oxygenase reaction and forms α-methyl kynurenine. Sourkes and Townsend (1955) and Civen and Knox (1960) reported that in rat liver preparations α-methyl tryptophan inhibits the oxygenase reaction. The latter workers also stated that the 5- and 6- methyl-DL-derivatives were 10% oxidized to the respective kynurenine analogues. Moyed (1960) observed that 5-methyl-DL-tryptophan inhibited tryptophan biosynthesis in Escherichia coli by a false feedback mechanism but could not act in the positive role of L-tryptophan and inhibited growth in this manner. Trudinger and Cohen (1956) noted that 4-methyl tryptophan strongly inhibited the synthesis of anthranilate and indole removal by washed cells of E. coli. The conversion of anthranilate to indole was not affected by the 4-methyl analogue.

On the basis of the above evidence it may be suggested that the analogues of tryptophan studied in the inhibition of pyocyanine production affect either the tryptophan oxygenase reaction and/or tryptophan synthesis at either the anthranilate synthetase or indole desmolase stages. If the tryptophan analogues are oxidized to kynurenine analogues inhibition may also occur at
later stages of tryptophan catabolism. The effect of D-tryptophan may also be speculated to be due to either a feedback role inhibiting L-tryptophan synthesis or when oxidized to D-kynurenine it may function to inhibit competitively the hydroxylation reaction of the L-isomer and thereby inhibit 3-hydroxyanthranilate formation. This could readily account for the effect of 3-hydroxyanthranilate on D-tryptophan inhibited pyocyanine production. The most probable source of anthranilate in the organism is via L-tryptophan catabolism since the synthetic pathway from shikimic acid to indole has been demonstrated only with considerable difficulty indicating close proximity of enzymes and substrates of the pathway (Gibson et al. (1962)). Therefore the role of the methyl analogue of tryptophan in inhibiting synthetic steps and/or degradation would be expected to be overcome by both 3-hydroxyanthranilate and N-methyl anthranilate as was the case. The lack of effect of anthranilic acid in overcoming methyl analogue inhibition may be related to its tendency to retard growth slightly. (table 7).

Due to the enhancement of pigment production by L-tryptophan, 3-hydroxyanthranilate and N-methyl anthranilate, the ability of the two former compounds to overcome D-tryptophan inhibition and the effect of all three on the inhibition of pyocyanine production by tryptophan methyl analogues it may be suggested that these compounds are precursors of the pigment.

iv) Use of auxotrophic strains of P. aeruginosa.

In efforts to confirm the above suggestion mutant strains of P. aeruginosa ATCC 9027 were used. The two auxotrophic strains obtained required tryptophan (Try-) and methionine (Org. S-)
respectively for growth. The latter strain was unable to make use of inorganic sulfate added to the medium as concluded by the fact that growth was obtained when either methionine or cysteine but not when homocysteine or serine were included (R.M. McKelvie - personal communication). The point of blockage in the Try- auxotroph was determined to be between the conversion of anthranilate to indole as shown in table 8.

Table 8. Point of blockage in the Try- auxotroph of *P. aeruginosa* ATCC 9027.

<table>
<thead>
<tr>
<th>Additions</th>
<th>ConcH (µg/ml)</th>
<th>20 hours</th>
<th>40 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Pyocyanine</td>
<td>Growth</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthranilate</td>
<td>20</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>Indole</td>
<td>20</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>5</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>10</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>20</td>
<td>+3</td>
<td>trace</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>50</td>
<td>+3</td>
<td>+</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>100</td>
<td>+3</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 8 also shows the concentration of L-tryptophan that would support growth but not pyocyanine production. Unfortunately the auxotrophs were rather labile and reversion to the wild type occurred in the control medium in approximately 40 hours and although the presence of some growth in media with low concentrations of required substrate tended to inhibit this reversion - termed "dark repair" - results after this time were viewed with some scepticism.

Experiments with the Try- auxotroph were designed to stimulate pigment production in media containing i) enough L-tryptophan to support growth but only meager pyocyanine production and ii) sufficient L-tryptophan to produce limited pigment concentrations. Results are given in table 9. It may be seen that although
levels of production are low the effect of 3-hydroxyanthranilate was to stimulate pigment production as were N-methyl anthranilate additions. This latter compound appeared to be less effective perhaps because the accumulation of anthranilate at the point of blockage in this auxotroph and the possibility of N-methylation with L-methionine. The use of this auxotroph in growing cultures helps confirm the role of 3-hydroxyanthranilate and N-methyl anthranilate in pyocyanine production.

The addition of L-tryptophan to the auxotroph at 18 hours stimulated growth of the organism and stimulated pyocyanine production excessively. This suggests that the cellular growth of the organism may be involved in some way in pyocyanine synthesis.

In an attempt to bypass the growth effect in the auxotrophic (Try-) strain it was grown in Roux flasks containing basal-buffered medium plus 100 ug/ml DL-tryptophan, harvested at 20 hours, at which time only slight amounts of the pigment had been formed, and reassembled to give non-proliferating cultures. L-glutamate was used, as described previously, as sole carbon and nitrogen source and the proposed precursors of pyocyanine were added. In no case did pyocyanine form in these resting cultures even though when DL-tryptophan was added to growing cultures the strain was able to synthesize the pigment.

The use of the Org. S- auxotroph, in growing cultures, in attempts to determine the source of the methyl group of pyocyanine, was not too successful, in that, although the results did indicate that the methyl group could be derived from L-methionine by addition onto anthranilate producing N-methyl anthranilate they were not significant enough to draw conclusions from them alone (Table 10).
After 40 hours the majority of cultures had obviously reverted to the prototrophic strain.

Table 9. Effect upon pyocyanine production of additions of anthranilate derivatives, L-methionine and L-tryptophan to an auxotrophic (Try-) strain of *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Additions (18 hours)</th>
<th>Conc(\text{(\mu)g/ml})</th>
<th>Pyocyanine ((\mu)g/ml)</th>
<th>35 hours</th>
<th>48 hours</th>
<th>35 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td></td>
<td>7</td>
<td>14</td>
<td>70</td>
<td>124</td>
</tr>
<tr>
<td>L-methionine</td>
<td>20</td>
<td></td>
<td>7</td>
<td>24</td>
<td>70</td>
<td>124</td>
</tr>
<tr>
<td>Anthranilale</td>
<td>20</td>
<td></td>
<td>0</td>
<td>0</td>
<td>64</td>
<td>92</td>
</tr>
<tr>
<td>3-OH Anth.</td>
<td>20</td>
<td></td>
<td>16</td>
<td>30</td>
<td>92</td>
<td>202</td>
</tr>
<tr>
<td>3-OH Anth.</td>
<td>50</td>
<td></td>
<td>32</td>
<td>50</td>
<td>101</td>
<td>92</td>
</tr>
<tr>
<td>NMA</td>
<td>20</td>
<td></td>
<td>14</td>
<td>48</td>
<td>68</td>
<td>130</td>
</tr>
<tr>
<td>NMA</td>
<td>50</td>
<td></td>
<td>8</td>
<td>16</td>
<td>70</td>
<td>136</td>
</tr>
<tr>
<td>3-OH Anth. + NMA</td>
<td>20 each</td>
<td></td>
<td>30</td>
<td>54</td>
<td>84</td>
<td>152</td>
</tr>
<tr>
<td>3-OH Anth. + Anth.</td>
<td>20 each</td>
<td></td>
<td>28</td>
<td>48</td>
<td>77</td>
<td>108</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>30</td>
<td></td>
<td>42</td>
<td>134</td>
<td>76</td>
<td>346</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>50</td>
<td></td>
<td>72</td>
<td>174</td>
<td>84</td>
<td>416</td>
</tr>
</tbody>
</table>

(A) - 30 \(\mu\)g/ml L-tryptophan added to basal medium at the time of inoculation of auxotrophic (Try-) *P. aeruginosa* ATCC 9027.

(B) - 50 \(\mu\)g/ml L-tryptophan added to basal medium.
Table 10. The effect upon pyocyanine production of L-methionine and anthranilate derivatives added to growing cultures of an auxotrophic (Org. S-) strain of *P. aeruginosa* grown in (a) basal medium plus 30 μg/ml L-methionine, and (b) basal medium plus 70 μg/ml L-methionine.

<table>
<thead>
<tr>
<th>Additions (20 hours)</th>
<th>Conc(l μg/mL)</th>
<th>Pyocyanine (μg/ml) at 33 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>21.5</td>
</tr>
<tr>
<td>L-methionine</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>L-methionine</td>
<td>30</td>
<td>27.5</td>
</tr>
<tr>
<td>L-methionine</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>NMA</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>NMA</td>
<td>30</td>
<td>27.5</td>
</tr>
<tr>
<td>L-methionine + NMA</td>
<td>20 each</td>
<td>27.5</td>
</tr>
<tr>
<td>L-methionine + NFA</td>
<td>20 each</td>
<td>25</td>
</tr>
<tr>
<td>L-methionine + 3-OH Anth.</td>
<td>20 each</td>
<td>0</td>
</tr>
</tbody>
</table>

* NFA = N-formyl anthranilate.

Resting cell cultures of this Org. S- auxotroph, for reasons not understood, gave sporadic production even in controls.

**Physiological factors**

The results discussed so far indicate that 3-hydroxyanthranilate plays the role of at least partial precursor to the pigment, pyocyanine. The pigment has been shown by several workers to play distinct physiological roles in the respiration of the bacterial cells. It was therefore felt that demonstration of a correlation of the concentrations of another respiratory agent derived from 3-hydroxyanthranilate with the levels of pyocyanine would be of significance. The presence of oxidized nicotinamide nucleotides can readily be measured by increase in optical density.
of a conjugation product with potassium cyanide at 325 μ. It can be seen that the level of oxidized nicotinamide nucleotide increased at a rate parallel to the liberation of pyocyanine (figure 19). This result demonstrates the possibility that there may be some physiological importance to the liberation of pyocyanine under conditions of cell degradation and autolysis. It is possible that the strongly aerobic conditions required for the growth of the organism are deficient and the liberation of pyocyanine is an effort to offset this fact. Kurachi (1960) reported that pyocyanine may act as a sole hydrogen acceptor, while Senez et al. (1958) have shown the inter-relationship of pyocyanine and NAD in hydrocarbon oxidation.

The formation of nicotinamide nucleotide from 3-hydroxyanthranilate has not been reported in Pseudomonas, although it has in Xanthomonas (Davis et al. (1951); Harris et al. (1957)), but the close correlation between pyocyanine and oxidized nicotinamide nucleotide would indicate that this is a pathway in this organism.
Fig. 19. The rate of production of pyocyanine (O--O), and cellular level of oxidized nicotinamide nucleotides (□--□) in Ca\(^{2+}\)-ketogluconate grown cultures of \textit{P. aeruginosa} G-3.
General discussion and conclusions

It was found that, in confirmation of reports by Harris (1951), Kurachi (1958) and MacDonald (1965), the production of pyocyanine commenced at the end of the logarithmic growth phase. A viable cell count of the culture during pigment production indicated that the maximal number of cells was achieved shortly after commencement of synthesis of the pigment. Following this highest level of growth there was a rapid decrease in viable cells paralleled by a decrease in cellular dried weight. It would appear that following rapid growth of the culture there was an equally rapid rate of cell autolysis. The liberation of ammonia coincided with the autolysis and pyocyanine synthesis. The production of ammonia did not appear to be due to amino oxidase reactions since α-keto acids could not be detected. Marcus and Feeby (1962) demonstrated that L-amino acid oxidase of snake venom is distinctly inhibited by the presence of pyocyanine because of the pigment's ability to intercept hydrogens from the reaction and prevent the necessary reduction of the flavin enzymes of the system.

In media containing levels of alanine low enough so that at the end of the logarithmic growth phase none remained in the supernatant little or no pyocyanine production occurred, indicating the need for an exogenous nitrogen source for the stimulation of pyocyanine production. This disagrees with the data of Grossowicz et al. (1957) who reported that, in resting cell suspensions, substrate levels were inversely proportional to the rate of pigment production.
Although cell degradation occurred at a rapid rate during pigment production, it is improbable that the precursors of pyocyanine are liberated via degradative enzymes because of the fact that in order to account for the yield of pyocyanine any single precursor would have to comprise approximately 12% of the cell dried weight. It is suggested, therefore, that there is a specific pathway, feeding precursors towards pigment biosynthesis and that this pathway is activated at the end of the logarithmic growth phase or during cell autolysis.

The effect of various amino acids and anthranilic acid derivatives on phenazine pigment production has been reported by a number of workers (Carter and Richards (1961); Levitch (1961); MacDonald (1963); Kurachi (1958); and Millican (1962)). These workers used a wide variety of conditions and an equally wide range of concentrations of proposed precursors, and so it was felt that, under standard conditions, new attempts should be made to stimulate and enhance production of pyocyanine. Compounds used in attempts to stimulate pyocyanine were: D- and L- tryptophan, L-methionine, 3-hydroxy-, N-methyl-, and N-formyl anthranilate, o-amino benzyl alcohol, and anthranilate itself. N-formyl anthranilate has been proposed as a metabolic degradation product of L-tryptophan in Neurospora by Matchett et al. (1965) and it was thought that it might, if present, be reduced at some stage of pyocyanine biosynthesis and act as the methylated moiety of the final molecule. The use of o-amino benzyl alcohol was suggested by Dr. M.F. Mallette as a precursor, because of the possibility that the condensation of an anthranilate type moiety might proceed via an aldehyde functional group. The formation of this group,
in this aerobic microorganism, might occur oxidatively from the alcohol in preference to its formation reductively from anthranilic acid. In the experiments undertaken the addition of either N-formyl anthranilate or 6-amino benzyl alcohol to pyocyanine producing cultures inhibited rather than stimulated production.

\[ \text{L-tryptophan, 3-hydroxyanthranilate} \pm \text{L-methionine, N-methyl anthranilate and anthranilate + L-methionine were found to enhance pigment production. The levels of enhancement were in considerable excess of the amount anticipated by stoichiometric condensations. It was felt that possibly the presence of high levels of pigment precursors stimulated, in some way, the level of the condensation enzymes.} \]

In aged cultures (<3 days) the media contained compounds which when added aseptically to young cultures enhanced pigment production indicating that, in early stages of pigment synthesis, the precursors are limiting, and that in aged cultures the condensing or other associated enzymes may be limiting. Initially most of the enhancement experiments were carried out using one strain of \textit{P. aeruginosa} (G-8), but increased significance of the data was obtained when other strains, with the exception of Jap. B1, showed similar stimulation effects.

The complicated picture of growth and pigment production was simplified by the use of resting cell suspensions. It was found that three strains were able to produce pigment under these conditions: G-8 (Greek cheese isolate), 5-64 (strain of Grossowicz et al.) and ATCC 9027. In these resting cell suspensions 3-hydroxyanthranilate, N-methyl anthranilate and L-tryptophan all enhanced production. In strain 5-64 the enhancement could be
further extended by making combined additions of 3-hydroxy-
anthranilate and N-methyl anthranilate. In ATCC 9027 L-tryptophan:
additions induced pyocyanine production to begin several hours
earlier than the control or other samples.

Methyl analogues of tryptophan and D-tryptophan were
shown to inhibit the production of pyocyanine. It was also found
that in growing cultures, the compounds which enhanced pyocyanine
production reversed these inhibitory effects.

It was found that D-tryptophan inhibited pigment prod-
duction and that this inhibition could be partially reversed by
the addition of either L-tryptophan or 3-hydroxyanthranilate *
L-methionine. N-methyl anthranilate had no effect on the inhibition.
The data of Behrman (1962) and the above information indicate
that the D-isomer is attacked by a specific isomeric oxygenase
to yield D-kynurenine, thus suggesting that this latter compound
competitively inhibits the hydroxylation of the L-isomer. This
suggestion readily accounts for the results obtained.

The methyl analogues of tryptophan used have been
reported to inhibit the tryptophan oxygenase reaction as well as
some of the steps of tryptophan biosynthesis (Hayaishi (1955);
Umbriet (1955); Sourkes and Townsend (1955); Civen and Knox
(1960); Moyed (1960); and Trudinger and Cohen (1956)). Reversal
of the inhibitory effects of these compounds was obtained by
the addition of L-tryptophan, 3-hydroxy- and/or N-methyl anth-
ranilate. N-methyl anthranilate could be replaced by the combined
addition of anthranilate and L-methionine in both inhibition
reversal and the enhancement experiments. The addition of either
anthranilate or methionine separately had little effect upon inhibition by the tryptophan analogues. Separate additions of these compounds to control media showed only low enhancement effects.

The use of auxotrophic strains of *P. aeruginosa* helped to establish the precursors of the pyocyanine molecule. A tryptophan requiring auxotroph of ATCC 9027 which was isolated by R.M. McKelvie was found to have a genetic block at the enzymatic steps between anthranilate and indole. When the auxotroph was grown on limiting levels of L-tryptophan and the proposed precursors added to the medium at the end of the logarithmic growth phase, pyocyanine synthesis was enhanced. The greatest enhancement was given by 3-hydroxyanthranilate. N-methyl anthranilate provided some enhancement, but the level of stimulation was less, perhaps because of the accumulation of anthranilate prior to the enzymatic block. L-tryptophan additions stimulated growth and the level of pyocyanine production was markedly increased. Resting cell suspensions of this auxotrophic organism failed to produce any pigment in 40 hours even when incubated with precursor compounds.

The use of an organic sulfur requiring auxotroph helped to support the role of N-methyl anthranilate in pyocyanine biosynthesis. Although levels of pyocyanine were low N-methyl anthranilate stimulated production 50% over the amount obtained with limiting levels of substrate methionine and higher than the amounts of pigment obtained by high concentration L-methionine additions. The use of resting cell suspensions of this auxotroph did not yield reproducible results.

The data obtained suggested that the biosynthesis of the phenazine pigment, pyocyanine, occurs at the end of the log-
arithmic growth phase and is coincident with the loss of cell viability and with autolysis. The synthesis of the molecule requires that alanine be present in the supernatant while there is still an excess of substrate carbohydrate. It is suggested that the autolysis of the cell causes the activation or liberation of an enzyme system which stimulates the synthesis of pyocyanine from anthranilate derivatives formed, in turn, from the degradation of L-tryptophan. A scheme of pyocyanine biosynthesis is proposed in figure 20. Reactions presented following condensation of the two anthranilate derivatives are speculative and are based on the fact that decarboxylation reactions are known to occur readily in obligate aerobes (Miller (1961); Evans (1965)). This suggested pathway is consistent with most of the published data concerning pyocyanine production and biosynthesis.

It was reported by Holliman (1961) and other workers that the level of oxygen supplied to the organism is critical. The presence of some oxygen is required, but excessive amounts result in no pigment synthesis. This phenomenon is readily explained by the fact that the organism requires oxygen for growth, for the tryptophan oxygenase reaction (Evans (1963)), and for the proposed hydroxylation and decarboxylation steps, whereas the presence of excess oxygen would allow degradation of anthranilate and 3-hydroxyanthranilate via oxygenase reactions (Evans (1963)) at earlier stages. The use of still cultures with large surface areas and high ionic strength of media (Ingram (1939)) seem to effectively control oxygen levels.

The possibility of an aromatic amino acid precursor
Figure 20. Proposed pathway of pyocyanine biosynthesis.

- Anthranilate → Indole
- L-Tryptophan
- N-formyl-L-kynurenine
- L-Alanine
- L-Alanine
- L-methionine
- Homocysteine
- N-methyl-anthranilate
- 3-hydroxykynurenine
- 3-hydroxyanthranilate
- Pyocyanine
was suggested by McDonald (1963), who demonstrated that the addition of quinic acid and shikimic acid to the medium strikingly competed with labelled glycerol as precursors of the pigment, and by Kurachi (1959), who noted stimulation of pigment production by the addition of combinations of anthranilate and L-methionine to the medium. Carter and Richards (1961) suggested that carboxyl labelled anthranilate was incorporated into the phenazine pigment of *P. chlororaphis* and accounted for at least one of the rings of the molecule. The data of Ingram et al. (1962) indicated that the labelling of the pyocyanine was symmetrical, when the medium contained glycerol-1,3-C\(^{14}\), and may be interpreted to be due to the condensation of two identically labelled rings. The pathway proposed for glycerol assimilation by McDonald (1963) does not account for the labelling obtained by Ingram et al.

It is of interest to note that the synthesis of nicotinamide derivatives from glycerol-1,3-C\(^{14}\) in *E. coli* (Ortega and Brown (1959)) proceeds with the highest specific activity (2 glycerol molecules/nicotinic acid moiety) of any compound studied (including succinate and pyruvate). It has been reported by Davis et al. (1951) and by Harris et al. (1957) that in *Xanthomonas* the synthesis of nicotinamide and its derivatives proceed directly from 3-hydroxyanthranilate. The role of 3-hydroxyanthranilate in pyocyanine production has added significance in that levels of pigment and oxidized nicotinamide derivatives increase together during incubation. This fact may be of physiological importance because of the function of both compounds in the respiration of the organism.

The medium for maximum pyocyanine production proposed
by Goodall (1961) may be accounted for by the suggested pathway of pigment synthesis. The addition of iron (Fe^{++}) is required for the cytochromes of the organism and also the tryptophan oxygenase enzyme of Pseudomonas has been reported by Hayaishi (1962) to be an iron porphyrin enzyme. The fact that alanine is the preferred amino acid for pigment production may be due to the fact that the normal route of tryptophan degradation does not involve 3-hydroxykynurenine (except for low levels which must be formed in coenzyme synthesis) and the degradation of kynurenine involves the cleavage reaction producing alanine and anthranilate (Behrman (1962)). The presence of high levels of alanine in the supernatant may act by mass law effect or a feedback mechanism to cause kynurenine, at least partially, to enter an alternate pathway - the first step of which would be, presumably, hydroxylation.

The net effect of the degradation of tryptophan in the presence of alanine would be the accumulation of 3-hydroxyanthranilate and anthranilate in the cell. The latter compound could be methylated and then the condensation of these two degradation products of tryptophan would form a carboxylated phenazine nucleus prior to pyocyanine formation.
Summary

1) A study was made on the relative abilities of strains of P. aeruginosa to produce pyocyanine.

2) It was found that pyocyanine production commenced at the end of the logarithmic growth phase and was coincident with a rapid loss of cell viability and autolysis.

3) The presence of alanine in the supernatant was shown to be obligatory for pigment formation.

4) It was found that pyocyanine production could be stimulated in both growing cultures and washed cell suspension by the addition of low levels (20 μg/ml) of L-tryptophan, 3-hydroxyanthranilate, N-methyl anthranilate or the combined addition of L-methionine and anthranilate.

5) Synthesis of pyocyanine was inhibited by the addition of α-methyl-DL-, 4-methyl-DL-, and 6-methyl-L-tryptophan and D-tryptophan. The ability to inhibit synthesis was found to be α->4->D->6-.

6) The addition of L-tryptophan or 3-hydroxyanthranilate + L-methionine, but not N-methyl anthranilate was found to reverse D-tryptophan inhibition.

7) All compounds found to stimulate pigment production were able to reverse the inhibitory effects of the methyl analogues of tryptophan.

8) The use of auxotrophic strains of P. aeruginosa, requiring tryptophan, and organic sulfur respectively, supported the postulate that the anthranilate precursors of pyocyanine were
derived from L-tryptophan.

9) A pathway of pyocyanine biosynthesis consistent with the majority of published data on pigment production is proposed.

10) A correlation was shown between the liberation of pyocyanine and oxidized nicotinamide nucleotide levels in the cell. Similarities in biosynthesis and function are discussed.
Bibliography


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